**1. Title:**

The use of nanovibration to discover specific and potent bioactive metabolites

that stimulate osteogenic differentiation in mesenchymal stem cells

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<http://dx.doi.org/10.5525/gla.researchdata.952>

***Abstract***

Bioactive metabolites have wide-ranging biological activities and are a potential source of future research and therapeutic tools. Here, we use nanovibrational stimulation to induce the osteogenic differentiation of MSCs, in the absence of off-target, non-osteogenic, differentiation. We show that this differentiation method, which does not rely on the addition of exogenous growth factors to the culture media, provides an artefact-free approach to identifying bioactive metabolites that specifically and potently induce osteogenesis. We first identify a highly specific metabolite as the endogenous steroid, cholesterol sulphate. Next, a screen of other small molecules with a similar steroid scaffold identified fludrocortisone acetate as being both specific and having highly potent osteogenic-inducing activity. Further, we implicate cytoskeletal contractility as a measure of osteogenic potency and cell stiffness as a measure of specificity. These findings demonstrate that physical principles can be used to identify bioactive metabolites and then metabolite potency can be optimised by examining structure-function relationship.

**3. Funder information:**

This work was funded by BBSRC project BB/P00220X/1 and EPSRC projects EP/P001114/1 and EP/N013905/1 and EPSRC grant "3D OrbiSIMS: Label free chemical imaging of materials, cells and tissues" EP/P029868/1.

**4. Dataset and readme information**

**Data folders**

**Nanovibrational Apparatus.** Nanovibrational bioreactor design was as previously described7. Briefly, 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, detailed in a previous publication36, 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 30 nm and 1000 Hz frequency.

**Figure 1a** The nanovibrational bioreactor.

**Interferometric measurement.** Vibrational amplitude was measured using a laser interferometry system previously used to accurately measure nanoscale displacements generated by the bioreactor platform used here7, 36. 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. Similarly, for 3D collagen I gel cultures, prismatic tape was adhered to the surface of the gels. 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. For 2D and 3D comparisons of nanovibrational amplitude 65 measurements were taken at multiple locations on the plates.

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

**Figure 1c.** Laser interferometry

**Suppementary figure 1.** Laser interferometry experimental apparatus

**Rheology Measurements**

Rheological measurements were carried out using an Anton Paar 301 rheometer. Strain sweeps were carried out using a parallel plate system with a 25 mm sand blasted plate and a gap of 2.8 mm. 2 mL collagen gels were prepared beforehand in a 12 well plate and then transferred to the rheometer plate for measuring. Strain sweep tests were performed at an angular frequency of 10 rad s-1 and a strain of 0.1 - 5000%. All experiments were performed at 25°C.

*Calculation of Youngs Modulus*

An average of the storage shear modulus was taken within the viscoelastic region (0.1-10 % strain) giving 13.7 Pa. Collagen gels are homogeneous and isotropic from the mechanical point of view, and a reference estimate for the Young’s modulus in the linear region can be obtained from the shear measurements using the following equation:

$$G^{\*}=\frac{E}{2(1+v)}$$

Where G’ is the storage shear modulus, 𝑣 is the Poisson’s ratio and E’ is the Young’s

modulus. Assuming full incompressibility for the material (Poisson’s ratio 𝑣 = 0.5 ), this gives a value of 41.7 Pa for the Young’s modulus.

At ~200% strain some slipping was observed this explains the increase in the size of the error bars in the region beyond this point.

**Data folder: Rheology Figure 1B**

**Figure 1b.** Cross-sectional drawing depicting 2D and 3D culture set-ups

**Application of Nanovibration in 2D and 3D culture.** Stro1+ MSCs were isolated from human bone marrow37. The term “mesenchymal stem (stromal) cell” is now widely used and, indeed, has come to often represent adherent fibroblastic population of cells, even those that are not stem cells based on rigorous criteria. For avoidance of confusion, in this manuscript, we refer throughout to mesenchymal stem cells to reference more appropriately skeletal stem cells – a clonogenic population of non-hematopoietic bone marrow stromal cells able to recreate cartilage, bone, haematopoiesis-supporting stroma and marrow adipocytes on the basis of in vivo transplantation studies38. MSCs were cultured in either expansion media (DMEM (Sigma-Aldrich), 10 % FBS (Sigma), 1 % Sodium Pyruvate (11 mg ml-1, Sigma), 1 % Gibco MEM NEAA (non-essential amino acids, Thermo Fisher Scientific), 2 % antibiotics (6.74 U ml-1 penicillin-streptomycin (Sigma) and 0.2 μg ml-1 fungizone) (Sigma) or osteogenic differentiation media (DMEM expansion media was supplemented with 100 μmol ascorbic acid (Sigma), 100 nmol dexamethasone (Sigma) and 10 mmol glycerol phosphate (Sigma)). Nanovibration (30 nm displacement; 1000 Hz) was applied to MSCs in 2D and 3D culture and compared to unstimulated control cells cultured in expansion media and MSCs cultured in osteogenic differentiation media without nanovibration.

In 2D culture, cells were seeded at 4 x 103 cells per cm2 in standard cell culture plates in either expansion or osteogenic media. For 3D culture, type I collagen gel was prepared by addition of 10x modified Eagle’s medium (Sigma), FBS, expansion media and 2.05 mg ml-1 rat tail type I collagen (First Link) in 0.16% acetic acid. The pH of the collagen solution was neutralised through the addition of 0.1 M NaOH on ice until a constant pH 7 was reached. The appropriate number of MSCs were then added to give a cell density of 4 x 104 cells ml-1 and the solution and mixed by pipette to provide a homogenous cell suspension. Solutions were pipetted into culture 24-well plates (1ml gel/cell solution giving a depth of 5.2mm) and allowed to gel in humidified incubators (37 °C, 5 % (v/v) CO2) for 2 hours. Subsequently, wells were flooded with the relevant media. Plates containing cells for nanovibration were then magnetically attached to the nanovibration bioreactor in cell culture incubators.

**Effect of nanovibration or osteogenic culture on MSC differentiation.** To compare the effects of nanovibration or osteogenic media stimulation on MSC osteogenic differentiation cells were seeded as above into 2D and 3D culture conditions. Over a 28-day time course cells were stimulated continuously with either nanovibration (30 nm/ 1000 Hz), osteogenic media or unstimulated controls. Samples were taken for qRT-PCR as below at days 0, 7, 10, 14, 21 and 28 (n=4 in triplicate) to determine changes in expression of early (Runx2; OSX) and late (OPN; OCN) osteogenic marker genes. For analysis of off-target gene expression induction markers of adipogenesis (PPARγ) and chondrogenesis (SOX9) were analysed by qRT-PCR.

**Data folder. 2D and 3D QPCR**

**Figure 1.** Nanovibration drives osteogenic differentiation in 2D and 3D MSC cultures. **1d**. osteogenic marker expression **1f.** non-osteogenic gene expression

**Supplementary Figure 2,** Osteogenic marker expression in MSCS cultured in 2D and 3D and exposed to nanovibrational stimulation, osteogenic or control media

**Figure 3a** Screening metabolites for osteogenic bioactivity in MSCs

**CS Stimulation culture.** To assess the bioactivity of the steroid library, MSCs were seeded at 4 x 103 cells per cm2 in standard cell culture plates and allowed to attach overnight (37 °C, 5 % (v/v) CO2). Media was then exchanged for media supplemented with the relevant small molecule at 0.001, 0.01, 0.1, 1 or 10 μM. Initial live-dead screening was then performed to assess metabolite toxicity. Non-toxic compound concentrations were selected for bioactivity screening by qRT-PCR after 14 days culture. Through this, 1 μM concentrations were selected for further experimental comparisons in culture time courses up to 21 days.

**Figure 4.** Screening cholesterol sulphate analogues for osteogenic bioactivity in MSCs.

**Alamar Blue Assay.** At determined intervals during culture, cell culture media was removed and cells washed with pre-warmed, sterile PBS. 10 % (v/v) Alamar Blue resazurin (Bio-Rad) was diluted in phenol-red free media (D5030, Sigma) and added to each hydrogel. Cells were incubated in Alamar Blue working solution for 4 hours (at 37 °C, 5 % (v/v) CO2). After incubation, supernatant was transferred to 96-well plates and absorbances read at 570 nm and 600 nm to determine metabolism of Alamar Blue. 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: Alamar blue Figure 4a.** Original data readings in .xls format

**Figure 4a.** Alamar blue analysis of MSC viability in the presence of selected steroids at 1 μM.

**In-Cell Western Assays.** MSCs in 24-well plates were fixed using 10 % (v/v) formaldehyde for 20 minutes at room temperature. Cells were then permeabilised with 0.1 % (v/v) Triton-X in PBS for 10 minutes at room temperature and blocked using 1 % milk protein in PBS-0.1 % (v/v) Tween20 (PBST). Primary antibodies to target proteins diluted in blocking buffer (1:200) were incubated with cells overnight at 4 °C with gentle agitation (Table 2). After incubation, cells were washed 5 times with PBST. As normalisation controls, CellTag 700 stain (LiCOR) was diluted in blocking buffer (1:1000). To this solution, the relevant secondary antibodies were added (1:2000, LiCOR). Cells were incubated with this solution for 1.5 hours at room temperature with gentle agitation, followed by 5 washes with PBST. Quantitative spectroscopic scanning and analysis was carried out using the LiCOR Odyssey Sa. All dyes and secondary antibodies were purchased from LiCOR. For analysis, internally normalised fluorescent intensities were normalised against unstimulated controls to generate fold change fluorescent intensities.

**Data folder: ICW.** Protein expression changes in nanokicked vs control samples or in the presence or absence of metabolite inhibitors were assessed by Incell western.

* **Subfolder: ICW myosin Figure 3c and 5d.** Quantitative spectroscopic plate image .png and quantitative analysis of the intensity readings is provided in a .xlsb file which was used to generate Figure 3c and Figure 5d.

**Figure 3c.** Analysis of p-myosin / total myosin, as measured by Western blot, showing reduced intracellular tension with cholesterol sulphate.

**Figure 5d.** Analysis of p-myosin / total myosin shows increased intracellular tension with OGM and fludrocortisone acetate treatment compared to control.

* **Subfolder:** **Supplementary figure 10A ICW**. Scan images are provided in .Tiff format. The analysed data is provided in .xls files for the data used to generate figure 5A graphs.

**Supplementary figure 10a.** Protein expression over a 14-day time course, during which MSCs were cultured with: 1 μM metabolite analoguess or OGM or DMSO (control) or control media.

**Quantitative polymerase chain reaction with Reverse transcription (qRT-PCR).** RNA was extracted from 2D and 3D cultures through Trizol extraction (LifeTechnologies). Media was removed and cells washed in sterile PBS on ice. Equal volumes of Trizol reagent was added to cells and incubated for 10 minutes at room temperature. Trizol was transferred to 1.5 ml tubes and 0.2 ml chloroform added to each tube per 1 ml of Trizol. Trizol/ chloroform solutions were vortexed and centrifuged (13000 xg/ 4 °C). Following centrifugation, the upper aqueous layer was transferred to a new 1.5 ml tube and an equal volume of 70 % (v/v) ethanol added. This solution was mixed by repeated inversion of the tubes. RNA was then extracted from this solution using the Qiagen RNAeasy extraction kit (including DNAse step) according to the manufacturer’s instructions. RNA was eluted in nuclease-free water and quantified using the Nanodrop and normalised across all samples. cDNA (1000ng per sample) was prepared by reverse transcription using the Qiagen Quantitect Kit according to manufacturer’s instructions. cDNA concentration was normalised to 5 ng μl-1 by dilution in nuclease-free water. Using the 7500 real-time PCR system from Applied Biosystems, qRT-PCR was performed using the Quantifast SYBR green qRT-PCR kit (Qiagen) and specific human gene target primers (Eurofins Genomics) (Table 1-further information in Supplementary Table 1),validated by dissociation/melt curve analysis. 10 ng of cDNA was loaded into each QRT-PCR reaction. QRT-PCR products were quantified using the 2-DDCt method57 and normalised to the housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which was confirmed to remain stable in all culture conditions.

***Table 1. List of Primers.***

|  |  |  |
| --- | --- | --- |
| **Gene** | **Forward Primer** | **Reverse Primer** |
| RUNX2 | GGTCAGATGCAGGCGGCCC | TACGTGTGGTAGCGCGTGGC |
| OSX | GGCAAAGCAGGCACAAAGAAAG | AATGAGTGGGAAAAGGGAGGG |
| OCN | CAGCGAGGTAGTGAAGAGACC | TCTGGAGTTTATTTGGGAGCAG |
| OPN | AGCTGGATGACCAGAGTGCT | TGAAATTCATGGCTGTGGAA |
| ALP | ATGAAGGAAAAGCCAAGCAG | CCACCAAATGTGAAGACGTG |
| PPARy | GACAGGAAAGACAACAGACAAATC | GGGGTGATGTGTTTGAACTTG |
| FABP4 | CCTTTAAAAATACTGAGATTTCCTTCA | GGACACCCCCATCTAAGGTT |
| GLUT4 | CTGTCCACCAAGCCCTCTC | CATCCCCAGTCTCCACTGTT |
| SOX9 | GCTCTGGAGACTTCTGAA | GGTACTTGTAATCCGGGTG |
| COL2A1 | GGCTTCCATTTCAGCTATG | CAGTGGTAGGTGATGTTC |
| ACAN | GGCTTCCACCAGTGTGAC | GTGTCTCGGATGCCATACG |
| GAPDH | TCAAGGCTGAGAACGGGAA | TGGGTGGCAGTGATGGCA |
| OSN | AGAATGAGAAGCGCCTGGAG  | CTGCCAGTGTACAGGGAAGA  |

**Data Folder: 2D and 3D QPCR.** Following different times points of stimulation, in the presence or absence of different metabolite supplements and /or inhibitors, in the bioreactor the MSCs were assessed by qRT‑PCR.

* **Subfolders: QPCR data Figure1d and QPCR data Figure 1f and suppl Fig3f.** Original data files from the ABI 7500 cycler programme in .xls format.

**Figure 1d.** qRT-PCR of osteogenic marker expression in MSCs cultured in different 2D and 3D conditions over a 28-day time course

**Figure 1f and suppl Fig 3f.** qRT-PCR of non osteogenic gene expression.

* **Subfolder: QPCR data Figure 3A, suppl Fig6.** Original data files from the ABI 7500 cycler programme in .xls format.

**Figure 3a.** A comparison of osteogenic marker expression in MSCs supplemented for metabolites that are depleted during OGM and nanovibration-induced osteogenic differentiation.

**Supplementary Fig 6**. Metabolite titration for RUNX2.

* **Subfolder: QPCR data Figure 4B-E.** Original data files from the ABI 7500 cycler programme in .xls format.

**Figure 4b, d, e.** Comparison of osteogenic marker gene expression in MSCs stimulated with metabolite analogues at 1 μM.

* **Subfolder QPCRs raw data and analysis mifepristone set Fig 5b.** MSCs were cultured for 3 weeks in the presence of glucocorticosteroid inhibitor Mifepristone.

**Figure 5b. Glucocorticoid inhibition** resulted in few changes in osteogenic marker expression in control cultures after 3 weeks of culture.

**Supplementary figure 2 same data as Subfolder: QPCR data Figure1d and 1f**

**Immunocytochemistry.** Cells to be immunostained were fixed in 10 % (v/v) formaldehyde/PBS at 4 °C for 1 hour. Cells were permeabilised with 0.1 % (v/v) Triton-X in PBS for 10 minutes at room temperature and blocked with 1 % (w/v) BSA in PBS with 0.1 % (v/v) Tween20 (PBST) for 1 hour at room temperature. Following blocking, the relevant primary antibodies (Table 2) were incubated with cells in blocking buffer over night at 4 °C. Cells were washed 3 times in PBST and incubated with biotinylated secondary antibodies in blocking buffer (1: 50, Vector Laboratories) for 1 hour at room temperature. Cells were again washed 3 times in PBST and incubated with FITC- or Texas Red-conjugated streptavidin in blocking buffer (1:50, Vector Laboratories). Where appropriate, cell F-actin was labelled though 1 hour incubation at room temperature with rhodamine conjugated phalloidin (1:1000 in blocking buffer). Nuclei were stained using Vectashield moutant with DAPI nuclear stain (Vector Laboratories).

**Data folder: Immunostaining**

* **Subfolder: Fig 5d and Suppl fig 8 CS actin images.** Cell images from the different culture conditions provided in .Tiff format.

**Figure 5d.** Fluorescent actin images of control MSCs and MSCs cultured with fludrocortisone acetate, showing more organised stress fibres in cells cultured with fludrocortisone acetate.

**Supplementary Figure 8** Actin cytoskeleton staining of MSCs.

* **Subfolder: Supplementary Figure 10B Immunofluorescence images.** Cell images from the different culture conditions provided in .Tiff format.

**Supplementary Figure 10b** Immunofluorescent staining for ALP and OPN after 14 days culture.

***Table 2.*** *List of Primary Antibodies.*

|  |  |  |
| --- | --- | --- |
| **Target** | **Company** | **Catalogue Number** |
| RUNX2 | Santa Cruz Biotechnology | Sc-390351 |
| Osteonectin | Millipore | AB1858 |
| Osteopontin | Santa Cruz Biotechnology | Sc-21742 |
| Osterix | Santa Cruz Biotechnology | Sc-393325 |
| Alkaline Phosphatase | Abcam | Ab354 |
| Osteocalcin | Santa Cruz Biotechology | Sc-365797 |
| Total Myosin | Cell Signallng | 3672s |
| P(S19)Myosin light chain | Cell Signalling | 3675s |

**Histological Staining. *Oil Red O.*** Cells to be stained were fixed in 10 % (v/v) formaldehyde/PBS at 4 °C for 1 hour. Cells were then washed with distilled water three times and rinsed with 60 % (v/v) isopropanol. Oil Red O solution was then added to the cells and incubated at room temperature for 15 minutes. Dye solution was removed and cells were washed again with 60 % (v/v/) isopropanol, washed three times in distilled water and imaged an inverted microscope (Olympus, Pennsylvania, USA) operated through Surveyor software (v.9.0.1.4, Objective Imaging, Cambridge, UK) and processed using ImageJ (v.1.50g, NIH, USA). ***Alizarin Red staining.*** Cells to be stained were fixed in 10 % (v/v) formaldehyde/PBS at 4 °C for 1 hour. After washing with PBS, fixed cells were stained with 2 % (w/v) Alizarin Red solution (pH 4.1-4.3) for 15 minutes at room temperature. After staining, cells were washed in deionised water and imaged an inverted microscope (Olympus, Pennsylvania, USA) operated through Surveyor software (v.9.0.1.4, Objective Imaging, Cambridge, UK) and processed using ImageJ (v.1.50g, NIH, USA).

**Data folder: Histological Staining.**

* **Subfolder: Oil Red O Figure 4c.** Scanned plate images in .Tiff format

**Figure 4c.** Oil Red O staining of lipid vesicles was observed in (+)-4-cholesten-3-one, triamcinolone and OGM stimulated MSCs.

* **Subfolder: Supplementary figure 10d. Alizarin red.** Scanned plate images in .Tiff format

**Supplementary figure 10d.** Alizarin red staining after 28 days of culture with: 1 μ M metabolite analogs, osteogenic media, DMSO (control) or control media.

**Metabolomics.** MSCs were stimulated with nanovibration for 7 and 14 days in 2D and 3D (collagen gels; 2 mg ml-1) culture. Non-stimulated samples cultured in expansion media and osteogenic media were used as controls. Metabolites were extracted using a 1:3:1 chloroform/ methanol/ water extraction buffer and vigorously shaken at 4 °C for 1 hour. Following this, metabolite extraction solution was collected, transferred to 1.5 ml tubes and centrifuged for 3 minutes at 13000xg at 4 °C. 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 XCMS40 (peak picking), MzMatch41 (filtering and grouping) and IDEOM42 (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 time43. 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**

* **Subfolder:** Metabolomics Figure 2, 3, suppl Fig4,5 Transfer-NWPEpfsUpKVsj2qJ contains MzXML files

**Figure 2a, b, c** suppl Fig4 Metabolic profiles of MSCs in 3D culture for 7 days with osteogenic media or nanovibration.

**Figure 3b,c** suppl Fig5 Screening metabolites for osteogenic bioactivity in MSCs.

* **Subfolder: Metabolomics Fig 5a** contains MzXML files

**Figure 5a.** Metabolomics Figure 5a . The effects of glucocorticoid inhibition (mifepristone) and mineralocorticoid inhibition (canrenone) on the top-ranked metabolite-driven biochemical networks.

**Chemistry.** Dexamethasone, cholesterol sulphate, fludrocortisone, fludrocortisone acetate, triamcinolone, cholic acid, and (+)-4-cholesten-3-one were obtained from commercial suppliers and used as received. Cholesta-1,4-dien-3-one was prepared according to a literature procedure on related steroids44 to a solution of (+)-4-cholesten-3-one (100 mg, 0.26 mmol) in dioxane (1.6 mL) was added tert-butyldimethylsilyl chloride (2.0 mg, 0.013 mmol) then the mixture was cooled to 0 °C. To the solidified solution was added DDQ (66 mg, 0.29 mmol). The mixture was allowed to warm to room temperature, then stirred for 3 days. The solvent was removed *in vacuo* and the residue dissolved in CH2Cl2 (40 mL), then washed with sat. aq. Na2S2O3 (40 mL), NaHCO3 (40 mL) and brine (40 mL). The organic phase was dried over Na2SO4, filtered and concentrated *in vacuo* to give a yellow oil. Purification by flash chromatography (petroleum ether:ethyl acetate, 9:1) afforded the title compound as a white solid (33 mg, 33%). Analytical data were in accordance with literature values45.

1H NMR (400 MHz, CDCl3) δ (ppm): 7.05 (1H, d, *J* = 10.1 Hz), 6.22 (1H, dd, *J* = 10.1, 1.9 Hz), 6.06 (1H, s), 2.50–2.42 (1H, m), 2.37–2.32 (1H, m), 2.06–2.01 (1H, m), 1.96–1.79 (2H, m), 1.69–1.46 (6H, m), 1.36–1.26 (4H, m), 1.23 (3H, s), 1.20–0.97 (9H, m), 0.90 (3H, d, *J* = 6.5 Hz), 0.86 (6H, dd, *J* = 6.6, 1.8 Hz), 0.74 (3H, s).

**Supplementary figure 12.** 1H NMR of Cholesta-1,4-dien-3-one.

**Alkaline Phosphatase Activity Assay.** To assess alkaline phosphatase (ALP) activity in cultured cells a colorimetric assay was used (Abcam; ab8369). This kit uses p-nitrophyenyl phosphate (pNPP) as a phosphatase substrate which turns yellow (ODmax = 405 nm) when dephosphorylated by ALP. Increases in ALP activity in cultured MSCs are indicative of the formation of osteogenic cell phenotypes. The assay was performed according to the manufacturer’s instructions. Briefly, cells to be assayed were trypsinised, counted, pelleted and washed in ice cold PBS. Cells were then resuspended in 50 μl Assay Buffer per 1x105 cells. Cells were homogenised on ice and centrifuged at 13000 xg for 15 minutes at 4 °C. The supernatant was transferred to a new tube. Supernatant volume to be added was optimised based on standard curve concentrations and reaction volume adjusted to 80 μl/ well. 50 μl of 5 nM pNPP solution was added to each well and incubated at 25 °C for 60 minutes protected from light. Stop solution was then added to each well and OD405 nm measured on a microplate reader. Corrected mean absorbance values were calculated by subtracting blank readings and ALP activity determined by applying the generated standard curve and using the following equation:

ALP activity (μmol/min/ml or U/ml) = (B/ΔT\*V)\*D

Where, B = amount of pNP in sample well calculated from standard curve (μmol), ΔT = reaction time (minutes), V = original reaction sample volume (ml), D = Sample dilution factor.

**Data folder: ALP activity assay Fig 1e, suppl Fig3e and suppl Fig 10c**.

* **Subfoder: ALP activity assay Figure 1E.** Plates were scan on the microplate reader MultiscanFC and software used: SkanIt Software 4.1 for Microplate Readers RE, ver. 4.1.0.43. Data is provided in .xlsx format. Analysed data was used to generate Figure 1e.

**Figure 1e.** ALP activity assay. ALP activity measured in NK, control and OGM media.

* **Subfolder: Supplementary Figure 10c ALP activity assay**. Plates were scan on the microplate reader and software used: SkanIt Software 4.1 for Microplate Readers RE, ver. 4.1.0.43. Data is provided in .xlsx format. Analysed data was used to generate supplementary figure 5c.

**Supplementary figure 10c** ALP activity assay after 28 days stimulation in culture by 1μ M metabolite analogs, osteogenic media and DMSO (control) vs control media.

**Bioactive compound specificity.** To determine the osteogenic specificity of bioactive compounds a ranking system was developed and employed. Osteogenic (RUNX2, OSX, ALP, OPN), adipogenic (PPARγ, FABP4, GLUT4) and chondrogenic (SOX9, ACAN, COL2A1) gene expression was used to determine induction of differentiation along each lineage. Fold change gene expression after stimulation with each compound for 21 days was determined at 1 µM concentration ­­­­­­­­and fold changes grouped and scored as following- Fold change 1-2, 1 point; 2-5, 2 points; 5-10, 3 points; 10-20, 4 points; over 20, 5 points. Scores for each gene were recorded and mean values for each category and each metabolite calculated out of 5. These scores were then plotted against each other in pairs to determine a relative osteogenic versus chondrogenic and osteogenic versus adipogenic gene expression induction, providing information on potency and specificity of small molecule action.

**Figure 4b, c** Screening cholesterol sulphate analogs for osteogenic bioactivity in MSCs.

**Supplementary Figure 8.** Actin cytoskeleton staining of MSCs when cultured in the presence of OGM, Fludrocortisone or cholesterol sulphate.

**Pathway inhibition.** In order to assess the target specificity of the glucocorticoid and mineralocorticoid stimulation, we used the inhibitors mifepristone (M8046, SIGMA) and canrenone (SML 1497, SIGMA) respectively. Cells were seeded at 2x103 cells/cm2 for the specified duration of experiments (7 days for the metabolomics experiments and 3 weeks for the long-term effect of mifepristone induced glucocorticoid inhibition in osteogenic marker expression). Mifepristone was used at 100µM and canrenone was used at 100µM supplemented in the medium with every feed (twice per week) for the duration of each experiment.

**3D OrbiSIMS.**3D chemical image analysis of the sample series was performed using dual beam (mode 926) ToF spectrometry employing a 30 keV Bi3+ primary ion source (0.3 pA target current) and a 10 keV Ar1450+ sputter ion source (3 nA target current). A sputter crater of 400 × 400 µm was etched with the central 200 × 200 µm area analysed at a resolution of 256 × 256 pixels. In each case >3 cells were analysed per sample area. Cells were also depth profiled using single beam OrbiTrap analysis (mode 426) in order to acquire relatively high resolution mass spectrometry data (>240,000) for the sample series. In this case a 20 keV Ar3000+ primary ion source (240 pA target current) was employed with a sputter crater of 284 × 284 µm with the central 200 × 200 µm area analysed. Three analytical repeat areas were analysed for each sample. A random raster function was applied throughout as well as charge compensation with the application of a low energy electron floodgun.

**Data folder: 3D OrbiSIMS data Fig3c, 5c, suppl Fig 7 and suppl Fig 11**

File format .ITM and requires SurfaceLab 7, IONTOF GmbH software.

**Figure 3c.** 3D OrbiSIMS of MSCs exposed to cholesterol sulphate.

**Figure 5c.** Fludrocortisone acetate products

**Supplementary figure 7** 3D OrbiSIMS of MSCs exposed to cholesterol sulphate.

**Supplementary figure 11** 3D OrbiSIMS data showing lack of detectable dexamethasone and fludrocortisone acetate structures in the MSCs after 4 days of culture.

**Single cell force spectroscopy (SCFS)**

***Experimental approach***. Single cell mechanics was evaluated using a nanoindentation device (Chiaro, Optics11, Amsterdam, NL) mounted on top of an inverted phase contrast microscope (Evos XL Core, Thermofisher, Paisley, UK) following a previously described approach46. hMSCs (Promocell, passage 2) were left to incubate for 72 h at 37 °C and 5% CO2 with the corresponding media (basal media, osteogenic media, basal media with metabolites). They were then washed one time with basal media just before the measurement was started. All the measurements were acquired at room temperature, keeping the measuring time under 90 minutes, to avoid changes of the mechanical properties associated to cell degeneration. A total of 35 cells from 2 biological replicates were measured for each condition. The selected cantilever had a stiffness of 0.032 N/m and held a spherical tip of 3.25 µm radius (serial number P190610). A tight 3x3 map with 500nm spacing was acquired (total of 9 indentations) aiming at the cellular soma (above the nucleus). Single indentations were acquired at the same speed of 2 µm/s exploiting the whole range of the vertical actuator, 10µm. After every experiment, the probe was washed in ethanol 70% for 10 min.

**SCFS data analysis**. The collected curves were bulk analysed using a custom software programmed with Python 3 (Python Software Foundation, www.python.org) and the Numpy/Scipy Scientific Computing Stack47. Curves were first aligned using a baseline detection method based on the histogram of the force signal48 and the corresponding indentation was calculated for each curve. In order to quantify the mechanical properties, data were fitted with the Hertz model49. While the hypothesis behind the theoretical derivation of the Hertz formula (isotropy, homogeneity and pure elasticity of the sample) are fairly satisfied by a cellular system, it has been shown that the corresponding Young’s modulus can provide a robust indicator of the elasticity if the experimental procedure is carefully designed50. To ensure consistency of the results, all the experimental parameters were kept constant during an experimental session for all different conditions (in particular, the same probe and calibration were used), and the results were reported indicating changes of elasticity relative to the control. The average absolute value for the control is also reported, but relative changes are typically more reliable and meaningful50.

**Cortical and Bulk stiffness**. The calculation of the Young’s modulus of single cells based on nanoindentation experiments strongly depends on the indentation depth of the corresponding measurement. This effect is partially due to artefacts such as the finite thickness of the sample51 and the parabolic approximation for the calculation of the Hertz formula52. Keeping the maximum indentation used in the calculation under about 10-15% of the thickness and 20-25% of the radius of the indenter is a rule of thumb typically used in literature (in our case, 600nm-700nm would match these requirements). Nevertheless, also for indentations lower than this threshold, a trend in the measured Young’s modulus as a function of the indentation depth appears, which is associated to the inhomogeneity of the cell53. Here we exploited this approach, trying to isolate the elasticity of the cortical region from the bulk of the cell. The actomyosin cortex is a very thin network of cytoskeletal elements lying directly beneath the plasma membrane and present in all mammalian cells54. The thickness of this rigid and compact structure challenges current microscopy approaches, and a precise measurement is often complex, but existing estimates typically range between 200nm and 300nm55. In the current work, we selected an indentation depth of 270nm to identify the cortical region, and we call “cortical elasticity” the value of the Young’s modulus obtained evaluating all the indentation curves up to this threshold. Similarly, we call “bulk elasticity” the value of the Young’s modulus calculated up to an indentation of 640nm (10% of the tip diameter, the maximum to remain in the Hertzian regime).

**Data folder: SCFS data Figure 3C.** Data is provided in.txt format. There is also a .txt file providing experimental explanation.

**Figure 3c.** OribiSIMS image of cholesterol sulphate within MSCs, d=1, r=3. Cell stiffness (assayed as Young’s modulus and measured by nanoindentation) for MSCs cultured in control, OGM and cholesterol sulphate containing media (Chol S1 = 1 μM cholesterol sulphate, Chol S2 = 2 μM cholesterol sulphate). These results show that cholesterol sulphate reduces the stiffness of the cortical and bulk cell regions.

**Figure 5d**Cell stiffness (assayed as Young’s modulus and measured by nanoindentation) for MSCs cultured in control, OGM and Fludrocortisone acetate containing media (FA= 1 μM).

**Statistics.** Statistical analysis of the effects of nanovibration and osteogenic media on ostegenic gene expression through qRT-PCR was performed by one-way ANOVA with Holm-Sidak’s multiple comparison test, data are means ±SEM or ±SD. Statistical analysis of off-target gene expression induction by nanovibration or osteogenic media was conducted through Kruskal-Wallis with Dunn’s multiple comparisons test. Alkaline phosphatase assay data was statistically analysed through Kruskal-Wallis with Dunn’s multiple comparisons test. Bioactive small molecule-mediated induction of osteogenic genes was statistically analysed through Kruskal-Wallis with Dunn’s multiple comparisons test. AlamarBlue experiments were statistically compared by one-way ANOVA with Geisser-Greenhouse correction and Tukey multiple comparison test. All statistical analysis was performed using GraphPad Prism Software (v8.0.0; GraphPad Software Inc.)

Please note that we denote replicates as follows: number of donors that were used for the particular experiment (i.e. experimental repeats) = d, replicates (i.e. number of wells) = r and technical replicates for qPCR etc to test pipetting error = t (if used). Full information is given in table 3.

***Table 3.*** *Replicates used for each experiment.*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Figure** | **Experiment** | **Cells from X Number of Donors** | **Well Replicates per group** | **Technical Replicates per group** |
| 1D, S2 | Nanovibration/ OGM osteogenic gene expression analysis- qRT-PCR | 1 | 4 | 3 |
| 1E, S3E | Alkaline Phosphatase Activity assay | 3 | 2 | 3 |
| 1F, S3F | Off-target gene expression in nanovibration, OGM stimulated cells- qRT-PCR | 3 | 3 (pooled) | 3 |
| 2 | Metabolomics | 1 | 4 | 1 |
| 3A | Metabolite compounds osteogenic gene expression- qRT-PCR | 1 | 3 | 3 |
| 3C | Nanoindentation | 2 | 2 | >100 |
| 4A | Alamar Blue | 2 | 2 | 3 |
| 4B | Metabolite compounds induction of osteogenic gene expression qRT-PCR  | 4 | 3 | 3 |
| 4C | Off-target gene expression effects of compounds | 4 | 3 | 3 |
| 4D, 4E | Off/ on-target gene expression effects of compounds | 4 | 3 | 3 |
| 5B | Glucocorticoid receptor inhibition- qRT-PCR | 1 | 4 | 2 |
| 5C, S7 | 3D OrbiSIMS | 1 | 3 | 3 |
| 5D | Nanoindentation | 2 | 2 | >100 |
| 5D | Myosin | 1 | 6 | 1 |
| S8 | Actin staining | 1 | 3 | 1 |
| S10A | In-cell western of osteogenic protein expression compound supplementation | 1 | 2 | 2 |
| S10B | Immunofluorescent staining | 1 | 2 | 5 |
| S10C | Colorimetric alkaline phosphatase activity assay | 2 | 5 | 2 |
| S10D | Alizarin red staining | 3 | 1 | 5 |
| S6 | Effects of compound concentration- qRT-PCR | 4 | 3 | 3 |
| S9 | Effects of compounds on fibroblasts | 1 | 3 | 3 |

5. Raw data can be found at http://dx.doi.org/10.5525/gla.researchdata.952

6. No restrictions

7. No preference

8.