Supplementary information for:

Stimulation of 3D osteogenesis by mesenchymal stem cells via a nanovibrational bioreactor.

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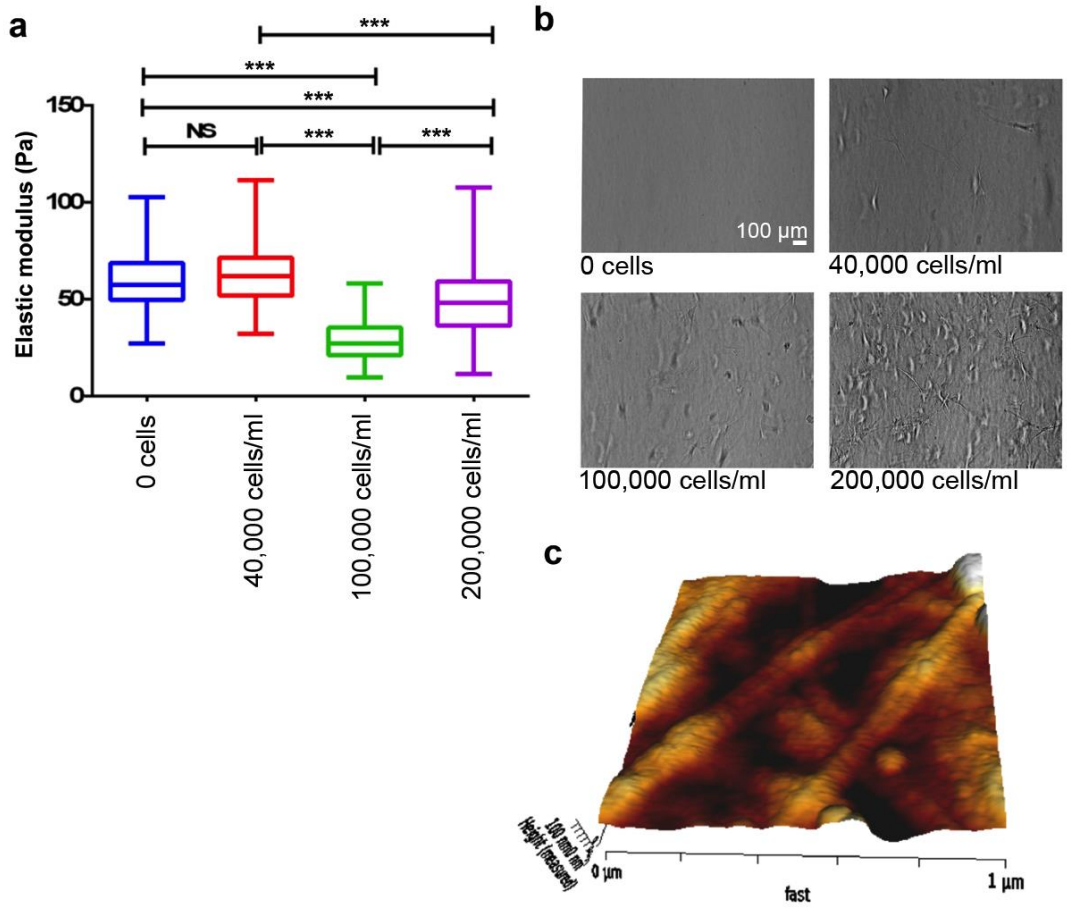
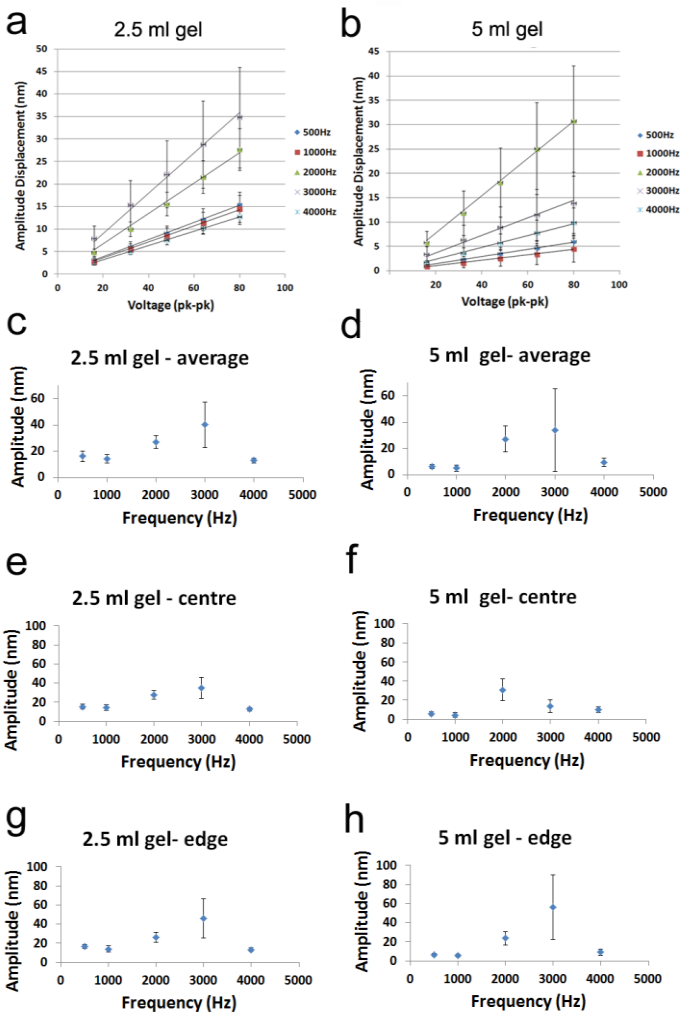
Supplementary table 1. *Material properties of the collagen gels, with and without MG63 cells. Values for shear moduli were taken from rheological measurement and used to calculate elastic and bulk moduli. Gels were seeded with 40,000 MG63 osteoblasts / ml). Results are mean ± SD, N=3. Note MG63 cells were used for interferometry as a model cell to prevent using primary human cells with equipment in a non-class II environment. Further note that modulus falls sharply when >100,000 cell/ml were seeded.*

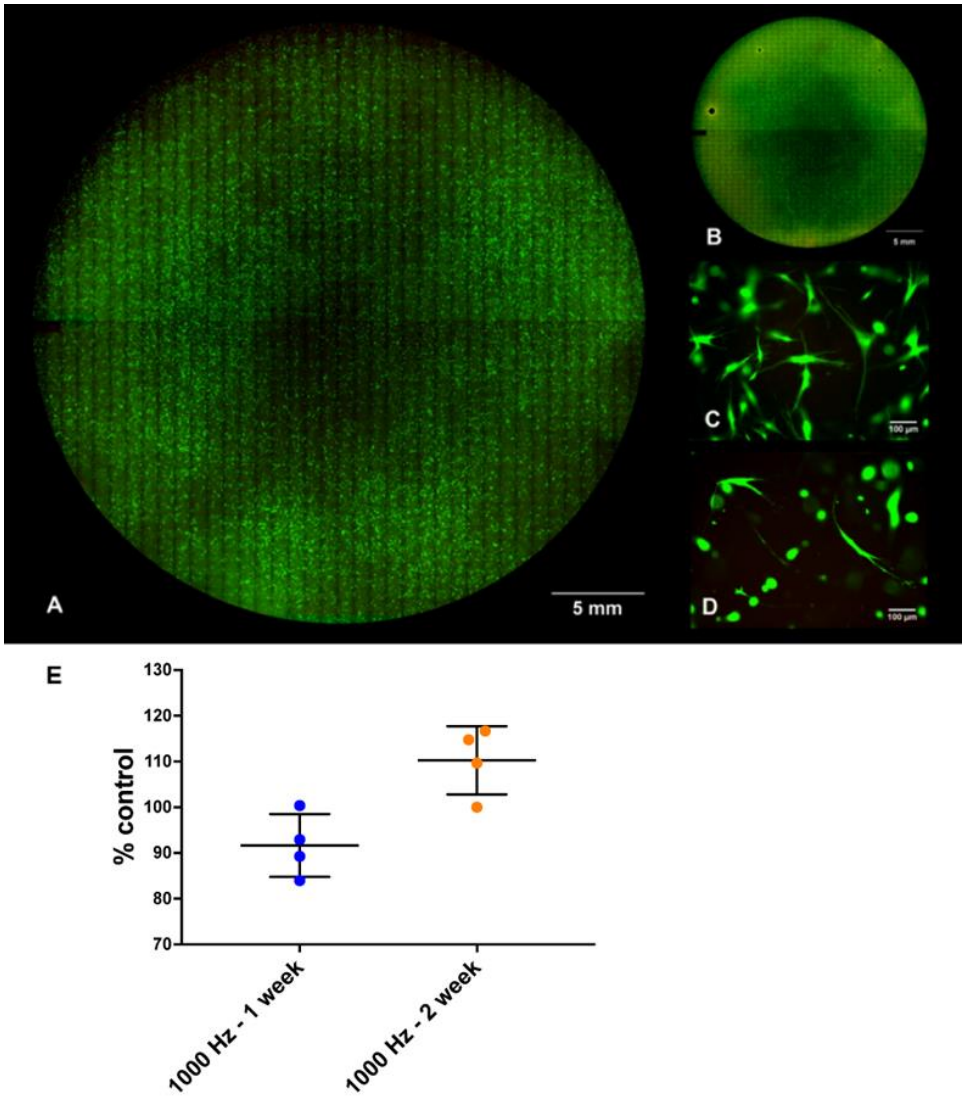
	Shear Moduli, G (Pa)	Elastic Moduli, E (Pa)	Bulk Moduli, K (MPa)	Poisson Ratio, ν
Collagen	36 ± 7.4	108 ± 22.2	1800 ± 370	0.5
Collagen/cells	24.5 ± 2.3	73.5 ± 6.3	1225 ± 105	0.5



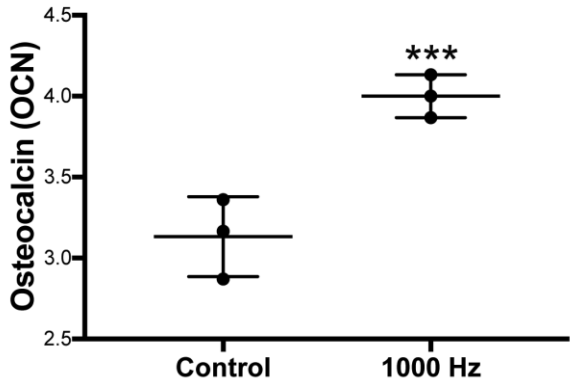
Supplementary Figure 1. *AFM stiffness measurements with increased cell loading. Values for elastic moduli were taken using AFM in tapping mode. Gels were seeded with 40,000 MG63 osteoblasts /ml in 2.5 ml gels. (A) data showed that stiffness was reduced if >40,000 cells were used (n=3, results show median with second and third quartiles (box) and upper and lower quartiles (whiskers), stats by ANOVA with Tukey test where *=p<0.05, **=p<0.01, and ***=p<0.001). (B) Increase in cell density in the gels could be seen using phase microscopy and (C) AFM revealed cells within collagen fibrils (gel with 200,000 cells/ml shown).*



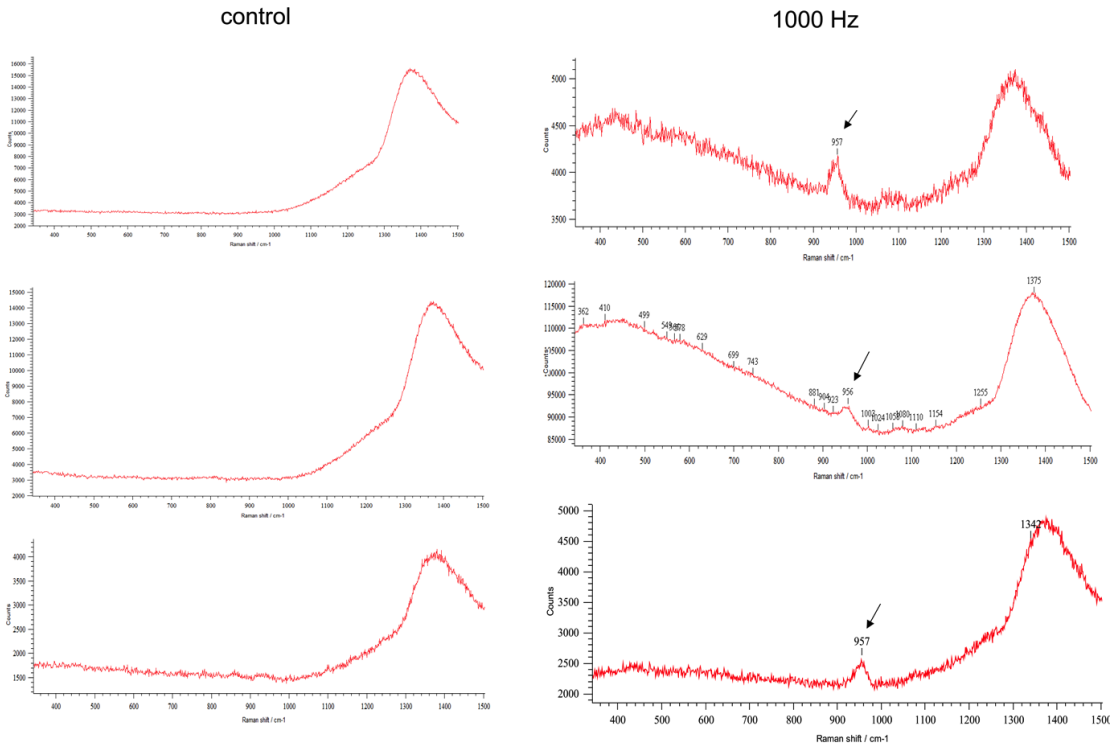
Supplementary Figure 2. *Comparison of 2.5 mL and 5.0 mL collagen gel fill on the nanovibration effect. (a&b) Collagen gel interferometric measurements change in displacement (nm) relative to voltage at a particular frequency, measurements performed on the collagen gel surface in a 6 well plate. (a) Assessment performed on a 2.5 mL collagen gel fill. (b) Assessment performed on a 5 mL collagen gel fill. Frequencies assessed were 500, 1000, 2000 and 4000 Hz and mean measurements appeared to be linear across voltages measured i.e. 16, 32, 48, 64, and 80 V (peak to peak). (c to h) Collagen gel interferometric measurements - displacements in nm were measured in 6 well plates, measurments were performed at 80 V for 500, 1000, 2000,3000 and 4000 Hz. (c) Summary of displacement in nm for a 2.5 mL gel fill (d) Summary of displacement in nm for a 5 mL gel fill. (e) 2.5 mL gel fill meansurements for centre only. (g) 2.5 mL gel fill measurements for edges only. (f) 5 mL gel fill measurement for centre only. (h) 5 mL gel fill measurement for edges only. N = 6 measurements for (c-d), and N = 3 measurements for (a-b, e-h).*



Supplementary Figure 3. *Viability and metabolic activity of MSCs seeded into collagen gels with/without nanovibrational stimulation. A&C are low and high magnification images of live/dead staining (live cells are green, dead cells are red) for MSCs in collagen gels after 14 days of nanovibrational stimulation showing a highly viable cell population. B&D are low and high magnification images of live/dead staining for MSCs in collagen gels with no nanovibrational stimulation after 14 days culture showing a highly viable cell population. Alamar blue metabolic analysis showed that the nanovibration stimulated MSCs had higher activity at 14 days culture compared to unstimulated 3D culture controls. D=1, r=4, results = mean±SD, stats by ANOVA and Kruskal Wallis test where *=p<0.05.*



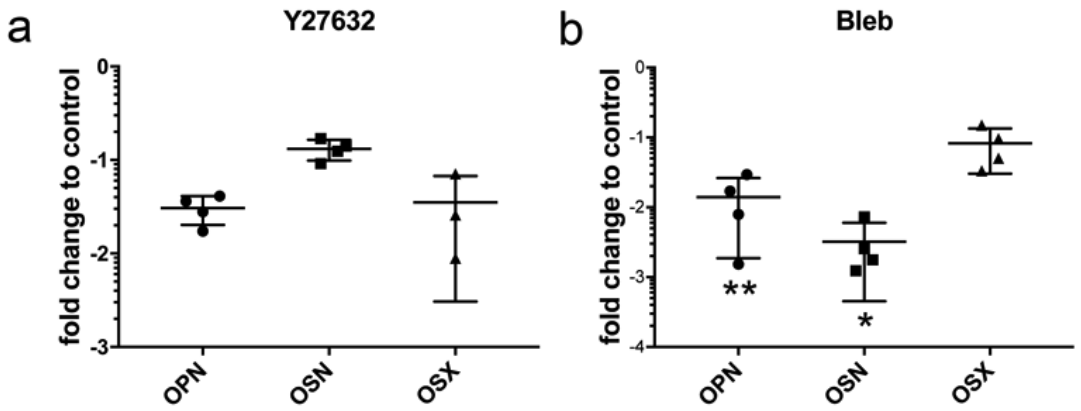
Supplementary Figure 4. *Osteogenic assessment of nanovibration on bone protein expression. MSCs exposed to nanovibrations at 1000 Hz for 21 days in 3D assessed against unstimulated controls for quantitative protein expression of osteocalcin OCN using in cell western showing significant increase by nanovibrational stimulation. Seeding = 100,000 MSCs per gel, d=1, r= 6, results are mean ± SD, ***p< 0.05 by unpaired T-Test.*



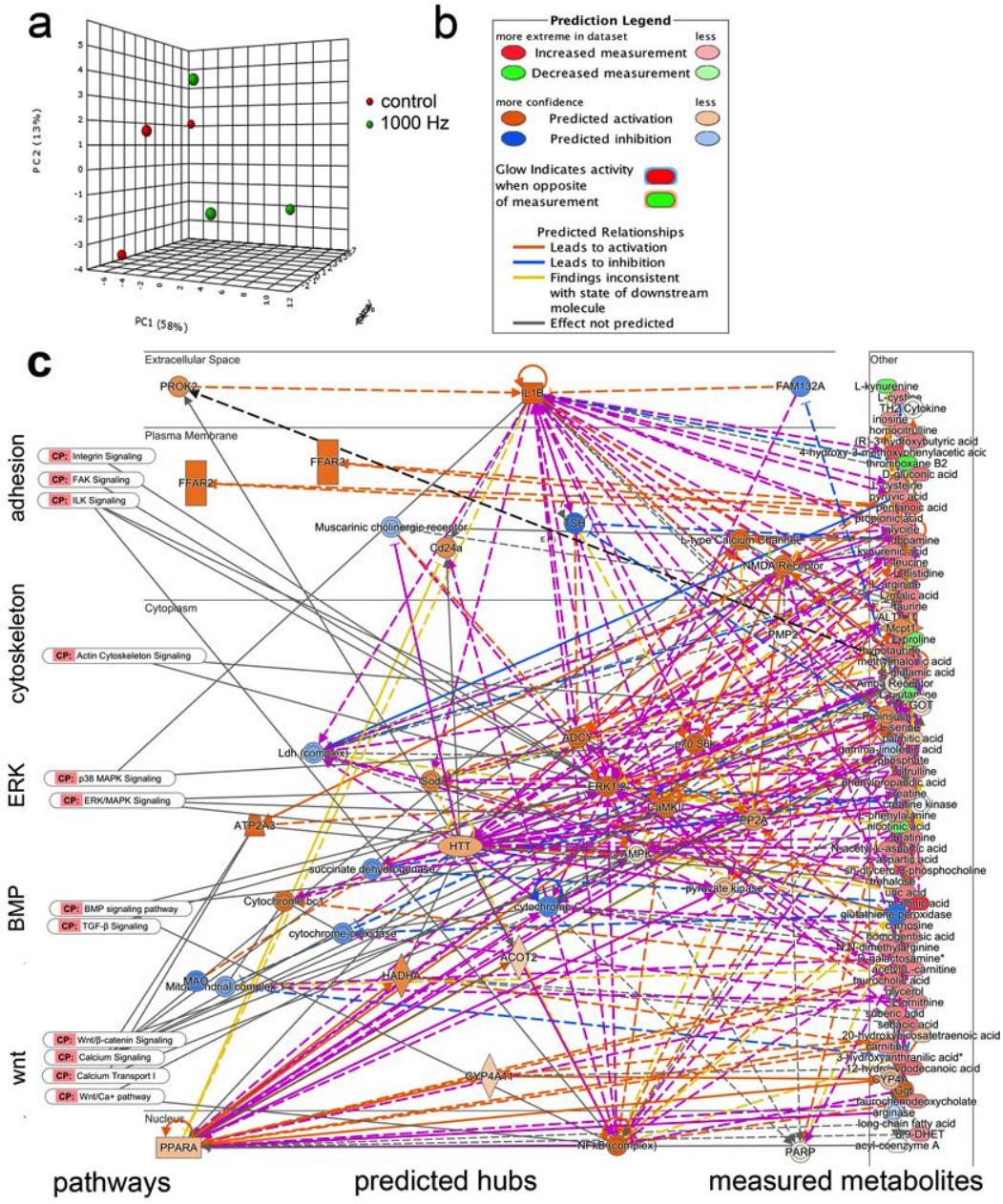
Supplementary Figure 5. *Further Raman Spectra. Arrows indicate phosphate peak.*

Supplementary table 2. *Quantitative results of Raman scattering. Raman spectra results for nanovibrated samples relative to the reference standard of cortical compact bovine mineralised bone. Values are shown for the Raman shift peak height, area and full width at half height maximum (FWHM). The lower the value the greater the crystalline alignment and hence an indication of mineral density[1,2].*

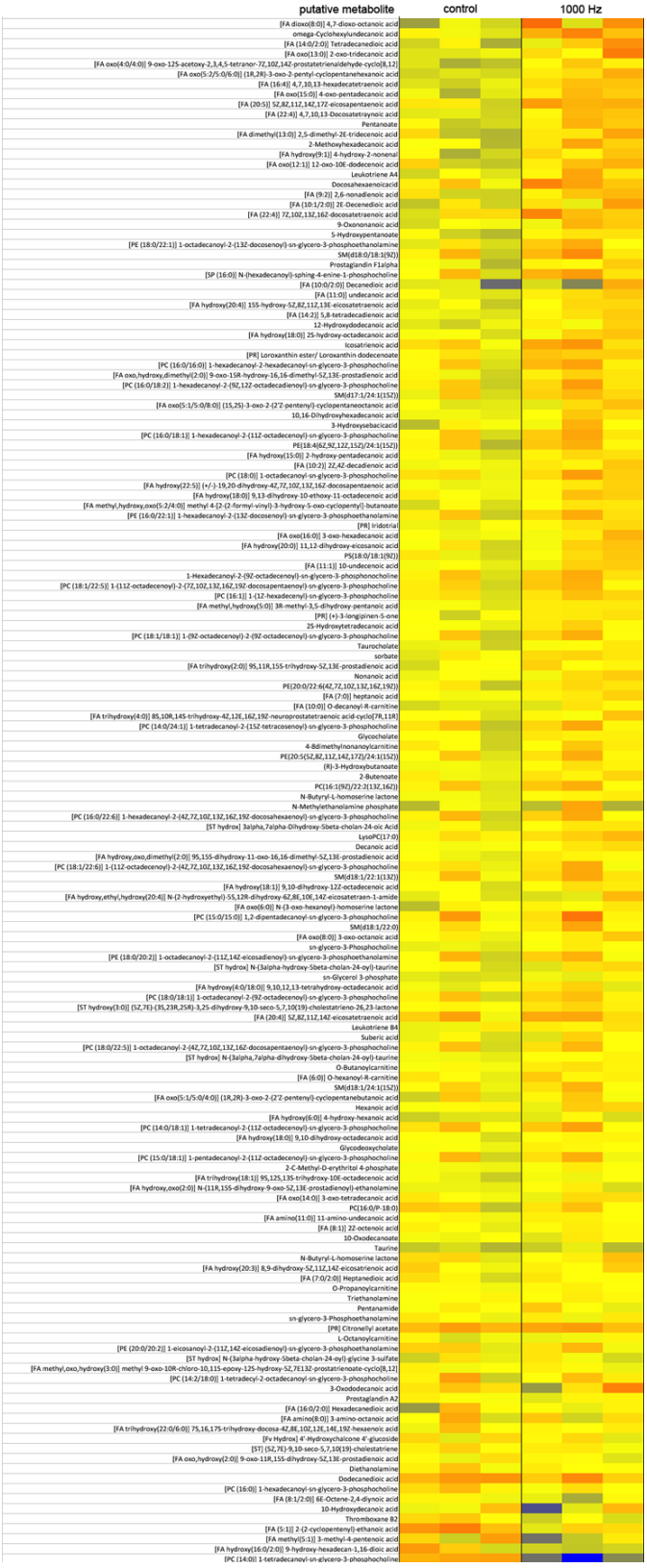
	Bovine Cortical Bone	1000 Hz (mean value)
Peak Raman Shift	960 cm^{-1}	960 cm^{-1}
Height	134510	2260
Area	3.030×10^7	2.3×10^6
FWHM	16.754	28.495



Supplementary Figure 6. *Analysis of osteoblast genes at 14 days of culture after inhibition of intracellular tension. Data looking at osteopontin (OPN), osterix (OSX) and osteonectin (OSN) shows down regulation of OPN and OSN with blebbistatin inhibition at this time point. Results are mean ± SD (d=1, n=4, t=2), stats by ANOVA with Kruskal Wallis and Dunn's post-test where *=p<0.05 and **=p<0.01. Values are on a log scale.*

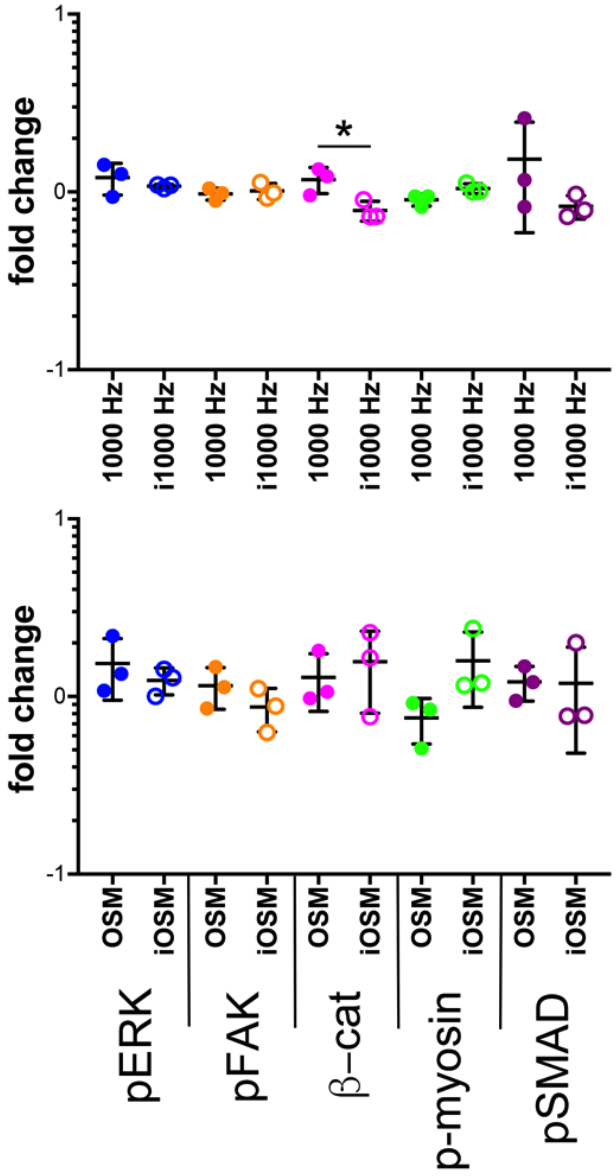


Supplementary Figure 7. *Metabolomics links to canonical signalling pathways. (a) Principle component analysis showing separation between control and nanostimulated cells after 5 days of 3D culture. (b&c) Key for, and analysis of, day 5 metabolomics data showing predicted interactions with canonical signalling pathways involved in focal adhesion, cytoskeletal tension, ERK 1/2 signalling, BMP signalling, and Wnt signalling (d = 1, r = 3).*

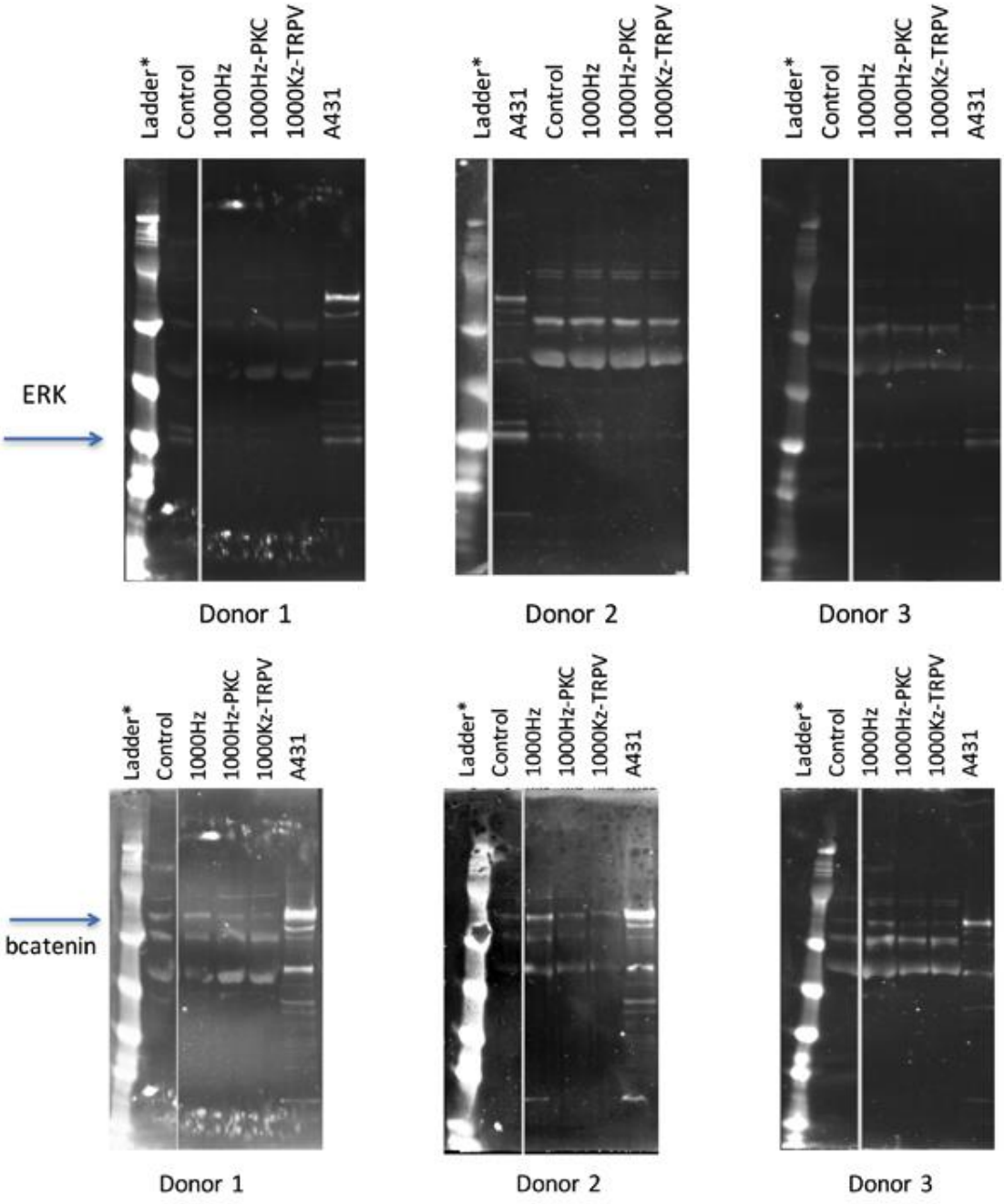


Supplementary Figure 8. *Heatmap of lipid metabolism. More up-regulations in the lipid class are seen in MSCs cultures in 3D with nanovibrational stimulation. Red=up, blue = down and yellow = no change. D = 1, r = 3.*

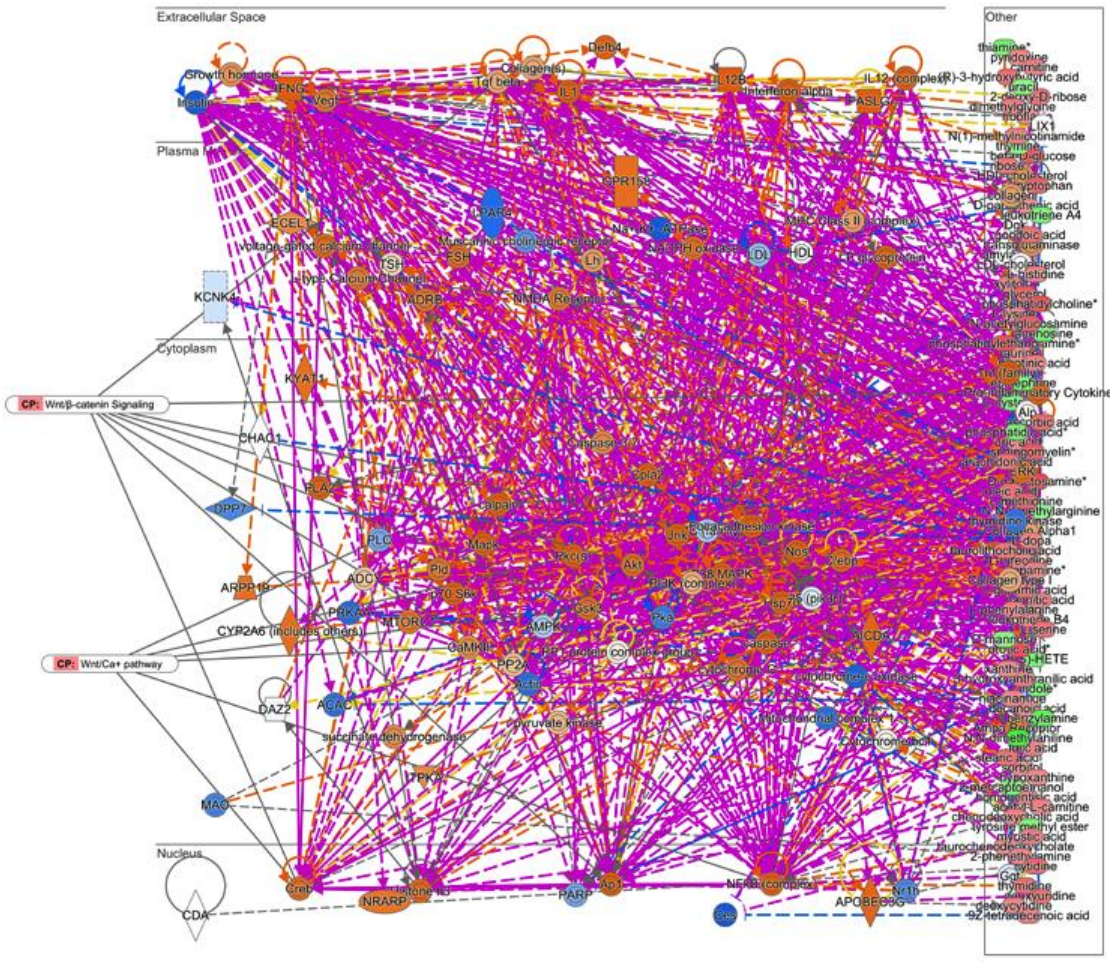
Supplementary Figure 9. *Heatmap of carbohydrate metabolism. More up-regulations in the carbohydrate class are seen in MSCs cultures in 3D with nanovibrational stimulation. Red=up, blue = down and yellow = no change. D = 1, r = 3.*



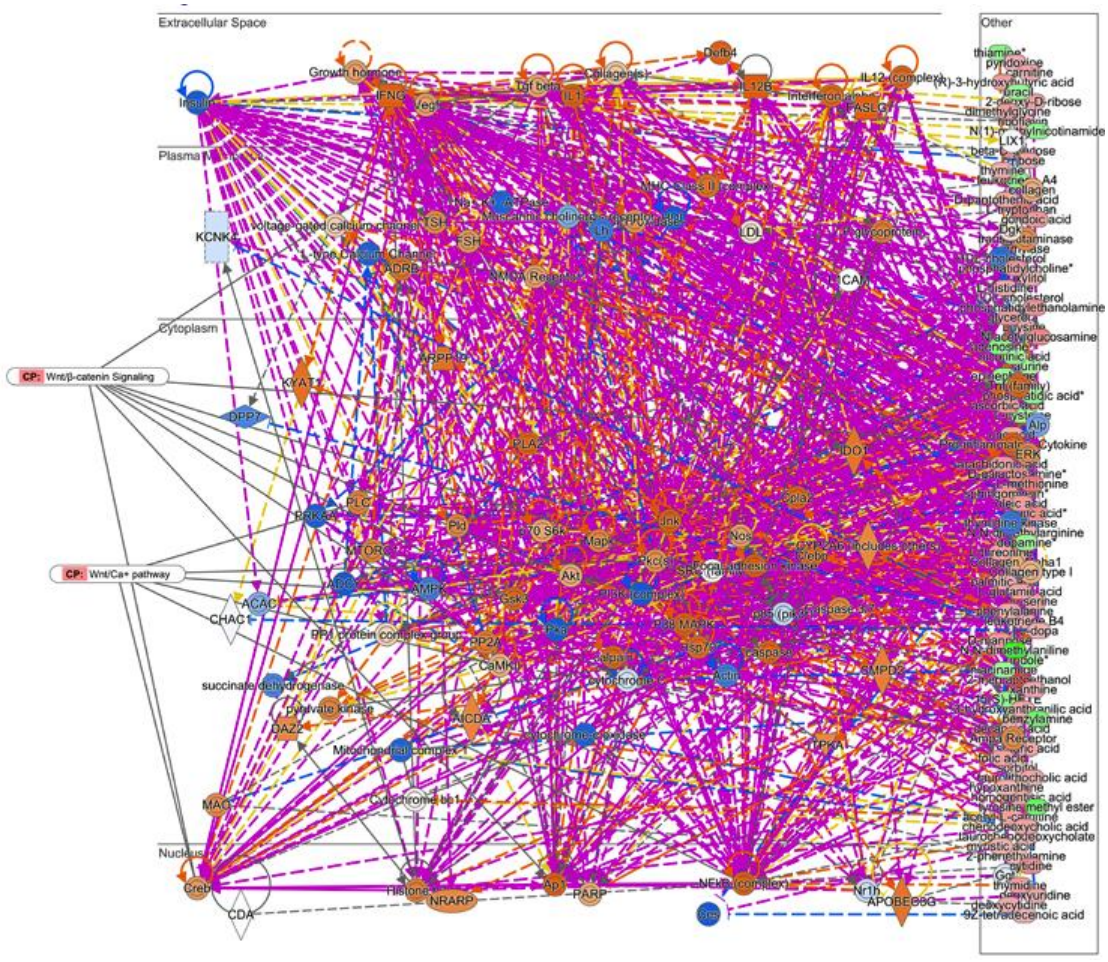
Supplementary Figure 10. *Western blot analysis for 3D cultures. (a) Comparison between 3D MSCs with nanovibrational stimulation and with similar treatment but with PKC inhibition (i) showing a decrease in β-catenin. (b) Comparison between 3D control MSCs and with similar treatment but with PKC inhibition (i) showing no significant change. D=3, r=3 (4 pooled/lane), *=p<0.05 by Tukey test. Values are on a log scale.*



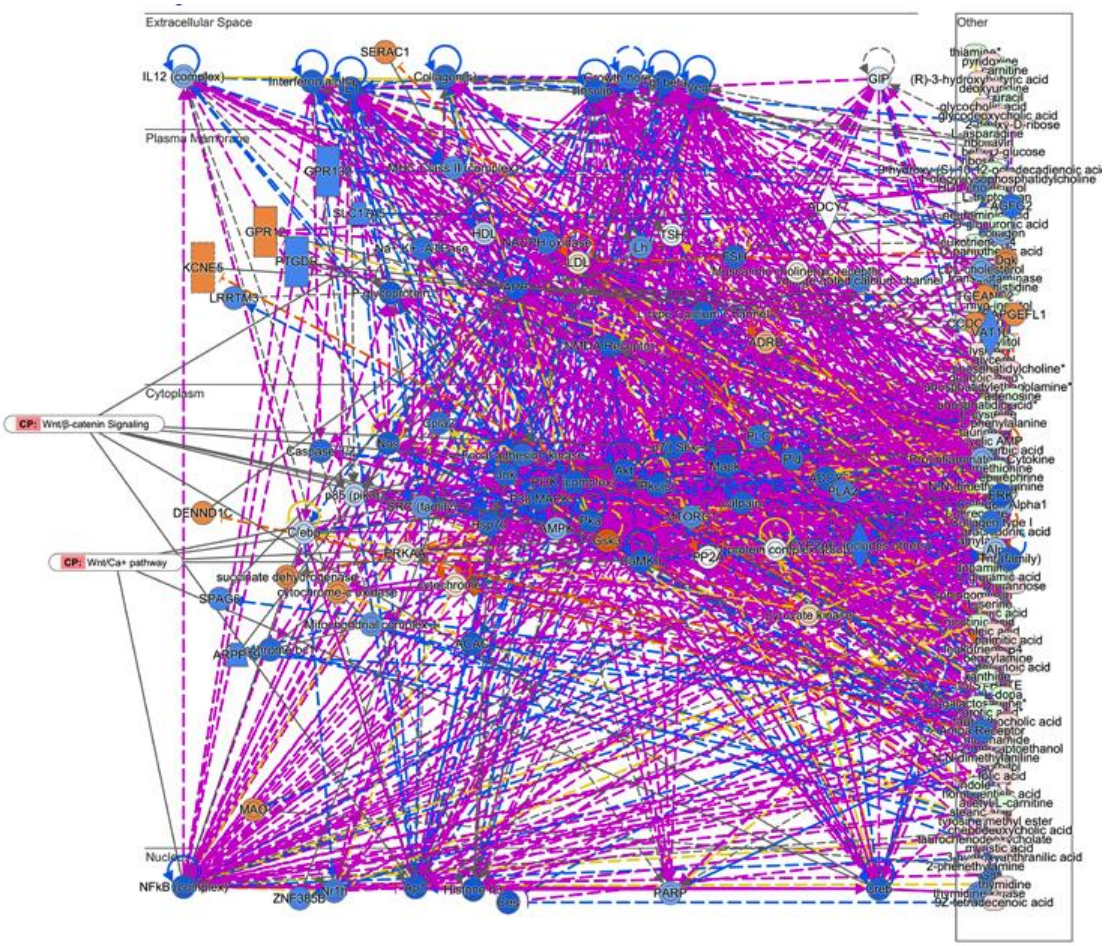
Supplementary Figure 11. *Western blots corresponding to Figure 5b.*



Supplementary Figure 12. *Metabolomics links to wnt signalling for nanovibrationally stimulated MSCs after 5 days of culture. Metabolite changes (left hand side, red=up-regulation, green = down-regulation) leads to a majority of predicted up-regulations (orange = up-regulation, blue = down-regulation) for biochemical hubs feeding into Wnt signalling. D = 3, r = 4.*



Supplementary Figure 13. *Metabolomics links to wnt signalling for NS-PKC stimulated MSCs after 5 days of culture. Metabolite changes (left hand side, red=up-regulation, green = down-regulation) leads to more of a balance of predicted up-regulations and down-regulations (orange = up-regulation, blue = down-regulation) for biochemical hubs feeding into Wnt signalling. D = 3, r = 4.*



Supplementary Figure 14. *Metabolomics links to wnt signalling for NS-TRPV stimulated MSCs after 5 days of culture. Metabolite changes (left hand side, red=up-regulation, green = down-regulation) leads to a prevalence of down-regulations (orange = up-regulation, blue = down-regulation) for biochemical hubs feeding into Wnt signalling. D = 3, r = 4.*

Supplementary Table 3. *RT-PCR Reverse transcription thermal programme.*

Temp	Time
42ºC	15 min
93ºC	3 min
4ºC	Hold

Supplementary Table 4. *Primers for genes used to perform qPCR.*

Gene Name	Forward Primer	Reverse Primer
GAPDH	TCAAGGCTGAGAACGGGAA	TGGGTGGCAGTGATGGCA
Actin	GTGGGCCGCCCTAGGCACCAG	CACTTTGATGTCACGCACGATTTC
18s	GCAATTATTCCCCATGAACG	GGGACTTAATCAACGCAAGC
RUNX2	GGTCAGATGCAGGCGGCCC	TACGTGTGGTAGCGCGTGGC
BMP2	CTTCTAGCGTTGCTGCTTCC	AACTCGCTCAGGACCTCGT
ALKP	ATGAAGGAAAAGCCAAGCAG	CCACCAAATGTGAAGACGTG
OPN	AGCTGGATGACCAGAGTGCT	TGAAATTCATGGCTGTGGAA
OCN	CAGCGAGGTAGTGAAGAGACC	TCTGGAGTTTATTTGGGAGCAG
COLI	CCATGTGAAATTGTCTCCCA	GGGGCAAGACAGTGATTGAA
OSX	GGCAAAGCAGGCACAAAGAAAG	AATGAGTGGGAAAAGGGAGGG
Osteonectin	AGAATGAGAAGCGCCTGGAG	CTGCCAGTGTACAGGGAAGA
BMPR1a	CTACACTGGACACCAGAGCC	GGTCAGCAATGCAGCAACTC
PIEZO1	TCGCTGGTCTACCTGCTCTT	GGCCTGTGTGACCTTGGA
PIEZO2	CCCGGAGTTTGAAAATGAAG	CAGTGCCTCTTCTGAATCAATTT
TRPA1	TGGACACCTTCTTCTTGCATT	TCTTCTCCATTAGCTCAATTTGG
TRPV1	AGAGTCACGCTGGCAACC	GGCAGAGACTCTCCATCACAC
KCNK4	Biorad assay	qHsaCED0047530
KCNK2	CGCTCAGAACTCCAAACCGA	CATGAGGCTGCTCCAATGCT

Supplementary Table 5. *Information regarding biological experiment replicate numbers linked to the figures.*

Figure	Experiment	Cells from X number of donors	Biological replicates	Technical replicates
1f	qPCR	3	4	2
1g	ICW	1	3	2
1h	ICW	3	4	1
3a	qPCR	1	6	2
3b	Von Kossa	3 (4 week) 1 (6 week)	4 5	1 1
3c	Raman	1	3	1
3d-f	µCT	1	3	1
4a	qPCR	1	3	2
4b-e	qPCR	3	3	2
4f-h	qPCR	2	4	2
5a	Western	3	4 (pooled)	3
5b	Western	3	4 (pooled)	3
5c-e	Metabolomics	1	3	1
6	qPCR	3	4	2
S3a-d	Live/dead	1	4	1
S3e	Alamar blue	1	4	3
S4	ICW	1	6	1
S6	qPCR	1	3	1
S7&8	Metabolomics	3	4	1
S9	Western	3	4 (pooled)	3

Supplementary references.

1. Tarnowski, C.P., Ignelzi, M.A., Jr. & Morris, M.D. Mineralization of developing mouse calvaria as revealed by Raman microspectroscopy. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 17, 1118-1126 (2002).
2. Gentleman, E*., et al.* Comparative materials differences revealed in engineered bone as a function of cell-specific differentiation. *Nat Mater* (2009).