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# Genetical stability and osteogenic ability of mesenchimal stem cells on demineralized bone matrices

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## ABSTRACT

**Aim** Tissue engineering is a rapidly expanding field with regard to the use of biomaterials and stem cells in the orthopedic surgery. Many experimental studies have been done to understand the best characteristics of cells, materials and laboratory methods for safe clinical applications. The aim of this study was to compare the ability of 2 different human demineralized bone matrices (DBMs), the one enriched and the other not enriched with hyaluronic acid, to stimulate in vitro the proliferation and the osteogenic differentiation of human adipose-derived stem cells (ADSCs) seeded onto an osteoconductive scaffold.

**Materials and Methods** ADSCs were isolated, by enzymatic digestion, from abdominal adipose tissue of 5 patients undergoing cosmetic lipoaspiration surgery. ADSCs were then seeded onto a 3D scaffold in the presence of the two different osteoinductive matrices of human demineralized bone and evaluated for proliferation and osteogenic differentiation. The safety of the methods was verified using array-Comparative Genomic Hybridization (array-CGH).

**Results** ADSCs were able to differentiate in osteogenic sense. Both DBMs showed the ability to induce osteogenic differentiation of the cells.

**Conclusion** array-CGH showed no changes at genome level, thus confirming the safety of materials and methods.

KEYWORDS Adipose-derived stem cells; Array-CGH; Demineralized bone matrices; Osteogenesis; Tissue engineering.

## INTRODUCTION

Bone regeneration is a multifactorial process in which osteoconduction, osteoinduction, and osteogenesis play a key role (1). Osteoconduction refers to 3D materials (scaffolds), which favour adhesion, migration and proliferation of the cells able to directly synthesize new bone (osteogenesis) (2). Osteoinductive factors, mainly bone morphogenetic proteins (BMPs), growth factors (GFs) and demineralized bone matrices (DBMs), guarantee the recruitment of osteoprogenitor cells and the differentiation of stem cells in osteoblasts. Autologous bone graft is considered the gold standard for bone repair and regeneration because of its osteogenic, osteoinductive, and osteoconductive properties related to the presence of cells from bone and bone marrow, proteins in the matrix, and a 3D structure that provides a viable scaffold for the growth of the surrounding bone (3, 4). However, the use of autologous bone graft presents several limits, mainly dependent on the low amount of harvested bone, increase in operative time, pain, infection, hematoma, and blood loss (5-11).

A recent approach able to stimulate the regeneration of fully functioning bone tissue is based on the use of mesenchymal stem cells (MSCs) derived from adipose tissue cultured on an osteoconductive scaffold in presence of DBMs (12, 13).

Ceramics, mainly hydroxyapatite (HA), are widely used as scaffolds due to their physical and chemical similarity to the mineralized cancellous bone which guarantees the osteoconductivity (14). HA has a 3D porous structure determined by a network of crystalline mineral delimiting numerous interconnected pores of various sizes. Pores smaller than 10 µm allow the movement of extracellular fluids, while the ones larger than 50 µm support the cells growth and proliferation (15-18).

Ceramics are considered an excellent vehicle for osteoinductive GFs and osteogenic cells, since they are biocompatible, not toxic, and they do not cause immunological responses (19).

DBMs are produced by removal of minerals from cadaver cortical bone and are considered allografts (20). They consist of collagen (mainly type I but also type IV and type X), non-collagenous proteins, a minimum percentage of $Ca_3(PO_4)_2$ (1-6%), and importantly BMPs and GFs essential

for bone growth and regeneration. The use of DBMs has some advantages, such as absence of rejection and foreign body reaction because antigenic surface structure of bone is destroyed during demineralization. DBMs are commercially available in various forms, including wafers, modeling paste, adhesive strips, and injectable compounds. Moreover, they can be molded even intraoperatively, they are intraoperative washing resistant, and they allow rapid vascularization after application. Problems related to the use of DBMs consist in the potential transmissibility of viruses (which has never been reported). DBMs are very expensive and they have different repair capability among different batches of product, due to variability in the quality of donor bone (20).

Recently, the interest towards the use of human adipose-derived stem cells (ADSCs) in bone repair and regeneration is increasing. ADSCs are isolated from the stromal-vascular fraction of adipose tissue obtained during abdominoplastic surgical procedures, or during subcutaneous liposuction (21). ADSCs share many properties with bone marrow stem cells (BMSCs), especially the same differentiation potential. Notably, ADSCs possess many advantages associated to the easy harvesting, security, abundance and higher yield (high number of cells available from a single patient) (22-24). Moreover, ADSCs are easily cultivated and expandable *in vitro* and have greater resistance in culture compared to BMSCs. ADSCs fulfill the International Society for Cellular Therapy (ISCT) criteria as they are plastic-adherent, express CD73, CD90 and CD105, lack CD34 antigens, and have a tri-lineage mesenchymal differentiation (25).

Many studies have shown possible stem cells involvement in the process of oncogenesis in which malignant cells have the ability to proliferate and divide indefinitely without control (26, 27). It has been hypothesized that some pathways for self-renewal, finely regulated in the healthy stem cells, are altered in neoplastic diseases (28-34). Therefore, array-Comparative Genomic Hybridization (array-CGH) analysis was performed in order to evaluate the genetic stability of ADSCs-based cultures. Array-CGH is a new technique that allows to identify copy number imbalances (gain and loss) of genetic material which are the basis of many disorders and diseases including cancer. Infact, array-CGH has many advantages with respect to classic cytogenetic methods, mainly due to higher resolution, higher reproducibility, and rapid and precise mapping of the whole genome (35).

The aim of the study was to evaluate the ability of 2 DBMs (TBM® e DBX®) to stimulate in vitro the proliferation and the osteogenic differentiation of mesenchymal stem cells isolated from adipose tissue. The safety of the methods was also investigated by means of molecular genetic tests.

## MATERIALS AND METHODS

### Scaffold

Orthoss® Blocks 1x1x2 cm (Geistlich Pharma AG, Wolhusen, Switzerland) were used in this study. Orthoss® is a natural carbonated HA of bovine origin. It is a highly osteoconductive material because of its particular structure, very similar to human cancellous bone, with interconnected macropores (100-300 µm), micropores, and nanopores (10-20 µm), resulting in a higher inner surface and excellent hydrophilic property (36).

### Demineralized Bone Matrices (DBMs)

Accell TBM® (Integra LifeScience Corporation, Irvine, CA, USA) is composed of 100% lyophilized human DBM which contains a broad spectrum of natural GFs. It is obtained through the proprietary Accell® DBM processing and it is provided in a sheet or "wafer" format. Accell TBM® is sterilized by electron beam irradiation.

DBX® Putty (Synthes Inc., West Chester, PA, USA) is composed of DBM (93% by volume) in sodium hyaluronate (4%) and phosphate dibasic buffer. It consists of collagen and bone GFs (mainly TGF-β), responsible of the osteoinductive properties. DBX is completely replaced by new host bone after 4 to 6 months. DBX® is aseptically produced processing the tissues in ISO class 5 static certified clean rooms.

### Isolation and expansion of human ADSCs

Adipose tissue was collected from the abdominal region of healthy patients (age, 35–58 years) who underwent liposuction cosmetic procedures. All patients gave written consent. The lipoaspirate was washed with Phosphate Buffered Saline (PBS, EuroClone, Milan, Italy) and digested using a solution of 0.075% collagenase from Clostridium histolyticum type II (Sigma-Aldrich, St. Louis, MO, USA) in Hank's Balanced Salt Solution (HBSS, Lonza S.r.l., Milano, Italy), for 3 h at room temperature and in slow agitation. At the end of the digestion, the collagenase activity was blocked with an equal volume of complete DMEM (cDMEM). cDMEM consisted of Dulbecco's modified Eagle's medium (DMEM, Lonza, Italy) supplemented with 10% Fetal Bovine Serum (FBS, Bidachem S.p.A., Milano, Italy) and 1% Penicillin/Streptomycin (P/S, EuroClone). After centrifugation for 4 min at 1200 rpm, the pellet was washed in PBS and filtered with a 70 µM cell strainer (BD Biosciences, Mississauga, Ontario, Canada). The cell suspension was resuspended in cDMEM, transferred to a 25-$cm^2$ tissue culture flask, then incubated at 37°C and 5% $CO_2$ for 15 days. Culture medium was changed every 2 days (37).

### Cell counting

The viable cells were counted using the trypan blue exclusion test (38). At the confluence point, ADSCs were detached from the flasks with a solution of 0.25% trypsin ad 0.02% EDTA (EuroClone). After the addition of cDMEM, the cells were centrifuged for 4 min at 1200 rpm. The pellet was resuspended in cDMEM, then, 20 µl of the suspension were added to 80 µl of trypan blue for each culture. Cell counting was done using a Burker's chamber.

### Cell characterization

The expression of stemness markers was evaluated by

immunofluorescence. The cells, seeded on a glass slide, were fixed for 10 min at room temperature with 4% paraformaldehyde in PBS, pH 7.4. The slides were treated with 1% Bovine Serum Albumin (BSA, Sigma-Aldrich) for 30 min to block non-specific sites, and then incubated at 37°C for 1 h with the following primary antibodies: anti-human CD73 (Abcam, Cambridge, UK), anti-human CD90 (Abcam), anti-human CD105 (Santa Cruz Biotechnology Inc., CA, USA), anti-human fibroblasts (FU) (Abcam), and anti-human CD34-FITC (Macs, Miltenyi Biotec GmbH, Germany). Subsequently, a second incubation was done at 37°C for 1 h with secondary antibodies: goat anti-mouse IgG DyLight 488 labeled (KPL, Gaithersburg, MD, USA) or goat anti-rabbit IgG (H+L), DyLight 549 labeled (KPL, Gaithersburg, MD, USA).

### 3D cell cultures

Cells were seeded at a density of 106/cm$^2$ on the 3D Orthoss®scaffold alone (control), with TBM® (Orthoss® + TBM®) or with DBX® (Orthoss® + DBX®). Two different media were used: standard medium (cDMEM) or osteogenic differentiation medium (cDMEM supplemented with 0.1 µM dexamethasone, 200 µM L-ascorbic acid, and 10 mM β-glycerol phosphate). All 3D cultures were incubated at 37°C and 5% $CO_2$ for 28 days, changing the medium every 2 days. The cell cultures were prepared as represented in Figure 1.

### MTT assay

The biocompatibility of the materials was assessed at day 28 verifying cell viability and proliferation by the MTT (3-4,5-dimethylthiazol-2YL-2,5-bromuro diphenyltetrazolium) assay (39). The test is based on the ability of functional mitochondria to oxidize the MTT solution, giving a blue-violet product. In detail, the supernatant was aspirated from the tissue culture plate and 1 mL of 0.5 mg/mL MTT solution in PBS was added. After 3 h of incubation, the supernatant was aspirated, each scaffold was transferred to a microtube and 0.5 mL of 10% dimethyl sulfoxide in isopropanol was added to extract the formazan in the samples for 30 min at 37°C. For each sample, absorbance values at 570 nm were recorded in duplicate on 200 µL aliquots deposited in microwell plates using a multilabel plate reader (Victor 3 Perkin Elmer, Milano, Italy).

### Real-time PCR

Total RNA was extracted on day 28 using the TRIzol® Reagent (Invitrogen, Carsbad, CA, USA). The samples were quantified using the NanoDrop spectrophotometer (NanoDrop™ 1000, Thermo Scientific). For the first-strand cDNA synthesis, 500 ng of total RNA was reverse transcribed using M-MLV RT (Moloney Murine Leukemia Virus Reverse Transcriptase, Invitrogen) according to the manufacturer's protocol. Human primers were selected for each target gene with Primer 3 software. Real-time PCRs were carried out using the designed primers at a concentration of 300 nM and FastStart SYBR Green Master (Roche Diagnostics, Mannheim, Germany) on a Rotor-Gene 3000 (Corbett Research, Sydney, Australia). Thermal cycling conditions

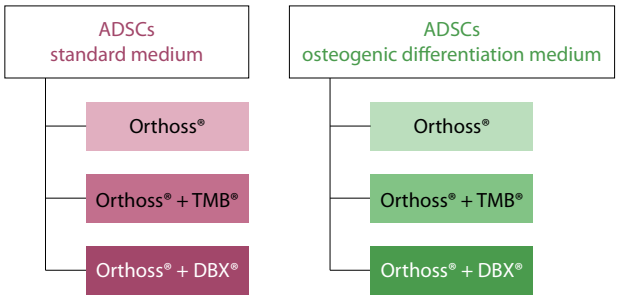


FIG. 1 Schematic representation of the prepared 3D cell cultures.

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. Values were normalized to the expression of the β-actin 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.

### array-CGH

In order to identify possible chromosomal aberrations, such as deletions, amplifications, and aneuploidy, array-CGH was performed using the Human Genome CGH Microarray (Agilent Technologies, Santa Clara, CA, USA) with a median probe spatial resolution of 44 Kb. array-CGH was performed following the manufacturer's protocol. Briefly, 1 µg of DNA from human ADSCs cultures (sample) and 1 µg of pooled sex-matched reference DNA (Promega, Madison, WI, USA) were digested with AluI and RsaI for 2 h at 37°C. After inactivation of the enzymes at 65°C for 20 min, each digested sample was labeled by random priming (Genomic DNA Enzymatic Labelling Kit, Agilent Technologies) for 2 h using Cy5-dUTP for sample DNA and Cy3-dUTP for reference DNA. Labeled products were then column-purified (Microcon YM-30 filters, Millipore Corporation, Billerica, MA, USA). After probe denaturation and pre-annealing with Cot-1 DNA, hybridization was performed at 65°C with rotation for 24 h. At the end of the incubation, slides were washed and analyzed using the Agilent scanner. Data and graphics elaboration was done using the CGH Analytics software (V3.1 Agilent Technologies).

### Statistical analysis

Statistical analysis was performed using the software SPSS 20.0.0. The comparison between groups was done using the Mann-Withney-U test. A $p<0.05$ was considered significant (*).

## RESULTS

### Cell isolation and characterization

Human ADSCs were isolated from lipoaspirate by an enzymatic digestion, then grown as monolayer for 15 days in cDMEM at 37°C and 5% $CO_2$. ADSCs showed

a fibroblastic-like morphology when observed under phase contrast microscope (Fig. 2). In agreement with the ISCT25, ADSCs were phenotypically characterized by immunofluorescence at passage three. The cells were positive for anti-CD73, -CD90 and -CD105 antibodies, negative for anti-CD34 and anti-FU antibodies (Fig. 3).

## Biocompatibility of the scaffolds

ADSCs were able to proliferate onto the scaffolds, both in standard medium and osteogenic differentiation medium, as demonstrated by the MTT assay performed 28 days after cell seeding (Fig. 4). These results demonstrated that the scaffolds were biocompatible and not toxic for the cell growth.

## Real-time PCR

The gene expression level of some osteoblast markers was analyzed at day 28 by means of Real-time PCR in order to verify the osteogenic properties of the materials used in the present study. The expression of selected genes (osteopontin, osteonectin, osteocalcin, type I collagen) was evaluated in relation to the expression of the reference gene (β-actin).

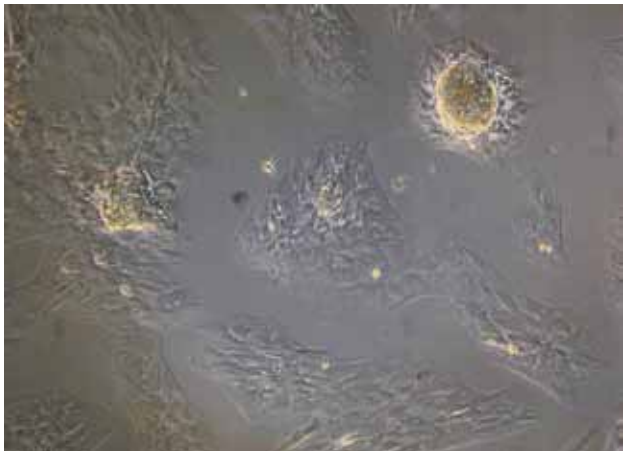

FIG. 2 Morphology of ADSCs isolated and cultured at 37°C with 5% $CO_2$ at passage three. Cells show a spindle or a triangle-like morphology (20X magnification).

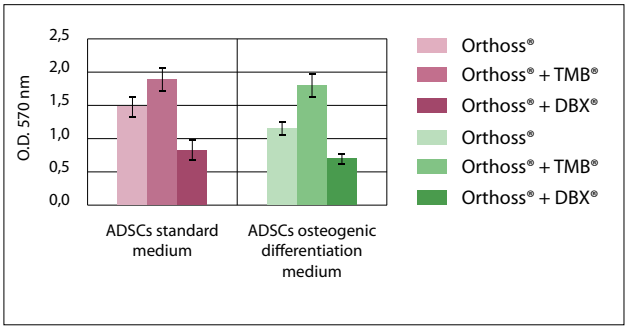


FIG. 4 MTT assay of 3D ADSCs cultures in standard medium (left) and osteogenic differentiation medium (right). After 28 days of culture, the cells show the same ability to proliferate in presence of both TBM® and DBX®.

Cells seeded on Orthoss® alone were used as control. In presence of standard medium, we found a significantly higher expression of selected genes in ADSCs seeded with TBM® compared to control and ADSCs grown with DBX®. On the contrary, there was not a significant difference in gene expression in the populations seeded with DBX® and control (Fig. 5). Osteogenic differentiation of ADSCs was, instead, similar independently of the biomaterials used (Fig. 6).

## array-CGH

In order to verify the safety of both materials and methods, we performed array-CGH analyses of all the 3D ADSCs cultures after 28 days from seeding.

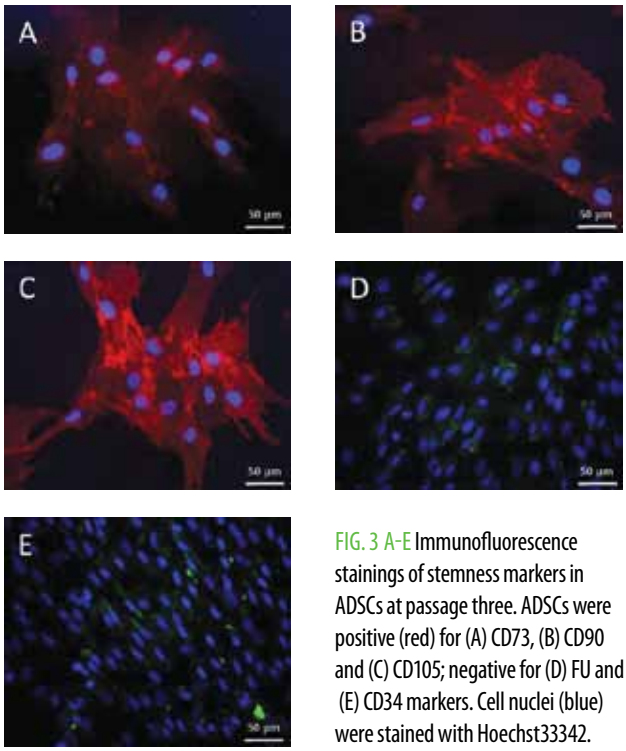


FIG. 3 A-E Immunofluorescence stainings of stemness markers in ADSCs at passage three. ADSCs were positive (red) for (A) CD73, (B) CD90 and (C) CD105; negative for (D) FU and (E) CD34 markers. Cell nuclei (blue) were stained with Hoechst33342.

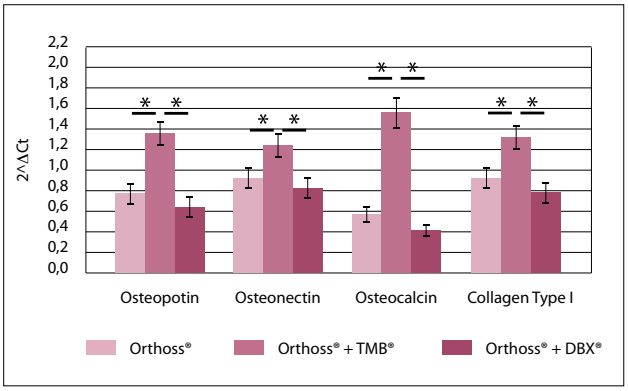


FIG. 5 Gene expression of some osteoblast markers in ADSCs cultured for 28 days in standard medium. Gene expression levels are higher in ADSCs seeded in Orthoss® + TBM® (* = $p<0.05$) compared to ADSCs seeded in Orthoss® alone or Orthoss® + DBX®.

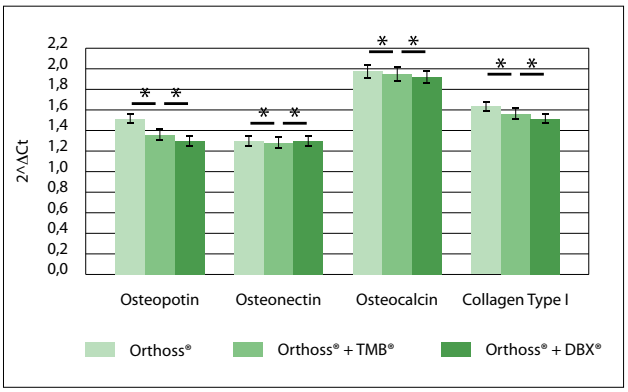


FIG. 6 Gene expression of some osteoblast markers in ADSCs cultured for 28 days in osteogenic differentiation medium. Gene expression levels are similar in ADSCs seeded in Orthoss® + TBM® (* = $p<0.05$) compared to ADSCs seeded in Orthoss® alone or Orthoss® + DBX®.

No chromosomal aberrations were detected in the experimental sets, confirming the biosafety of the Orthoss® scaffold, of both the DBMs as well as of the cell culture method used.

## DISCUSSION

Bone regeneration is a complex and multifactorial process that mainly needs the presence of osteoconductive (3D scaffold), osteoinductive (BMPs, GFs, and DBM), and osteogenic (bone-forming cells) factors (1). These properties are typically found in autologous bone graft; however, the disadvantages associated to its harvesting limit its use. For this reason, an alternative approach has been extensively studied with the aim to obtain a fully functioning bone. This strategy is based on the ability of MSCs to proliferate, self-renew and differentiate. MSCs can be harvested from many tissues, among which adipose tissue represents an abundant and accessible source (40, 41). Indeed, ADSCs have similar ability to proliferate and differentiate of BMSCs, in particular in osteogenic sense (42). Moreover, ADSCs possess additional advantages compared to BMSCs, mostly related to the great availability and yield (22).

On the basis of these knowledges, in the present study we tested the ability of ADSCs to differentiate towards the osteogenic phenotype in vitro when seeded on a 3D scaffold in presence of osteogenic factors.

ADSCs were isolated, by enzymatic digestion, from the lipoaspirate of 5 healthy patients undergoing abdominal cosmetic liposuction. After an incubation period of 15 days, positivity to CD73, CD90, and CD105 stem cell markers was verified by immunofluorescence. Negativity to FU and CD44 was also assessed. Then, ADSCs were seeded on Orthoss®, an HA scaffold necessary for 3D cell culture (1). ADSCs were also 3D cultured in presence of 2 different DBMs: TBM®, entirely composed of demineralized bone matrix, and DBX®, adjuvanted with sodium hyaluronate, able to stimulate the osteogenic differentiation.

In order to compare the osteoinductive ability of the 2 DBMs, cells were cultured in cDMEM or in osteogenic differentiation medium for 28 days. At the end of this period, the biocompatibility of the scaffolds was tested with the MTT assay by comparing viability of the cells cultured with or without differentiation medium. Proliferation rates were higher, although not significant, in the group of cells cultured in standard medium compared to the ones treated with osteogenic factors. This result could be explained by a slower ability of the cells to differentiate in standard medium, where they maintain stemness characteristics and, therefore, a greater replicative capacity.

Moreover, we observed a lower proliferation rate of ADSCs seeded on Orthoss® + DBX® compared to Orthoss® + TBM® and also to the group only cultured on Orthoss® (control group). On day 28, the osteoinductive capacity of DBMs were evaluated by measuring the gene expression levels of osteopontin, osteonectin, osteocalcin and type I collagen. Real-time PCR results revealed the expression of all the genes analyzed, thus confirming the mature osteogenic phenotype of the cells. In detail, the results showed a significantly greater gene expression level in cells cultured with Orthoss® + TBM® compared to the control and to the Orthoss® + DBX® groups. On the contrary, there was no difference between the control and the Orthoss® + DBX® groups. This would suggest a greater ability for TBM® to induce osteogenic differentiation *in vitro*.

ADSCs were able to differentiate in osteogenic sense also in the group seeded on Orthoss® alone. It has been demonstrated that Orthoss® not only has osteoconductive ability but also osteoinductive properties due to its porous structure that ensures to the cells a habitat extremely similar to that of native bone (43, 44). The main difference between TBM® and DBX® consists in the presence of hyaluronic acid in the second material. As this molecule is abundantly found in vivo in the dermis, we hypothesized that the lesser osteoinductive capacity of DBX® compared to TBM® may be due to an interaction of hyaluronic acid with its receptor (CD44), normally expressed by MSCs. This interaction could alter the normal process of MSCs osteoinduction interfering with the normal pathways of differentiation and promoting the activity of the peroxisome proliferator activated receptor γ (PPAR γ), factor required for the adipogenic differentiation (48). Finally, we evaluated the safety of the method used with array-CGH analysis. This test is particularly important for further clinical applications. array-CGH is preferentially used to test genetic alterations, index of neoplastic transformation, because of its higher resolution than other cytogenetic and molecular methods. The analysis was performed on the entire genome of all 3D cultures, after 28 days of in vitro

culture. Results showed no alterations at the genome level. These data strongly supported the safety of both biomaterials and stem cells cultured in vitro, according to the methods used in this study.

## CONCLUSION

Based on the results of this study, it can be concluded that human ADSCs are able to adhere, proliferate and differentiate in osteoblast-like cells, also in absence of osteogenic factors. This confirms the osteoinductive ability of both the DBMs used. Moreover, the absence of genetic alterations demonstrates the safety of materials and cultures and supports their potential clinical application for bone reconstruction. The use of in vitro cultured autologous ADSCs is, therefore, promising for the clinical treatment of bone defects of various dimensions reducing the problems related to the use of non-autologous material and to the availability of great amount of materials.

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