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OSTEOPLANT® ACTS ON STEM CELLS DERIVED FROM BONE MARROW INDUCING OSTEOBLASTS DIFFERENTIATION

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Osteoplant, an equine flexible cortical and spongy bone tissue, is a promising material for bone grafting in orthopedic, maxillofacial and dental surgery. Osteoplant is completely resorbable, inducing oteoclast activation and promoting the substitution of the scaffold with new bone. To study how Osteoplant can induce osteoblast differentiation in mesenchymal stem cells, the expression levels of bone related genes and mesenchymal stem cells marker were analyzed, using real time Reverse Transcription-Polymerase Chain Reaction. Osteoplant is an inducer of osteogenesis on human stem cells, as showed by the activation of bone related genes ALPL, SPP1 and RUNX2, and by the down-regulation of the mesenchymal stem cells marker ENG.

Large bone defects still represent a major problem in orthopedics and maxillofacial surgery. Autogenous bone grafts are traditionally employed in bone-repair treatments. However, bone graft is associated with an unacceptably high incidence of complications such as chronic pain, numbness, and poor cosmesis (1). Furthermore, operative time and length of hospitalization are often increased (2).

A composite graft that combines a synthetic scaffold with osteoprogenitor cells from bone marrow aspirate may potentially deliver the advantages of autogenous bone grafts without the procurement morbidity (3).

Biomaterials used in bone regeneration are designed to be gradually resorbed by the osteoclast and replaced by new bone formed through osteoblastic activity (4).

A new promising material for bone grafting is Osteoplant, an equine flexible heterologous deantigenic cortical and spongy bone tissue.

Osteoplant is osteoconductive and useful to fill bone defects in orthopedic, maxillofacial and dental surgery.

Once hydrated, this scaffold is flexible and therefore precisely adaptable to defect that needs to be filled. Osteoplant is a completely resorbable biomaterial that induces oteoclast activation promoting the substitution of the scaffold with new bone (5).

Here we investigated the mechanism by which Osteoplant promotes osteoblast differentiation and bone regeneration, in bone marrow derived stem cells (BMSC) and Human Osteoblasts (HOb).

The quantitative expression of the mRNA of specific genes, like transcriptional factors (RUNX2 and SP7), bone related genes (SPP1, COL1A1, COL3A1, ALPL, and FOSL1) and mesenchymal stem cells marker (ENG) were examined by means of real time Reverse Transcription-Polymerase Chain Reaction (real time RT-PCR) in treted BMSC and HOb.

Gene expression in BMSC was then compared with the gene expression in Human Osteoblasts (HOb) treated

Key words: Osteoplant, bone marrow, stem cells, gene expression

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with Osteoplant, to evaluate the potential effect of this biomaterial in osteoblasts differentiation.

MATERIALS AND METHODS

Bone marrow stem cells culture

BMSC were purchased from Lonza (Walkersville, MD, http://www.lonza.com) and were routinely cultured in MSC-Growth Medium (MSC-GM; Lonza) in a humidified atmosphere of 5% CO2 at 37°C.

Primary Human Osteoblasts cell culture

Fragments of bone derived from skull of healthy volunteers were collected during operation. The pieces were transferred in 75 cm^2 culture flasks containing DMEM medium (Sigma Aldrich, Inc., St Louis, Mo, USA) supplemented with 20% fetal calf serum, antibiotics (Penicillin 100 U/ml and Streptomycin 100 micrograms/ml - Sigma Aldrich, Inc., St Louis, Mo, USA) and amminoacids (L-Glutamine - Sigma Aldrich, Inc., St Louis, Mo, USA).

Cells were incubated in a humidified atmosphere of 5% CO2 at 37°C. The medium was changed the next day and every 3 days thereafter. After 15 days, the pieces of bone tissue were removed from the culture flask. Cells were harvested after 30 days of incubation.

Cell culture

For the assay, BMSC and HOb at second passage were seeded with Osteoplant (Bioteck SRL, Vicenza, Italy) at the concentration of 10 mg/1 ml Another set of wells containing untreated cells was used as control. The medium was changed every 3 days. The cells were maintained in a humidified atmosphere of 5% CO2 at 37°C. Cells were collected, for RNA extraction at 15 and 30 days.

RNA processing

Reverse transcription to cDNA was performed directly from cultured cell lysate using the TaqMAN Gene Expression Cells-to-Ct Kit (Ambion Inc., Austin, TX, USA), following manufacturer's instructions. Briefly, cultured cells were lysed with lysis buffer and RNA released in this solution. Cell lysate were reverse transcribed to cDNA using the RT Enzyme Mix and appropriate RT buffer (Ambion Inc., Austin, TX, USA).

Finally, the cDNA was amplified by real-time PCR using the included TaqMan Gene Expression Master Mix and the specific assay designed for the investigated genes.

Real time PCR

Gene expression was quantified using real time PCR. Forward and reverse primers and probes for the selected genes were designed using primer express software (Applied Biosystems, Foster City, CA, USA) and are listed in Table I.

All PCR reactions were performed in a 20 µl volume using the ABI PRISM 7500 (Applied Biosystems, Foster City, CA, USA). Each reaction contained 10 µl 2X TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA, USA), 400 nM concentration of each primer and 200 nM of the probe, and cDNA. The amplification profile was initiated by 10-minute incubation at 95°C, followed by two-step amplification of 15 seconds at 95°C and 60 seconds at 60°C for 40 cycles. All experiments were performed including non-template controls to exclude reagents contamination.

The gene expression levels were normalized to the expression of the housekeeping gene RPL13A and were expressed as fold changes relative to the expression of the untreated cells. Quantification was done with the delta/ delta calculation method (6).

Statistical analyses

Comparison of the gene expression between BMSC and HOb was performed with "Two tailed ANOVA "statistic analyses using Excel spreadsheets (Microsoft Office 2003).

RESULTS

Osteoinductive properties of Osteoplant were studied measuring the gene expression levels of osteoblasts related genes in treated BMSC, at 15 and 30 days of treatment.

Gene expression in BMSC was then compared with the gene expression in treated HOb.

Real time RT-PCR showed after 15 days of treatment in BMSC the up-regulation of FOSL1, ALPL and SPP1 genes and the down-regulation of SP7, ENG, COL3A1 and COL1A1. Expression of RUNX2 didn't change during the treatment (Fig. 1a).

After 30 days of treatment, the up-regulated genes were FOSL1, RUNX2, COL3A1, ALPL and SPP1. COL1A1 and SP7 were the only down-regulated genes (Fig. 1b).

HOb after 15 days of treatment showed the over-expression of SP7, COL3A1, ALPL, SPP1 genes and the down-regulation of ENG, FOSL1 and RUNX2 genes (Fig. 2a).

After 30 days, the bone related genes SP7, FOSL1, RUNX2 and ALPL were up-regulated. COL1A1, COL3A1, ENG, and SPP1 were decreased (Fig. 2b).

Comparing, by "Two tailed ANOVA", the relative expression of the analyzed genes in BMSC and HOb we observed that the significantly differentially expressed genes at 15 days of treatment were SP7, ENG, COL3A1, COL1A1, ALPL and SPP1 (Table II). After 30 days of treatment, the significantly differentially expressed genes were FOSL1, RUNX2, COL1A1, ALPL and SPP1 (Table III).

DISCUSSION

Synthetic and biological materials are increasingly used to provide temporary or permanent scaffolds for bone regeneration (7). Bone regeneration approaches attempts to create tissue replacement by culturing autologous

Table I. *Primer and probes used in real time PCR*

Gene symbol	Gene name	Primer sequence (5'>3')	Probe sequence (5'>3')
SPP1	osteopontin	F-GCCAGTTGCAGCCTTCTCA R-AAAAGCAAATCACTGCAATTCTCA	CCAAACGCCGACCAAGGAAAACTCAC
COL1A1	collagen type I alpha1	F-TAGGGTCTAGACATGTTCAGCTTTGT R-GTGATTGGTGGGATGTCTTCGT	CCTCTTAGCGGCCACCGCCCT
RUNX2	runt-related transcription factor 2	F-TCTACCACCCCGCTGTCTTC R-TGGCAGTGTCATCATCTGAAATG	ACTGGGCTTCCTGCCATCACCGA
ALPL	alkaline phospatasi	F-CCGTGGCAACTCTATCTTTGG R-CAGGCCCATTGCCATACAG	CCATGCTGAGTGACACAGACAAGAAGCC
COL3A1	collagen, type III, alpha 1	F-CCCACTATTATTTTGGCACAACAG R-AACGGATCCTGAGTCACAGACA	ATGTTCCCATCTTGGTCAGTCCTATGCG
BGLAP	osteocalcin	F-CCCTCCTGCTTGGACACAAA R-CACACTCCTCGCCCTATTGG	CCTTTGCTGGACTCTGCACCGCTG
CD105	endoglin	F-TCATCACCACAGCGGAAAAA R-GGTAGAGGCCCAGCTGGAA	TGCACTGCCTCAACATGGACAGCCT
FOSL1	FOS-like antigen 1	F-CGCGAGCGGAACAAGCT R-GCAGCCCAGATTTCTCATCTTC	ACTTCCTGCAGGCGGAGACTGACAAAC
SP7	osterix	F-ACTCACACCCGGGAGAAGAA R-GGTGGTCGCTTCGGGTAAA	TCACCTGCCTGCTCTTGCTCCAAGC
RPL13A	ribosomal protein L13	F-AAAGCGGATGGTGGTTCCT R-GCCCCAGATAGGCAAACTTTC	CTGCCCTCAAGGTCGTGCGTCTG

Table II. *Differentially expressed genes between BMC and HOb after 15 days of treatment*

Genes	BMC	HOb	Differentially expressed genes
	Log10 RQ	Log10 RQ	p<0,005
SP7	-0,08	0,43	**0,00682**
ENG	-0,30	-0,18	**0,01825**
FOSL1	0,10	-0,44	0,25662
RUNX2	-0,01	-0,57	0,07348
COL3A1	-1,24	0,09	**0,00001**
COL1A1	-1,43	0,00	**0,00280**
ALPL	0,21	1,10	**0,00014**
SPP1	1,16	2,07	**0,00016**

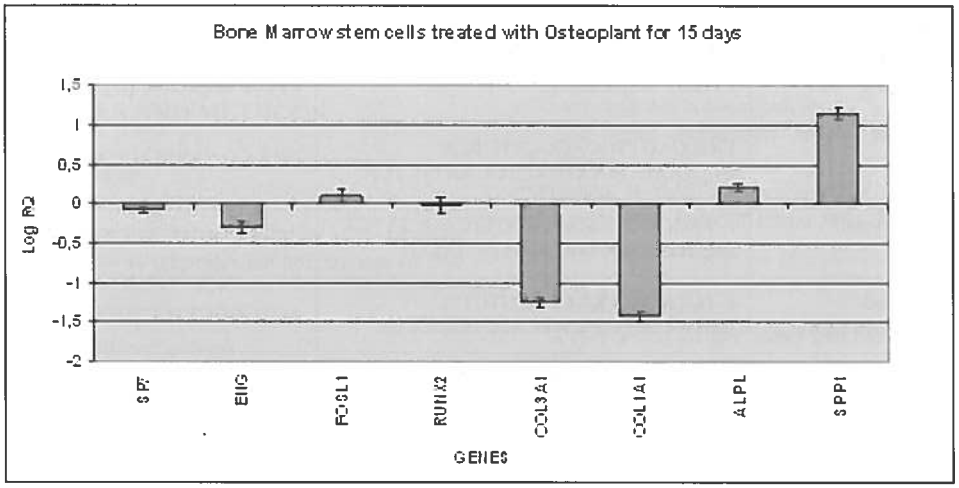
Table III. *Differentially expressed genes between BMC and HOb after 30 days of treatment*

Genes	BMC	HOb	Differentially expressed genes
	Log10 RQ	Log10 RQ	p<0,005
SP7	-0,34	0,39	0,10235
ENG	0,02	-0,73	0,89702
FOSL1	0,71	0,21	**0,00002**
RUNX2	1,63	1,61	**0,00055**
COL3A1	0,07	-0,86	0,45156
COL1A1	-1,28	-0,76	**0,00001**
ALPL	0,48	2,15	**0,00118**
SPP1	2,50	-0,78	**0,00001**

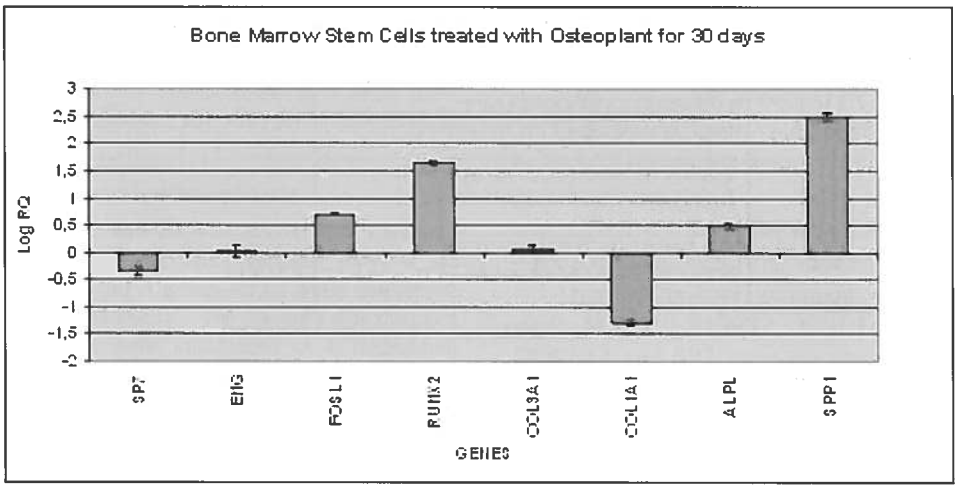
cells onto three-dimensional biodegradable scaffold that facilitate cell progenitor migration, proliferation and differentiation (8).

In this study we focus our interest on a new promising material for bone grafting (Osteoplant) which is an equine flexible heterologous deantigenic cortical and spongy bone tissue.

To study the osteoinductive properties of Osteoplant, we analyzed changes in expression of bone related genes (RUNX2, SPP1, COLIA1, COL3A1, ALPL and FOSL1) and mesenchymal stem cells marker (ENG), comparing the genetic profile of BMSC with HOb.



a



b

Fig. 2. *Gene expression in BMSC treated with 10 mg/ml of Osteobiol for 15 days (a) and 30 days (b).*

"Two TAILED ANOVA" showed that after 15 days of treatment the significantly differentially expressed genes among the two groups were SP7, ENG, COL3A1, COLA1, ALPL and SPP1. After 30 days of treatment, the significantly differentially expressed genes were FOSL1, RUNX2, COL1A1, ALPL and SPP1

ALPL and SPP1 genes were up-regulated during the entire treatment in BMSC.

Alkaline phosphatase regulates mineralization of bone matrix and is widely used as a marker of osteoblasts differentiation. Increasing in ALPL expression should be associated with osteoblast differentiation.

SPP1 encodes osteopontin, the most representative non collagenic component of extracellular bone matrix (7). Osteopontin is actively involved in bone resorbitive processes directly by ostoclasts (9). Osteopontin produced by osteoblasts, show high affinity to the molecules of hydroxylapatite in extracellular matrix and it is chemo-attractant to osteoclasts (10). Osteoplant seem to act enhancing bone resorption processes in the early stages of

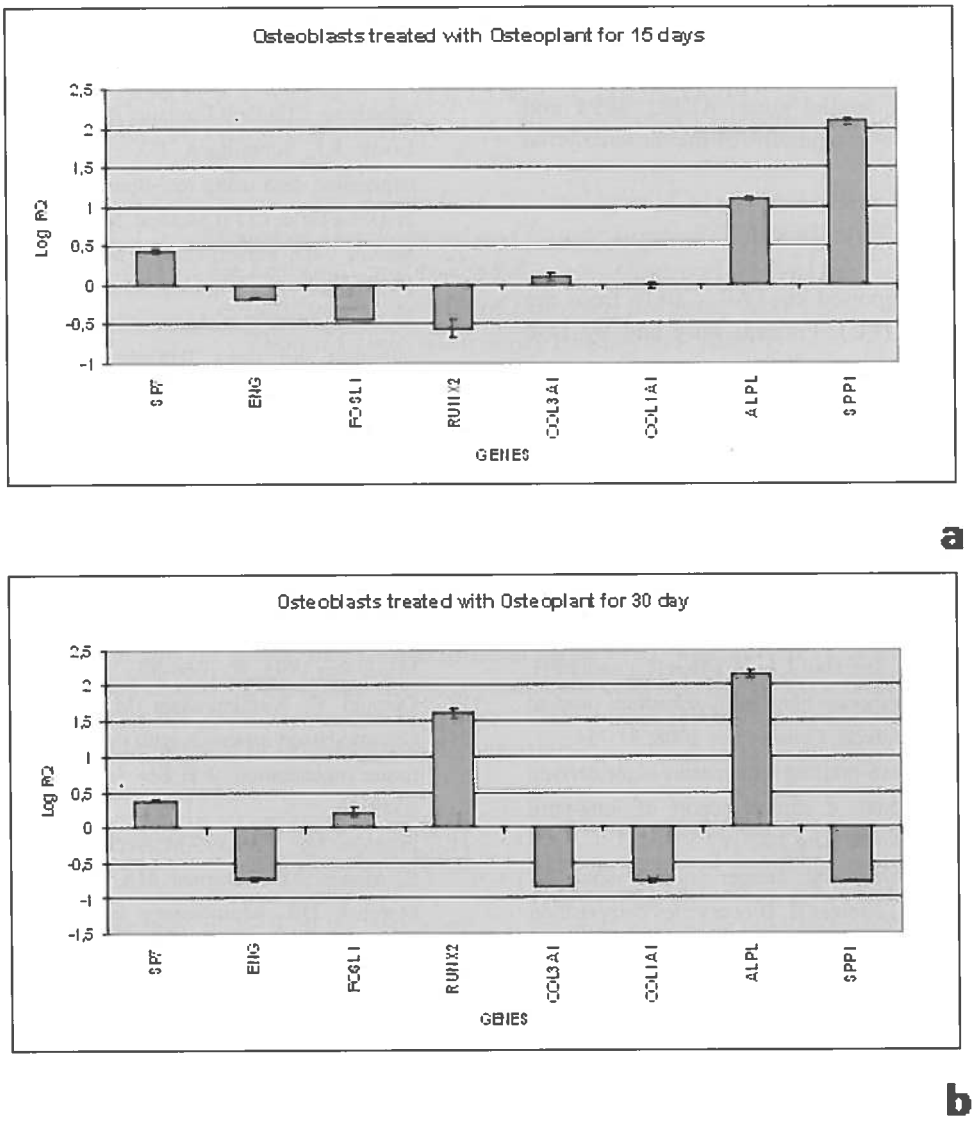


Fig. 3. *Gene expression in HOb treated with 10 mg/ml of Osteobiol for 15 days (a) and 30 days (b).*

cell differentiation.

After 30 days of treatment the transcriptional factor RUNX2 was up-regulated in BMSC. RUNX2 is the most specific osteoblast transcription factor and is a prerequisite for osteoblast differentiation and consequently mineralization.

Another transcriptional factor up-regulated in the first stage of BMSC treatment was SP7, a zinc finger transcriptional factor that regulates bone formation and osteoblast differentiation in vitro and in vivo.

Osteoplant also modulate the expression of genes encoding for collagenic extracellular matrix proteins like collagen type 1α1 (COL1A1) and collagen type 3α1 (COL3A1).

ENG (CD105), a surface markers used to define a bone marrow stromal cell population capable of multilineage differentiation (11), was down-regulated in treated BMSC respect to control. The disappearance of the ENG in BMSC during Osteoplant treatment suggests the osteodiffrentiative potential of this biomaterial on stem cells.

The present study shows the effect of Osteoplant on BMSC osteo-differentiation. Osteoplant is an inducer of osteogenesis on human stem cells, as showed by the activation of bone related genes ALPL, SPP1 and RUNX2, and by the down-regulation of the mesenchymal stem cells marker ENG.

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