**1. Title:**

Nanovibrational stimulation inhibits osteoclastogenesis and enhances osteogenesis in co-cultures

**2. Authors:**

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**Abstract**

Models of bone homeostasis could be useful in drug discovery, particularly if the model is one that replicates bone regeneration with reduction in osteoclast activity. Here we use nanovibrational stimulation to achieve this in a 3D co-culture of primary human osteoprogenitor and osteoclast progenitor cells. We show that 1000 Hz frequency, 40 nm amplitude vibration reduces osteoclast formation and activity in human mononuclear CD14+ve blood cells and enhances osteogenesis/reduces osteoclastogenesis in a co-culture of primary human bone marrow stromal cells and bone marrow hematopoietic cells. Further, we use metabolomics to identify Akt (protein kinase C) as a potential mediator. Akt is known to be involved in bone differentiation via transforming growth factor beta 1 (TGFβ1) and bone morphogenetic protein 2 (BMP2) and it has been implicated in reduced osteoclast activity via Guanine nucleotide-binding protein subunit α13 (Gα13). With further validation, our nanovibrational bioreactor could be used to help provide humanised 3D models for drug screening.

**3. Funder information**

This work was funded by The Royal College of Surgeons Edinburgh Pump Priming Grant.

**4. Dataset and readme information**

**Data folders**

**Nanovibration bioreactor**

Standard cell culture plates (Corning, NY) were magnetically attached (NeoFlex® Flexible Neodymium Magnetic Sheet, 3M, Minnesota, United States) to the vibration plate (dimensions 128 x 176 mm). This plate was secured on the underside to an array of low-profile, multilayer piezo actuators (NAC2022, Noliac A/S CTS, Denmark). To power the piezo array, a custom power supply unit, consisting of a signal generator integrated circuit (AD9833, Analog Devices, Massachusetts, USA) to provide a 1000 Hz sine wave modulation and a parallel configuration of class AB audio amplifiers (TDA7293, STMicroelectronics, Geneva, Switzerland) to amplify the sine wave signal. This results in the vibration plate oscillating at an amplitude of 40 nm and 1000 Hz frequency.

Figure 1 Nanovibrational stimulation setup and measurement.

**Interferometry**

Vibrational amplitude was measured using laser interferometry to accurate measurement of nanoscale displacements generated by the bioreactor. A USB interferometer (Model SP-S, SIOS Messtechnik GmbH, Ilmenau, Germany) was mounted on a frame with the laser aimed downwards at the measurement site. For 2D tissue culture plastic surface displacement measurements, self-adhesive, reflective tape was bonded at measurement sites to reflect the laser for accuracy. Analysis of the interference pattern between the reflected laser light and the reference signal in the interferometer’s INFAS software (where the time series interference signal is converted to frequency space by fast Fourier transform (FFT)) allows the displacement of the target surface to be determined from the produced frequency spectrum. This model of interferometer is sensitive to displacements of 0.1 nm, however, seismic noise (produced by people walking and moving around near the apparatus) can reduce this sensitivity. To prevent this noise affecting the measurements, the interferometric apparatus was mounted on an optical bench supported by polystyrene blocks to provide noise dampening. Each well in a 24 well plate was measured three times and mean displacements for each well calculated.

**Data folder: Interferometry Figure 1.** Interferometry measurements provided in .xls format.

**Figure 1:** Laser interferometry set-up and results.

Supplementary figure 1. Nanovibrational measurements

**Application of nanovibration in CD14+ culture**

**2D cell culture:** Buffy coats – the fraction of blood containing white blood cells and platelets – were received from the Scottish National Blood Transfusion Service. These were diluted 1:1 with phosphate buffered saline (1 x PBS) and mixed by inversion. In a 15 ml conical centrifuge tube 10 ml of buffy coat/PBS were overlaid on 4 ml of Ficoll-Paque (Sigma-Aldrich, Dorset, UK) at room temperature and centrifuged at 400 x gravity (*g*) for 30 minutes. After centrifugation, the monocyte layer was aspirated using a Pasteur pipette and transferred to a 50 ml conical centrifuge tube, before resuspending the cells with addition of 1 x PBS to 50 ml total volume. The cells were washed twice with PBS and centrifuged at 200 x *g* for 10 minutes for platelet exclusion. The monocyte pellet was resuspended with 10 ml specific cell separation media (2% foetal bovine serum (FBS) and 1 mM EDTA in 1 x PBS) and mixed. A cell count was then performed with a 10 x dilution (10 μl cells added to 80 μl 1 x PBS and 10 μl trypan blue) using a haemocytometer. Cells were further centrifuged at 350 x *g* for 5 minutes and resuspended to obtain 1 x 108 cells/ml. The suspension was transferred to a 6 ml round bottomed polystyrene tube. Magnetic selection was performed using EasySep Human CD14 positive selection kit (Stemcell Technologies, Cambridge, UK). 100 μl/ml of CD14 positive selection cocktail was added and incubated at room temperature for 15 minutes. Magnetic nanoparticles were vortexed and 50 μl/ml added before incubating at room temperature for 10 minutes. The suspension was made up to 2.5 ml with the addition of cell separation buffer and the tube placed in a specialised magnet for 5 minutes. The magnet was then inverted to pour off the negative fraction. The tube was removed from the magnet and a further 2.5 ml cell separation buffer added prior to placing back in the magnet for a further 5 minutes. This process was repeated a further two times. The cells were finally re-suspended at 1 x 106 cells/ml in alpha minimum essential media (α-MEM) supplemented with 10% FBS, 0.02 mM L-glutamine, 10 U/ml Penicillin, and 0.1 μg/ml Streptomycin. Following suspension at 1 x 106 cells/ml, 25 ng/ml of recombinant human M-CSF (Peprotech, London, UK) was added. Cells were plated in 24 well plates and incubated overnight at 37°C and 5% CO2. After approximately 18 hours incubation 25 ng/ml of human RANKL (Peprotech, London, UK) was added to a proportion of the wells. Those wells with M-CSF and no RANKL were used as a negative control of osteoclastogenesis. Medium was refreshed on days 4 and 7 if required.

**3D cell culture:** CD14+ cells were isolated as per the 2D technique laid out above. They were then centrifuged at 400 x *g* for 5 minutes to produce a cell pellet. The cells were then resuspended in 10% FBS and 10 x DMEM (Sigma-Aldrich, Dorset, UK). The collagen gel was then prepared, with the total volume required to produce a concentration of 1 x 106 cells/ml. This stage of the experiment was performed on ice to avoid the gel setting early. Sodium hydroxide (NaOH) was combined with rat tail collagen (Sigma-Aldrich, Dorset, UK). The resuspended cells were then added to the NaOH/collagen mix. 1 ml of gel/cell suspension was then pipetted onto each well of a 24 well plate. Plates were then placed in an incubator (37°C and 5% CO2) for 30 minutes to allow the gels to set. 1 ml of α-MEM (supplemented with 10% FBS, 0.02 mM L-glutamine, 10 U/ml Penicillin, and 0.1 μg/ml Streptomycin) containing 25 μl/ml M-CSF was then added on top of the gels. After 18 hours RANKL, at a concentration of 25 μl/ml, was added to a proportion of the wells. Those wells with M-CSF and no RANKL were used as a negative control of osteoclastogenesis. Media was replaced on days 4 and 7 as required.

**Application of nanovibration in co-culture**

**2D:** Human bone marrow was aspirated from patients undergoing elective hip and knee arthroplasty and stored for transfer (1 x PBS, 0.53 mM EDTA, and antibiotics (6.74 U/ml Penicillin-Streptomycin, 0.2 μg/ml Fungizone)). The bone marrow aspirate was diluted with 10 ml modified Dulbecco’s Modified Eagle Medium (mDMEM) (DMEM (D5671), 10% FBS, 100 mM sodium pyruvate, 200 mM L-glutamine (Invitrogen, Paisley, UK), and antibiotics). This was then centrifuged at 350 x *g* for 10 minutes, repeated twice. The cell pellets were re-suspended in mDMEM and overlaid on a Ficoll-Paque gradient. This was then centrifuged at 450 x *g* for 45 minutes (with no speed brake) and the subsequent mononuclear interface layer aspirated and resuspended in mDMEM. The cells were washed a further three times and plated at a density of 1 x 106 in a 75 cm2 vented cell culture flask and incubated at 37°C with 5% humidified CO2. At day 3 non-adherent cells were removed and cultured separately at a density of 1 x 106 in a 75 cm2 vented flask and incubated at 37°C with 5% humidified CO2, with three times weekly media change. This non-adherent fraction contained mainly bone marrow haematopoietic cells (BMHC), macrophages and osteoclast precursors. The remaining adherent cells were assumed to be MSCs, osteoprogenitors, osteoblasts and osteocytes and were cultured for a further 7 - 10 days until an approximately 80% confluent layer was obtained. The adherent cells were then detached with 0.05% trypsin/0.53 mM EDTA, centrifuged and resuspended in mDMEM to a concentration of 3 x 104 cells/ml. 1 ml of cell suspension/well was pipetted onto a 24 well plate. The media was replaced on day 3. At day 7, 1 ml of BMHC suspension/well was added at a concentration of 1.2 x 105/ml. This co-culture was maintained with media changes three times a week.

**3D culture:** Human bone marrow was processed as described in for 2D above. At day 3, non-adherent cells were removed and cultured separately. Both adherent and non-adherent flasks were cultured until a confluent layer was achieved. In the 2D model, adherent cells were plated first, with non-adherent cells being added after 7 – 10 days. Given it would not be possible to add additional cells once the gel had set, cells from both flasks were combined prior to combining with the gel. The adherent cells concentration was 3 x 104 cells/ml and the non-adherent concentration 1.2 x 105 cells/ml as per the 2D model. Cells were detached with 0.05% trypsin and counted. They were then centrifuged at 400 x *g* for 5 minutes to produce a cell pellet. The cells were then resuspended in 10% FBS and 10 x DMEM before adding to the NaOH/collagen mix. Further NaOH was added drop-wise while agitating the mix until the colour changed from yellow to pink, indicating the correct pH had been achieved. 1 ml of gel/cell suspension was then pipetted onto each well of a 24 well plate. Plates were then placed in an incubator (37°C and 5% CO2) for 30 minutes to allow the gels to set. 1 ml of mDMEM was subsequently added on top of the gels and replaced three times per week.

**Alamar Blue assay**

Cell viability was assessed at defined time points using an alamarBlue assay (Thermo Fisher Scientific, Loughborough, UK) according to the manufacturer’s instructions. A 10% solution was produced (1 ml alamarBlue, 9 ml media). Media was removed from the wells and 500 μl of 10% alamarBlue added. Both the stimulated and control samples were then incubated at 37°C for 4 hours in the dark. 150 μl/well of 10% alamarBlue was then transferred in triplicate to a 96 well plate. The remainder of 10% alamarBlue was discarded before washing three times with HEPES saline and adding fresh media. The absorbance was measured at 570 nm and 600 nm using a Multiskan FC Microplate Photometer (Thermo Fisher Scientific, Loughborough, UK). The percentage of alamar Blue reduction was calculated as follows:

% reduction of Alamar Blue = ((O2 x A1) – (O1 x A2) / (R1 x N2) - (R2 x N1)) x 100

where O1 and O2 are the molar extinction coefficients of oxidised Alamar Blue at wavelengths 570 nm and 600 nm respectively. R1 and R2 are the molar extinction coefficients of reduced Alamar Blue at wavelengths 570 nm and 600 nm respectively. A1 and A2 are the observed absorbance readings for test wells at wavelengths 570 nm and 600 nm respectively. N1 and N2 are the observed absorbance readings for the negative control wells at wavelengths 570 nm and 600 nm respectively.

**Data folder: Alamarblue.** Original data readings in .xls format.

**Subfolder Fig 2a:** CD14+ alamarBlue original data.

**Subfolder Fig 3a:** Co-culture alamarBlue original data.

**Figure 2a:** (a) No detrimental effect on CD14+ cell viability was seen, as measured by Alamar blue

**Figure 3a:** (a) No detrimental effect on CD14+ cell viability, as measured by Alamar blue, was seen.

**TRAP staining**

TRAP (Tartrate-Resistant Acid Phosphate) was used as per manufacturer’s instructions (Acid Phosphatase, Leukocyte (TRAP) kit, No.387A, Sigma-Aldrich, Dorset, UK). Fast Garnet solution (25 μl Fast Garnet and 25 μl Sodium Nitrite) was mixed by inversion for 30 seconds and left to stand for 2 minutes. 400 μl of fixative (12.5 ml Citrate solution, 32.5 ml Acetone and 5 ml Formaldehyde) was added to the base of each well for 30 seconds. This was then washed three times with warm water. 400 μl TRAP staining solution (4.5 ml warm water, 50 μl Fast Garnet solution, 50 μl Napthol, 200 μl Acetate, 250 μl Tartrate) was added and incubated at 37°C for 30 minutes in the dark. The TRAP staining solution was then removed. Samples were washed three times with warm water and allowed to air dry. Digital images of the wells were acquired using an EVOS® FL Auto 2 Cell Imaging System (Thermo Fisher Scientific, Loughborough, UK). Osteoclasts were identified as TRAP positive cells with ≥3 nuclei and quantified by both number and area using Fiji software (Image J). Cell numbers were determined by counting all osteoclasts in the entire well of each replicate. Area was calculated by manually delineating the cell border of 100 cells/well and using the area calculation tool in Image J.

**Data folder: TRAP stain.** Original images of TRAP stained wells used for cell count and area measurements.

**Subfolder Fig 2b, c:** CD14+ original TRAP stained images.

**Subfolder Fib 3b, c:** Co-culture original TRAP stain images.

**Figure 2b:** (b) following 7 days of nanovibrational stimulation numbers of fused, multinucleate osteoclasts observed by TRAP staining were reduced. Similarly, the mean osteoclast area (c) was also reduced with nanovibrational stimulation after 7 days of culture.

**Figure 3b:** (b) the number of osteoclasts were seen to reduce and (c) area of osteoclasts was decreased with nanovibrational stimulation.

**Scanning electron microscopy**

Cells were fixed with buffered fixative (1.5% glutaraldehyde, 0.1 M sodium cacodylate) for 1 hour at 4°C. Samples were then rinsed with 0.1 M sodium cacodylate for 3 x 5 minutes. Postfix in 1% osmium tetroxide for 1 hour at room temperature was then performed followed by 3 x 10 minutes wash with distilled water. A dehydration process was performed using an ethanol series (30, 50, 70 and 90% for 5 minutes each x 2, followed by 100% for 5 minutes x 4, and dried absolute ethanol for 5 minutes x 4). Hexamethyl-disilazane (HMDS) was then applied to samples and left overnight for drying. An 18 nm gold palladium coating was overlaid on the sample surface using a Polaron SC515 SEM Coating System (Quorum Technologies, Sussex, UK). Finally, samples were attached to aluminium stubs and analysed on a Carl Zeiss Sigma variable-pressure analytical SEM (Carl Zeiss Ltd, Cambridge, UK). The accelerating voltage was 10 kV, with a working distance of 5 nm and 30 μm aperture.

**Data folder: Scanning electron microscopy.** Original scanning electron microscopy images.

**Subfolder Fig 2e:** CD14+ culture scanning electron microscopy images.

**Subfolder Fig 3f:** Co-culture scanning electron microscopy images.

**Figure 2e:** SEM images at day 7 showing less osteoclasts were present following nanovibrational stimulation.

**Figure 3f:** SEM images after 28 days of culture showed that while many osteoclasts could be seen in control co-cultures, fewer were observed, along with better spread BMSCs, following nanovibrational stimulation.

**Resorption analysis**

The Osteo Assay Surface is a multiple well plate. The base of each well is coated with a synthetic inorganic bone surface. As osteoclasts resorb this surface coating, the plate can be used to determine the function of osteoclasts by measuring the area resorbed. CD14+ cells were selected as per the technique described above. Cells were then seeded at a concentration of 1 x 106 /ml onto a 24 well Osteo Assay Surface plate (Corning, Flintshire, UK). The RANKL was added at a concentration of 25 ng/ml after approximately 18 hours incubation. Media was refreshed twice weekly. After 7 days incubation, the cell layer was removed by adding 10% chlorine solution (Sigma-Aldrich, Dorset, UK) to the wells at room temperature for 10 minutes. The wells were examined to ensure all cells had been removed before rinsing thoroughly with deionised water and leaving to dry. Images were then captured using an EVOS® FL Auto Cell Imaging System and the area of resorption calculated using Fiji software (Image J).

**Data folder: Resorption analysis Fig 2f.** Original images of Osteo Assay plate following osteoclast mediated resorption.

**Figure 2f:** (f) Resorption assay at day 7 showing less active osteoclasts following nanovibrational stimulation.

**RT-qPCR**

**2D RNA extraction:** After culture for defined time points, media was removed from the samples and the wells gently washed with sterile 1 x PBS. They were then lysed using RLT buffer (QIAGEN, Manchester, UK). Lysates from wells were homogenised and then transferred to 1.5 ml nuclease free tube. RNA was extracted using RNeasy Micro Kit (QIAGEN, Manchester, UK) following the manufacturer’s instructions. 14 μl of RNase-free water was used to elute RNA. Nucleic acid quantification was performed using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Loughborough, UK). Reading distilled water absorbance acted as a blank, and the samples were read at 260 nm to give quantification and the ratio of 260/280 used for purity estimation. RNA was then either immediately used for cDNA generation or stored at -80°C for later use.

**3D RNA extraction**: After culture for defined time points, media was removed before gently washing with sterile 1 x PBS. The gel was then disrupted with a pipette tip and transferred to an eppendorf. 350 μl TRIzol (Thermo Fisher Scientific, Loughborough, UK) was added and the tube vortexed to further disrupt the gel. Samples were incubated for 10 minutes to allow for cell lysis and subsequently centrifuged at 12000 x *g* for 15 minutes at 4°C. The pellet was discarded and the RNA in solution transferred to a clean eppendorf. 100 μl chloroform was added before mixing vigorously for 20 seconds then incubating at room temperature for 3 minutes. Samples were centrifuged at 12000 x *g* for 15 minutes at 4°C and the upper aqueous phase transferred to a clean tube. 1 μl glycoblue (Thermo Fisher Scientific, Loughborough, UK) was added to aid with visualisation of the pellet and 250 μl isopropanol (Sigma-Aldrich, Dorset, UK) mixed in to samples. The eppendorfs were then incubated at -20°C for 1 hour and then centrifuged at 12000 x *g* for 15 minutes at 4°C. The supernatant was discarded without disrupting the blue pellet. The RNA pellet was then washed twice with 70% ethanol and centrifuged 7500 x *g* for 5 minutes at 4°C. The ethanol was removed and the tube air-dried. The pellet was re-suspended in 20 μl RNase-free water and incubated at 60°C for 10 minutes to ensure full re-suspension. Nucleic acid quantification was performed using a Nanodrop 1000 spectrophotometer. Reading distilled water absorbance acted as a blank, and the samples were read at 260 nm to give quantification and the ratio of 260/280 used for purity estimation. RNA was subsequently either immediately used for cDNA generation or stored at -80 °C for later use.

**cDNA generation:** 100 – 300 ng of RNA was used to generate cDNA using QuantiTect Reverse Transcription Kit (QIAGEN, Manchester, UK) following the manufacturer’s instructions. RNA, RNase free water and gDNA Wipeout Buffer were combined to produce a volume of 14 μl and incubated for 2 minutes at 42°C. A master mix containing Quantiscript Reverse Transcriptase, Quantiscript RT buffer and RT Primer Mix was then produced, providing a total reaction volume of 20 μl. The samples were incubated at 42°C for 15 minutes and then 95°C for 3 minutes to terminate the reaction. cDNA was finally further diluted to a concentration of 5 ng/μl and stored at -20°C.

**RT-qPCR:** Using a 96 well PCR plate, each sample was run in duplicate. A master mix was prepared using 10 μl SYBR Green (QIAGEN, Manchester, UK), 0.1 μl forward primer, 0.1 μl reverse primer and 7.8 μl nuclease free water. This was added to each duplicate alongside 2 μl of cDNA, producing a volume of 20 μl per PCR well. The plate was then sealed using an adhesive PCR plate cover and centrifuged at 200 x g for 1 minute. Primers were provided by Eurofins (Eurofins, Hamburg, Germany) and the sequences shown in Table 2:1. An Abi7500 thermal cycler PCR machine (Thermo Fisher Scientific, Loughborough, UK) was used to run the samples. The primer sequences for the genes were validated by dissociation curve/melt curve analysis. The GapDH housekeeping gene primer/probe set was used (ABI predesigned amplification reagent) for normalisation. The ΔCT of each sample was calculated with normalisation against GapDH. Blanks (no cDNA) were used to act as controls.

**Table 1:** List of Primers

|  |  |  |
| --- | --- | --- |
| **Target Gene** | **Forward Sequence** | **Reverse Sequence** |
| **ALP** | AGAACCCCAAAGGCTTCTTC | CTTGGCTTTTCCTTCATGGT |
| **Cathepsin-K** | GCCAGACAACAGATTTCCATC | CAGAGCAAAGCTCACCACAG |
| **IL-6** | GATGAGTACAAAAGTCCTGATCCA | CTGCAGCCACTGGTTCTGT |
| **M-CSF** | GAACTGCCAGTGTAGAGGGAAT | GCTGGTCAGACAACATCTGG |
| **OSCAR** | CCAGCTCTAGCGGGTATCTG | GACGGAGTGATGTCTGTGTGAC |
| **OPN** | AGCTGGATGACCAGAGTGCT | TGAAATTCATGGCTGTGGAA |
| **OPG** | GAAGGGCGCTACCTTGAGAT | GCAAACTGTATTTCGCTCTGG |
| **RANKL** | TGATTCATGTAGGAGAATTAAACAGG | GATGTGCTGTGATCCAACGA |
| **TNF- α** | CAGCCTCTTCTCCTTCCTGAT | GCCAGAGGGCTGATTAGAGA |
| **CSF-1** | TCC CAG TGA TAG AGC CCA GT | CAG GGT CCA GTG AGG TGA TG |
| **NFATc1** | CAC CAA AGT CCT GGA GAT CCCA | TTC TTC CTC CCG ATG TCC GTCT |
| **Osterix** | GGC AAA GCA GGC ACA AAG AAAG | AAT GAG TGG GAA AAG GGA GGG |
| **Piezo 1** | CCT GGA GAA GAC TGA CGG CTAC | ATG CTC CTT GGA TGG TGA GTCC |
| **RANK** | GCT GTA ACA AAT GTG AAC CAGGA | GCC TTG CCT GTA TCA CAA ACT |
| **TRAP** | GGACTGAAGGGACTCCTGAAT | GGTCCCTGAGCCTTTATTCC |

**Data folder: QPCR**

**Subfolder: Fig 2g:** Original data for 2D CD14+ culture in .xls format

**Subfolder: Fig 3h:** Original data for 2D and 3D co-culture in .xls format

**Figure 2g:** qPCR for nanovibrated vs control CD14+ cells for transcripts related to osteoclastogenesis and inflammation. A trend towards repression of these genes in the nanovibrated cultures was observed.

Supplementary Figure 2. QPCR data for Figure 2g – CD14 culture. Data shows mean and individual data points.

**Figure 3h:** heatmap presentation of RT-qPCR 2D and 3D co-culture data. In the 2D culture, a significant reduction in IL-6 at Day 14 and OSCAR at Day 21 was observed in the nanovibration group. In 3D, cathepsin-k and OPN were significantly increased in the nanovibration group at Day 7.

**Supplementary Figure 4**. QPCR data for Figure 3h 2D (top) – 2D co-culture. Data shows mean and individual data points.

**Supplementary Figure 5**. QPCR data for Figure 3h 2D (bottom) – 3D co-culture. Data shows mean and individual data points.

**ELISA**

Cells were cultured for defined time periods, with regular media changes. The supernatant was removed and stored pending analysis at -80°C. The media was last changed 72 hours prior to removal for analysis. 96 well ELISA plates were prepared according to the manufacturer’s instructions (R&D Systems, Abingdon, UK). The capture antibody was diluted to the working concentration and 100 μl/well added. The plates were sealed and left overnight at room temperature. Wells were then aspired and washed with 400 μl wash buffer (0.05% Tween 20 in 1 x PBS) x 3. Plates were then blocked with 300 μl/well reagent diluent (1% BSA in 1 x PBS) for 1 hour. A further wash x 3 with wash buffer was performed. 100 μl/well of either samples or standard was added, with duplicate technical replicates, and incubated at room temperature for 2 hours. A further wash x 3 was performed. 100 μl/well of detection antibody diluted in reagent diluent was added for 2 hours. A further wash x 3 was performed. 100 μl/well of Streptavidin-HRP was added for 20 minutes, avoiding direct light exposure. A further wash x 3 was performed. 100 μl/well of substrate solution (1:1 mixture of Colour Reagent A and B) was added for 20 minutes, avoiding direct light exposure. Finally, 50 μl/well of Stop Solution was added. The optical density was calculated at 450 nm using a Multiskan FC Microplate Photometer. A standard curve was created with Prism v6 (GraphPad Software, California, USA) and the results interpolated from this.

**Data folder: ELISA Fig 2h.** Raw data for ELISA analysis of CD14+ culture in .xls format

**Figure 2h:** At the protein level, IL-6 was seen to be repressed.

**Metabolomics**

Cells were cultured for defined time periods. All the media was removed from the wells and then gently washed with 1 x PBS at 4°C. All the PBS was removed before adding 500 μl/well of chilled extraction solvent (1:3:1 chloroform: methanol: water). Solvent was also placed in a well without cells to produce a blank. Plates were then sealed with parafilm and vigorously agitated on a rotary shaker for 1 hour at 4°C. The solvent was removed from the wells and placed in an eppendorf before centrifuging at 1300 rpm for 3 minutes. The supernatant was removed and placed in a new eppendorf. 50 μl of each sample was placed in a separate tube to produce a pooled sample of each condition. Metabolomics was performed through hydrophilic interaction liquid chromatography mass spectroscopy analysis (UltiMate 3000 RSLC, ThermoFIsher) with a 150 x 4.6 mm ZIC-pHILIC column running at 300 μl min-1and Orbitrap Exactive (ThermoFIsher). A standard pipeline, consisting of XCMS (peak picking), MzMatch (filtering and grouping) and IDEOM (further filtering, post-processing and identification) was used to process the raw mass spectrometry data. Identified core metabolites were validated against a panel of unambiguous standards by mass and retention time. Further putative identifications were allotted mass and predicted retention time. Means and standard errors of the mean were generated for every group of picked peaks and the resulting metabolomics data were uploaded to Ingenuity pathway analysis software for pathway analysis.

**Data folder: Metabolomics.** IDEOM file containing raw data used for analysis.

* one folder containing the raw files (our mass spectrometer works in polarity switching mode so there is one raw file per sample)
* two folders for the mzXML files in negative and positive mode, respectively (polarities get split when converting to the mzXML format).
* The FRAG folder contains the fragmentation data files in pos/neg mode in the mzML format.

**Figure 3i:** Untargeted metabolomic analysis for 2D and 3D co-culture. Lipid-based pathways were upregulated, particularly at day 14 in the 3D culture; steroid and cholesterol pathways are indicated by \*. This suggests that cell growth and differentiation is more energetically demanding in 3D culture compared to 2D culture.

***Supplementary figure 3.******Osteoclast response to nanovibrational stimulation in 2D.*** *Ingenuity pathway analysis of metabolite data after 7 days of culture inferred inhibition of NFκB signalling with nanovibration*

***Supplementary Figure 6.* *Metabolite network analysis implying differential Akt regulation.*** *Untargeted metabolite analysis for 2D and 3D nanovibrational cultures compared to controls at days 14 and 21 of culture all linked to Akt signalling.*

**Actin ring staining**

Cells were cultured for 28 days. Medium was removed and cells were fixed with 3.7% methanol-free formaldehyde (Sigma-Aldrich, Dorset, UK) for 10 minutes at room temperature in the dark. Samples were then washed twice with 1 x PBS and permeabilised with 0.1% Triton x-100. A further wash x 2 with 1 x PBS was performed. Rhodamine-Phalloidin (1:40 PBS, Thermo Fisher Scientific, Loughborough, UK) was added to sufficiently cover the cells prior to incubating in the dark at room temperature for 20 minutes. Samples were given a final wash x 2 with 1 x PBS. Cover slips were applied and fluoroscopy images acquired using an EVOS® FL Auto 2 Cell Imaging System.

**Data folder: Immunostaining**

**Subfolder Fig 3e:** original images of actin ring stain in control and nanovibration group.

**Figure 3e:** Actin/DAPI immunofluorescence after 28 days of culture showed that osteoclast cells identified by multiple nuclei and by actin rings tended to have fewer nuclei following nanovibrational stimulation.

**Von Kossa staining**

Samples were cultured for 28 days. They were then fixed (4% formaldehyde/1 x PBS with 2% sucrose) at 37°C for 15 minutes. 5% silver nitrate was added to cover the cells before exposing to UV light for 30 minutes. Samples were then rinsed three times with deionised water. 5% sodium thiosulphate was then added for 10 minutes to remove excess silver nitrate. They were then rinsed three times with deionised water. Counterstaining with 0.1% nuclear fast red for 10 minutes was then performed before a further rinse x 3 with deionised water. A final rinse with 70% ethanol was performed. Digital images of the entire wells were acquired using an EVOS® FL Auto 2 Cell Imaging System. The percentage surface area stained was then calculated using Fiji software (Image J).

**Data folder: Von Kossa Fig 3g:** Original microscopy images of cell cultures following Von Kossa staining.

**Subfolder MSC culture:** original microscopy images of isolated MSC culture

**Subfolder 2D co-culture:** original microscopy images of 2D co-culture

**Subfolder 3D co-culture:** original microscopy images of 3D co-culture

**Figure 3g:** Looking at osteogenesis after 28 days of culture using von Kossa staining in 2D BMSC monoculture, 2D co-culture and 3D co-culture, osteogenesis was enhanced in all conditions with nanovibrational stimulation; typical von-Kossa images from the co-cultures are shown below their corresponding graphs (2D = left, 3D = right).

**Statistics**

Statistical analyses were preformed using GraphPad Prism software (version 6). Normally distributed data were analysed with the t-test. Conversely, if the data were not normally distributed the Mann-Whitney U test was utilised. For assessing statistical significance of metabolite changes, Fisher’s exact test was used.

**Table 2:** List of replicates for each experiment

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Figure** | **Experiment** | **Number of Donors** | **Well replicates per Donor** | **Technical replicates per well** |
| Fig 2b, 3b | TRAP cell count | 3 | 3 | 1 |
| Fig 2c, 3c | TRAP cell area | 3 | 3 | 1 |
| Fig 2a, 3a | AlamarBlue | 3 | 3 | 3 |
| Fig 2e, 3f | Scanning electron microscopy | 1 | 3 | 1 |
| Fig 2f | Resorption analysis | 3 | 3 | 1 |
| Fig 2g, Supp Fig 2 | RT-qPCR CD14+ culture 2D   * Day 1 * Day 2 * Day 3 * Day 7 | * 3 * 1 * 3 * 1 | * 3 * 3 * 3 * 3 | * 2 * 2 * 2 * 2 |
| Fig 3h, Supp Fig 4 | RT-qPCR co-culture 2D   * Day 7 * Day 14 * Day 21 * Day 28 | * 1 * 3 * 3 * 1 | * 4 * 4 * 4 * 4 | * 2 * 2 * 2 * 2 |
| Fig 3h, Supp Fig 5 | RT-qPCR co-culture 3D   * Day 7 * Day 14 * Day 21 * Day 28 | * 2 * 4 * 4 * 2 | * 4 * 3 * 3 * 3 | * 2 * 2 * 2 * 2 |
| Fig 2h | ELISA | 3 | 4 | 2 |
| Fig 3i, Supp Fig 3, Supp Fig 6 | Metabolomics | 3 | 4 | 1 |
| Fig 3e | Actin ring stain | 1 | 3 | 1 |
| Fig 3g | Von Kossa   * MSC * 2D * 3D | * 3 * 3 * 3 | * 5 * 4 * 4 | * 1 * 1 * 1 |