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Differences in osteoblast miRNA induced by cell binding domain of collagen and silicate-based synthetic bone

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Summary

PerioGlas (PG) is an silicate-based (i.e. anorganic) material used for grafting periodontal osseous defects since the ninety whereas P-15 is an analog of the cell binding domain of collagen (i.e. organic material) that is successfully used in clinical trial to promote bone formation. However, how PG (i.e anorganic material) and P-15 (i.e. collagen) differentially alter osteoblast activity to promote bone formation is unknown. We therefore attempted to get more insight by using microRNA microarray techniques to investigate the translation process in osteoblasts differentially exposed to PG and P-15. We identified 3 up-regulated miRNA (i.e. mir-30b, mir-26a, mir-92) and 8 down-regulated miRNA (i.e. mir-337, mir-377, mir-25, mir-200b, mir-129, mir-373, mir-133b, mir-489). The data reported are, to our knowledge, the first study on translation regulation in osteoblatsts differentially exposed to cell binding domain of collagen and to silicate-based material. Both enhance the translation of several miRNA belonging to osteogenetic genes, but P-15 acts preferentially on homeobox genes.

PerioGlas® (PG) is an alloplastic material used for grafting periodontal osseous defects since the ninety. In animal models it achieves histologically good repair of surgically created defects. In monkey [1–3] PG demonstrates biocompatibility and osteoconductive activity. It is mostly resorbed and replaced by bone and the remaining granules are in close contact with bone. In rabbit model, PG is able to improve bone healing at the interface between titanium dental implants and bone [4] whereas in ovariectomized rats a neoformation of bone trabeculae into extraction sockets is improved [5].

In clinical trial, bioactive glass is effective as an adjunct to conventional surgery in the treatment of intrabony defects [6] as well in the treatment of dental extraction sites before dental implant placement, to implement bone regeneration and to augment early fixation of implant [7]. However, PG has no regenerative properties as regard cementum and periodontal ligament [8].

P-15 (Ceramed, Lakewood, CO) is an analog of the cell-binding domain of collagen [9]. Type I collagen represents approximatively one third of the body proteins [9]. Collagen, moreover, is a major determinant of the architecture and tensile strength of the tissues, and it modulates cell proliferation, migration, differentiation, and specific gene expression [9]. P-15 competes for cell surface sites for attachment of collagen and, when immobilized on surfaces, it promotes adhesion of cells [10]. P-15 has been shown to facilitate physiological processes in a way similar to collagen, to facilitate the exchange of mechanical signals, and to promote cell differentiation

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[11–13]. Like other bone augmentation materials, P-15 associated with anorganic-derived bone matrix (ABM), has been shown to be helpful in the treatment of periodontal defects, and sinus-lifting procedures [14–19].

Because the mechanism by which PG (i.e anorganic material) and P-15 (i.e. collagen) differentially alter osteoblast activity to promote bone formation is unknown, we therefore attempted to address this question by using microRNA microarray techniques.

MicroRNAs (miRNAs) represent a class of small, functional, noncoding RNAs of 19–23 nucleotide (nt) cleaved from 60- to110-nt hairpin precursors [20, 21]. Hundreds of miRNAs have been identified in plants and animals. The miRNAs are involved in various biological processes, including cell proliferation and cell death during development, stress resistance, and fat metabolism, through the regulation of gene expression [22] in a post-transcriptional RNA silencing pathways. The RNA interference (RNAi) the microRNA (miRNA) pathway, regulate gene expression by inducing degradation and/or translational repression of target mRNAs. These pathways are generally initiated by various forms of double-stranded RNA (dsRNA), which are processed by Dicer, an RNase III family endonuclease, to 21–22 nt long RNA molecules that serve as sequence-specific guides for silencing [23, 24].

MicroRNAs are transcribed as long primary transcripts (pri-miRNAs), which are processed by a nuclear RNase III Drosha-containing complex into short hairpin intermediates (pre-miRNAs). Pre-miRNAs are transported to the cytoplasm where they are further processed by a second Rnase III-family enzyme called Dicer to generate 22-bp RNA duplexes with 2-nt 3′ overhangs [25–28].

MicroRNAs are loaded onto an Argonaute containing effector ribonucleoprotein (RNP) complex, referred to as miRNP or RISC (RNA-induced silencing complex), which is capable of recognizing cognate mRNAs and inhibiting protein expression.

We used a recently developed methodology for miRNA gene expression profiling based on the hybridization of a microchip, the Ncode Multi-Species miRNA Microarray (Invitrogen, Carlsbad, CA, USA), a slide printed with approximately 900 unique probe of miRNA sequences for Homo sapiens, Mus musculus, Rattus norvegicus, Drosophila melanogaster, Caenorhabditis elegans, and Danio rerio.

By the analysis of the the 329 Human miRNAs sequences spotted on the array, we compared miRNA expression and consequently gene regulation in Human MG63 cells treated with P-15 vs. MG63 cells treated with PG.

Materials and methods

Cell culture

Osteoblast-like cell (MG63) were cultured in sterile Falcon wells (Becton Dickinson, New Jersey, USA) containing Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) (Sigma, Chemical Co., St Louis, Mo, USA) and antibiotics (Penicillin 100 U/ml and Streptomycin 100 micrograms/ml – Sigma, Chemical Co., St Louis, Mo, USA). Cultures were maintained in a 5% CO_2 humidified atmosphere at 37 °C.

MG63 cells were collected and seeded at a density of 1×10^5 cells/ml into 9 cm^2 (3 ml) wells by using 0.1% trypsin, 0.02%EDTA in Ca+ +- and Mg- free Eagle's buffer for cell release. One set of wells were added with PerioGlas® (US Biomaterials Corp., Alachua, FL) at the concentration of 0.04 g/ml. Another set of wells were added with P-15 (Ceramed, Lakewood, CO) at the concentration of 10 µl/ml. P-15 was previously prepared by adding 2 ml serum-free medium in 1 g of P-15 for 1 h at room temperature. After 24 h, when cultures were sub-confluent, cells were processed for RNA extraction.

miRNA Microarray

MicroRNA were extracted from the cells using the PureLink™ miRNA Isolation Kit (Invitrogen). About 400 ng of miRNA from each sample (treated and control) were used for hybridization of NCode™ Multi-Species miRNA Microarray, a slide containing 329 Human miRNAs sequences in duplicate.

NCode™ miRNA Labeling System (Invitrogen) was used for labeling and hybridizing miRNA to microarray, according to the manufacturer's instructions. Briefly, a poly(A) tail was added to each miRNA, using a poly A polymerase and an optimized reaction buffer. Then a capture

sequence was ligated to the miRNA using a bridging oligo (dT). Following a purification step, the tagged miRNAs was hybridized to the microarray and incubate overnight.

After an incubation of 18–20 h, the array was washed and hybridized with Alexa Fluor® 3 capture reagents (for P-15) and Alexa Fluor® 5 capture reagents (for PG) in the first experiment and then switched. After another wash the array was scanned using a standard microarray scanner (Axon Instruments, Sunnyvale, CA).

After scanning, each spot is identified by means of GAL (GenePixR Array List) file downloaded from www.invitrogen.com/ncode, that lists the identities and locations of all the probes printed on the array.

Images were quantified by GenePix 6.0 software (Axon Instruments, Sunnyvale, CA). Signal intensities for each spot were calculated by subtracting local background from total intensities. The data were normalized by using the DNMAD and Preprocessing (http://gepas.bioinfo.cipf.es/cgi-bin/tools) [29, 30]. This generates an average value of the two spot replicates of each miRNA.

To select the differentially expressed miRNA, the data obtained were analyzed using the *SAM* package (significance analysis of microarray) [31].

For target predictions and validations miRNA were processed using miRBase Target, a web resource (http://microrna.sanger.ac.uk/targets/v4/) developed by the Enright Lab at the Wellcome Trust Sanger Institute. This source use an algorithm called miRanda to identify potential binding sites for a given miRNA in genomic sequences.

The gene target list was then processed by FatiGO (http://fatigo.bioinfo.cnio.es), a web interface which carries out simple data mining using Gene Ontology The data mining consists on the assignation of the most characteristic Gene Ontology term to each cluster of regulated genes.

Results

Hybridization of miRNA to the sequences spotted on the slide allowed us to perform systemic analysis of microRNAs and to provide primary information as regard regulation of translation process induced by P-15 vs. PG. We identified three up-regulated miRNA (i.e. mir-30b, mir-26a, mir-92) and eight down-regulated miRNA (i.e. mir-337, mir-377, mir-25, mir-200b, mir-129, mir-373, mir-133b, mir-489) for FDR (false discovery rate) = 0 and score > 7. Figure 1 is the graphical output of SAM (Statistical Analysis for Microarray) and it shows differentially expressed miRNA. Because miRNA potentially regulates thousand genes, in this study we select only genes related to osteogenesis and bone remodeling (Table 1). Genes with opposed regulation were excluded.

Data verification by means messengers microarray technique

To verify the effect of miRNAs on messengers a subsequent experiment with conventional mRNAs microarray technique is performed. The method is the same of that used in previously studies [32–34]. Briefly, messenger RNAs extraction was performed by MG63 cells treated in the same experimental condition used for miRNAs. Then cDNA was synthesized from mRNA and indirect cDNA labeling performed. A total of 20 K human DNA microarrays slides (MWG Biotech AG, Ebersberg, Germany) were used. The experiment was repeated twice and the dyes switched. A GenePix 4000a DNA microarrays scanner was used and a SAM program was then performed. Finally results obtained from miRNAs and mRNAs were matched and a list of genes is reported in Table 2.

Discussion

PG is a silicate-based synthetic bone augmentation material that has been used to fill periodontal defects with bonding and integration to both soft tissue and bone. Previous studies in animal models have shown that PG achieves histologically good repair of surgically created defects [1–5]. In clinical trial, PG is effective as an adjunct to conventional surgery in the treatment of intrabony defects [6] as well in the treatment of dental extraction sites [7].

P-15 is an highly conserved linear peptide with a 15-amino acid sequence identical to the sequence contained in the residues 766–780 of the alpha chain of type I collagen [35]. In order to get more inside how P-15 vs. PG alter osteoblast activity to promote bone formation, we used a new method, miRNA microarray.

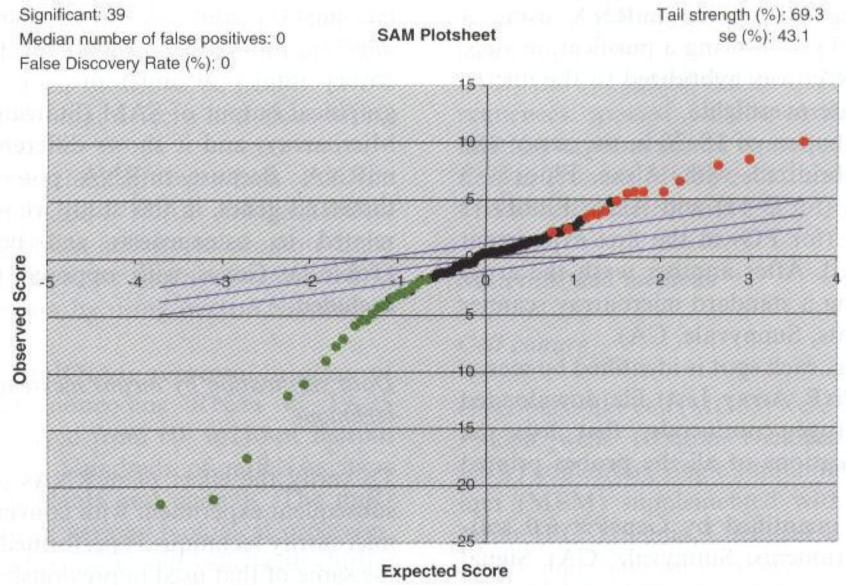

Figure 1. SAM (statistical analysis of microarray) plot of MG63 cultured for 24 h with PerioGlas at a concentration of 0.04 g/ml vs. MG63 cultured for 24 h with P-15 at the concentration of 10 µl/ml. Expected differentially expressed miRNAs are reported in x axe, whereas observed differentially expressed miRNAs are in y axe. Down-regulated miRNAs (green dots) are located in the lower left side of the diagram; up-regulated miRNAs (red dots) are in the upper right side; miRNAs whose expression is different but statistically not significant are represented by black dots. Parallel lines drawn from lower-left to upper-right squares are the cut-off limits. The solid line indicates the equal value of observed and expected differentially expressed miRNAs.

Hybridization of miRNA derived from MG63 cultured with 10 µl/ml of P-15 vs. MG63 cultured with 0.04 g/ml of PG to the sequences spotted on the slide allowed us to perform systemic analysis of miRNAs and to provide primary information as regard regulation of translation induced by P-15 vs. PG (Table 1).

Most notable up-regulated genes in P15 (due to down-regulated miRNA) are homeobox, i.e. genes spatially and temporally regulated during embryonic development which regulate morphogenesis. Among them is Noggin or NOG. It inactivates members of the TGF-beta superfamily signaling proteins, such as BMP4. By diffusing through extracellular matrices more efficiently than members of the TGF-beta superfamily, noggin may have a principal role in creating morphogenic gradients [36]. CHRD, another hoxgene, dorsalizes early vertebrate embryonic tissues by binding to ventralizing TGF-beta-like BMPs and sequestering them in latent complexes [37]. HOXD13 is another homeobox gene whose mutation has been associated with severe limb and genital abnormalities [38].

Table 1. Down- and up- regulated genes.

miRNA	Biological function	Target genes
Up-	Skeletal development	GDF10, CALCA, MATN3, ANXA2, ARSE
	Cartilage development	SNAI1
	Bone remodeling	KAZALD1
Down-	Skeletal development	CHRD, HOXD13, EBP, INHBA, CMKLR1, COMP, ADAMTS4, KLF10, AEBP1, COL11A2, PAPSS1, COLL11A1, ALPL, DLX6, COL2A1, POSTN, PAX1, SUFU
	Cartilage development	NOG, MGP, AHSG, AMELY, CDH11, SPP1, PMF1
	Bone remodeling	CALCR, CADR, OSTF1, BMP1, IGF1

Table 2. Messenger RNAs up- and down-regulated by miRNAs and confirmed by using standard microarray technique.

miRNA	*Down-regulated genes in microarray*
mir-337	ARRDC1, BCKDK, DAPK3, OPRS1, PTPRC
mir-377	B4GALT6, CLCN3, EIF4E, PTPRC
mir-200b	CALCR, FBXW7, GHRHR, LAMA4
mir-489	PTPRC, SOX18, STK24, TGFBR3
miRNA	*Up-regulated genes in microarray*
mir-26a	ZNF547, MSX1, CALCA, MATN3, ANXA2

These mRNAs are different from those of the previous table because slides for messenger microarray technique does not carried the previous genes.

Additional up-regulated genes code for extracellular matrix components, expecially collagen or related molecules like COL11A1, COL11A2, COL2A1 and COMP. ALPL, instead, is a non-specific alkaline phosphatase that acts on matrix mineralization [39].

Other up-regulated genes in P-15 vs. PerioGlas are hormons or their receptors like calcitonin receptor (CALCR) and insulin-like growth factor 1 (IGF1) whereas additional mRNAs act on osteoblast (i.e. osteoclast stimulating factor 1 – OSTF1) or on bone formation (i.e. BMP1).

Selected down-regulated genes in P-15 vs. PerioGlas (due to up-regulated miRNAs) act on skeletal morphogenesis (i.e. GDF10, a member of the BMP family) or on osteoclast formation and bone resorption (i.e. ANXA2 an autocrine factor).

The genes discussed are only a limited number among those differentially regulated by miRNA reported in Table 1. We briefly analyzed some of those with a better known function and directly related to bone formation, skeletal development, cartilage remodeling and bone production.

It is worth noting that MG63 are a cell line and not normal osteoblasts. Notwithstanding the advantages of using a cell line is related to the fact that the reproducibility of the data is higher because there is not the variability of the patient studied. Primary cell cultures provide a source of normal cells but they also contain contaminating cells of different types and cells in variable differentiation states. Moreover, we have chosen to perform the experiment after 24 h in order to get information on the early stages of stimulation which correspond to the early phase of grafting. This is of paramount importance for successfully clinical procedure.

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