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Zirconium oxide regulates RNA interfering of osteoblast-like cells

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Abstract Zirconium oxide (ZO) has outstanding mechanical properties, high biocompatibility and high resistance to scratching. Since dental implants are made with ZO and the genetic effects of ZO on osteoblasts are incompletely understood, we used microRNA microarray techniques to investigate the translation process in osteoblasts exposed to ZO. By using miRNA microarrays containing 329 probes designed from Human miRNA sequences, we identified in osteoblast-like cells line (MG-63) cultured on ZO disks several miRNA whose expression was significantly modified. The most notable regulated genes acting on osteoblasts are: NOG, SHOX, IGF1, BMP1 and FGFR1. The data reported below represent the first study on translation regulation in osteoblasts exposed to zirconium and one in which the effect of ZO on bone formation has been detected.

1 Introduction

Zirconium oxide (ZO) is a bio-inert material that exhibit high mechanical strength, excellent corrosion resistance and good biocompatibility [1]. It is frequently used in manufacture of hip head prostheses [2].

The characteristics of tooth-colour like, the ability to be machined and the low plaque affinity make ZO especially suitable in esthetically important areas of the oral cavity [3, 4].

The biocompatibility and osseointegration of ZO has been largely demonstrated in vitro and in vivo. Many authors observed that ZO has no cytotoxic effects when fibroblasts were co-cultured with it [5, 6]. Additional studies reported that ZO is not able to generate mutations of the cellular genome [7, 8]. No adverse responses were reported following the insertion of ZO into bone or muscle in "in vivo" models [9, 10].

Starting from these points we decided to get more information as regard bone–ZO interaction: the translation process is investigated in osteoblast-like cells exposed to ZO by using miRNA microarray.

MicroRNAs (miRNAs) represent a class of small, functional, non-coding RNAs of 19–23 nucleotide (nt) cleaved from 60- to 110-nt hairpin precursors [11, 12]. Hundreds of miRNAs have been identified in plants and animals. 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 [13] in a post-transcriptional RNA silencing pathway.

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 probes of miRNA sequences for *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Danio rerio*.

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By analyzing 329 Human miRNAs sequences spotted on the array, we compared miRNA expression and consequently gene regulation in Human MG63 cells cultured on zirconium disks versus untreated MG63 cells.

2 Materials and methods

2.1 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 μg/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 contained sterile disks of zirconium oxide with a diameter of 3 cm covering the same area as the reference wells. The chemical composition of the disks primarily showed the presence of ZrO_2 with traces of C, Na, Ca, and Si. After 24 h, the medium (3 ml of MEM with 10% FCS) had changed. More details on zirconium surfaces are available in previously published manuscripts [14–16]. Finally, after 24 h, when the cultures were sub-confluent, the cells were processed for RNA extraction.

2.2 miRNA microarray

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

The NCode™ miRNA Labeling System (Invitrogen) was used for labeling and hybridizing miRNA to microarray, according to the manufacturer's instructions. Briefly, a poly

Table 1 RNAs down- and up-regulated by miRNAs and confirmed by using standard microarray techniques

Up-regulated miRNA miRNA	Down-regulated genes in microarray Gene symbol
mir-337	IGSF1, HTF9C, GJB1, DEAF1, BCKDK, ATPAF2, TMEM8, SLC35A2, MUC1
mir-423	ARHGDIB, ACE, RCN2
mir-497	KCNJ4, IGSF1, CPD, TMEM8, PRKAKA, KIAA0241
mir-214	KIAA0241, KCNJ4, GJB1, FGFR1, AP2A2, TRPM4, SALL2, SHC3, PDE6G
mir-377	DNAJB1, MUC1
mir-296	KDELR1, HTF9C, GPC1, DEAF1, BRD2, MUC1, PRKAKA
mir-99b	SLC35A2
mir-193b	IGFBP6, HTF9C, SYN1, SUPT5H, OAZ2, MUC1
mir-25	RAD21, COL4A6, ACOX1, KIAA1109, FBXW7
mir-324	KIAA0241, KCNJ4, FGFR1, DEAF1, BRD2, BMIP1, BCKDK, PDE6G, OAZ2
mir-518a	MAP4, KIAA1109, IDS, GJB1, GATM, FGFR1CXCL12, CNNM2, CDC2L2, SLC35A2
mir-320	IGSF1, DAB2, WATP, SUPT5H, SLC35A2, RAD21, PLEK, OAZ2, MUC1
mir-23b	KIAA1109, IL6R, CNNM2, WTAP, TIMM10, ST7L, SALL2
mir-93	CNNM2, BNIP1, ARS2, PLAC1
mir-23a	KIAA1109, IL6R, GJB1, COL4A6, CNNM2, WTAP, TIMM10, ST7L, SALL2
mir-422b	KCNJ4, BNIP1, BHLHB3, PDE6G, MUC1
mir-330	IDS, FGFR1, ELF1, CDC2L2, OAZ2
mir-197	MPZL1, MLT10, IGFBP6, HTF9C, FBXW7, CNNM2, AMPD2, SYN2
Down-regulated miRNA	Up-regulated genes in microarray
mir-302c	IL8
mir-369 5p	ACAD8
mir-10b	–

Some of these mRNAs are different from those in the previous table because the slides for the messenger microarray techniques did not carry all previously listed genes

(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 were hybridized to the microarray and incubated overnight.

After an incubation of 18–20 h, the array was washed and hybridized with Alexa Fluor® 3 capture reagents (for the control) and Alexa Fluor® 5 capture reagents (for the treated cells). After another wash the array was scanned using a standard microarray scanner (Axon Instruments, Sunnyvale, CA).

After scanning, each spot was identified by means of the 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 the local background from the total intensities. The data were normalized by using DNMAD and Preprocessing (http://gepas.bioinfo.cipf.es/cgi-bin/tools) [17, 18]. 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) [19].

For target predictions and validations the miRNAs 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 uses 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 in assigning the most appropriate Gene Ontology term to each cluster of regulated genes.

2.3 Data verification by means messengers microarray technique

To verify the effect of miRNAs on messengers a subsequent experiment with conventional mRNA microarray technique was performed. The method was the same as that used in previous studies [20–22]. Briefly, messenger RNA extraction was performed by MG63 cells treated in the same experimental conditions used for miRNAs. Then the cDNA was synthesized from the mRNA and indirect cDNA labeling performed. 20 K human DNA microarrays slides (MWG Biotech AG, Ebersberg, Germany) were used. The experiment was repeated twice and the dyes then switched. A GenePix 4000a DNA microarray scanner was used and the SAM program was then performed. Finally, the results obtained from the miRNAs and mRNAs were matched and a list of genes is reported in Table 1.

3 Results

The hybridization of miRNAs (derived from MG63 cultured on zirconium disks) to the sequences spotted on the slide allowed us to perform a systemic analysis of microRNAs and to provide primary information as regards the regulation of the translation process induced by ZrO_2. There were 18 up-regulated miRNA (i.e. mir-337, mir-423, mir-497, mir-214, mir-377, mir-296, mir-99b, mir-193b,

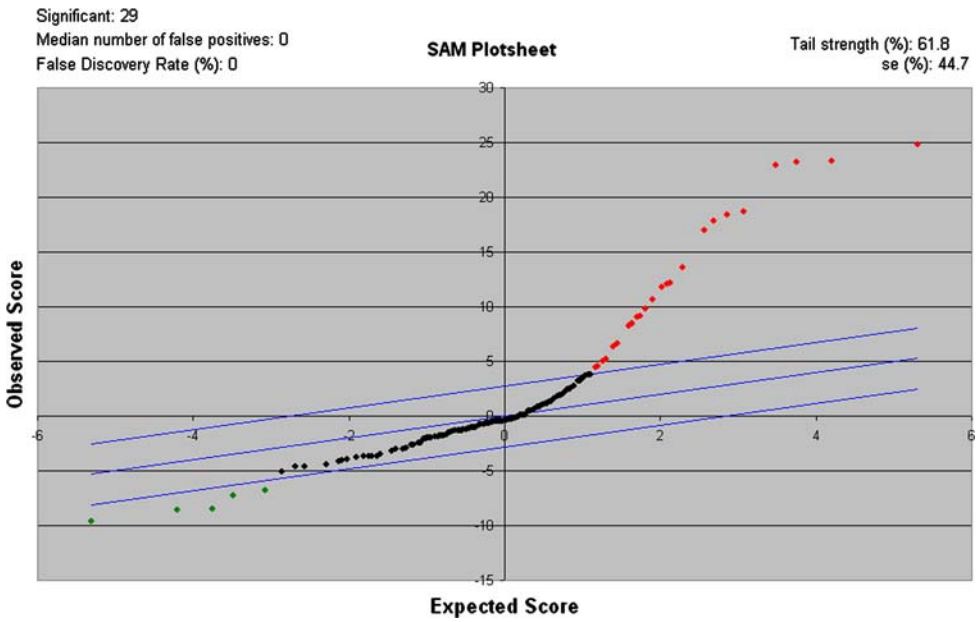

Fig. 1 SAM (significance analysis of microarray) plot of MG63 cultured for 24 h on zirconium disks

Table 2 Down- and up-regulated genes

miRNA	Biological function	Genes
Up	Skeletal development	CHRD, COMP, ADAMTS4, TCOF1, SHOX, FGFR1, IGF1, GHRHR , PMF1, FGFR3
	Bone formation	BMP1, AHSG, AMBN
	Cartilage development	NOG, MATN1
Down	Skeletal development	TRAPPC2

mir-25, mir-324, mir-518a, mir-320, mir-23b, mir-93, mir-23a, mir-422b, mir-330, mir-197) and 3 down-regulated miRNA (i.e. mir-302c, mir-369 5p, mir-10b) for FDR (false discovery rate) = 0 and score > 8. Figure 1 is the graphical output of SAM (Statistical Analysis for Microarray) and it shows differentially expressed miRNA. Since miRNA potentially regulates thousands of genes, in this study we selected only those genes related to osteogenesis and bone remodeling and regulated in the same way by approximately two independent miRNAs (Table 2). Genes with the opposite type of regulation were excluded.

4 Discussion

ZO is a bioactive material with an high affinity for bone tissue [9, 14] and excellent biocompatibility [1–3]. Its mechanical properties and its color so similar to tooth color make this material suitable for dental implants [1].

Biological, mechanical, and clinical studies published to date indicate that ZO implants are both well tolerated and sufficiently resistant [9, 10, 23]. Oliva et al. [24] evaluated the success rate of 100 zirconia dental implants with two different rough surfaces. After 1 year of follow-up zirconia dental implants demonstrated to be a viable alternative for tooth replacement.

ZO creates less flogistic reaction in tissue than other restorative materials such as titanium [25]. This datum was confirmed by a study comparing soft tissue around zirconia and titanium healing caps: inflammatory infiltrate, microvessel density, and vascular endothelial growth factor expression were higher around the titanium caps than around the zirconia ones [26].

ZO is not cytotoxic [27–29]. In a study conducted by Covacci et al. [8] the potential toxicity of ZO and the cell capability to grow and adhere upon the ceramic surfaces were tested, while mutation and transformation assays were performed after detachment of cells from the disks. Results confirm the very good ZO cytocompatibility and show that this ceramic do not have mutagenic and carcinogenic effects on cells.

Osteoblast-like cell cultured with pure zirconia ceramics show a continuous cellular growth, alkaline phosphatase activity and mRNA expression of Cycline D1, integrin beta 1, osteonectin (ON) and beta-actin demonstrating the good biocompatibility of ZO [30]. Because the mechanism by which ZO acts on osteoblasts is poorly understood, we therefore attempted to get more informations on the translation regulation by analyzing differences in microRNAs expressed in osteoblast-like cells (i.e. MG63) cultured on zirconium disks versus untreated cells.

MicroRNAs (miRNAs) are a recently discovered class of small, ~19–23-nucleotide non-coding RNA molecules. They are cleaved from 70 to 110-nucleotide hairpin precursors and play an important role in the post-transcriptional regulatory process. MicroRNAs are not translated into proteins: instead, they regulate the expression of other genes by either cleaving or repressing the translation of their messenger RNA (mRNA) targets.

Recent advances in spotted oligonucleotide microarray labeling and detection have enabled the use of this high-throughout technology for miRNA screening.

Microarray is a molecular technology that enables the analysis in parallel of a very large number of DNA or RNA fragments, spanning a significant fraction of the human genome. Gene expression is performed by a process of (i) miRNA extraction, (ii) labeling (different dyes are used for reference untreated cells and investigated cells—i.e. cultured on zirconium disks), (iii) and hybridization on slides containing miRNA probes. Then the slides are scanned with a laser system, and two false color images are generated for each hybridization with miRNA from the investigated and reference cells. The overall result is the generation of a so-called genetic portrait. It corresponds to up or down-regulated miRNA in the investigated cell system.

Hybridization of miRNA derived form MG63 cultured on zirconium disks to the sequences spotted on the slide allowed us to perform a systemic analysis of miRNAs and to provide primary information as regards the regulation of translation induced by zirconium (Table 2).

The vast majority of detected genes are down-regulated and among them are some homebox genes (i.e. genes that regulate the morphogenesis of entire segments of the body). Noggin or NOG 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 [31]. SHOX (Short stature HOmeoboX-containing gene) is involved in idiopathic growth retardation and in the short stature phenotype of Turner syndrome patients [32].

Another group of down-regulated genes are hormones, receptors and cytokines. IGF1 (or insulin-like growth factors) mediates many of the growth-promoting effects of growth hormone [33]. Interestingly, also GHRHR is down-regulated. GHRHR, expressed in the pituitary, is a receptor for growth hormone-releasing hormone. The binding of this hormone to the receptor leads to the synthesis and release of growth hormone.

BMP1 or procollagen C proteinase is a secreted metalloprotease requiring calcium and needed for cartilage and bone formation [34]. Bone related receptors are FGFR1 and 3 which are FGFR family members. The interaction between receptors and fibroblast growth factors sets in motion a cascade of downstream signals, ultimately influencing mitogenesis and differentiation. FGFR1 is involved in limb induction, whereas FGFR3 plays a role in bone development and maintenance [35].

Finally, COMP is a noncollagenous extracellular matrix protein and its mutations cause osteochondrodysplasias pseudochondroplasia and multiple epiphyseal dysplasia [36].

The genes discussed above are only a limited number among those differentially regulated by miRNA reported in Table 2. We briefly analyzed some of those with better known functions and directly related to bone formation, skeletal development, cartilage remodeling and bone production. In addition, the fact that several genes related to bone formation have a translational negative control can be related to the early stage in which they were analyzed (i.e. MG63 cultured for 24 h until they were sub-confluent). This phase is characterized by elevated cellular kinetics and low differentiation and extracellular matrix production.

It is worth noting that MG63 is a cell line and not a normal osteoblast. Notwithstanding this, the advantages of using one cell line are related to the fact that the reproducibility of the data is higher due to the lack of variability in 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 because early events in implant-bone interaction are critical for osseointegration. In addition, this time point raises additional relevance if one considers that immediate loading is presently the main topic in implantology. In conclusion, the global effect of ZOC on bone formation was detected and the reported data could represent a model for comparing different substances with similar effects.

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