EXPRESSION OF SP7, RUNX1, DLX5, AND CTNNB1 IN HUMAN MESENCHYMAL STEM CELLS CULTURED ON XENOGENEIC BONE SUBSTITUTE AS COMPARED WITH MACHINED TITANIUM.

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EXPRESSION OF SP7, RUNX1, DLX5, AND CTNNB1 IN HUMAN MESENCHYMAL STEM CELLS CULTURED ON XENOGENEIC BONE SUBSTITUTE AS COMPARED WITH MACHINED TITANIUM.
PURPOSE

The aim of this research was to investigate the gene expression profile of 4 transcription factors in human mesenchymal stem cells (hMSC) cultured with a xenogeneic bone substitute and a support of machined titanium.

MATERIAL AND METHODS

In vitro studies were performed on hMSC cells, which grew in contact with cortical porcine bone and machined titanium disks for 10 days. RNA quantification for genes DLX5, CTNNB1, RUNX1, and SP7 was assessed by quantitative real-time polymerase chain reaction. For cells supported by titanium, immunocytochemistry of osteocalcin (OC) was also performed.

RESULTS

In the osteoblast-induced cells (OIC), DLX5, CTNNB1, and RUNX1 were significantly upregulated (+2.38-, +3.51-, and +7.08-fold, respectively), whereas SP7 was downregulated (−26.32-fold). None of the genes seemed to be upregulated or downregulated by the corticocancellous porcine bone. In cells grown on titanium support, DLX5 and RUNX1 were respectively upregulated (+3.12-fold) and downregulated (−2.14-fold). For titanium support, the presence of both catenin beta-1 and OC was verified.

CONCLUSION

The 2 genes RUNX1 and SP7 resulted differently expressed in cells cultured on metallic supports if compared with the expression recorded for OIC. An induction of the osteogenic phenotype was observed when cells were cultured on machined titanium, but not on xenogeneic material. (Implant Dent 2014;23:1–9)

Key Words: bone substitutes, gene expression, transcription factor proteins, xenogeneic support.
Alveolar bone, after tooth extraction, has been observed generally to undergo negative bone remodeling and to exhibit a wide range of dimensional changes. Whatever the causes, be they trauma, inflammatory episode, or periodontal disease, the major clinical objectives in dentistry have been the prevention or, at least, the minimization of the alveolar bone loss due to the extreme difficulty of restoring the lost bone volume through augmentation procedures, and to guarantee their successful outcomes. A functional rehabilitation of an edentulous area of a patient could be performed through a fixed prosthesis supported by an osseointegrated dental implant; implants could be successfully inserted immediately after several possible augmentation techniques, including the use of growth and differentiation factors, particulate and block grafting materials, distraction osteogenesis, and guided bone regeneration.

An increased knowledge of genes involved in alveolar bone resorption and remodeling has allowed new insight regarding promising therapeutic approaches. Identifying and understanding the dynamics of these important osteogenic environmental cues in alveolar socket healing and osseointegration processes are of critical importance. Greater knowledge about factors expressed during bone repair could serve as a foundation for novel therapeutic alternatives, addressing clinically challenging situations that often compromise the proper restoration of the bone’s function and structure. The biology of bone defect healing and bone substitute integration has been somewhat investigated by several studies; however, most of these focused on histological aspects of the healing process, and only a few tried to investigate gene expression. As regards dental implant osseointegration, only in longer bones has the gene expression profile of bone-titanium interaction been deeply investigated using different approaches. Furthermore, although the formation of alveolar bone is more easily accomplished within a sterile environment, such as that obtained for the placement of 2-stage implants in oral rehabilitation, integration of the newly formed bone over a surface of titanium fixtures carries an additional challenge that relates to the physicochemical characteristics of the implant surface. Little is known about how bone grafting materials and titanium dental implant surfaces modulate the nature of intracellular events in the hard tissue forming cells.

So far, at our present state of knowledge, no studies have shown the in vitro interaction of human marrow stem cells with xenogeneic bone, and a scant number of in vitro investigations have examined the path of intracellular events triggered by the interaction with titanium dental implant surfaces. Indeed, in a large part of these studies, a greater effort to validate the gene expression by microarrays was performed, but in only a few analyses, a quantitative validation of deregulated messenger RNAs (mRNAs) AU6 was employed.

The use of real-time quantitative polymerase chain reaction (qRT-PCR) technology, the most powerful tool for quantitative nucleic acids analysis, allowed the evaluation of the gene expression levels for several conditions.

In recent years, search/statistic algorithms have permitted the use of our preexisting knowledge regarding gene and protein interactions for investigating the genes involved in osseous remodeling; among the genes playing a role in bone volume augmentation, a number of them seemed to modulate bone formation, acting on the differentiation and maturation of the osteogenic cells, and on bone matrix formation: 3 of the transcription factors affecting these steps (RUNX1, DLX5, and SP7), plus the growth factor CTNNB1, were investigated. The expressions of the 3 Runx-related RUNX genes (RUNX1, RUNX2, and RUNX3) were described during the development of teeth and other craniofacial tissues; in summary, Runx1 and Runx2 may play roles during skeletal development, the former having the primary function of mediating early events of endochondral and incipient intramembranous bone formation, whereas Runx2 appeared as a potent inducer for the differentiation of chondrocytes and osteoblasts, mainly in the late stages. DLX5 gene encodes a bone-specific transcription factor, a member of the distalless homeobox family. Such molecules are sequence-specific DNA-binding proteins. All DLX genes are supposed to play a very important role in chondrogenesis and/or osteogenesis. In particular, DLX5 is expressed already in the very early stages of bone formation, and it is conceivable that this gene acts as a leading factor in controlling this process. Osterix (Oxs or Sp7), a zinc finger transcription factor, is specifically expressed by osteoblasts during bone development; its inactivation causes a lack of bone mineralization. The CTNNB1 encoded protein embeds the actin cytoskeleton and may be responsible for conveying the contact inhibition signal that causes cells to stop dividing. b-catenin is permitted to build up in the cytosol and can be subsequently moved into the nucleus to perform a variety of functions.

The primary aim of this article was to compare the gene expressions of human mesenchymal stem cells (hMSCs) cultured in the presence of 2 different supports, one an organic bone substitute and the other a machined titanium surface, with the gene expression of osteoblasts through a qRT-PCR analysis of 4 different genes (RUNX1, CTNNB1, SP7, and DLX5).
MATERIALS AND METHODS

CELL CULTURE

The biomaterial testing was performed through commercial hMSC (supplied by LCG Standards, LONZA, Milan, Italy). All other reagents were commercial products of analytical grade. Cells were cultivated as follows; in brief, hMSC were seeded in an expansion medium in 25-cm² plastic tissue culture flasks (Primaria Tissue Culture Flask; Becton Dickinson, Franklin Lakes, NJ) at 37°C and in a humidified atmosphere containing 10% of CO₂. The medium was replaced every 3 days, until cell colonies were identified. Standard expansion medium (Invitrogen Corporation, New York, NY) was supplemented with 10% of fetal calf serum, L-glutamine (2 mM), streptomycin (100mg/mL), and penicillin (100 U/mL).

1. CPB group: hMSC cocultivated in the presence of the organic bone substitute support for 10 days in the standard medium (Fig. 1, B).

2. MTD group: hMSC cocultivated in the presence of the titanium surface support for 10 days in the standard medium (Fig. 1, C–F).

3. Osteoblast-induced cells (OIC, positive control group): OIC from hMSC, treated for 10 days with modified osteogenic medium containing 10 nM Dex (Sigma Chemical Co., St. Louis, MO), 10 mM β-glycerophosphate (Sigma Chemical Co.), and 50 µM ascorbate-2-phosphate (Sigma Chemical Co.).

4. Negative control (NC) group: untreated hMSC, cultured without any support or additional supplementation of the standard medium.

EXPERIMENTAL DESIGN

Modification induced by support on hMSC was tested for 2 different types of material:
• An organic bone substitute consisted of cortical porcine bone (CPB) (Lamina; Osteobiol-Tecnoss, Coazze, Italy) (Fig. 1, A and B)
• A titanium surface consisted of Machined Titanium in the form of Disks (MTD) (Biotec, Vicenza, Italy) (Fig. 1, C–F).

Cells were seeded on 2 supports at about 40,000 cells per square centimeter, in a medium containing either CPB or MTD support. Cells were cultivated in 6 multiwell plates (Corning; Becton and Dickinson), and for each group, all experiments were done twice in triplicate. The medium was changed 3 times per week during the culture period. On the basis of the physical supports and mediums employed, the following experimental groups were introduced:

1. CPB group: hMSC cocultivated in the presence of the organic bone substitute support for 10 days in the standard medium (Fig. 1, B).

2. MTD group: hMSC cocultivated in the presence of the titanium surface support for 10 days in the standard medium (Fig. 1, C–F).

3. Osteoblast-induced cells (OIC, positive control group): OIC from hMSC, treated for 10 days with modified osteogenic medium containing 10 nM Dex (Sigma Chemical Co., St. Louis, MO), 10 mM β-glycerophosphate (Sigma Chemical Co.), and 50 µM ascorbate-2-phosphate (Sigma Chemical Co.).

4. Negative control (NC) group: untreated hMSC, cultured without any support or additional supplementation of the standard medium.
RNA ISOLATION AND SINGLE QRT-PCR

By means of a dedicated RNA mini-kit (Qiagen, San Diego, CA) for each group, the RNA was purified, and after appropriate elution, the RNA yield was obtained measuring the absorbance 260 to 280 nm ratio through spectrophotometric assay, where as RNA integrity was assessed by denaturing agarose gel electrophoresis.

Sets of primers (forward and reverse) and probes for all transcription factors (runt-related transcription factor 1, catenin beta-1, transcription factor Sp7, homeobox protein DLX5) and endogenous control GAPDH (glyceraldehyde-3-phosphate dehydrogenase), 36 obtained through a specific software (Primer Express Software; Applied Biosystems, Foster City, CA) for RUNX1, CTNNB1, SP7, and DLX5 genes, were designed on the basis of the gene sequence obtained from web-available databases, specifically the official Human Genome Organization Gene Nomenclature Committee, or HGNC (available at http://www.genenames.org/), and the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING: available at http://string-db.org/).

Total RNA (0.5 µg) was reverse transcribed into complementary DNA (cDNA) by incubation at 37°C for 1 hour using 0.8 mM of deoxynucleotide mix (Sigma Chemical Co.), 200 U of Moloney murine leukemia virus reverse transcriptase (MMLV-RT; Sigma Chemical Co.), 40 units of RNAse inhibitor (Invitrogen Ltd., Paisley, United Kingdom), and 0.05 mg/µL random primers in a total volume of 50 µL using a dedicated system (GeneAmp PCR system 9700; Applied Biosystems).

The resulting cDNA was diluted in 200 µL and then amplified by qRTPCR with a sequence detection system (Applied Biosystems 7900HT; Applied Biosystems).

The qRT-PCR reactions were performed in triplicate using 5 µL of cDNA, 2.5 µL of the specific probe, and the forward and reverse specific primers, in concentrations optimized in preliminary reactions, in a total volume of 25 µL.

All the amplifications were carried out with normalization of gene expression against the GAPDH control reagent and the quantification of gene expression expressed as the fold change being performed using the relative quantitation method ∆∆CT calculation, in which the (n-fold) amount of target was given as $2^{-\Delta\Delta CT}$, and for which an optimal doubling of the targetDNA during each performed RT-PCR cycle is assumed.

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An ICC analysis of OC was performed to evaluate the osseogenic lineage differentiation for the titanium group. For the present experiment, 105 cells were seeded in 4 wells chamber slides support (Becton and Dickinson) (Fig. 1). After the period of treatment, the cells were fixed using a 70% of ethanol solution and stained with the specific monoclonal mouse antiosteocalcin antibody (ab13418; Abnova, Jhongli City, Taiwan) (dilution 1:200).

The antibody revelation was performed using HRP Detection systems (Cell Marque, Rocklin, CA). The nuclei of cells were stained with 80% of hematoxylin solution.Weevaluated the presence of a specific marker related to osseogenic lineage, such as OC, by the presence of a brown color deriving from a positive reaction of peroxidase. The group having cells cultured without any support and osteogenic medium was employed as a NC group, also for ICC analysis; to assess the specificity of immunoreaction of OC, the solution of primary antibody of OC was replaced with phosphate-buffered saline solution (PBS13). The IF intensities were evaluated for cells in the NC group and for cells cocultivated with the machined titanium surface. All stained samples were acquired and evaluated by D-Sight system (Menarini, Florence, Italy). Original magnifications (340 and 3100) were employed for IF data acquisition. Ten high-density fields for each experiment were analyzed.
Table 1. Results of qRT-PCR of the Expression of 4 Genes, DLX5, CTNNB1, RUNX1, and SP7, Obtained in the Analysis of the 3 Groups, CPB, MTD, and OIC

<table>
<thead>
<tr>
<th>Genes</th>
<th>DLX5</th>
<th>CTNNB1</th>
<th>RUNX1</th>
<th>SP7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cortical Porcine Bone Group</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ct&lt;sub&gt;tar&lt;/sub&gt;/soi/Ct&lt;sub&gt;ref&lt;/sub&gt;/soi</td>
<td>1.141 ± 0.041</td>
<td>1.137 ± 0.052</td>
<td>1.163 ± 0.049</td>
<td>1.251 ± 0.063</td>
</tr>
<tr>
<td>∆∆Ct</td>
<td>0.287 ± 1.179</td>
<td>-0.128 ± 1.073</td>
<td>-0.427 ± 2.385</td>
<td>0.493 ± 1.5054</td>
</tr>
<tr>
<td>2&lt;sup&gt;−∆∆Ct&lt;/sup&gt;</td>
<td>1.028 ± 0.610</td>
<td>1.397 ± 1.107</td>
<td>3.135 ± 3.468</td>
<td>1.014 ± 0.741</td>
</tr>
<tr>
<td>n-fold</td>
<td>+1.03</td>
<td>+1.40</td>
<td>+3.14</td>
<td>+1.01</td>
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</table>

<table>
<thead>
<tr>
<th>Genes</th>
<th>DLX5</th>
<th>CTNNB1</th>
<th>RUNX1</th>
<th>SP7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Machined Titanium Disk Group</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ct&lt;sub&gt;tar&lt;/sub&gt;/soi/Ct&lt;sub&gt;ref&lt;/sub&gt;/soi</td>
<td>1.069 ± 0.019</td>
<td>1.129 ± 0.095</td>
<td>1.232 ± 0.049</td>
<td>1.238 ± 0.054</td>
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<tr>
<td>∆∆Ct</td>
<td>-1.517 ± 0.670</td>
<td>-0.268 ± 2.389</td>
<td>1.387 ± 1.096</td>
<td>0.347 ± 1.216</td>
</tr>
<tr>
<td>2&lt;sup&gt;−∆∆Ct&lt;/sup&gt;</td>
<td>3.120 ± 1.371</td>
<td>2.506 ± 2.194</td>
<td>0.468 ± 0.271</td>
<td>1.080 ± 0.963</td>
</tr>
<tr>
<td>n-fold</td>
<td>+3.12</td>
<td>+2.51</td>
<td>-2.14</td>
<td>+1.08</td>
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</table>

<table>
<thead>
<tr>
<th>Genes</th>
<th>DLX5</th>
<th>CTNNB1</th>
<th>RUNX1</th>
<th>SP7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Osteoblast - Induced Cell</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ct&lt;sub&gt;tar&lt;/sub&gt;/soi/Ct&lt;sub&gt;ref&lt;/sub&gt;/soi</td>
<td>1.090 ± 0.008</td>
<td>1.074 ± 0.015</td>
<td>1.074 ± 0.015</td>
<td>1.074 ± 0.015</td>
</tr>
<tr>
<td>∆∆Ct</td>
<td>-1.228 ± 0.272</td>
<td>-1.795 ± 0.226</td>
<td>-2.822 ± 0.101</td>
<td>4.760 ± 0.405</td>
</tr>
<tr>
<td>2&lt;sup&gt;−∆∆Ct&lt;/sup&gt;</td>
<td>2.375 ± 0.423</td>
<td>3.507 ± 0.589</td>
<td>7.084 ± 0.487</td>
<td>0.038 ± 0.010</td>
</tr>
</tbody>
</table>
| n-fold                 | +2.38           | +3.51           | +7.08           | -26.32         

**STATISTICAL ANALYSES**

All sample-related data were entered into a database (Access; Microsoft Corp., Redmond, WA), allowing calculations to be performed automatically.

Descriptive statistical analyses were performed using a statistical tools package (Statistics Toolbox, Matlab 7.11; The MathWorks, Natick, MA). In all Figures, data are depicted through box and whiskers plot; the box has lines at the lower, median, and upper quartile values, whereas the whiskers are lines extending from each end of the box to show the extent of the rest of the data. All measurements in the text and Tables are described as mean and SDs: m ± SD. A normal distribution of data resulting from the qRT-PCR analysis for all groups investigated was carried out, but not confirmed, through the Lilliefors test; because the measurements acquired were not normally distributed, multiple comparisons among the unmatched groups considered were determined by the Kruskal-Wallis test with Bonferroni correction, after which pair-comparisons were performed. Wilcoxon rank sum tests were used to assess pair-wise statistical differences between the groups, whereas upregulation and downregulation were verified by the Wilcoxon 2-sided signed rank test.

For IF analysis, the data were subjected to Pearson x2 analysis with YATES correction, when necessary.

The level of statistical significance was set at 0.05 for all analyses.
RESULTS

FEASIBILITY OF CELL CULTURES
Vitality and integrity of the cells after the culture on the 2 types of support employed were verified: for the former, cells were stained using CytoRed (Fig. 1, G), whereas the latter, that is, the integrity of the cells, was observed by contrast-phase light microscopy (Fig. 1, H).

REGULATION OF THE TRANSCRIPTION FACTORS
The results of the RT-PCR showed that statistical differences were recorded among the groups presented. Normalized qRT-PCR data throughout the reference GAPDH gene of the expressions of the target genes (DLX5, CTNNB1, RUNX1, and SP7) are shown FT12 in Table 1 and Figure 2. Comparisons among the 4 groups (NC, CPB, MTD, and OIC), performed by nonparametric 1-way analysis of variance, were significant for 3 genes, DLX5, RUNX1, and SP7 (with P as 0.0003, 0.0027, and 0.0045, respectively), revealing different patterns of expression in each of the presented groups.

The results of pair-comparison tests for the direct ratio GENE/GAPDH performed for all genes and groups are shown in Figure 2. For the 2 genes DLX5 and RUNX1, most of the pair-comparisons showed significant differences, except between the NC and CPB groups, and, only for the RUNX1 gene, between the OIC and MTD groups.

Data regarding CYNNB1 verified the presence of just 1 significant difference (hMSC vs OIC). For the last gene, SP7, the data regarding the OICs were different at a statistically significant level from all the other groups, whereas no other comparisons seemed significant. Obtained values of DDCt and respective normalized mean numberfold of downregulation or upregulation are presented in Table 1.

Again the Kruskal-Wallis tests were significant for all genes except for the CTNNB1, with P as 0.0062, 0.0138, and 0.0033 for the expression of the genes DLX5, RUNX1, and SP7, respectively.

As is shown in Figure 2, for data regarding the gene expression of DLX5, significant differences between the CPB group and both the MTD and OIC groups were observed; moreover, in these latter 2 groups, DLX5 presented an upregulation related to that of the NC. The CTNNB1 gene expression analysis revealed just 1 significant difference, between the CPB and that having the OICs, in which CTNNB1 was also significantly upregulated. RUNX1 genes, expressed in cells grown on titanium support and in the OICs, were significantly downregulated and upregulated, respectively, and also a significant difference was found between the expression data of the 2 groups.

The SP7 gene resulted as significantly downregulated in the OIC group, and the n-fold of downregulation was seen to be significantly different in respect to those of both supports (lamina and machined titanium).
Fig. 2. RNA expression of genes, DLX5, CTNNB1, RUNX1 and SP7 in a comparison among the 4 groups untreated hMSC NC, CPB, MTD, and OIC, detected by qRT-PCR: direct ratio between genes and GAPDH expression and DDCT analysis in logarithmic scale. Significant differences: **P < 0.005; *0.005 < P < 0.05; °downregulated genes of cells grown on supports in respect to that of the NC group (untreated AU9 hMSC); ^upregulated genes of cells grown on supports in respect to that of the NC group (untreated hMSC).

Fig. 3. Immunocytochemistry of OC; NC test (A). The antibody was replaced with PBS 31solution. Weak staining (B), middle staining (C), and strong staining (D) of OC in hMSCs. Magnification 3100. Immunofluorescence of beta-catenin; 3 different scores of IF of beta-catenin: score 1 (E), score 2 (F), and score 3 (G).
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The ICC of OC was tested in hMSC treated with the titanium support employed. In the experiments, the Fig. 2.

RNA expression of genes, DLX5, CTNNB1, RUNX1, and SP7 in a comparison among the 4 groups untreated hMSC NC, CPB, MTD, and OIC, detected by qRT-PCR: direct ratio between genes and GAPDH expression and DDCT analysis in logarithmic scale. Significant differences: **P , 0.005; *P , 0.05; °downregulated genes of cells grown on supports in respect to that of the NC group (untreated AU9 hMSC); ^upregulated genes of cells grown on supports in respect to that of the NC group (untreated hMSC).

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Following 4 different degrees of brown F3 staining were found: absence (Fig. 3, A), weak (Fig. 3, B), middle (Fig. 3, C), and strong (Fig. 3, D). To assess the specificities of OC staining, a comparison was performed with respect to the NC. All the machined titanium treated cells showed a positive OC staining ranging from weak to strong degree (Fig. 3, C and D).

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A higher protein expression of beta-catenin in hMSC cells treated with machined titanium support compared with the NC cells was verified T2 (Table 2). About 100 cells in both the NC and the treated MT cells were counted.

The samples treated with MT support showed a number of beta-catenin “score 3” cells with respect to the control of 30.43% and 1.82%, respectively. A score 3 value was assigned to those cells showing a violet staining deriving from a merging between the red color (beta-catenin) and the blue color (DAPI), associated with cytoplasm and nucleus, respectively (Fig. 3, E).

The intensities of scores 1 and 2 are illustrated in Figures 3, F and G. The ranked scores of the 2 groups (machined titanium support and NC cells) showed significant statistical differences (P< 0.0001).

### Table 2. Immunofluorescence of Beta-Catenin, Recorded Total Number and Percent of Cells Ranked on IF Scoring and Type of Treatment, With Results of χ² Analysis

<table>
<thead>
<tr>
<th>Type of Treatment</th>
<th>No. of Cells</th>
<th>Score</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Px²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>110</td>
<td></td>
<td>24</td>
<td>57</td>
<td>27</td>
<td>2</td>
<td>&lt;0.0001; df = 3; YATES correction applied</td>
</tr>
<tr>
<td></td>
<td>100.00%</td>
<td></td>
<td>21.82%</td>
<td>51.82%</td>
<td>24.55%</td>
<td>1.82%</td>
<td></td>
</tr>
<tr>
<td>MTD</td>
<td>92</td>
<td></td>
<td>4</td>
<td>29</td>
<td>31</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100.00%</td>
<td></td>
<td>4.35%</td>
<td>31.52%</td>
<td>33.70%</td>
<td>30.43%</td>
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</tbody>
</table>
DISCUSSION

Clinicians have attempted to investigate the biologic influences of several types of support on bone healing after a surgical procedure; each support was supposed to have its own specific characteristics.

Positive or negative outcomes regarding the augmentation of the alveolar bone depend primarily on the physical variables and chemical nature of the grafting material employed. Xenogeneic biomaterials are interesting as bone substitutes because they have several characteristics and a morphology that are similar to human bone. An innovative xenogeneic grafting biomaterial, consisting of porcine bone, has been clinically employed as a bone substitute: owing to its advantageous properties, porcine bone has been successfully integrated into daily clinical practice.5,13,15,16 Dental implants are predictably successful when proper guidelines are followed.

The failure rate is very low, and, where failure occurs, it is most likely due to infection or osseointegration loosening. Indeed, the most frequent cause of early implant failure is insufficient bone formation immediately after fixture insertion. This, therefore, represents a field in which additional research is needed, and this issue is becoming ever more critical because clinicians are pressing more and more for accelerating healing times.

As a consequence, most efforts to modify implant surface features have been directed toward reducing the time interval between implant placement and loading. Acquiring new insight into the interaction between the biomaterial implanted and the bone of the recipient site seems to be crucial to developing new implant surface chemistries and morphologies that will enhance adhesion and integration of the fixture to the surrounding tissue.39

Since the succession of intracellular events triggered by the interaction with titanium dental implant surfaces and bone substitutes is still unclear, despite decades of encouraging clinical outcomes, in this article the gene expression of cells cultured with such compounds was compared with that of human osteoblasts.35

The process of osteogenesis depends on complex regulatory networks, involving molecular signals and transcription factors. Text-mining and gene-deletion studies have allowed the identification of transcription factors required for the specification and/or differentiation of the osteoblastic lineage. These include, for example, runt-related transcription factor family (RUNX), catenin beta-1, zinc finger transcription factors (SP), and distal-less homeobox family (DLX).36,27 In the cells cultured on the machined metallic support, the RUNX1 gene seemed downregulated (~2.14-fold), whereas the genes in the cells which were chemically induced toward osteoblastic lineage seemed upregulated of +7.08-fold; the values of the expression also presented a difference at a statistically significant level between MTD and OIC groups, attesting that hMSCs in contact with titanium support modified the expression of RUNX1 in a diametrically opposite direction in respect to the induced osteoblasts. RUNX factors also Table 2.

Immunofluorescence of Beta-Catenin: Recorded Total Number and Percent of Cells Ranked on IF Scoring and Type of Treatment, With Results of x2 Analysis Type of Treatment No. of Cells Score 0 1 2 3 Px2 Control 110 21.82% 51.82% 24.55% 1.82% MTD 92 4 29 31 28 100.00% 4.35% 31.52% 33.70% 30.43% Fig. 3. Immunocytochemistry of OC; NC test (A). The antibody was replaced with PBS 31 solution. Weak staining (B), middle staining (C), and strong staining (D) of OC in hMSCs. Magnification 3100. Immunofluorescence of beta-catenin; 3 different scores of IF of betacatenin: score 1 (E), score 2 (F), and score 3 (G). 3 HUMAN MESENCHYMAL STEM CELLS CULTURED BARONE ET AL arrange signaling responses from the extracellular matrix by forming coregulatory complexes, for example with Smad proteins, mediators of bone morphogenetic protein (BMP)/transforming growth factor-b signaling.40 Runx2 overexpression inhibits osteoblast maturation and decreases bonematrix protein gene expression. Thus, RUNX2 commits pluripotent mesenchymal cells to the osteoblast lineage, triggers the expression of major bone matrix protein genes, and keeps the osteoblasts in an immature stage.41,42

Considering only significant results for both the direct ratio analysis and the ∆∆CT calculation, DLX5 resulted as upregulated of +3.12 and +2.38, respectively, for titanium support and the induced osteoblasts; these values of upregulation resulted as being significantly different compared with that of the cells grown on the bone lamina. The present data showed that, at least as regards a follow-up of 10 days, the expression of the gene was modified, but only for the bone support, whereas the titanium support seemed to stimulate cells similarly to chemical osteoblast induction. Several recent in vitro studies have pointed out the fact that many early and late markers of osteoblastic differentiation are potential direct targets of Dlx5, thus suggesting that it might take part in multiple stages of chondrogenesis and osteogenesis by controlling the expression of bone-related genes.31

Some other studies have indicated that DLX5 is not only an activator of osteoblast proliferation and early differentiation but can also affect later stages of osteogenesis. Alkaline phosphatase and OCa re known to be responsive to DLX5.43,44 As regards the SP7 gene, it seemed downregu-
lated in the induced osteoblasts by $-26.32$-fold, and this
downregulation was significantly different in respect to
cells grown on both the bone lamina and the machined
titanium support, indicating that the present genes were
activated by the 2 supports employed. Osterix(Osx orSp7)
has the ability to coordinately modulate Runx2 and Dlx5
proteins at levels appropriate for optimal osteoblast dif-
ferentiation and function.$^{32,33}$

However, although osterix is necessary, it is not suffi-
cient$^{45}$: for endochondral ossification, physical and func-
tional interaction between Osterix and Runx2 were also
required.$^{46}$

The present data showed that the CTNNB1 seemed sig-
ificantly upregulated (+3.51-fold) only in the induced
osteoblasts.

Even if an upregulation of cells cultured on the metal-
lic support was not statistically verified, the presence of
b-catenin was confirmed by IF analyses, where the cells
cultured on the titanium surface showed higher scores of
IF; these results indicate that b-catenin protein was both
modulated and expressed after treatment.

The Wnt/b-catenin pathway modulates a number of bio-
logical events during embryonic development, including
bone formation. The Wnt/ b-catenin signaling pathway is
triggered during fracture repair, and deregulation of this
pathway affects the physiological bone-healing response.
In early pluripotent mesenchymal stem cells, Wnt/ b-cat-
enin signaling needs to be precisely regulated to promote
the differentiation of osteoblasts. Once mesenchymal
stem cells are engaged to the osteoblast lineage, acti-
vation of Wnt/b-catenin signaling enhances bone forma-
tion.$^{34}$

This study indicates that, notwithstanding the limited in-
formation obtained from the present chosen genes, when
mesenchymal stem cells have grown on titanium and
porcine bone, or when they were induced toward osteo-
blastic phenotype, significantly different patterns of expres-
sion result; the CPB seemed not to influence the gene
expression: none of the investigated genes were either
upregulated or downregulated.

Moreover, the results would suggest that the behavior of
hMSCs cultured on the bone materials used were not dif-
ferent from that recorded for the untreated hMSCs (the
NC).

These data were interesting for the clinical aspects of the
bone regeneration techniques involving this xenogeneic
material used as simple scaffold.

In 2 short-term studies, the xenogeneic corticocancellous
porcine bone was successfully used as a grafting material
both for sinus elevation and for ridge preservation after
tooth extraction$^{47,48}$; moreover, histologic and biomolecu-
lar analysis seemed to confirm precisely the biocompat-
ibility and osteoconductivity of the porcine material in
alveolar bone grafting.$^{49}$ The hMSCs cultured on titanium
support certainly did not behave as the OICs, in which
all genes seemed upregulated or downregulated; how-
ever, 2 of the transcription factors seemed significantly
modulated.

Results in the literature have suggested that the nature of
the metallic support had a great influence on bone heal-
ing after dental implant insertion. Several studies have
indicated that implant surface topography affects osteo-
blast gene expression$^{50,51}$; specifically, a dual acid-etched
titanium surface seemed to stimulate the expression of
markers of osteoblastic phenotype more than amachined
titanium surface.$^{52}$

Data obtained by Donos et al.$^{24}$ attested that AU7 hMSCs
cultured on rough surfaces underwent a decrease in cell
number early in culture, yet simultaneously expressed
higher levels of the osteogenic markers, although the re-
searchers did find that the mRNA expression decreased
after a long period of smooth titanium treatment (14 and
21 days).$^{25}$

Additionally, the "commitment of lineage" in the present
treated cells by ICC analyses was demonstrated. In a re-
cent study, the authors showed that a polished titanium
surface affected the quantity of OC in the medium after 7
days, in respect to its control.$^{25}$

As regards bone grafting materials, additional experi-
ments should clarify if such compounds are capable of
promoting the production of appreciable amounts of
BMPs, as well as vascular endothelial growth factor and
specific bone proteins in the subsequent phases of bone
development, other than osteoblast differentiation and
maturation of hMSC in the early stage of osteogenesis.

Therefore, the achievement of a complete osseointe-
gration has to be demonstrated. Moreover, as concerns
titanium implant surfaces, additional study on rougher
surfaces should clarify if the activation of genes may in-
duce the production of the relative protein and/or show
a different behavior.
CONCLUSION

In the comparisons between the group of induced osteoblasts and that of the cells cultured on the titanium support, similar behaviors were obtained for the upregulation of the gene DLX5.

The 7 genes RUNX1 and SP7 resulted differently expressed in cells cultured on metallic supports if compared with their expression recorded for induced osteoblasts: for the former gene, the titanium seemed to induce a downregulation, whereas a downregulation seemed evident, for the latter gene, in the induced osteoblasts. CPB seemed to have minimal impact on gene expression. An induction of the osteogenic phenotype was observed when cells were cultured on machined titanium.

REFERENCES


DISCLOSURE

The authors claim to have no financial interest, direct or indirect, in any entity that is commercially related to the products mentioned in this article.