Journal of Oral Science, Vol. 49, No. 4, 299-309, 2007

Original

Genetic effects of a titanium surface on osteoblasts: a meta-analysis

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(Received 20 August and accepted 1 November 2007)

Abstract: Titanium is used worldwide to make osseointegrable devices. The favorable characteristics that make this material desirable for implantation are i) its mechanical proprieties and ii) its biocompatibility. The latter has been demonstrated by *in vivo* studies with animal models and clinical trials over a forty-year period. However, the exact genetic effect of the titanium layer on cells is still not well characterized. To detect the genetic effect of titanium surfacing on osteoblasts without porosity as a confounding variable, a meta-analysis of genetic expression profiling obtained from several titanium surfaces was performed. One hundred seventy-three genes were selected, 84 up-regulated and 95 down-regulated. Among them, the most notable were genes that participate in osteoblast differentiation and bone regeneration, such as TIMP1, PTN, and RUNX1. Titanium surfacing has i) a direct genetic effect on osteoblasts, ii) induces several bone-related genes, and iii) is relevant to the osseointegration process. These properties should be taken into consideration in separating the effect of porosity (i.e. the micro-dimension) from implant coating (i.e. the nano-dimension). (J. Oral Sci. 49, 299-309, 2007)

Keywords: biocompatibility; bone; cell culture; osseointegration; surface modification; titanium.

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Introduction

Titanium and its alloys are employed as implant materials because of their desirable mechanical properties and "biocompatibility" (1). For decades, oral, maxillofacial and orthopaedic surgeons have utilised dental implants, screws and plates, and prostheses to substitute lost teeth, fix bone fragments, and replace joints, respectively. Also, many surgical instruments, such as drills and saws, are made with titanium alloys. The biocompatibility of titanium has been demonstrated by in vivo studies with both animal models and clinical trials, for more than forty years (2,3).

Prostheses are characterized my macro-, mini-, micro- and nano-design. Macro-design is the shape of the prosthesis: some examples include the cylindrical or root form of dental implants. Mini-design is the dimension of the threads or the shape of the neck of the dental fixture. The dimensions range from 1 to 0.1 mm. Micro-design is the shape of the implant surface, as exemplified by the "grooves and holes" resulting from surface treatments such as machination, acid etching and sand-blasting. These treatments determine the roughness of the surface and the "holes" have a cellular dimension. Nano-design is determined by the molecular composition of the surfaces, such as those composed of hydroxyapatite, zirconium and titanium. Usually, mechanical proprieties are related to macro- and mini-design, whereas biological properties are related to micro- and nano-design. Mechanical properties are responsible for primary implant stability, whereas biological properties are important in the osseointegration process (4).

When titanium is exposed to oxygen, a stochastic distribution of three titanium-oxide isoforms (i.e. rutile,

brookite and anatase) occurs on the surface of the prosthesis. These isoforms are responsible for the material biological properties (4).

Although previous studies have demonstrated that titanium surfacing is capable of modulating osteoblast gene expression (5-7), they failed to separate the effect of roughness (i.e. the micro-dimension) from that of composition (i.e. the nano-dimension). The present study investigated the effect of the titanium layer on osteoblast gene expression (5-7) independently from porosity. For this purpose, a meta-analysis was performed by using implant surfaces with various surface textures.

Materials and Methods

The data set was derived from previously published studies (5-7) in which osteoblast-like cells were cultured on different titanium surfaces.

Cell culture

Osteoblast-like cells (MG63) were cultured in sterile Falcon wells using Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) (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. One set of wells contained sterile 3-cm-diameter circular metal disks of Ti6Al4V alloy covering an area of 35 cm^2. The surface roughness (SR) was 0.77 ± 0.28 for the machined disks. A second set was composed of implants blasted with tricalcium phosphate and then subjected to light nitric acid treatment to remove excess blast material and pacify the surface (BioLok International, Inc., Deerfield Beach, FL, USA). The SR was 1.93 ± 0.37 for the BioLok implants. A third set contained disks with a different surface (nanoPORE, Out-Link, Sweden & Martina, Due Carrare, Padova, Italy) covering the same area (35 cm^2). The SR was 1.05 ± 0.34 for the nanoPORE disks. A fourth set contained titanium disks with a titanium pull spray superficial – TPSS surface (Oralplant, Cordenons, PN, Italy). The SR values were 2.74 for the TPSS surface. Finally, a fifth set comprised only medium and cells. After 24 h, the cultures reached sub-confluence, and cells from all specimens were processed for RNA extraction.

DNA microarray slide preparation and screening

Ten micrograms of total RNA was extracted from each cell sample using RNAzol. cDNA was then synthesized using Superscript II (Invitrogen, Life Technologies, Milan, Italy) and amino-allyl dUTP (Sigma Chemical Co.). Mono-reactive Cy3 and Cy5 esters (Amersham Pharmacia, Little Chalfont, UK) were used for indirect cDNA labeling. Untreated cell RNA was labeled with Cy3 and used as a control against the Cy5-labeled treated cDNA in the first experiment, and then switched. Human 19.2 K DNA microarrays were used (Ontario Cancer Institute). For 19.2 K slides, 100 µl of the sample and pool control cDNAs in DIG Easy hybridization solution (Roche, Basel, Switzerland) were used for sandwich hybridization of the two slides constituting the 19.2. K set at 37°C overnight. Washing was performed three times for 10 min with 1 × SSC, 0.1% SDS at 42°C, and three times for 5 min with 0.1 × SSC at room temperature. Slides were dried by centrifugation for 2 min at 2,000 rpm. The experiment was repeated twice for each sample and the dyes were then switched. A GenePix 4000a DNA microarray scanner (Axon, Union City, CA, USA) was used to scan the slides, and the data were extracted with GenePix Pro. After removing the local background, genes with expression levels of less than 1,000 were not included in the analysis, since ratios are not reliable at that detection level (5-7).

DNA microarray statistical analysis

After scanning the slides containing the 19,200 human genes in duplicate, data analysis was performed using customized R language-based script (8) employing the Bioconductor packages (9) for quality control analysis, data normalization, hierarchical clusters and identification of differentially expressed transcripts. Specifically, the *arrayQuality* package performed array quality on spotted arrays, and the *limma* (Linear Models for Microarray Analysis) and *marray* packages were used together for chip normalization and background correction. These Bioconductor packages provide alternative functions for reading and normalizing spotted microarray data. The *marray* package provides flexible location and scale normalization routines for log-ratios from two-colour arrays. The *limma* package overlaps with *marray* in functionality but is based on a more general separation between within-array and between-array normalization. We chose the *rma* method to correct the background, the *robust spline* and *quantile* options to normalize within and between arrays, and the *vsn* package from the calibration and transformation of the probe intensities to evaluate the between-group variability. The *genefilter* package was used to separate genes with high variance according to the interquartile range method (IQR). The unsupervised two-way (genes against samples) hierarchical clustering method using about 3,000 EST (Expressed Sequence Tag) of our data set, previously filtered, was used to test the internal consistency, to explore the relationship

between samples and underlying features of gene expression, and to check if the individual samples clustered together according to their features. The unsupervised cluster analysis was followed by Significant Analysis of Microarrys (SAM) (10). We used the SAM one-class option to identify genes that were distributed between two categories (titanium vs controls). One-class analyses use a modified t-statistic. Welch's t-statistic (unequal variance) or the t-statistic assuming equal variances can be computed. The SAM analysis was performed using the R package "samr" as described previously (10). The list of ranked genes obtained was 78 up-regulated and 95 down-regulated genes (delta value 1.098, false discovery rate = FDR 0.016, Tables 1 and 2).

Another biological question regarding genes, gene product interactions or biological processes played by networks of components, was exploited by using the web application tool FatiGO (11) (http://www.babelomics.org.) and DAVID (12) (http://david.abcc.ncifcrf.gov/). FatiGO is a tool for finding significant differences in the distribution of GO terms between groups of genes, which tend to be evidence of the common function, gene co-expression, functional properties and biological roles of genes and gene-product interaction. In brief, FatiGO takes two lists of genes and converts them into two lists of GO terms using the corresponding gene-GO association table. The program implements Fisher's exact test for 2×2 contingency tables for comparison of two groups of genes and extracts a list of GO terms whose distribution among the groups is significantly different. The results of the test are corrected for multiple testing to obtain an adjusted *P*-value based on three different ways of accounting for multiple testing. The DAVID tool suite mainly provides typical batch annotation and gene-GO term enrichment analysis to highlight the most relevant GO terms associated with a given gene list. Functional Annotation Clustering, a feature of the DAVID Functional Annotation Tool, uses an algorithm to measure the relationship between the annotation terms based on the degree of their co-association genes to group the similar, redundant, and heterogeneous annotation contents from the same or different resources into annotation groups.

PCR Validation

Validation was performed in each single study (5-7).

Results

The meta-analysis combines the result of several studies and provides information on transcriptional changes associated with titanium-osteoblast interactions independently from surface texture. Several genes were identified whose expression was definitely up- or down-regulated.

Up-regulated genes (Table 1)

Some of the genes found to be up-regulated are involved in cell communication, such as PLCG2 and EPHA7. PLCG2 is an enzyme of the phospholipase C family. EPHA7 belongs to the ephrin receptor subfamily of the protein-tyrosine kinase family. Ephrins regulate a variety of developmental processes; in particular EPHA7 has a potential role in tooth formation (13).

Other up-regulated genes are involved in cell cycle regulation, such as RASSF2 encoding a protein containing a Ras association domain, MAPK4 a protein kinase, and PPM1B a protein phosphatase known to be a negative regulator of cell stress response pathways. Over-expression of this phosphatase is reported to cause cell-growth arrest or cell death (14).

WDR26 is a member of the WD repeat protein family, involved in a variety of cellular processes, including cell cycle progression, signal transduction, apoptosis, and gene regulation. TIMP1, a metallopeptidase inhibitor, is a very interesting gene expressed in human cementum and dentine (15) TIMP1 overexpression is involved in the regulation of bone remodelling in normal developing human bone. In combination with TIMP2 are major serum factors that stimulate the induction of TIMP-1 mRNA in quiescent human gingival fibroblasts (16). Pleiotrophin (PTN) is an extracellular matrix protein. The overexpression of PTN regulates bone morphogenetic protein (BMP)-induced ectopic osteogenesis (17), and in transgenic mice leads to increased bone formation (18). PTN play an important role as an osteoblast-stimulating factor (19). It has multiple effects on bone formation and its effects are dependent on its concentration and the timing of its expression.

MSX1 is a member of the muscle segment homeobox gene family that has roles in limb-pattern formation, craniofacial development – particularly odontogenesis – and tumor growth inhibition.

Interesting up-regulated genes are molecules involved in cell adhesion, such as tenascin TNC and TNXB (which are two extracellular matrix proteins), ZFHX1B (a transcriptional repressor involved in the TGFbeta signaling pathway) and TRPM7.

Down-regulated genes (Table 2)

The most notable down-regulated gene was RUNX1, which is essential for hematopoiesis, and contains RUNX binding sites in its promoter region, suggesting a possible cross-regulation with RUNX2 and potential regulatory roles in bone development. It has been shown that both

Table 1 Up-regulated genes

Up-regulated genes (84)			
ID	**Name**	**logFC**	***P* value**
TIMP1	TIMP METALLOPEPTIDASE INHIBITOR 1	5.417615	0.009397
SERPINB6	SERPIN PEPTIDASE INHIBITOR, CLADE B (OVALBUMIN), MEMBER 6	5.07146	0.003139
PPM1B	PROTEIN PHOSPHATASE 1B MAGNESIUM-DEPENDENT, B ISOFORM	4.850225	3.17e-05
TOP1	TOPOISOMERASE (DNA) I	4.531555	0.006914
UTX	UBIQUITOUSLY TRANSCRIBED TETRATRICOPEPTIDE REPEAT	3.638635	0.003826
ZNF516	ZINC FINGER PROTEIN 516	3.557045	0.001381
PHGDH	PHOSPHOGLYCERATE DEHYDROGENASE	3.503755	0.001813
RASSF2	RAS ASSOCIATION (RALGDS/AF-6) DOMAIN FAMILY 2	3.49974	2.67e-05
SLC29A1	SOLUTE CARRIER FAMILY 29 (NUCLEOSIDE TRANSPORTERS), MEMBER 1	3.30619	0.000739
SRM	SPERMIDINE SYNTHASE	3.28561	0.002733
RBM34	RNA BINDING MOTIF PROTEIN 34	3.19493	0.006898
ADRA2C	ADRENERGIC, ALPHA-2C-, RECEPTOR	3.1525	0.001942
SDC2	SYNDECAN 2	3.13583	0.004286
TRPM7	TRANSIENT RECEPTOR POTENTIAL CATION CHANNEL	3.11311	0.0066
OSBPL8	OXYSTEROL BINDING PROTEIN-LIKE 8	3.099305	0.006584
ADRA2C	ADRENERGIC, ALPHA-2C-, RECEPTOR	3.054575	0.000987
MGAT2	MANNOSYL (ALPHA-1,6-)-GLYCOPROTEIN BETA-1,2-N-ACETYLGLUCOSAMINYLTRANSFERASE	2.91397	0.007355
STAT6	SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 6, INTERLEUKIN-4 INDUCED	2.900315	0.001458
C5orf32	CHROMOSOME 5 OPEN READING FRAME 32	2.869305	0.002758
HERC3	HECT DOMAIN AND RLD 3	2.854395	0.004891
B4GALT1	UDP-GAL:BETAGLCNAC BETA 1,4- GALACTOSYLTRANSFERASE, POLYPEPTIDE 1	2.83587	0.007555
SRRM1	SERINE/ARGININE REPETITIVE MATRIX 1	2.740745	0.002378
SNTG1	SYNTROPHIN, GAMMA 1	2.72913	0.006646
OSBPL8	OXYSTEROL BINDING PROTEIN-LIKE 8	2.717815	0.000702
IGHA1	IMMUNOGLOBULIN HEAVY CONSTANT ALPHA 1	2.71197	0.002661
SRM	SPERMIDINE SYNTHASE	2.696045	0.002005
DAPK3	DEATH-ASSOCIATED PROTEIN KINASE 3	2.684485	0.002231
HERC3	HECT DOMAIN AND RLD 3	2.62453	0.002702
MAPK4	MITOGEN-ACTIVATED PROTEIN KINASE 4	2.584045	9.75e-05
C5orf3	CHROMOSOME 5 OPEN READING FRAME 3	2.58241	0.002624
TNC	TENASCIN C (HEXABRACHION)	2.564835	0.005904
EPHA7	EPH RECEPTOR A7	2.48622	1.85e-05
AKAP8L	A KINASE (PRKA) ANCHOR PROTEIN 8-LIKE	2.377685	0.000631
SERPINF2	SERPIN PEPTIDASE INHIBITOR, CLADE F	2.36245	0.005531
WT1	WILMS TUMOR 1	2.34614	0.002189
FBXO46	F-BOX PROTEIN 46	2.334265	0.00537
CHAF1A	CHROMATIN ASSEMBLY FACTOR 1, SUBUNIT A (P150)	2.31692	0.001385
DNM1L	DYNAMIN 1-LIKE	2.2738	0.005383
RBM34	RNA BINDING MOTIF PROTEIN 34	2.25862	0.004685
TXNDC13	THIOREDOXIN DOMAIN CONTAINING 13	2.247395	0.002724
HSD11B1	HYDROXYSTEROID (11-BETA) DEHYDROGENASE 1	2.187985	0.009112
AKAP8L	A KINASE (PRKA) ANCHOR PROTEIN 8-LIKE	2.17055	0.001053
EIF2B1	EUKARYOTIC TRANSLATION INITIATION FACTOR 2B, SUBUNIT 1 ALPHA, 26KDA	2.137495	0.00633
C7orf49	CHROMOSOME 7 OPEN READING FRAME 49	2.1274	0.008271
TNS1	TENSIN 1	2.117205	0.003253
BAT2	HLA-B ASSOCIATED TRANSCRIPT 2	2.09537	0.00484
FLJ12529	PRE-MRNA CLEAVAGE FACTOR I, 59 KDA SUBUNIT	2.087675	0.004307

C8orf42	CHROMOSOME 8 OPEN READING FRAME 42	2.02265	0.008999
SYNE2	SPECTRIN REPEAT CONTAINING, NUCLEAR ENVELOPE 2	1.991205	0.008695
DOCK2	DEDICATOR OF CYTOKINESIS 2	1.98719	0.00595
TNFRSF1A	TUMOR NECROSIS FACTOR RECEPTOR SUPERFAMILY, MEMBER 1A	1.97418	0.008295
PTN	PLEIOTROPHIN (HEPARIN BINDING GROWTH FACTOR 8, NEURITE GROWTH-PROMOTING FACTOR 1)	1.95861	0.008544
TBRG1	TRANSFORMING GROWTH FACTOR BETA REGULATOR 1	1.950275	0.006328
DUSP12	DUAL SPECIFICITY PHOSPHATASE 12	1.94335	0.00524
C7orf49	CHROMOSOME 7 OPEN READING FRAME 49	1.90811	0.00845
C2orf40	CHROMOSOME 2 OPEN READING FRAME 40	1.897465	0.00423
TRIM39	TRIPARTITE MOTIF-CONTAINING 39	1.89442	0.005319
ZFHX1B	ZINC FINGER HOMEOBOX 1B	1.891785	0.004907
CRHBP	CORTICOTROPIN RELEASING HORMONE BINDING PROTEIN	1.86726	0.003855
MSX1	MSH HOMEOBOX 1	1.86138	0.005807
TNXB	TENASCIN XB	1.8267	0.007472
MORC3	MORC FAMILY CW-TYPE ZINC FINGER 3	1.82291	0.005722
FBXO33	F-BOX PROTEIN 33	1.822175	0.00567
PHGDH	PHOSPHOGLYCERATE DEHYDROGENASE	1.81096	0.00894
ELF1	E74-LIKE FACTOR 1 (ETS DOMAIN TRANSCRIPTION FACTOR)	1.809235	0.008751
HSPA1A	HEAT SHOCK 70KDA PROTEIN 1A	1.80841	0.007735
ZFHX1B	ZINC FINGER HOMEOBOX 1B	1.785345	0.00847
EPN2	EPSIN 2	1.78051	0.006089
COX15	COX15 HOMOLOG, CYTOCHROME C OXIDASE ASSEMBLY PROTEIN (YEAST)	1.73406	0.008474
FBXL7	F-BOX AND LEUCINE-RICH REPEAT PROTEIN 7	1.71686	0.00738
MLLT3	MYELOID/LYMPHOID OR MIXED-LINEAGE LEUKEMIA (TRITHORAX HOMOLOG, DROSOPHILA); TRANSLOCATED TO, 3	1.70554	0.00455
HP1BP3	HETEROCHROMATIN PROTEIN 1, BINDING PROTEIN 3	1.68755	0.005487
PLCG2	PHOSPHOLIPASE C, GAMMA 2 (PHOSPHATIDYLINOSITOL-SPECIFIC)	1.680165	1.18e-05
RBBP9	RETINOBLASTOMA BINDING PROTEIN 9	1.66329	0.008802
CLN5	CEROID-LIPOFUSCINOSIS, NEURONAL 5	1.62409	0.007194
LOC541471	HYPOTHETICAL LOC541471	1.60204	0.006121
SNAI2	SNAIL HOMOLOG 2 (DROSOPHILA)	1.600015	0.009477
TTC23	TETRATRICOPEPTIDE REPEAT DOMAIN 23	1.58859	0.005047
PLEKHA4	PLECKSTRIN HOMOLOGY DOMAIN CONTAINING, FAMILY A (PHOSPHOINOSITIDE BINDING SPECIFIC) MEMBER 4	1.57538	0.006385
USF2	UPSTREAM TRANSCRIPTION FACTOR 2, C-FOS INTERACTING	1.539145	0.00927
WDR26	WD REPEAT DOMAIN 26	1.52026	5.68e-05
SERPINB5	SERPIN PEPTIDASE INHIBITOR	1.52009	0.006913
TRIM39	TRIPARTITE MOTIF-CONTAINING 39	1.349445	0.009265

RUNX1 and RUNX2 are expressed in pre-chondrogenic mesenchyme of the developing embryo (20). Smith et al. (21) demonstrated that RUNX1 and RUNX2 are expressed at different stages of skeletal development, with a possible role for RUNX1 in mediating early events of endochondral and intramembranous bone formation, while RUNX2 is a potent inducer of the late stages of chondrocyte and osteoblast differentiation.

GO and functional analysis

The largest number of significantly and differentially expressed (222 overespressed and 268 down-regulated) genes were selected for analysis by this procedure using FatiGO. As a result of this approach, we found significantly different terms, including a group of genes that were directly or indirectly related to osteoblast differentiation and bone regeneration. The more representative terms found were, *tissue remodelling* (the reorganization or

Table 2 Down-regulated genes

Down-regulated genes (95)			
ID	**Name**	**logFC**	***P* value**
WWC2	WW AND C2 DOMAIN CONTAINING 2	-17.3048	0.005626
ZFPM2	ZINC FINGER PROTEIN, MULTITYPE 2	-12.5233	0.001607
CYP2C8	CYTOCHROME P450, FAMILY 2, SUBFAMILY C,POLYPEPTIDE 8	-8.2086	0.004695
UBD	UBIQUITIN D	-7.99515	0.001513
C2orf27	CHROMOSOME 2 OPEN READING FRAME 27	-7.65285	0.004228
ZER1	ZER-1 HOMOLOG (C. ELEGANS)	-6.70495	0.001164
TESC	TESCALCIN	-6.44225	0.005956
CCDC52	COILED-COIL DOMAIN CONTAINING 52	-6.1456	0.000427
BEAN	BRAIN EXPRESSED, ASSOCIATED WITH NEDD4	-6.1283	0.003784
KDELR1	KDEL (LYS-ASP-GLU-LEU) ENDOPLASMIC RETICULUM PROTEIN RETENTION RECEPTOR 1	-5.6492	0.002604
TESC	TESCALCIN	-5.40945	0.006521
DUSP22	DUAL SPECIFICITY PHOSPHATASE 22	-5.28715	0.00962
ZER1	ZER-1 HOMOLOG (C. ELEGANS)	-5.25575	0.000362
GFM2	G ELONGATION FACTOR, MITOCHONDRIAL 2	-4.89635	0.001937
CLIC5	CHLORIDE INTRACELLULAR CHANNEL 5	-4.6089	0.003934
PKD2	POLYCYSTIC KIDNEY DISEASE 2 (AUTOSOMAL DOMINANT)	-4.2829	0.003954
TP53I11	TUMOR PROTEIN P53 INDUCIBLE PROTEIN 11	-4.26185	0.005698
SLIT3	SLIT HOMOLOG 3 (DROSOPHILA)	-4.2403	0.006122
TRAPPC6B	TRAFFICKING PROTEIN PARTICLE COMPLEX 6B	-4.10335	0.002901
GMFB	GLIA MATURATION FACTOR, BETA	-4.05825	0.009415
ZNF776	ZINC FINGER PROTEIN 776	-4.05445	0.004782
ADA	ADENOSINE DEAMINASE	-3.95985	0.004268
LOC92270	HYPOTHETICAL PROTEIN LOC92270	-3.9506	0.000421
BEAN	BRAIN EXPRESSED, ASSOCIATED WITH NEDD4	-3.8775	0.003488
BCHE	BUTYRYLCHOLINESTERASE	-3.83025	0.003544
GALNT5	UDP-N-ACETYL-ALPHA-D GALACTOSAMINE:POLYPEPT. N-ACETYLGALACTOSAMINYLTRANSFERASE 5	-3.8199	0.004283
AMOTL2	ANGIOMOTIN LIKE 2	-3.81125	0.006059
HMGCLL1	3-HYDROXYMETHYL-3-METHYLGLUTARYL-COENZYME A LYASE-LIKE 1	-3.8003	0.000456
PIAS1	PROTEIN INHIBITOR OF ACTIVATED STAT, 1	-3.72315	0.005224
MEG8	MATERNALLY EXPRESSED (IN CALLIPYGE) 8	-3.72305	0.005796
ADA	ADENOSINE DEAMINASE	-3.697	0.002522
EDNRB	ENDOTHELIN RECEPTOR TYPE B	-3.67965	0.009807
NOL1	NUCLEOLAR PROTEIN 1, 120KDA	-3.67605	0.005302
EBF3	EARLY B-CELL FACTOR 3	-3.67535	0.009403
BAT3	HLA-B ASSOCIATED TRANSCRIPT 3	-3.6527	0.003942
ATP2B2	ATPASE, CA++ TRANSPORTING, PLASMA MEMBRANE 2	-3.5347	0.005642
FLJ10159	HYPOTHETICAL PROTEIN FLJ10159	-3.5119	0.007467
BCAR3	BREAST CANCER ANTI-ESTROGEN RESISTANCE 3	-3.41045	0.00929
GNAO1	GUANINE NUCLEOTIDE BINDING PROTEIN (G PROTEIN)	-3.40345	0.004128
ACSL6	ACYL-COA SYNTHETASE LONG-CHAIN FAMIL	-3.36525	0.004946
CSMD3	CUB AND SUSHI MULTIPLE DOMAINS 3	-3.326	0.002872
SGIP1	SH3-DOMAIN GRB2-LIKE (ENDOPHILIN) INTERACTING PROTEIN 1	-3.2702	0.004617
KCNIP3	KV CHANNEL INTERACTING PROTEIN 3, CALSENILIN	-3.25595	0.002127
ADCK5	AARF DOMAIN CONTAINING KINASE 5	-3.15875	0.007517
FOS	V-FOS FBJ MURINE OSTEOSARCOMA VIRAL ONCOGENE HOMOLOG	-3.14605	0.005441
MAP3K5	MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE 5	-3.02065	0.001523
CCDC43	COILED-COIL DOMAIN CONTAINING 43	-2.97805	0.00311

MTDH	METADHERIN	-2.966	0.008365
GPR108	G PROTEIN-COUPLED RECEPTOR 108	-2.9649	0.007156
TAS2R14	TASTE RECEPTOR, TYPE 2, MEMBER 14	-2.9415	0.004592
ZBTB20	ZINC FINGER AND BTB DOMAIN CONTAINING 20	-2.93525	0.00624
MIER1	MESODERM INDUCTION EARLY RESPONSE 1 HOMOLOG (XENOPUS LAEVIS)	-2.93205	0.004753
CEBPZ	CCAAT/ENHANCER BINDING PROTEIN ZETA	-2.9298	0.003824
ZNF276	ZINC FINGER PROTEIN 276	-2.92435	0.000652
USP12	UBIQUITIN SPECIFIC PEPTIDASE 12	-2.81995	0.003695
C18orf17	CHROMOSOME 18 OPEN READING FRAME 17	-2.7684	0.009018
CLIP4	CAP-GLY DOMAIN CONTAINING LINKER PROTEIN FAMILY, MEMBER 4	-2.7134	0.005662
DGKG	DIACYLGLYCEROL KINASE, GAMMA 90KDA	-2.669	0.002463
N4BP1	NEDD4 BINDING PROTEIN 1	-2.62205	0.005761
DDB2	DAMAGE-SPECIFIC DNA BINDING PROTEIN 2, 48KDA	-2.60645	0.006808
PDK2	PYRUVATE DEHYDROGENASE KINASE, ISOZYME 2	-2.57495	0.003518
PGM5	PHOSPHOGLUCOMUTASE 5	-2.5714	0.009786
LHFPL4	LIPOMA HMGIC FUSION PARTNER-LIKE 4	-2.55355	0.006018
TM2D1	TM2 DOMAIN CONTAINING 1	-2.51575	0.006927
DEPDC1B	DEP DOMAIN CONTAINING 1B	-2.454	0.007616
RYR1	RYANODINE RECEPTOR 1 (SKELETAL)	-2.4084	0.003057
BCAR3	BREAST CANCER ANTI-ESTROGEN RESISTANCE 3	-2.38555	0.007147
PRKACA	PROTEIN KINASE, CAMP-DEPENDENT, CATALYTIC, ALPHA	-2.37805	0.005232
CSMD3	CUB AND SUSHI MULTIPLE DOMAINS 3	-2.3711	0.006228
CRSP6	COFACTOR REQUIRED FOR SP1 TRANSCRIPTIONAL ACTIVATION, SUBUNIT 6, 77KDA	-2.3588	0.00274
KIAA0649	KIAA0649	-2.3483	0.00399
LYRM1	LYR MOTIF CONTAINING 1	-2.2995	0.009018
MGC12760	HYPOTHETICAL PROTEIN MGC12760	-2.2995	0.004982
REV3L	REV3-LIKE, CATALYTIC SUBUNIT OF DNA POLYMERASE ZETA (YEAST)	-2.2192	0.004827
RUNX1T1	RUNT-RELATED TRANSCRIPTION FACTOR 1; TRANSLOCATED TO, 1 (CYCLIN D-RELATED)	-2.21487	1.22e-06
TAS2R14	TASTE RECEPTOR, TYPE 2, MEMBER 14	-2.1434	0.003525
TNFRSF19	TUMOR NECROSIS FACTOR RECEPTOR SUPERFAMILY, MEMBER 19	-2.12295	0.009171
E2F4	E2F TRANSCRIPTION FACTOR 4, P107/P130-BINDING	-2.08635	0.007842
GOLGA7	GOLGI AUTOANTIGEN, GOLGIN SUBFAMILY A, 7	-2.06395	0.006622
CSMD3	CHROMOSOME 9 OPEN READING FRAME 61	-2.03955	0.006163
GPATCH2	G PATCH DOMAIN CONTAINING 2	-2.03535	0.005281
HMGCLL1	3-HYDROXYMETHYL-3-METHYLGLUTARYL-COENZYME A LYASE-LIKE 1	-2.02645	0.005358
ESCO2	ESTABLISHMENT OF COHESION 1 HOMOLOG 2 (S. CEREVISIAE)	-1.9938	0.009067
ATG5	ATG5 AUTOPHAGY RELATED 5 HOMOLOG (S. CEREVISIAE)	-1.9912	0.002479
IFRG15	INTERFERON RESPONSIVE GENE 15	-1.94395	0.005125
LPL	LIPOPROTEIN LIPASE	-1.92395	0.006608
OAT	ORNITHINE AMINOTRANSFERASE (GYRATE ATROPHY)	-1.84135	0.00705
PLCD1	PHOSPHOLIPASE C, DELTA 1	-1.7921	0.008362
TAS2R14	TASTE RECEPTOR, TYPE 2, MEMBER 14	-1.7858	0.008996
LOC729096	SIMILAR TO BMS1-LIKE, RIBOSOME ASSEMBLY PROTEIN	-1.7137	0.004258
CRSP6	COFACTOR REQUIRED FOR SP1 TRANSCRIPTIONAL ACTIVATION, SUBUNIT 6, 77KDA	-1.68845	0.006394
PRKCE	PROTEIN KINASE C, EPSILON	-1.6005	0.009286
DKFZP	HYPOTHETICAL PROTEIN DKFZP313A2432	-1.5791	0.006615
TOMM40	TRANSLOCASE OF OUTER MITOCHONDRIAL MEMBRANE 40 HOMOLOG (YEAST)	-1.5579	0.007834
PARK2	PARKINSON DISEASE (AUTOSOMAL RECESSIVE, JUVENILE) 2, PARKIN	-1.53865	0.007039

renovation of existing tissues), *extracellular matrix organization and biogenesis* (the formation, arrangement of constituent parts, or disassembly of an extracellular matrix), *bone remodelling* (the continuous turnover of bone matrix and minerals that involves first, an increase in resorption and later, reactive bone formation), *anatomical structure morphogenesis* (the aggregation and bonding together of a set of macromolecules to form a complex) *macromolecule complex assembly biological process terms* (the process by which anatomical structures are generated and organized: morphogenesis pertains to the creation of form), and *anatomical structure development* (the process whose specific outcome is the progression of an anatomical structure from an initial condition to its mature state). Functional annotation clustering, the DAVID tool for grouping genes based on functional similarity which groups/displays similar annotations together, is based on the hypothesis that similar annotations should have similar gene members. In this way we have found different significant clusters, whose biological significance was ranked using the Group Enrichentment Score, the geometric mean (in -log scale) of a member's *P*-values in a corresponding annotation cluster. A significant cluster in 10th position (on 110 clusters) was formed by

Fig. 1 The lists of genes which are detected by using gene ontology analysis.

morphogenesis, organ morphogenesis, and organ development, including the above-mentioned genes (Fig. 1).

Discussion

The idea of surface modification is to retain the desired bulk properties while modifying only the outermost surface, which interacts with the surrounding tissues (22). Titanium has been demonstrated to be a good material for surgical application. For forty years it has been used to produce prostheses for joint replacement and for dental substitution. Titanium is well tolerated by bone and is able to induce osseointegration of implants. Recently titania film has been used to cover prosthetic implants in medical fields such as orthopaedics or dental surgery (23,24). Titania film creates an active surface that is able to improve the biocompatibility of titanium implants (25). In prostheses made of titanium, the titania film surface is composed mainly of two polymorphs of titanium: rutile and anatase. Rutile is considered the stable form of titania while anatase is metastable and converts to rutile at high temperatures (25). Anatase titania is more advantageous for medical applications than rutile. Compared to rutile, anatase exhibits stronger interactions between metal and support, and the surface of anatase titania can absorb more OH- and $PO4_3$- than that of rutile titania in body fluid, which favours the deposition of bone-like apatite (26,27).

Although some studies have been performed to detect the genetic effect of titanium surfacing on osteoblast genetic activity, they have not taken into account variable porosity (5-7). In fact it is well known that roughness increases implant osseointegration, and several implant types are sand-blasted and/or acid-etched to increase their surface texture (28).

Here the effect of the titanium layer on osteoblast gene expression (5-7) was studied independently from roughness. To reach this goal a meta-analysis was performed by using implant surfaces having various surface textures.

The most notable genes selected by Gene Ontology are shown in Fig. 1. Among the coagulation/vascular process-related factors were plasminogen and collagen type XVIII. Plasminogen is a circulating zymogen that is converted to the active enzyme plasmin by cleavage of the peptide, which is mediated by urokinase and a tissue plasminogen activator. The main function of plasmin is to dissolve fibrin clots. This is of particular interest, as platelet-rich plasma is widely used in clinical practice and has well established effects on bone regeneration (29). Collagen type XVIII, alpha 1 is one of the multiplexins: extracellular matrix proteins that contain multiple triple-helix domains interrupted by non-collagenous domains. The proteolytically produced C-terminal fragment of type XVIII collagen is endostatin, a potent antiangiogenic protein also used in cancer therapy (30).

Among the osseoinduction genes is pleiotrophin (i.e. PTN) which has potent effects on the regulation of osteoblast recruitment, proliferation and differentiation. A specific study examining the long-term effects of targeted PTN overexpression on bone development and repair in a transgenic mouse model demonstrated that PTN overexpression had enhancing effects on early bone development (31). TIMP1, as reported previously (15,16), is involved in the regulation of bone remodelling in normal developing human bone, whereas Split hand/foot malformation type 1 is another gene with a role in limb development (32). Other genes with a direct role in bone regulation are PLCG2 and MSX1. PLCG2 belongs to the phospholipase C family of enzymes: blockade of PLCgamma enzymatic activity blocks early osteoclast development and function and mediates RANKL-induced osteoclastogenesis (32).

MSX1 is a homeobox gene that controls cellular proliferation and differentiation during embryonic development. It also plays an important role in craniofacial skeleton formation. Msx1-deficient mice exhibit dental and craniofacial malformations, such as cleft palate, reduced mandible length, abnormalities of the nasal, frontal, and parietal bones, as well as arrested tooth development, suggesting that Msx1 has a role in outgrowth of these tissues (33,34). In humans, mutations in the Msx1 gene have been identified in cleft lip and palate associated with tooth agenesis (35,36).

Other up-regulated genes directly activated by titanium surfaces are involved in cell adhesion. Examples are tenascin TNC and TNXB. Tenascins are a family of extracellular matrix proteins showing prominent expression during tissue interactions in embryogenesis.

TNXB has anti-adhesive effects, as opposed to fibronectin, and regulates collagen synthesis and deposition (37). Its deficiency has been associated with the connective tissue disorder Ehlers-Danlos syndrome, characterized by altered metabolism of fibrillar collagens. TNC is involved in skeletogenesis: osteoblasts express three different splice variants of tenacin during bone development (38), whereas tenascin-C is produced during the initial or early stages of chondrogenesis in the chicken mandibular arch (39).

Other up-regulated genes that mediate cell adhesion are ZFHX1B and TRPM7. ZFHX1B is a transcriptional repressor involved in the TGFbeta signaling pathway and in the process of epithelial to mesenchymal transition via regulation of E-cadherin (40). TRPM7 regulates cell adhesion through the protease m-calpain by mediating

the local influx of calcium into peripheral adhesion complexes (41).

The genes discussed here are only a limited number among those differentially expressed and shown in Fig. 1. We briefly analysed some of those with better-known functions. Currently, the precise interaction between the discussed genes and, above all, which of them have the most relevant role in bone formation, is unclear. However, the identification of several genes induced by titanium and acting on bone formation is relevant in order to have a paradigm for comparison of chemically different layers or similar titanium surfaces with different textures.

Acknowledgments

This work was supported by grants from Unife 60% (F.C.), CARIFE (F.C.), and CARISBO (F.P.).

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