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Dental Pulp stem cells grown on dental implant titanium surfaces: An in vitro evaluation of differentiation and microRNAs expression

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Abstract: The surface roughness of dental implants influences the proliferation and differentiation rate of adult mesenchymal stem cells (MSCs). The aim of the present study was to evaluate whether specifically treated titanium implant surfaces influenced human dental pulp stem cells (DPSCs) differentiation in an osteogenic pattern through modulation of microRNAs expression. The degree of differentiation was evaluated after 7, 14, and 21 days, through the expression of microRNAs characterizing the osteogenesis (miR-133 and miR-135), of Runx2 and Smad5 (key factor transcriptions associated with osteoblast differentiation) and Osteocalcin, marker for the bone formation process. DPSCs were cultured on sandblasted and acid-etched titanium disks, with (Test) or without the presence of ions (Control). Early differentiation of DPSCs cultured on titanium could be detected at all the evaluated time points, respect to cells grown alone. Moreover, the Test surfaces seemed to induce a more marked cells differentiation. The obtained results demonstrated that microRNAs played a pivotal role in the differentiation of MSCs and could be used as marker of osteogenic differentiation. Furthermore, the evaluated ionized sandblasted and acid-etched surface seemed to markedly enhance the development of osteoblast cells. A faster osseointegration could be achieved in the presence of specifically treated implant surfaces, promising encouraging clinical outcomes. © 2016 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 00B: 000–000, 2016.

Key Words: bone regeneration, dental pulp stem cells, implant surfaces, microRNAs, osseointegration

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INTRODUCTION

Bone development and regeneration is strictly depended on complex interactions between bone-forming osteoblasts, bone-degrading osteoclasts and other cells of the bone microenvironment.[1] In this process, the bone microvasculature play a crucial role, since it regulates the bone cells physiology and allows a circulatory network that links together local signals and growth factors with cells of the bone microenvironment,[2,3] providing not only a temporal, but also a spatial relationship between angiogenesis and osteogenesis[2]. Due to these reasons, loading of scaffolds with stem cells (SCs) is recently proposed, in order to develop new bio-complexes that more properly replace and induce the bone defect repair.[4,5]

The differentiation of SCs in osteoblastic cells and osteocytes is obtained subsequently the exposure to hormones, growth factors (GFs), and mechanical loading, all of which determine sequential gene regulation by transcription factors (TFs), co-activators, associated proteins, and repressor molecules.[6] During osteogenesis, microRNAs also play a pivotal role in the development of this process.

MicroRNAs (miRNAs) are tiny RNAs composed by only 18–22 nucleotides, crucial in regulation of development, proliferation, differentiation, apoptosis and response to different extracellular signals and stress.[7] miRNAs are able to regulate the gene expression through the post-transcriptional silencing of mRNA expression and their subsequent down- regulation. Each miRNA may target hundreds of mRNAs, and some targets are affected by multiple miRNAs.[6]

Probably, miRNAs are fundamental in the maintenance of pluripotency and undifferentiation of adult stem cells (ASCs)[7]; indeed, several miRNAs appear to significantly modulate the differentiation of mesenchymal precursors in osteoblast cells, regulating the activity of transcription factors (TFs).[6]

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In the early osteogenesis, BMP2 (Bone morphogenic proteins) signaling appears to downregulate some miRNAs (miR-133 and miR-135) that suppress two essential TFs for osteogenesis forming a transcriptional complex (Runx2 and Smad5).[8] Runx2 become to be expressed, as genetic markers, during the differentiation of the osteoprogenitors into osteoblast cells and regulates many genes that determine the osteoblast phenotype, such as collagen1α1, osteopontin, bone sialoprotein (BSP), alkaline phosphatase (ALP) and osteocalcin. Osteocalcin, a late-stage marker of differentiation, is secreted solely by osteoblasts and seems to play a role in the body's metabolic regulation; it is also implicated in bone mineralization and calcium ion homeostasis and it is often used as a marker for bone formation process.[8]

The occurrence of bone tissue regeneration is fundamental during the osseintegration of a dental implant, that, among other factors, seems to be influenced by the implant micro-topography in terms of cell adhesion, proliferation and differentiation and of production of local factors.[9–12] The superficial roughness induces an increase in the retention of both the blood and fibrin clot, stimulating the bone healing and a major mechanical stability between the bone and the surface.[11,12]

The superficial topography, moreover, stimulates osteoblast gene expression and induces the differentiation in an osteogenic pattern.[13,14] The osteoblast differentiation, that is crucial in the occurrence of osseointegration process, directly depends on the nano- and microtopography of the implant surface.[15] Thus, modifications of surface macrostructure, microarchitecture and surface properties play a significant role in the bone response. Irrespective of the surface roughness achievement, was widely demonstrated that the treated surface seemed to promote a more intense bone growth, both qualitatively and quantitatively, and to ensure a better organization of bone tissue compared with the machined implants.[16] Moreover, an increase of the surface microtexture complexity seems to determine the formation of a more extensive and three-dimensionally complex fibrin scaffold.[17,18]

Recently, was demonstrated that dental pulp stem cells (DPSCs) seeded on porous surfaces, shown osteoblast differentiation, production of appreciable amounts of bone morphogenetic proteins as well as vascular endothelial growth factor and specific bone proteins.[19] Dental stem cells are mesenchymal stem cells (MSC) capable of both self-renewal and multi-lineage differentiation,[20] recently widely used in the dental tissue engineering. Indeed, the use of DPSCs, which are capable of producing woven bone, would accelerate the implant loading time.[21] Similar outcomes were obtained from human periosteum-derived cells (hPDCs) cultured on different surfaces treated at the micro- and nanotopographical level, where cell response, and apatite-forming ability were increased.[22]

The aim of the present study was to evaluate whether specifically treated titanium implant surfaces influenced the DPSCs differentiation in an osteogenic pattern and modulation of miRNAs expression. The degree of differentiation was evaluated through the expression of miRNAs characterizing the osteogenesis (miR-133 and miR-135), of Runx2 and Smad5 (key factor transcriptions associated with osteoblast differentiation) and the levels of Osteocalcin, marker for the bone formation process, in order to assess whether the modified surfaces were able to induce differentiation of DPSCs in osteoblast cells.

MATERIALS AND METHODS

Human dental pulp stem cells were extracted and characterized at the Department of Experimental Medicine, Section of Histology and Embryology, Second University of Naples (Naples, Italy), as previously reported.[23,24] Accordingly, only cells that co-expressed CD117, CD34, STRO-1 and flk-1 markers (5% of total cell population) were sorted in order to obtain a homogeneous population of DPSCs. The sorted cells were maintained in culture and analyzed by cytofluorimetry for CD34/CD45.[25] Therefore, DPSCs were stored at -80°C.

Cell culture

Once defrosted, cells were put in culture with a Standard Medium and incubated at 37°C and 5% CO_2. The Standard Medium was composed as follows: DMEM High glucose (Dulbecco's Modified Eagle Medium High glucose, #ECB7,501L, Euroclone, Milan, Italy) + 10% FBS (fetal bovine serum, FBS, #ECS0,1,00187, Euroclone, Milan, Italy) + 1% Pen/Strep (Penicillin/Streptomycin 100X, #ECB3,001D, Euroclone, Milan, Italy) + 1% L-Glutamine (100× (#25,030–024, GIBCO, Life Technology, Carlsbad, California, USA). Standard Medium was changed twice a week, just before cells become confluent (sub-confluent).

Once the cells reached an adequate number, they were washed twice with PBS (phosphate buffered saline, #ECB4,004L, Euroclone, Milan, Italy) and detached using 1 mL of Trypsin-EDTA 1X in PBS (#ECB3,052DK, Euroclone, Milan, Italy) for 5 min at 37°C. Cells were collected in a sterile tube and centrifuged for 5 min at 900 rpm. Once resuspended, the cells were counted using a Bürker chamber, and, thus, were used for the following experiments, as reported below.

The experimental surfaces

In the present study, innovative titanium disks (Implacil De Bortoli-Dental Product, São Paulo, Brazil) (5-mm diameter and 2-mm thick) were used. Disks were divided into two experimental groups according to the type of surface treatment: experimental titanium disks with a sandblasted and acid-etched surface (Control Group); experimental titanium disks with a sandblasted and acid-etched surface, then treated with inorganic ions (Test Group).[26]

Topographic analysis of the disk surfaces was performed by Stereo SEM (EVO MA 10 SEM, Zeiss, Germany). Roughness was evaluated quantitatively using dedicated software to convert conventional SEM images into three-dimensional data (Mex 4.2, Alicona Imaging GmbH, Graz, Austria). Basically, two images of the same field (200 × 300 μm) were acquired after eucentric rotation by a given angle. This was obtained by changing the angle between the sample and the

electrons, by tilting the table that held the sample. The tilting angle (5° at 1,000×) was 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 were the input data that the software converted into a single three-dimensional image, where each data point was characterized by the values of the x, y, z coordinates. The image obtained by this process allowed to measure the height of surface topography and to calculate the different roughness parameters. The following parameters were calculated: the roughness average of the selected area (Sa); the root mean square area roughness (Sq); the maximum height of area roughness (Sz); the maximum peak height of area roughness (Sh); the maximum valley depth of area roughness (Sv); the average mean spacing of profile irregularities of area roughness (Scx); the ratio of true area to projected area (Sdr).

Design of the study

Human DPSCs were cultured in vitro on sandblasted and acid etched titanium disks (Control) or on sandblasted and acid etched titanium disks treated by inorganic ions (Test), as described above, in presence or not of differentiation medium.

Five test and control disks were placed in each well of a six-multiwell plate used for every experimental point. On the top of each disk, a volume of 20 μL of Standard Medium (or Growth Medium-GM) with an amount of ~150,000 cells was set; then, the multiwell plates were incubated at 37°C and 5% CO_2. After 5 h (needed for cell adhesion on titanium disks), 1.5 mL of GM was added to each well before re-incubation; the same culture conditions were repeated with the addition of a Differentiative Medium (DM) rather than GM, in order to induce the cell differentiation in an osteogenic pattern. DM was obtained by supplementation of GM with 100 n*M* Dexamethasone, 100 m*M* β-glycerol phosphate and 50 μ*M* ascorbic acid; dexamethasone, a synthetic glucocorticoid, was used instead of vitamin D_3, since it appeared to optimize the differentiation from MSCs.

An amount of ~50,000 DPSCs without disks, but in the same conditions, were cultured in cell culture dishes (100 mm diameter), for each analyzed time point. All the experiments were performed with two samples of stem cells obtained by two different dental pulps, Sample 1 and Sample 2, in order to have reliable outcomes.

After 7, 14, and 21 days of culture, all titanium disks ($n = 30$) were removed from each six-multiwell plates and placed in cell culture dishes (100 mm diameter); thus, only the cells cultured in contact with the disks were detached using 1 mL of Trypsin-EDTA 1X in PBS (#ECB3,052DK, Euroclone, Milan, Italy) for 5 min at 37°C, and collected in order to evaluate their differentiation. For each experimental time point (7, 14, and 21 days) eight 6-multiwell plates (4 in GM and 4 in DM) were used for the analysis of miRNAs expression (miR-133 and miR-135), gene expression (Runx2 and Smad5), amount of cells expressing RUNX2 by flow cytometry and Osteocalcin levels released in the medium.

MicroRNAs expression. PureLink miRNA Isolation kits were used for the miR-133a, miR-133b, miR-135a extractions (# K1,570–01, Invitrogen, Life Technologies, Molecular Devices, Sunnyvale, USA). About 800,000 cells were resuspended in 300 μL binding buffer (the buffers were present in the PureLink miRNA kit), and 300 μL 70% alcohol was added to the lysate. This was forced into the spin cartridges of PureLink miRNA Isolation kits, which were then centrifuged at 12,000*g* for 1 min; after washing with 100% alcohol, these were centrifuged again, as previously described. Then 500 μL of wash buffer was added to the spin cartridges, which were centrifuged again at 12,000*g* for 1 min. This procedure was performed twice, and then the spin cartridges were centrifuged at 12,000*g* for 3 min, to remove residual buffer. Finally, they were eluted with 50 μL RNase-free sterile water. The RNA concentrations were determined using a spectrophotometric quantification. Retro-transcription and real-time PCR were carried out according to the Applied Biosystems TaqMan miRNA assay kit protocols (Life Technologies, Monza-Italy). Briefly, the retro-transcription involved 20 ng of a "small" RNA, as the "stem loop" primer specific for each miRNA, dNTPs and inverse transcriptase RNAse inhibitors (according to the Applied Biosystems High capacity cDNA reverse transcription kit, # 4.368814), using a Thermocycler (30 min at 16°C, 30 min at 42°C, 5 min at 85°C, then at 4°C). Then, real-time PCR for the miRNA expression levels was performed using the TaqMan probes and the specific TaqMan ®Universal Master Mix II, no UNG, in 96-well plates (#4.440040, Applied Biosystems, Life Technologies, Monza-Italy) with an Applied Biosystems PRISM 7900 HT Sequence Detection System, in triplicate. miR-16 was used as the endogenous control. The specific miRNA sequence probes used (Applied Biosystems) were:

has-miR-133a (UUUGGUCCCCUUCAACCAGCUG; # 002,246);
has-miR-133b (UUUGGUCCCCUUCAACCAGCUA; #002,247);
hsa-miR-135a-5p (UAUGGCUUUUUAUUCCUAUGUGA; #000, 460);
has-miR-16–5p (UAGCAGCACGUAAAUAUUGGCG; #000, 391).

The relative quantification of the miRNA targets was carried out using the ΔCt formula, according to the Ct method ($Ct_{\text{miRNA of interest}} - Ct_{\text{miR-16}}$). Upregulated miRNAs were showed with positive values and downregulated miRNAs with negative values. [27] Three independent experiments were performed in triplicates, for each DPSCs sample.

Real-time PCR analysis. For the real-time PCR, the cells were collected for RNA extraction, as previously described.[28,29] The total RNA was isolated using Tri Reagent (#T9,424, Sigma-Aldrich, Saint Louis, MO-USA), according to the manufacturer instructions.

For cDNA synthesis, 1 μg total RNA was directly processed with High-Capacity cDNA Archive kits (Applied

TABLE I. Stereo SEM analysis of titanium disks surfaces

Parameter	Test surface	Control surface
Sdr	1.68 ± 0.08	1.71 ± 0.11
Sa	0.92 ± 0.01	0.95 ± 0.04
Sq	1.14 ± 0.02	1.19 ± 0.03
Sz	6.68 ± 0.48	7.26 ± 0.08
Sh	3.44 ± 0.30	3.60 ± 0.09
Sv	3.24 ± 0.19	3.65 ± 0.01
Scx	12.73 ± 0.97	13.32 ± 0.15

Sa: the roughness average of the selected area; Sq: the root mean square area roughness; Sz: the maximum height of area roughness; Sh: the maximum peak height of area roughness; Sv: the maximum valley depth of area roughness; Scx: the average mean spacing of profile irregularities of area roughness; Sdr: the ratio of true area to projected area. Data are shown as mean ± standard deviation. All data are expressed in micrometers, except Sdr that, as a ratio, is a pure number.

Biosystems, Life Technologies, Monza-Italy), according to the manufacturer instructions. Singleplex real-time PCR was performed for the relative quantification of gene expression of RUNX2 (runt-related transcription factor 2) (# Hs0,0231 692_m1), SMAD5 (SMAD family member 5) (# Hs0,0195 437_m1), BGLAP (bone gamma-carboxyglutamate (gla) protein) (# Hs0,1587 814_g1) versus GAPDH (the glyceraldehyde-3-phosphate dehydrogenase gene) (# Hs0,2800 695_m1), using TaqMan technology on an ABI Prism 9,700HT Sequence Detection System instrument, connected to Sequence Detector Software (SDS, version 2.0) for data collection and analysis. The primer pairs and TaqMan probes for all of the target genes and for the GAPDH reference gene were provided as 20× mixtures that were ready to use at a final concentration of 1×. According to the manufacturer recommendations, 25 μL reactions were performed in a MicroAmp Optical 96-well reaction plate using 12.5 μL 2X TaqMan Universal PCR Master mix, with 1.25 μL 20× Inventoried Gene Expression Product for the mouse RUNX2 target gene, SMAD5, BGLAP versus GAPDH (FAM-dye-labeled TaqMan MGB probe). PCR was performed at 50°C for 2 min, and at 95°C for 10 min, and then run for 45 cycles at 95°C for 15 s and at 60°C for 1 min. All of the reactions were performed in triplicate, and each experiment was repeated three times. The results were exported from the ABI Prism 9700HT Sequence Detection System into Microsoft Excel files for further analysis. The relative quantification of target gene expression was evaluated with data from the SDS software, using the arithmetic formula 2–DDCt, according to the comparative Ct method, which represents the amount of target, as normalized to the GAPDH endogenous control. All materials, instruments and software were purchased from (Life Technologies, Monza-Italy). Three independent experiments were performed in quintuplicate, for each DPSCs sample.

Fluorescence activated cell analysis and sorting. Once detached and collected, cells were washed in PBS and for Runx-2 intracellular analysis, Fix&Perm Kit (Invitrogen, San Giuliano Milanese, Milan-Italy) was used according to guidelines. Five μl of primary Runx-2 antibody (Ms mAb to Runx-2 #ab5,4868, Abcam, Cambridge-UK) were added, and all was incubated for 30 min at 4°C. After washing with PBS, incubation with the secondary Runx-2 antibody (Alexa Fluor 488 donkey anti-mouse IgG (H + L), Life Technologies, Monza-Italy) was performed for 30 min at 4°C. The samples were then analyzed on a FACSCalibur flow cytometer (two-lasers, four-colour configuration) equipped with CellQuest 3.2.1.f1 software (BD Biosciences). The data were analyzed using the FlowJo v8.8.6 software (TreeStar, Ashland, OR, USA).

Quality control included regular checks with Rainbow Calibration Particles (6 peaks; BD Biosciences). In each

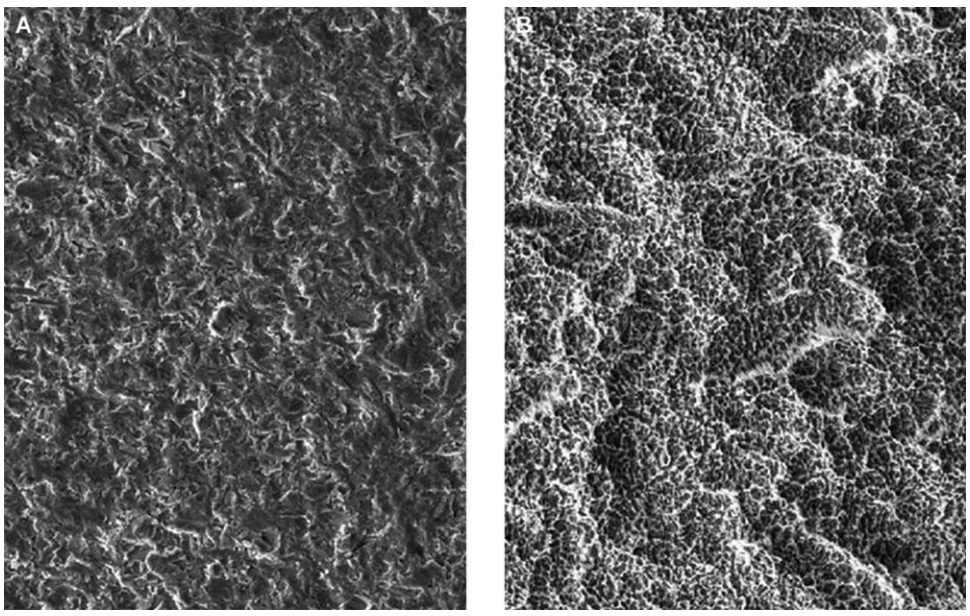

FIGURE 1. Stereo SEM images of titanium disks surfaces. Test disk (A) and Control disk (B); magnification 1,000×.

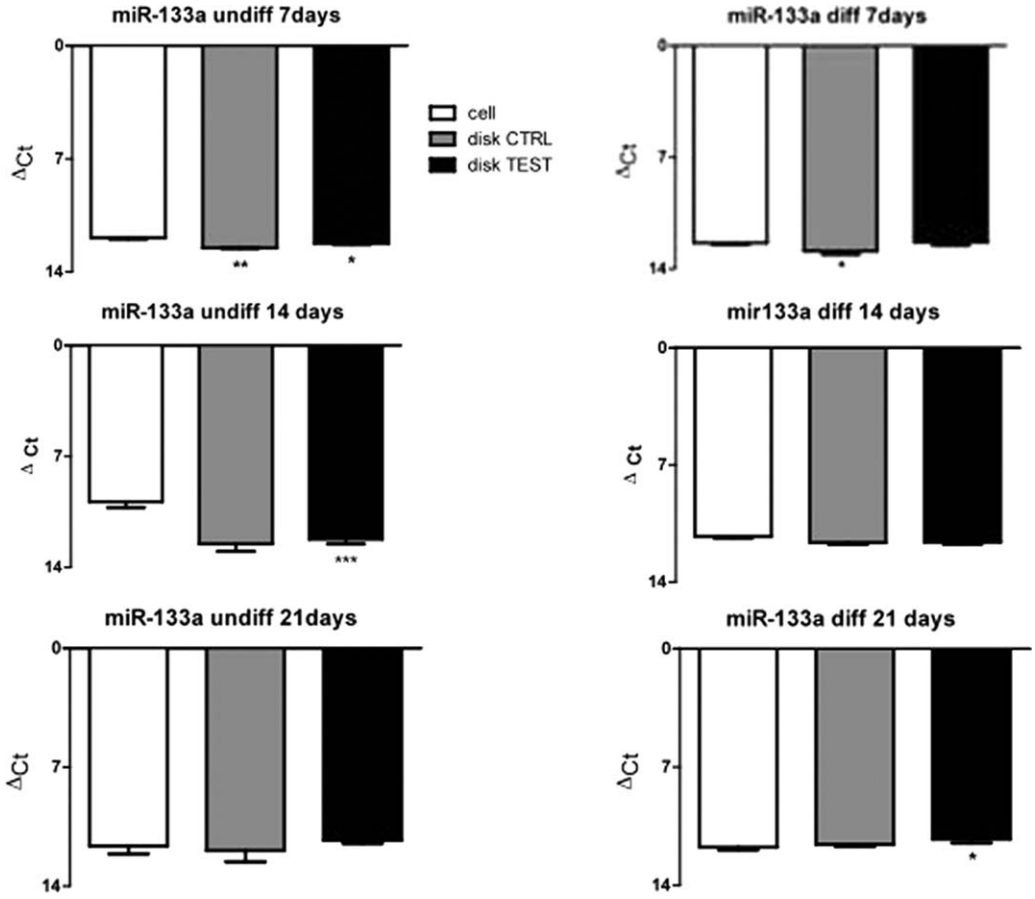

FIGURE 2. Expression of miRNA 133a. The graphs show the down-regulation of miRNA 133a compared with cells grown without disks after 7 and 14 days of DPSCs culture. At 21 days the down-regulation disappears. Data derived from three different experiments (each $n = 3$) were presented as means ± standard deviation.

analysis 1,000,000 total events were recorded. To evaluate nonspecific fluorescence when defining positive events, Fluorescence Minus One controls was used. Data expressing the positive intracellular Runx2 cells were presented as Mean Fluorescence Intensity (MFI). Three independent experiments were performed in quintuplicate, for each DPSCs sample.

Osteocalcin assay. Immunoenzymatic assay to quantify the Osteocalcin was performed using the MicroVue Bone Osteocalcin EIA (#8002 Quidel, San Diego, CA, USA) according to the guidelines. Briefly, 1× wash buffer (dilution of 10× wash buffer 1:10 with deionized water) was prepared, and standards and controls with 0.5 mL 1× wash buffer were reconstituted. For the assay procedure 25 μL of reconstituted standards, controls, and samples were put into assay 96-well microplate and 125 μL of anti-Osteocalcin were added to assay wells. After incubation of 120 ± 10 min at room temperature, enzyme conjugate with 1× wash buffer was prepared, and 150 μL of it was added into assay wells and incubated for 60 ± 5 min at room temperature. Finally, 150 μL substrate solution were added and incubated for 30–40 min at room temperature. Thus, 150 μL of stop solution was supplemented.

Optical density was read at 405 nm; the assay results were analyzed using a 4 parameter curve fit $y = (A\text{-}D)/(1+(x/C)B)+D$.

Three independent experiments were performed in quintuplicate, for each DPSCs sample.

Alizarin red staining. Once test and control titanium disks were removed after 21 days of culture in DM condition, cells adhering on the plates were washed twice in PBS and then fixed with 1 mL/well (six-multiwell plate) of 4% paraformaldehyde (#157–8, Electron Microscopy Sciences, Hatfield, PA-USA) for 15 min. Specimens were washed twice with deionized water and 1 mL/well of 1% Alizarin Red 40 nM (#A5,533, Sigma-Aldrich, Saint Louis, MO-USA) was added and incubated for 20 min at room temperature.

Specimens were then washed four times with deionized water for 5 min, and viewed under the light microscope at a magnification of 10×.

Statistical analysis

Statistical analysis was performed with unpaired t-test using the GraphPad Prism 5.0. software. For miRNAs expression and FACS, Test and Control group were correlated with cells alone; for Osteocalcin assay and RT-PCR Test group was

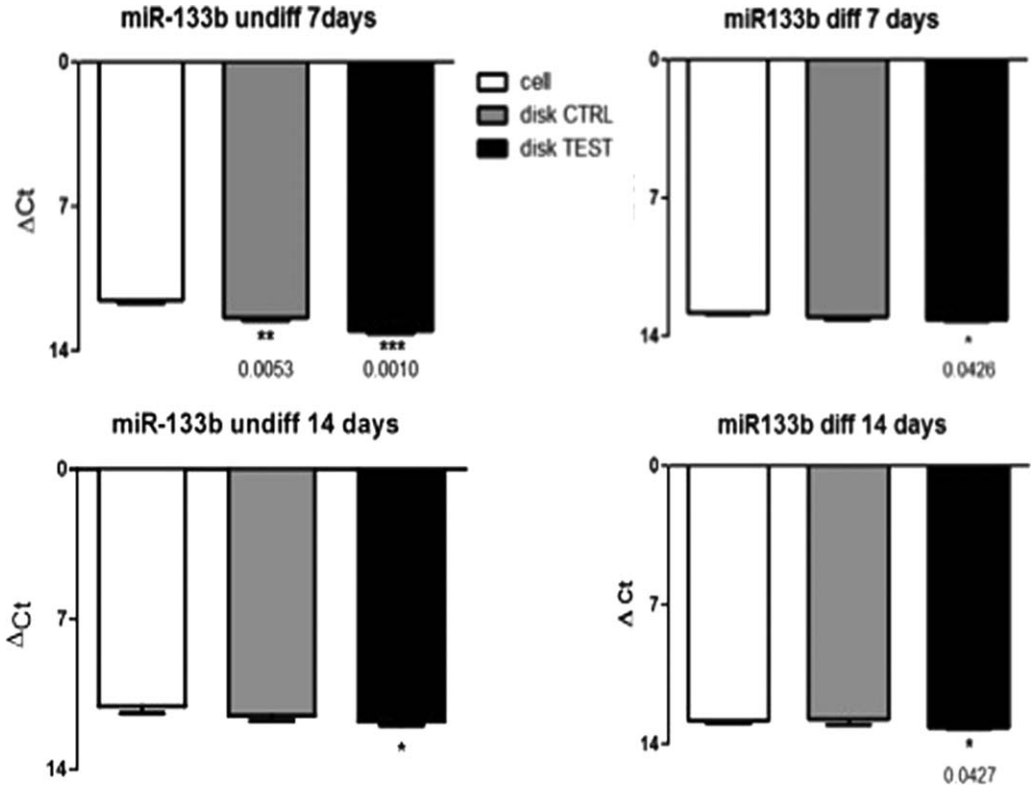

FIGURE 3. Expression of miRNA 133b. The graphs show the down-regulation of miRNA 133b compared with cells grown without disks after 7 and 14 days of DPSCs culture. Data derived from three different experiments (each $n = 3$) were presented as means ± standard deviation.

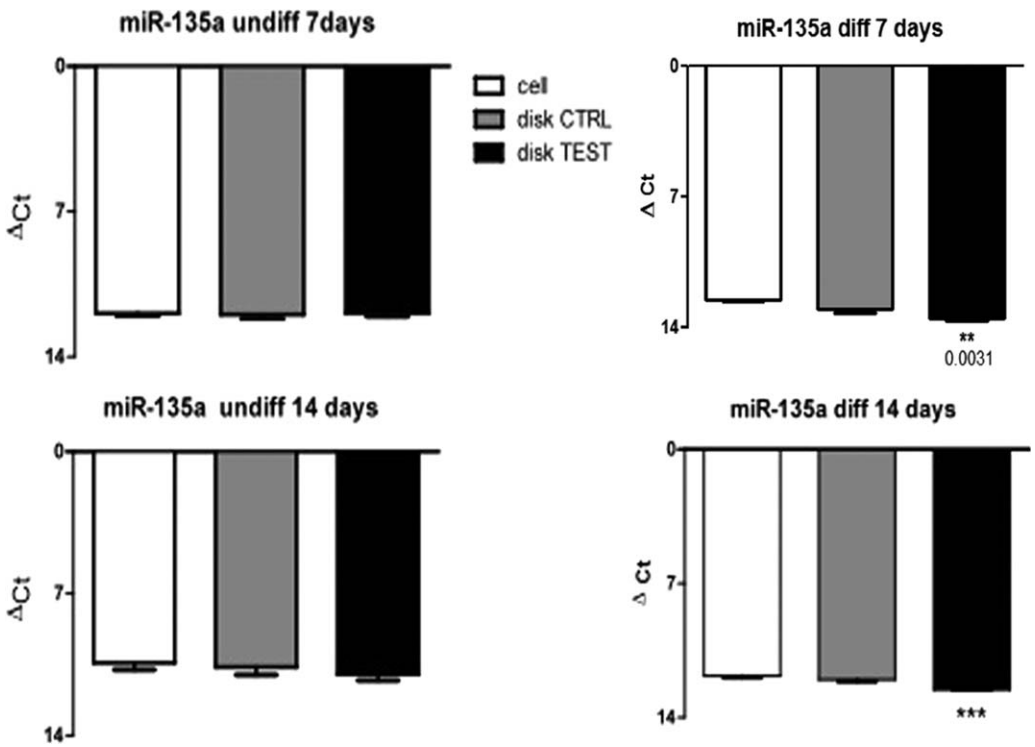

FIGURE 4. Expression of miRNA 135a. The graphs show the down-regulation of miRNA 135a compared with cells grown without disks after 7 and 14 days of DPSCs culture. Data derived from three different experiments (each $n = 3$) were presented as means ± standard deviation.

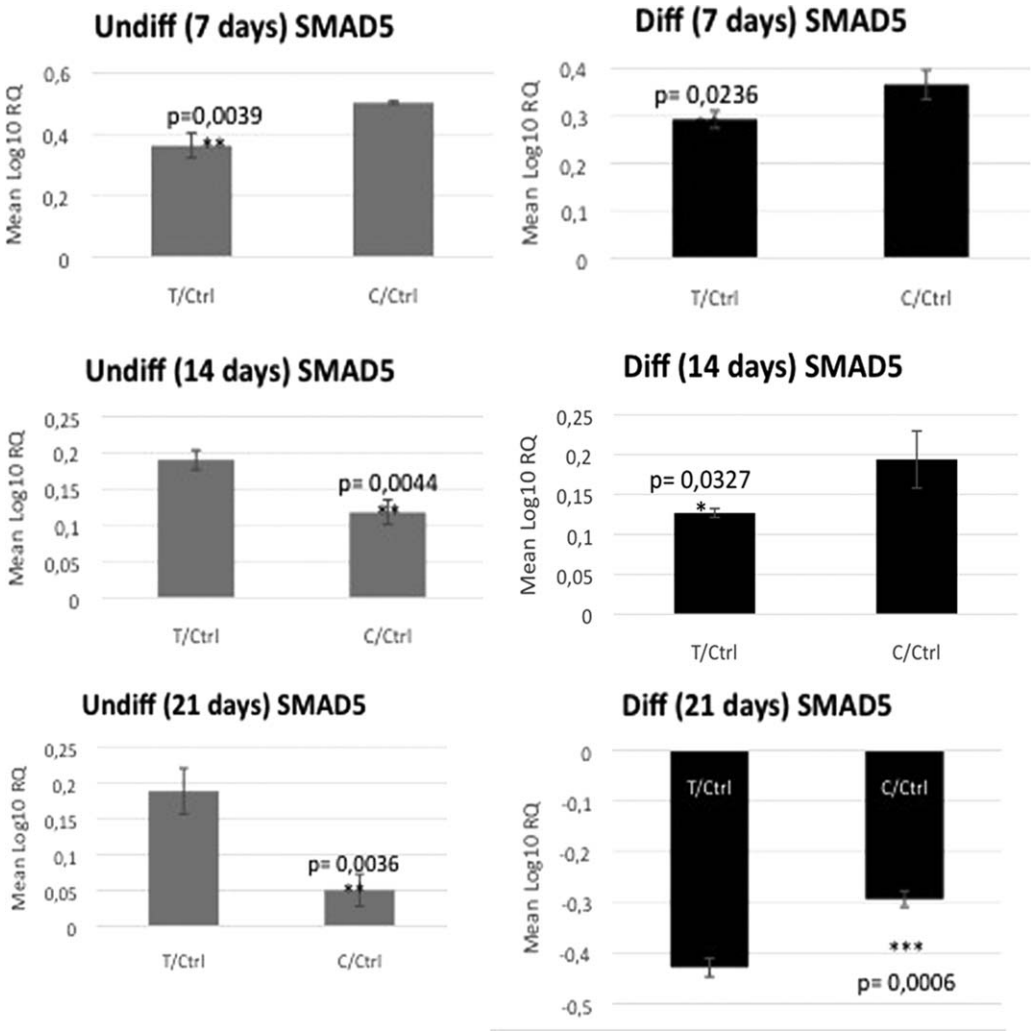

FIGURE 5. RT-PCR of Smad5. The graphs show the Smad5 expression after 7, 14, and 21 days in DM and GM of DPSCs cultured on Test and Control disks in relation with cells alone. Data derived from three different experiments (each $n = 5$) were presented as ratio (cells growth on disks vs cells alone) means ± standard deviation.

compared with Control group. The level of significance was set at 0.05.

RESULTS

The cytoflorimetry analysis of DPSCs demonstrated the persistence of negativity for CD45 and positivity for CD34, showing that these cells were of mesenchymal origin. After sorting, the population was found to be $CD90^{+}/CD133^{+}$ (data not shown); this evidenced that the population was still undifferentiated, as well as potentially multipotent, as previously reported.[23,24]

Topographic analysis of the Test and Control disks surfaces is reported in Table I.

Both the evaluated surfaces were highly rough, with an increase in the implant surface area of 65%. Specifically, the roughness parameters of Test surfaces were on average slightly lower than those measured on Control disks (Sa of 0.92 ± 0.01 and 0.95 ± 0.04, respectively); although, the surface topography of the latter was more irregular than the Test disk, in which the surface roughness is more uniform and widely spread (Figure 1).

The results obtained in the present study were presented as mean values of both DPSCs Sample 1 and Sample 2.

MicroRNAs expression

miRNAs 133a, 133b, and 135a expression analysis was performed after 7, 14 or 21 days of DPSCs culture. After 7 and 14 days of culture in GM, there was a significant down-regulation of miR-133a for Test and Control groups (11.89 ± 0.13 cells alone, 12.52 ± 0.06 control disks, 12.24 ± 0.10 test disks after 7 days; 9.87 ± 0.35 cell alone, 12.53 ± 0.47 control disks, 12.23 ± 0.31 test disks after 14 days) respect to the cells alone (*$p < 0.05$, ** $p < 0.005$ and *** $p < 0.0005$). Instead after 21 days, in GM none differences between cells cultured on disks and cells alone were observed in miR-133a expression, whereas, in DM condition, a slightly lower down-regulation was detected in Test group respect to the cells alone (Figure 2).

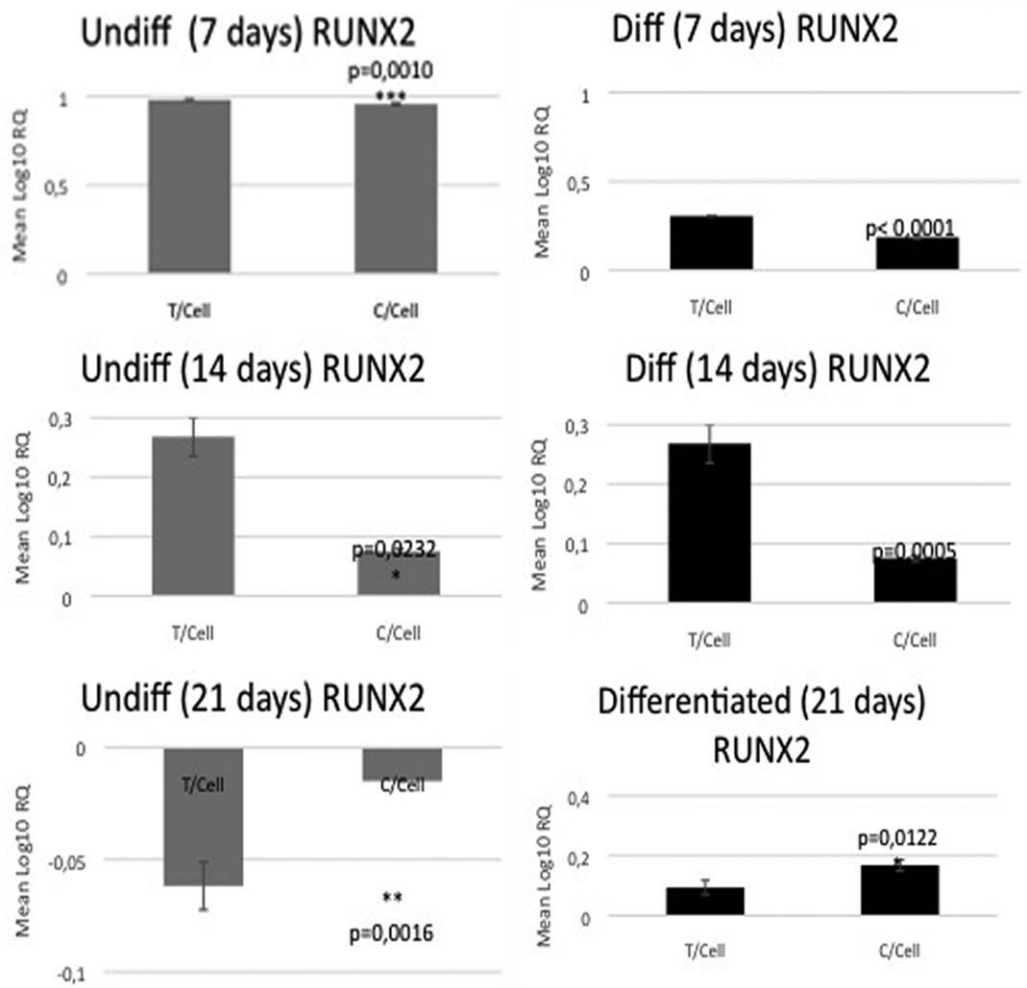

FIGURE 6. RT-PCR of Runx-2. The graphs show the Runx-2 expression after 7, 14, and 21 days in DM and GM of DPSCs cultured on Test and Control disks in relation with cells alone. Data derived from three different experiments (each $n = 5$) were presented as ratio (cells growth on disks vs cells alone) means ± standard deviation.

A greater down-regulation of miR-133b was observed at all the time points in the Test groups respect to the cells alone or cells grown on control disks (Figure 3).

A significant down-regulation of miR-135a in the Test group was shown at all the analyzed time points in the Test groups, except for 7 days of culture in GM in which there were no significant differences, respect to the cells alone or cells grown on control disks (Figure 4).

Real-time PCR analysis

RT-PCR Analysis was performed for Smad5 and Runx-2 after 7, 14, and 21 days of culture, both in GM and DM condition. Data were reported as the ratio of cells cultured on Test titanium disks (T/Ctrl) or Control (C/Ctrl) to cells alone.

The Smad5 expression was significantly higher at 14 and 21 days in GM condition, in the Test group/cells alone respect to Control group/cells alone ($p = 0.0044$ and $p = 0.0036$, respectively); whereas, a greater gene expression was observed in Control group/cells alone in DM condition ($p = 0.0236$, $p = 0.327$ $p = 0.0006$ at 7, 14, and 21 days respectively) and at 7 days in GM ($p = 0.0039$) (Figure 5).

Runx-2 expression was significantly higher both in GM and DM in Test group/cells alone at 7 ($p = 0.0010$ and $p = 0.0001$) and at 14 ($p = 0.0232$ and $p = 0.0005$) days of culture, respectively. Indeed, after 21 days of culture the Runx-2 gene expression in Test group/cells alone was significantly down-regulated in GM condition and in any case always less detected in respect to Control group/cells alone, both in GM ($p = 0.0016$) and DM condition ($p = 0.0122$) (Figure 6).

Fluorescence activated cell analysis and sorting

Characterization by flow-cytometry of Runx2 was performed after 7 and 21 days of DPSCs culture. At 7 days of culture, in GM the analysis always showed more positive intracellular Runx2 cells in both Test and Control groups respect to cells alone (**$p = 0.0052$ and *** $p < 0.002$); moreover, in the same time point, Runx2 expression was greater in cells of Test group than cells of Control one. Whereas after 21 days of culture, there was a lower amount of expressing

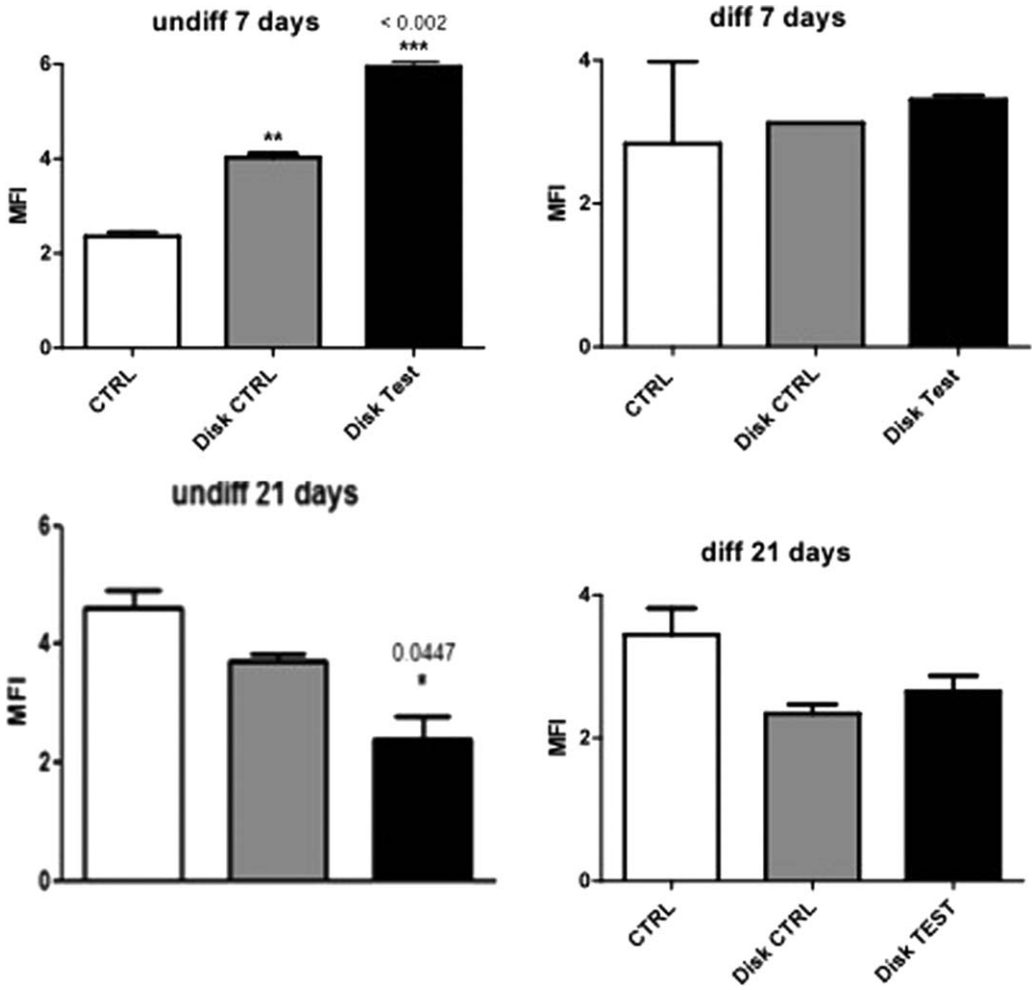

FIGURE 7. Flow cytometry of Runx2. The graphs show the Runx-2 expression after 7 and 21 days in GM and DM of DPSCs cultured on Test disks, Control disks and alone. Data derived from three different experiments (each $n = 5$), expressing the positive intracellular Runx2 cells, were presented as Mean Fluorescence Intensity (MFI) ± standard error of mean (SEM).

Runx2 cells grown on titanium disks compared with cells alone, with a significant statistical difference for the Test group (*$p = 0.0047$) (Figure 7).

Osteocalcin assay

Osteocalcin assay was performed after 14 and 21 days of culture, since it represents a late-stage marker of differentiation. The osteocalcin levels measured in supernatant were reported as the difference in ng/ml/10^5 between cells grown on disks and cells alone. The cells grown on disks always showed a greater amount of osteocalcin in medium respect to the cells alone, with higher levels in cells grown on Test disks respect to the Control one (*$p = 0.0208$ and ** $p = 0.0019$, 14 days in GM and in DM respectively). On the other hand, at 21 days of differentiation, the two groups did not show any statistical difference (Figure 8).

Alizarin red staining

The formation of calcification nodules obtained in cultures from DPSCs was showed by Alizarin Red Staining. The cells cultured on plate surfaces around Test or Control disks were analyzed after 21 days. Both analyzed samples showed nodules in differentiate medium (DM) condition positive for Alizarin Red (Figure 9). Moreover, weak primary mineral depositions could be detected even in absence of titanium disks (Figure 9).

DISCUSSION

Early differentiation of DPSCs into osteoblast cells was already largely described.[6,8,20,25] Moreover, since their mesenchymal origin, dental stem cells are able to differentiate in several cell lines. Particularly, it was demonstrated that when DPSCs differentiated into osteoblasts, they gave rise also to endotheliocytes[25]; indeed, during their differentiation, DPSCs were observed to change their surface antigen expression. After 40 days of culture, stem cells started to differentiate into two cytotypes: about 70% of them became osteogenic progenitor cells, being flk-1^+/STRO-1^-/$CD44^+$/RUNX-2^+, whereas the remaining 30% became flk-1^+/STRO-1^+/$CD44^+$/$CD54^+$ endothelial cells.[25] The presence of vasculogenic markers demonstrated a deep correlation and

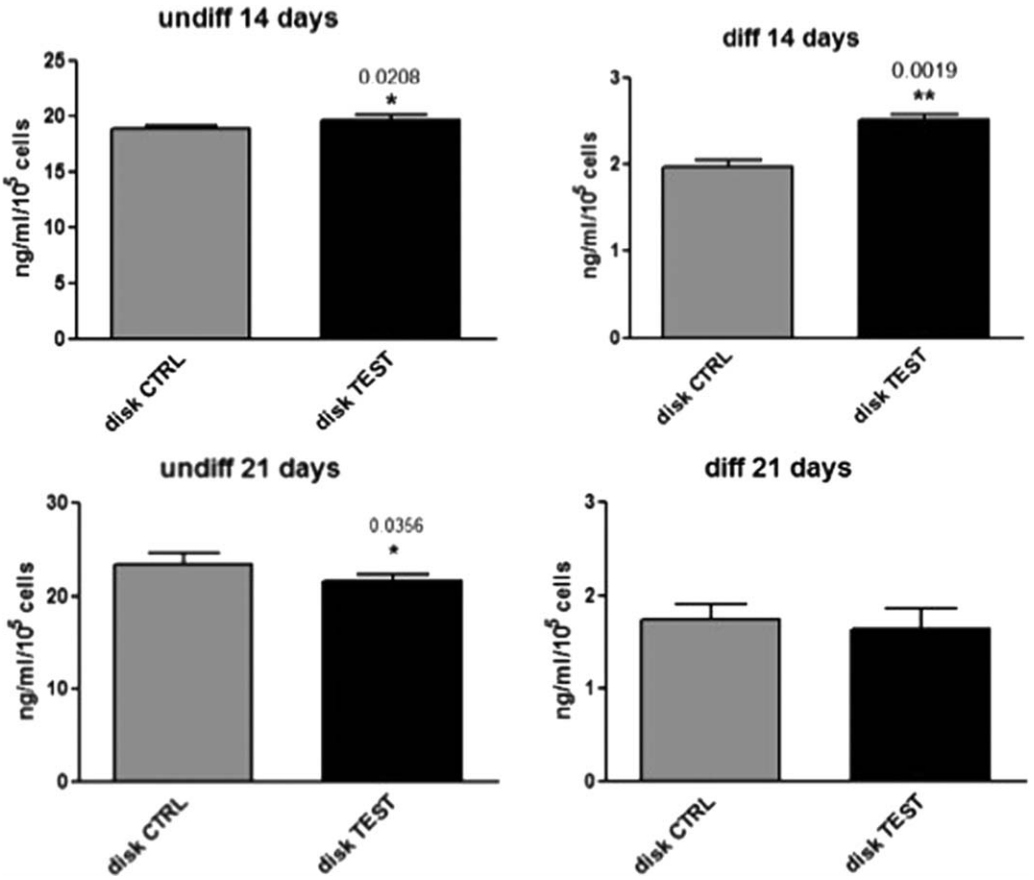

FIGURE 8. Osteocalcin assay. The graphs show the osteocalcin level measured in supernatant after 14, 21 days in DM and GM of DPSCs culture. Data, derived from three different experiments (each $n = 5$), are reported as the difference in ng/mL/10^5 cells between cells growth on disks and cells alone, and presented as means ± standard deviation.

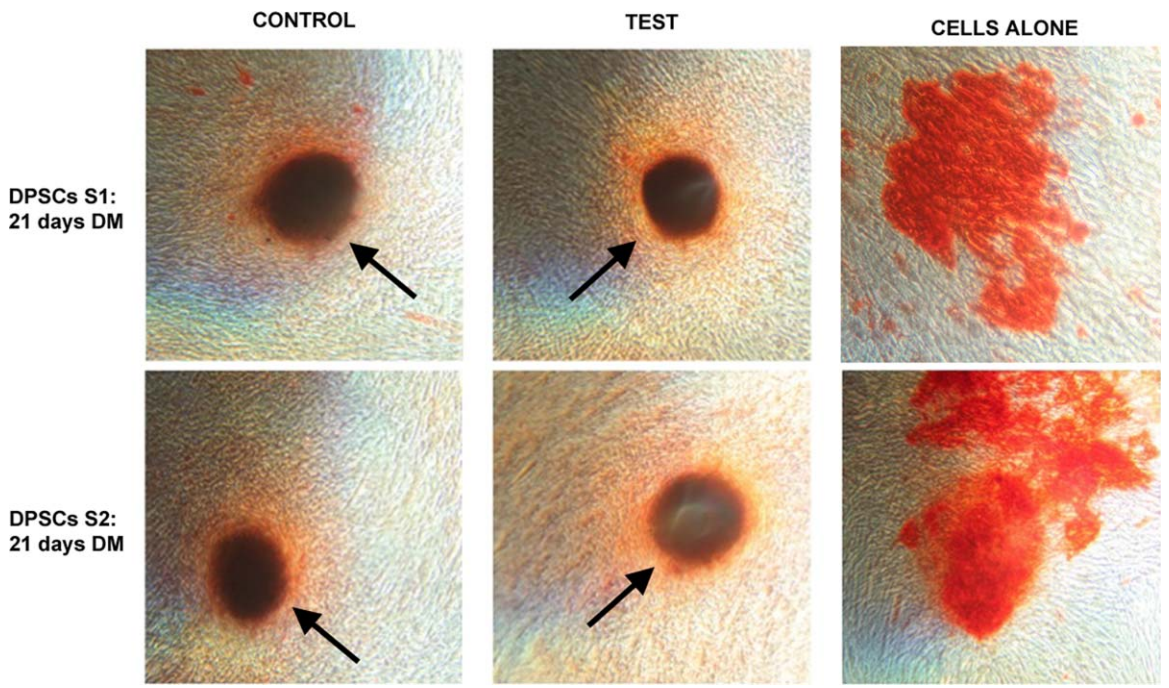

FIGURE 9. Alizarin Red Staining of DPSCs Sample 1 (S1) and Sample 2 (S2). Calcification nodules (arrows) after 21 days of culture in differentiate medium (DM) observed in the same plates of Test and Control disks or in control cultures (without titanium disks) (magnification 10×).

cooperation of both cytotypes, that are of fundamental importance in the production of woven bone in vitro, and able to form adult bone once transplanted in vivo.[25]

The peri-implant bone regeneration, among other factors, is directly related to the surface roughness of the titanium dental implant, in order to achieve a biomechanical interlocking and to provide a long-term osseointegration, and thus high implant success rate.[13,14,30] Since the superficial topography stimulates osteoblast gene expression and induces the differentiation in an osteogenic pattern,[13,14] many cellular types were cultured on titanium surfaces to evaluate the cell-material relationship.

Human bone marrow stem cells (BM-hMSCs) cultured on different implant surfaces, demonstrated a differentiation in osteoblast cells,[19] directly related to the surface microtopography in the early and late stages of differentiation. DPSCs and human osteoblasts cultured on acid-etched titanium (AET) and Laser Sintered Titanium (LST) surfaces in plane cultures or in a roller apparatus demonstrated a bone tissue formation after about 1 month of culture.[21] Briefly, rotating cultures demonstrated that both DPSCs and human osteoblasts after 30 days showed a diffuse bone formation on the surfaces; particularly, the bone deposition, positive for alizarin staining, was slightly more evident in the samples where cells were challenged with laser sintered titanium, due to the numerous microconcavities of the surface. In addition, DPSCs showed a significant better bone formation with respect to human osteoblasts, thanks to an extremely quick osteo-differentiation and secretion of significant amounts of morphogens such as VEGF and BMP-2. Moreover, these cells were capable of expressing significantly higher amounts of transcription factors for bone secretion with respect to human osteoblasts, demonstrating a good performance toward osteogenesis and better interaction with the surface topography.[21]

The good behavior of DPSCs was even demonstrated in the present study. The influence of titanium implant surfaces on stem cells differentiation in an osteogenic pattern, was evaluated through the expression of miRNAs characterizing the osteogenesis (miR-133 and miR-135), and bone formation-related genes, as Runx2 and Smad5, as well Osteocalcin protein expression, with or without the supplement of factors, such as dexamethasone, glycerol phosphate and ascorbic acid. Specifically, in the present study, the cells grown on the titanium disks in comparison with cells alone, showed a statistically significant down-regulation of miR-133a −133b and −135a (at 7 and 14 days) and a great osteocalcin release in the supernatant at 14 days, supposing that the cells cultured on titanium activated an earlier differentiation process, even in proliferation condition. However, it should be emphasized that this event was more markedly evident in Test group respect to Control one. On the other hand, after 21 days in both culture conditions, the down-regulation of miR-133a expression was higher in the cells alone than in the cells cultured on titanium. This result makes us hypothesize that the cells grown on titanium disks, could be already in a later differentiation stage, restarting to express miR-133, which again inhibits Runx2. These results suggest to use miRNAs expression as marker of osteogenic differentiation, that, to our knowledge, is not yet reported in the scientific literature.

The early microstructured titanium surfaces-dependent activation of BMP-2 pathway in human mesenchymal stem cells was previously demonstrated by Olivares-Navarrete et al.[31] Indeed, in this study was reported as these cells differentiated, through coordinated regulation, into osteoblasts on microstructured titanium surfaces without addition of medium supplements, suggesting that surface-dependent endogenous mechanisms were involved.[31]

The down-regulation of miR-133 and miR-135, mediated by BMP-2, is fundamental for the expression of Runx2 and Smad5. Smad5 gene expression showed, at all the analyzed time points, a higher significant expression in the cells cultured on disks compared with them grown alone. Instead, after 21 days in DM, a significantly greater down-regulation of the gene expression in the cells cultured on disks compared with cells alone was observed. These results suggest that probably those cells were markedly differentiated due to the presence of the titanium (more evident in the Test group) as well as differentiation factors.

The better performances in terms of Runx2 gene expression of cells grown on Test disks was observed after 7 and 14 days for both culture conditions. After 21 days in GM, a significant higher down-regulation of the cells cultured on Test disks was appreciated, demonstrating that these cells were, by this time, in the late stage of differentiation, since Runx-2 should be suppressed at this point.[32] The same trend was corroborated by the protein expression of Runx-2, where a higher number of DPSCs of the Test group expressing Runx2 after 7 days in GM and DM was observed, confirming an earlier differentiation. In the late stages (21 days), this pattern was inverted, stressing that cells cultured on titanium disks were already differentiated, regardless the presence of dexamethasone, glycerol phosphate and ascorbic acid in the medium.

The miRNAs down-regulation, widely demonstrated that the induction of differentiation was strictly dependent on the presence of titanium, rather than the presence of supplement factors. Moreover, the presence of a 3D scaffolds might play a key role in the induction of cells differentiation, as previously described with bone substitute materials.[4]

Also worth noting is that Runx2 was down-regulated in mature osteoblasts expressing osteocalcin.[33] Osteocalcin (OC), as a late-stage marker of differentiation, is secreted into the medium of MSCs from day 8 and increased substantially until day 16. According to a former study,[21] DPSCs cultured on highly rough titanium surfaces expressed greater osteocalcin levels after 15 days, with respect to those challenged with acid-etched surfaces. Since, DPSCs express osteocalcin only during their differentiation into osteoblasts, we can assume that the increased osteocalcin expression detected in the supernatant of cells cultured on Test disks, in respect to cells grown on Controls, meant that more cells had switched toward the osteogenic lineage. The levels became almost similar after 21 days in DM, probably

because the differentiation in an osteogenic pattern was already occurred in both groups. Furthermore, the small difference in osteocalcin amounts between cells grown on the disks and cells alone observed in cells cultured in DM, confirmed an activation of differentiation process also in cells alone.

The positivity for alizarin red staining showed mineral deposition obtained from DPSCs cultured in presence of Test and Control disks. The calcification nodules were observable after 21 days of culture in DM demonstrating an earlier differentiation of DPSCs in an osteogenic pattern, probably due to the presence in the same plates of titanium disks. These results were in line with previous outcomes showing that alizarin red stained calcification nodules obtained from DPSCs cultured for 30 days on a flask surface in GM condition.[21] Although, the more pronounced calcification nodules in presence of disks, confirmed an earlier differentiation process related to the titanium.

CONCLUSIONS

Within the limitation of the present study, the obtained results demonstrated that miRNAs could be used as marker of osteogenic differentiation. Moreover, the presence of a titanium-treated surface, which presented a more uniform and widely spread roughness, would affect the stem cells differentiation, more significantly than the only presence of differentiation medium. Specifically, the evaluated ionized sandblasted and acid-etched surface (Test) seemed to markedly enhance the development of osteoblast cells.

Therefore, a faster osseointegration could be achieved in the presence of this particular type of implant surfaces, promising encouraging clinical outcomes.

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REFERENCES

1. Collin-Osdoby P. Role of vascular endothelial cells in bone biology. J Cell Biochem 1994;55:304–309.
2. Buckwalter JA, Glimcher MJ, Cooper RR, Recker R. Bone biology. I: Structure, blood supply, cells, matrix, and mineralization. Instr Course Lect 1996;45:371–386.
3. Strocchi R, Orsini G, Iezzi G, Scarano A, Rubini C, Pecora G, Piattelli A. Bone regeneration with calcium sulfate: evidence for increased angiogenesis in rabbits. J Oral Implantol 2002;28:273–278.
4. Mangano C, Paino F, d'Aquino R, De Rosa A, Iezzi G, Piattelli A, Laino L, Mitsiadis T, Desiderio V, Mangano F, Papaccio G, Tirino V. Human dental pulp stem cells hook into biocoral scaffold forming an engineered biocomplex. PLoS ONE 2011;6: e18721.
5. Barboni B, Mangano C, Valbonetti L, Marruchella G, Berardinelli P, Martelli A, Muttini A, Mauro A, Bedini R, Turriani M, Pecci R, Nardinocchi D, Zizzari VL, Tete` S, Piattelli A, Mattioli M. Synthetic bone substitute engineered with amniotic epithelial cells enhances bone regeneration after maxillary sinus augmentation. PLoS ONE 2013;8:e63256.
6. Gordeladze JO, Reseland JE, Duroux-Richard I, Apparailly F, Jorgensen C. From stem cells to bone: phenotype acquisition, stabilization, and tissue engineering in animal models. LAR J 2009; 51:42–61.
7. Shookhoff JM, Gallicano GI. The emerging role of microRNAs in adult stem cells. Turksen K, editor. Stem cell biology and regenerative medicine, vol 1, Humana Press: Springer; 2011. pp 57–94.
8. Fakhry M, Hamade E, Badran B, Buchet R, Magne D. Molecular mechanisms of mesenchymal stem cell differentiation towards osteoblasts. World J Stem Cells 2013;5:136–148.
9. Buser D. Titanium for dental applications (II): implants with roughened surfaces. In: Brunette DM, Tengvall P, Textor M, Thomsen P. Titanium in Medicine. Material Science, Surface Science, Engineering, Biological Responses and Medical Applications. Berlin: Springer; 2001. pp 875–888.
10. Shalabi MM, Gortemaker A, Van't Hof MA, Jansen JA, Creugers NH. Implant surface roughness and bone healing: A systematic review. J Dent Res 2006;85:496–500.
11. Sykaras N, Iacopino AM, Marker VA, Triplett RG, Woody RD. Implant materials, designs, and surface topographies: their effect on osseointegration. A literature review. Int J Oral Maxillofac Implants 2000; 15:675–690.
12. Shibli JA, Grassi S, de Figueiredo LC, Feres M, Marcantonio E Jr, Iezzi G, Piattelli A. Influence of implant surface topography on early osseointegration: A histological study in human jaws. J Biomed Mater Res B Appl Biomater 2007;80:377–385.
13. Schneirder GB, Zaharias R, Seabold D, Keller J, Stanford C. Differentiation of preosteoblasts is affected by implant surface microtopographies. J Biomed Mater Res A 2004;64:462–468.
14. Schneider GB, Perinpanayagam H, Clegg M, Zaharias R, Seabold D, Keller J, Stanford C. Implant surface roughness affect osteoblast gene expression. J Dent Res 2003;82:372–376.
15. Mangano F, Mangano C, Macchi A, Perrotti V, Iezzi G, Piattelli A. La topografia della superficie implantare nella moderna implantologia. Parte I: Superfici lisce e micro rugose. Italian Oral Surg 2010;9:201–214.
16. Gehrke SA, Zizzari VL, Iaculli F, Mortellaro C, Tetè S, Piattelli A. Relationship between the surface energy and the histologic results of different titanium surfaces. J Craniofac Surg 2014;25: 863–867.
17. Traini T, Caputi S, Gherlone E, Degidi M, Piattelli A. Fibrin clot extension on zirconia surface for dental implants: A quantitative in vitro study. Clin Implant Dent Relat Res 2014;16:718–727.
18. Gandolfi MG, Taddei P, Siboni F, Perrotti V, Iezzi G, Piattelli A, Prati C. Micro-topography and reactivity of implant surfaces: An in vitro study in simulated body fluid (SBF). Microsc Microanal 2015;21:190–203.
19. Perrotti V, Palmieri A, Pellati A, Degidi M, Ricci L, Piattelli A, Carinci F. Effect of titanium surface topographies on human bone marrow stem cells differentiation in vitro. Odontology 2013;101: 133–139.
20. Bojic S, Volarevic V, Ljujic B, Stojkovic M. Dental stem cells-characteristics and potential. Histol Histopathol 2014;29:699–706.
21. Mangano C, De Rosa A, Desiderio V, d'Aquino R, Piattelli A, De Francesco F, Tirino V, Mangano F, Papaccio G. The osteoblastic differentiation of dental pulp stem cells and bone formation on different titanium surface textures. Biomaterials 2010;31:3543–3551.
22. Amin Yavari S, van der Stok J, Chai YC, Wauthle R, Tahmasebi Birgani Z, Habibovic P, Mulier M, Schrooten J, Weinans H, Zadpoor AA. Bone regeneration performance of surface-treated porous titanium. Biomaterials 2014;35:6172–6181.
23. Tirino V, Paino F, d'Aquino R, Desiderio V, De Rosa A, Papaccio G. Methods for the identification, characterization and banking of human DPSCs: Current strategies and perspectives. Stem Cell Rev 2011;7:608–615.
24. Tirino V, Paino F, De Rosa A, Papaccio G. Identification, isolation, characterization, and banking of human dental pulp stem cells. Methods Mol Biol 2012;879:443–463.
25. d'Aquino R, Graziano A, Sampaolesi M, Laino G, Pirozzi G, De Rosa A, Papaccio G. Human postnatal dental pulp cells co-differentiate into osteoblasts and endotheliocytes: A pivotal

synergy leading to adult bone tissue formation. Cell Death Differ 2007; 14:1162–1171.
26. Zizzari VL, Marconi GD, De Colli M, Zara S, Zavan B, Salini V, Fontana A, Cataldi A, Piattelli A. In vitro behavior of primary human osteoblasts onto microrough titanium surface. Implant Dent 2015; 24:377–383.
27. La Rovere RM, Quattrocelli M, Pietrangelo T, Di Filippo ES, Maccatrozzo L, Cassano M, Mascarello F, Barthélémy I, Blot S, Sampaolesi M, Fulle S. Myogenic potential of canine craniofacial satellite cells. Front Aging Neurosci 2014;6:1–12.
28. Mancinelli R, Pietrangelo T, Burnstock G, Fano F, Fulle S. Transcriptional profile of GTP-mediated differentiation of C2C12 skeletal muscle cells. Purinergic Signal 2012;8:207–221.
29. Mancinelli R, La Rovere RM, Fulle S, Miscia S, Marchisio M, Pierdomenico L, Lanuti P, Procino G, Barbieri C, Svelto M, Fanò-Illic G, Pietrangelo T. Extracellular GTP is a potent water-transport regulator via aquaporin 5 plasma-membrane insertion in M1-CCD epithelial cortical collecting duct cells. Cell Physiol Biochem 2014; 33:731–746.
30. Klokkevold P, Nishimura RD, Adachi M, Caputo A. Osseointegration enhanced by chemical etching of the titanium surface. A torque removal study in the rabbit. Clin Oral Implant Res 1997;8: 442–447.
31. Olivares-Navarrete R, Hyzy SL, Haithcock DA, Cundiff CA, Schwartz Z, Boyan BD. Coordinated regulation of mesenchymal stem cell differentiation on microstructured titanium surfaces by endogenous bone morphogenetic proteins. Bone 2015;73:208–216.
32. Phimphilai M, Zhao Z, Boules H, Roca H, Franceschi RT. BMP signaling is required for RUNX2-dependent induction of the osteoblast phenotype. J Bone Miner Res 2006;21:637–646.
33. Liu TM, Lee EH. Transcriptional regulatory cascades in Runx2-dependent bone development. Tissue Eng Part B Rev 2013;19: 254–263.