1. Title:

Analysis Of Osteoclastogenesis/Osteoblastogenesis On Nanotopographical Titania Surfaces

2. Authors:

Mr Robert K. Silverwood[1], Mr Paul G. Fairhurst[1], Dr Terje Sjöström[2], Mr Findlay Welsh[1], Dr Yuxin Sun[4], Prof Gang Li[4,5] , Dr Bin Yu[6], Mr Peter S. Young[1], Prof Bo Su[2], Mr Robert M.D. Meek[3], Prof Matthew J. Dalby[1], Dr Penelope M. Tsimbouri[1*]

1 Centre for Cell Engineering, Joseph Black Bld, University of Glasgow, G12 8QQ, Glasgow, Scotland, UK

email:*penelope.tsimbouri@glasgow.ac.uk

2 Biomaterials Engineering Group, School of Oral & Dental Sciences, University of Bristol, Lower Maudlin Street, Bristol BS1 2LY, UK

3 Department of Orthopaedics, Southern General Hospital, Glasgow, Scotland, UK

4 Department of Orthopaedics & Traumatology, Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong, Prince of Wales Hospital, Hong Kong, PR China

5 The Chinese University of Hong Kong Shenzhen Research Institute, Shenzhen, PR China

6 Department of Orthopaedic Surgery, Southern Medical University, Southern Hospital, Guangzhou, PR China

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4. Dataset and readme information

Substrate Fabrication (Figure 1)

14mm diameter Ti disks (ASTM grade 1, Titanium Metals UK Ltd.) were mirror polished. Poly(styrene-b-4-vinylpyridine) (PS-b-P4VP) with molecular weight 41.5-b-17.5x10^3 g/mol (Polymer Source) was dissolved in a mixture of toluene and THF (70/30 %) to a concentration of 0.5wt%. A thin film of the polymer solution was formed on the Ti disks by spin-coating at 2000rpm and subsequently solvent annealed in THF vapour for 3h. The Ti samples were then anodized at 10 V in 0.01M oxalic acid and finally the polymer mask was removed using an O_2 plasma. The resulting nanopillars had a height of 15nm and average distance of 30nm. Mirror polished Ti disks were used as control surfaces.

Human bone marrow stromal cells (BMSCs) and human bone marrow hematopoetic cells (BMHCs) isolation

Bone marrow (BM) was harvested from healthy patients (informed and signed consent) undergoing hip and knee arthroplasty and stored in (phosphate buffered saline (PBS), 0.53Mm EDTA and antibiotics (6.74 Uml-1 Penicillin-Streptomycin, 0.2µgml-1 Fungizone)). BM aspirate was washed and cultured in basal media (DMEM (Sigma), 10% foetal bovine serum, 100mM sodium pyruvate, 200 mM L-glutamine (Invitrogen) and antibiotics. The aspirate was then centrifuged at 376 xg for 10 minutes. This sequence was repeated twice. The cell pellets were resuspended in medium and overlaid on a Ficoll gradient. This was then centrifuged at 445 g for 45 minutes. The mononuclear interface layer aspirated and resuspended in medium. The cells were further washed and transferred in an appropriate sized cell culture flask and incubated at 37°C with 5% humidified CO2. At day 3, the non-adherent cells were removed and cultured separately as HSCs. The remaining adherent cells were cultured for a further 7-10 days until a confluent BMSC layer was identified. This whole process was repeated three times using BM from three different patients to ensure reproducibility.

Co-cultures on titania nanopillar surfaces (Figure 2)

Following 7-10 days of culture, the adherent *BMSCs* were detached by washing twice with HEPES saline followed by a 4 minute incubation at 37°C in a trypsin solution (0.05% trypsin/0.53mM EDTA). The enzyme was stopped by addition of fresh culture medium, the cells were then centrifuged, counted and resuspended in mDMEM to a concentration of 1 x 10^4 cells/ml. 1ml of the cell suspension was pipetted directly onto the prepared Ti substrates. After 7 days of culture, 1ml of *BMHCs* suspension was added at a concentration of 1.5 x 10^5 cells/ml. This created the co-culture.

Scanning Electron Microscopy (SEM) (Figure 5)

SEM was performed at day 14 and 28 of co-culture on two 15nm nanopillar and two polished Ti substrates for each time point. Cells were fixed in 4% gluteraldehyde, followed by a wash stage in 0.2M sodium cacodylate (pH 7.4). The substrates were then post fixed in 1% osmium tetroxide in sodium cacodylate buffer, and then washed again in sodium cacodylate buffer. The substrates were then immersed in a 1% tannic acid in 0.1 M sodium cacodylate solution for 60 minutes. This was

followed by a further wash in 0.2 M sodium cacodylate. Dehydration through an incremental alcohol series followed by hexamethyldisiloxane was conducted prior to sputter coating (20nm gold/palladium) and viewing with Carl Zeiss Sigma Variable Pressure Analytical SEM with Oxford Microanalysis. Imaging conditions on data bar on images, KV and working distance. Also 30um aperture, 450pA (probe Current). Files generated in JPEG or TIFF format that would open with any imaging software.

Atomic force microscopy (AFM) (Figure 1)

The surfaces were characterized using AFM (Veeco Multimode with Quadrex Nanoscope 3D system) and FEG-SEM (JEOL JSM 6330F). The nanodots height was retrieved from AFM cross-sectional profiles.

Histochemistry

A. TRAP Staining (Figure 6)

At day 28 of co-culture, histochemical analysis was performed on 2 nanopillar and 2 flat substrates. Cells were fixed with 4% formaldehyde for 30 seconds and then stained for tartrate resistant acid phosphatase (TRAP), (Acid Phosphatase Leukocyte No.387, Sigma-Aldrich). Samples were also counterstained for 10 minutes in haematoxylin solution and washed with H_2O. TRAP staining allows assessment of osteoclastogenesis, as TRAP is expressed by osteoclast cells and their progenitors. Imaging was performed using the Leica DM750M microscope, with a ThorLabs DCC1645C colour camera running uc480 viewer to capture pictures. Files generated in JPEG or TIFF format that would open with any imaging software.

B. Alizarin staining (Figure 4)

Alizarin red stain (pH 4) of 2% (w/v) was prepared (Alizarin red S (Sigma)) in dH_2O. Cells were fixed in 4% formaldehyde for 15 minutes then stained for 5 minutes before H_2O and PBS rinses. Samples were assessed by bright-field optical microscopy. Alizarin red staining assesses calcium deposition and thus osteoblastogenesis. Imaging was performed using the Leica DM750M microscope,

with a ThorLabs DCC1645C colour camera running uc480 viewer to capture pictures. Files generated in JPEG or TIFF format that would open with any imaging software.

Immunofluorescence (Figures 2 and 4)

At day 28, 2 discs of each substrate were analysed by immunofluorescence staining. Cells were fixed (4% formaldehyde/phosphate-buffered saline (PBS) with 1% sucrose) at 37°C for 15 min. The fixative was then removed and the samples were permeabilized (10.3g of sucrose, 0.292g of NaCl, 0.06g of MgCl2, 0.476g of HEPES buffer, 0.5 ml of Triton X, in 100 ml of 1xPBS, pH 7.2) at 4°C for 5 minutes. This was followed by a blocking step with 1% bovine serum albumin (BSA)/PBS at 37°C, for 5 minutes. Anti-osteopontin (1:100 in 1% BSA/ PBS, AKm2A1 (osteopontin, Autogen Bioclear, UK) mouse monoclonal anti-human antibody (IgG1)) was added for 1 hour (37°C). The samples were co-stained with fluorescein isothiocyanate (FITC)-conjugated phalloidin (1:100 Invitrogen, UK). The samples were then washed in 1xPBS/0.5% Tween 20 (3x5 minutes at room temperature). A secondary, biotin-conjugated antibody (1:50 in 1% BSA/ PBS, monoclonal horse anti-mouse (IgG), Vector Laboratories, Peterborough, UK) was added for 1h (37°C) followed by washing. A third, Cy-3 conjugated streptavidin, layer was added (1:50, Vector Laboratories, Peterborough, UK) at 4°C for 30 minutes, before the samples were given a final wash and mounted in Vectashield mounting medium (Vector labs) containing DAPI to stain the nucleus. Visualisation was via a fluorescence microscope (Zeiss Axiovert 200M, 10x magnification, NA 0.5). For cell count osteoclasts were defined as cells greater than 30µm diameter, with greater than 3 nuclei and presence of actin ring. Files generate Files generated in JPEG or TIFF format that would open with any imaging software.

Image analysis

For both TRAP and Alizarin red stains and immunofluorescence, comparisons of staining intensity between substrates was analysed by Image J software version 1.42q using the Aliz and TRAP macro_intensity_sequence.txt file provided.

The CellProfiler software suite (Broad Institute,USA) was used to process over 30 image sets, acquired using an inverted fluorescence microscope (Olympus). An image processing pipeline was generated to load the DNA (DAPI), actin (phalloidin-rhodamine), and antibody stain (fluorescein), followed by automated detection of cell morphology, area and cell aspect ratio.

After analysis by Image J or CellProfiler softwares, statistical analysis was performed by the student's t-test.

qRT-PCR (Figure 3)

Total RNA from both day 14 and 28 time points cultures was extracted using a Qiagen RNeasy micro kit according to the manufacturer's protocol. Equal amount of RNA from each sample was used for cDNA preparation using the QuantiTect RT-PCR kit from Qiagen following the Qiagen protocol. qRT-PCR was carried out using the Quantifast SYBR Green kit (Qiagen)and the 7500 Real Time PCR system from Applied Biosystems. The GapDH housekeeping gene primer set was used for normalisation. 3 replicates were tested at each time point with the calculated mean normalised against GapDH. OSCAR, Osteoprotegrin, RANKL, IL-6, TRAP, TNF-α, Cathepsin- K, OPN and Alkaline phosphatase primers were used for analysis. The primer sequences (Table 1) for the genes were validated by dissociation curve/melt curve analysis.

Table 1: PCR Primer Sequences

Target Gene	Forward Sequence	Reverse Sequence
OSCAR	CCAGCTCTAGCGGGTATCTG	GACGGAGTGATGTCTGTGTGAC
Osteoprotegrin	GAAGGGCGCTACCTTGAGAT	GCAAACTGTATTTCGCTCTGG
RANKL	TGATTCATGTAGGAGAATTAAACAGG	GATGTGCTGTGATCCAACGA
IL-6	GATGAGTACAAAAGTCCTGATCCA	CTGCAGCCACTGGTTCTGT
TRAP	GGACTGAAGGGACTCCTGAAT	GGTCCCTGAGCCTTTATTCC
TNF-alpha	CAGCCTCTTCTCCTTCCTGAT	GCCAGAGGGCTGATTAGAGA
Cathepsin-K	GCCAGACAACAGATTTCCATC	CAGAGCAAAGCTCACCACAG
Alkaline Phosphatase	AGAACCCCAAAGGCTTCTTC	CTTGGCTTTTCCTTCATGGT

Osteopontin	AGCTGGATGACCAGAGTGCT	TGAAATTCATGGCTGTGGAA
GapDH	GTCAGTGGTGGACCTGACCT	ACCTGGTGCTCAGTGTAGCC

QPCR data analysis was performed using the $2^{(-DDCT)}$ Method (doi:10.1006/meth.2001.1262, available online at http://www.idealibrary.com).

1) Using the Ct column data from the original data file, the average of the technical replicates from both house keeping and test gene were calculated.
2) The house keeping ct average was subtracted from the test gene equivalent (In our case normalize to GapDH).
3) The control group was selected and the average the biological replicates calculated.
4) The control average was subtracted from each one of the test gene equivalent replicates (in our case normalized to Flat).
5) The resulting values were converted using the $2^{(-DDCT)}$ formula.

Data file of calculations provided in Excel format.

Statistical analysis of qPCR was performed using the Graphpad Prizm software version 4c by the non-parametric Kruskal-Wallis test and Dunn's Multiple Comparison Test.

Implantation of surface-treated metal materials in rabbits (Figure 7)

In vivo studies were performed with the approval of the Animal Experimentation Ethics Committee of The Chinese University of Hong Kong. Five New Zealand male 8-week-old white rabbits underwent the surgery. Under the general aesthesia, two materials with different coating were randomized implanted on the anterior side of right femur of the animals. All the animals were terminated and femora with materials were harvested 2 months after the implantation surgery. All the samples were embedded into methylmethacrylate (MMA) before sliced. Bone to implant contact (BIC) was quantitatively measured by Osteomeasure software.

Material implant surgery

All the animals were anesthetized with a mixture of ketamine (35mg/kg) and xylazine (5mg/kg) intramuscularly. Femora were exposed via longitudinal skin

incision on the lateral side of hind limb. Three grooves, (0.8*0.8 cm in area; 0.1 cm in depth; 1 cm in interval), were created by an abrasive drill to make the bone surface rough. Two different kinds of material were implanted in the grooves randomly and covered with periosteum separately. Finally, wounds were closed in layers. All rabbits were injected with penicillin (800,000 units) intramuscularly once per day in 3 days after surgery.

Histological evaluation of rabbit tissues

Femora were harvested and fixed in 4% paraformaldehyde (pH 7.4) for 48 h after sacrifice. Then all the samples were placed in 70% ethanol for further dehydration in graded ethanol and embedded undecalcifiedly in MMA. The infiltration process was carried out by placing the bone specimens into a solution of MMA and dibutylphtalate (3:1) for 48 hours, followed by another 48 hours in MMA. Embedding of the infiltrated specimens was done in fresh MMA, dibutylphtalate (3:1) and 2.5% benzoyl peroxide solution at 20 □. Polymerisation was completed within 48 hours. Attempts were made to standardize the sectioning at a mid-sagittal plane of each specimen by cutting the specimen in half (transversely in a coronal plane) using a low-speed diamond saw (Leica SP1600, Leica Company, Germany), and the MMA sections were then polished to thin MMA sections (100 μm). For histological examination, MMA slices were stained with Stevenel's Blue, and counter stained with Van Gieson stain. These specimens were evaluated by optical microscope (Olympus BX41, Olympus Co., Japan) and image analysis software (Osteomeasure Software, Osteometric Inc., USA) to allow a quantitative measurement of the bone to implant contact (BIC).

Statistical analyses of in vivo rabbit experiments were performed by student Ttest within groups. All data are presented as $\bar{X}$±SEM. The level of statistical significance was set at $p < 0.05$.