Published in final edited form as:

Bone. 2018 January ; 106: 148–155. doi:10.1016/j.bone.2017.10.019.

Ex Vivo Replication of Phenotypic Functions of Osteocytes through Biomimetic 3D Bone Tissue Construction

Qiaoling Sun¹, Saba Choudhary², Ciaran Mannion⁴, Yair Kissin⁵, Jenny Zilberberg^{3,*}, and Woo Y. Lee^{1,*,#}

¹Department of Materials Science and Chemical Engineering, Stevens Institute of Technology, Hoboken, NJ, USA

²Department of Biomedical Engineering, Chemistry and Biological Sciences, Stevens Institute of Technology, Hoboken, NJ, USA

³Department of Biomedical Research, Hackensack University Medical Center, Hackensack, NJ, USA

⁴Department of Pathology, Hackensack University Medical Center, Hackensack, NJ, USA

⁵Department of Orthopedic Surgery, Hackensack University Medical Center, Hackensack, NJ, USA

Abstract

Osteocytes, residing as 3-dimensionally (3D) networked cells in bone, are well known to regulate bone and mineral homeostasis and have been recently implicated to interact with cancer cells to influence the progression of bone metastases. In this study, a bone tissue consisting of 3D-networked primary human osteocytes and MLO-A5 cells was constructed using: (1) the biomimetic close-packed assembly of 20–25 μ m microbeads with primary cells isolated from human bone samples and MLO-A5 cells and (2) subsequent perfusion culture in a microfluidic device. With this 3D tissue construction approach, we replicated ex vivo, for the first time, the mechanotransduction function of human primary osteocytes and MLO-A5 cells by correlating the effects of cyclic compression on down-regulated SOST and DKK1 expressions. Also, as an example of using our ex vivo model to evaluate therapeutic agents, we confirmed previously reported findings that parathyroid hormone (PTH) decreases SOST and increases the ratio of RANKL and OPG. In comparison to other in vitro models, our ex vivo model: (1) replicates the cell density, phenotype, and functions of primary human osteocytes and MLO-A5 cells and (2) thus provides a clinically relevant means of studying bone diseases and metastases.

[#]Corresponding author: Woo Young Lee, Ph.D., 1 Castle Point on Hudson, Stevens Institute of Technology, Hoboken, NJ 07030, 201-216-8307, wlee@stevens.edu.

^{*}Equally contributed to this work

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

Human primary osteocytes; 3D bone tissue; Mechanotransduction; SOST/sclerostin; Parathyroid hormone (PTH)

1. Introduction

Osteocytes reside as 3D-networked cells within mineralized extracellular matrix (ECM) cavities (“lacunae”) in bone tissue, and are interconnected by dendritic cell processes and gap junctions along ECM canals (“canaliculi”).^{1–4} Osteocytes function as master regulators of homeostatic bone remodeling,^{1–3} and play important roles in the metabolic regulation of minerals.⁴ Also, recent studies suggest that osteocytes, as 3D-networked cells, can interact with bone marrow cells⁵ as well as prostate cancer and multiple myeloma cells located on the bone marrow side.^{6–8} For bone homeostasis, osteocytes regulate: (1) osteoblastogenesis through releasing sclerostin and DKK1 and (2) osteoclastogenesis by secreting RANKL and OPG.^{1,9,10}

Our long-term motivation has been to construct the 3D-networked structure of human primary osteocytes, as a clinically relevant means of developing high-throughput in vitro bone tissue models. For clinical relevance, the use of human primary osteocytes is critically important since: (1) immortalizing human cells into cell lines by gene transfection perturbs the cells’ gene expression profiles and cellular physiology^{11–13} and (2) cell lines cannot capture the genotypic and phenotypic heterogeneity of primary cells.¹² Also, the ability of such ex vivo models to recapitulate the mechanotransduction function of osteocytes is critical as a physiological pathway of regulating bone formation.

It is well established that bones mechanically behave as “elastic sponges.”¹⁴ When they are cyclically compressed during physical body movements, the interstitial fluid within the lacunocanicular structure of bone squeezes in and out. As a result, flow-induced shear stresses are generated on osteocytes. Osteocytes are known to sense shear stresses through cell body and dendritic processes.^{15,16} Upon sensing mechanical stimuli, osteocytes reduce the production of sclerostin (encoded by SOST) and DKK1, which activate osteoblasts for new bone formation.^{17,18,19} Especially, the SOST/sclerostin signaling pathway has received much attention as a unique drug target for treating osteoporosis²⁰ and tumor-induced osteolytic lesions.²¹

In the past, this mechanotransduction function could not be equivocally replicated in vitro due to: (1) the relatively insufficient SOST and FGF23 expressions of osteocytic cell lines^{22–26} and (2) the difficulty of maintaining the phenotype of primary osteocytes due to their osteoblastic dedifferentiation and proliferation during 2D culture.^{27,28} Also, state-of-the-art 3D bone tissue models, developed by other investigators,^{29–32} cannot replicate physiologically relevant cell-to-cell distance and strong expressions of SOST and FGF23 as the key markers of mature osteocytes. Note that FGF23 is a hormone expressed by osteocytes to regulate phosphate homeostasis, and is gaining importance since it has been recently implicated to facilitate prostate cancer progression³³ and to be elevated in multiple myeloma patients.³⁴

We previously found that the 3D bone tissue structure consisting of 3D-networked osteocytes could be constructed via: (1) biomimetic assembly with microbeads with an osteocytic cell line (MLO-A5) and primary cells isolated from murine and human bones and (2) subsequent perfusion culture in a microfluidic device.^{28,35–37} These findings suggested that the 3D construction: (1) mimics the lacunocanalicular structure of human bone tissue, (2) mitigates the osteoblastic dedifferentiation and proliferation of primary osteocytes encountered in 2D culture, (3) promotes the significant gene expression of mature osteocytes (SOST and FGF23), and (4) therefore reproduces *ex vivo* the phenotype of terminally differentiated, non-proliferating 3D-networked osteocytes.

The major aims of this study were to: (1) examine our *ex vivo* 3D tissue model's ability to replicate the mechanotransduction function of primary human osteocytes and (2) demonstrate the model's utility by confirming the previously reported findings that continuous parathyroid hormone (PTH) treatment decreases SOST and increases FGF23.

2. Materials and Methods

2.1. Culture of MLO-A5 cells

The MLO-A5 post-osteoblast/pre-osteocyte cell line was a kind gift from Professor Lynda Bonewald (Indiana University). Cells were maintained in collagen-coated flasks in α -MEM (Gibco) supplemented with 5% (v/v) fetal bovine serum (FBS, ATCC), 5% (v/v) calf bovine serum (CBS, ATCC), and 1% (v/v) penicillin-streptomycin (P/S, MP Biomedicals) at 37°C and 5% CO₂, and subcultured when they reached about 80% confluence. Cells from passages 2 to 8 were used.

2.2. Isolation and culture of human primary bone cells

Discarded bone samples were obtained with informed consent from a patient (Male, 64 years old) undergoing knee joint replacement surgeries. Protocol (Pro5059) for collection of samples and consent of the patient was approved by the Institutional Review Board (IRB) of Hackensack University Medical Center. Bone samples were cleaned using a scalpel to remove soft tissue and cartilage regions and cut into 1–5 mm chips in length and width. In brief, bone chips were digested using a collagenase solution (300 active units/mL) in α -minimal essential medium (α -MEM) and an ethylenediaminetetraacetic acid (EDTA) solution of 5 mM with 1% (v/w) bovine serum albumin (BSA).

Human primary osteoblasts were isolated using the isolation procedures described previously elsewhere.³⁵ In brief, we used osteoblastic cells isolated at the fourth digestion step and cultured for 10 days in a collagen-coated 6-well plate with a seeding cell density of 5×10^5 – 1×10^6 per well using α -MEM supplemented with 10% FBS and 1% P/S at 37°C, and 5% CO₂. After 10 days of expansion, the cells were frozen down in liquid nitrogen. Prior to use, the thawed cells were used for 3D culture from passages 2 to 4.

2.3. Microfluidic device and fabrication

We used a polydimethylsiloxane (PDMS)-based microfluidic culture device which consisted of 6 perfusion culture chambers (6 mm $\times$ 200 μ m in thickness) as shown in Fig. 1A. As

shown in Fig. 1B and as illustrated in Fig. 2, three double-sided, biocompatible pressure-sensitive adhesive (PSA) layers (ARcare 90106, Adhesive Research), a polyester (PE) membrane layer with an average pore size of 3 μm (Sterlitech) and a layer of PE woven mesh with an opening of 80 μm (SpectrumLab) were used to fabricate the device. Note that PSA consists of a 25 μm -thick PE layer sandwiched by 60 μm -thick layers of a medical grade acrylic hybrid adhesive.

The PE membrane was used to retain cells, but allow culture medium to perfuse through. PSA helped seal the culture chambers much tighter to mitigate culture medium leakage when the culture chambers were pressurized. The woven PE mesh was used to help anchor the cells and microbeads to settle in mesh openings and form mechanically stable tissue samples. These materials were: (1) patterned and cut using a digital cutter (CAMEO, Silhouette) and (2) assembled with the PDMS layers and a glass slide.

2.4. Microbeads-guided 3D network construction

Human primary bone cells or MLO-A5 cells were mixed with biphasic calcium phosphate (BCP) microbeads (68% of hydroxyapatite and 32% of β -tricalcium phosphate; CaP Biomaterials) prior to assembling them into the 3D culture chamber. The microbeads were sieved to a size range of 20 to 25 μm , coated with collagen type I (Sigma-Aldrich) using a collagen/acetic acid solution (0.15 mg/mL) for 1 h at 37°C, and washed with phosphate-buffered saline (PBS) three times. A cell and microbead mixture was made by combining 2×10^7 cells/mL with 2×10^7 microbeads/mL and mixing thoroughly in an osteogenic α -MEM medium with 10% (v/v) FBS, 50 $\mu\text{g/mL}$ ascorbic acid (Sigma-Aldrich), 3 mM β -glycerophosphate (Sigma-Aldrich) and 1% (v/v) P/S. Ten microliters of the cells/microbeads mixture was pipetted into the culture chamber. As the medium was withdrawn through the membrane, cells and microbeads were assembled to a close-packed structure, as illustrated in Fig. 2B.

The culture device was then placed inside of a conventional incubator at 37°C (5% CO_2). The osteogenic α -MEM was supplied to the culture chambers using syringe pumps (KD Scientific) located outside of the incubator via polyethylene tubing. The reconstructed 3D samples were cultured up to 14 days at a flow rate of 1 $\mu\text{L/min}$. For 2D culture control, cells were seeded into a 24-well plate at a concentration of 10,000 cells/well. Medium was replaced every 1 to 2 days.

2.5. Cyclic compressive loading

After 14 days of culture, the 3D-constructed tissue was cyclically compressed by pressurizing the culture chamber. The chamber was pressurized by sealing the outlet tubing with an artery clamp (Fig. 2B). The syringe pump was programmed to cyclically feed and withdraw the culture medium at a rate of 0.5 mL/min at a frequency of 0.17 Hz as shown in Fig. 2C. The cells were loaded for 10 min per day for 2 days. This flow rate and frequency were chosen based on: (1) the adhesive strength of the device; higher flow rates damaged the device, and (2) the limited resolution of the syringe pump; 0.17 Hz was the highest possible frequency achievable. Similar frequency and loading duration were previously used by other in vitro studies to observe the response of bone cells to mechanical loading.^{30,38} For control

3D culture, the flow rate was programmed to fluctuate, but the medium outlet was not closed off so that the tissue samples did not undergo pressurization/depressurization cycles. The 3D samples were harvested immediately after the 10-min loading on the second day. RNA was extracted using a RNA Mini kit (Ambion) directly after the treatment using the procedures described previously.³⁵ For histology, 3D samples were harvested 24 h after the treatment and fixed with 4% paraformaldehyde (PFA, Affymetrix).

2.6. Ca²⁺ staining and time-lapsed imaging

Fluo-8 AM is a green fluorescent indicator that utilizes a fluorescein core to monitor Ca²⁺ concentration and flux in cells. Studies showed that the fluorescence intensity of Fluo-8 AM increases immediately in osteocytes when they are mechanically stimulated.³⁹ After 7 days of culture, the tissue samples were washed with Dulbecco's PBS 3 times and stained with Fluo 8 AM (AAT Bioquest, Inc). The stained samples were observed under a confocal microscope (Nikon E1000 with Nikon C1-Plus confocal system) within 2 h. Forty time-lapsed images were taken with time intervals of 3 s during cyclic compression loading. Fluorescence intensity analysis was performed by randomly selecting 6 cells and using Nikon NIS Element AR analysis software.

2.7. PTH treatment

After 14 days of perfusion culture, the 3D and 2D culture samples were treated with human PTH (1–34) (TOCRIS) at 50 nM in the osteogenic medium for 48 h. RNA was extracted using a RNA Mini kit (Ambion) directly after the treatment using the procedures described previously.³⁵ Also, the effluent culture medium was collected over a period of 24 h after the treatment. The 3D tissue samples were collected 24 h after the treatment and fixed with 4% PFA for histology.

2.8. RT-PCR

For RT-PCR quantification of gene expressions, target gene expression was normalized to GAPDH for MLO-A5 and 18S for human primary cells. Up to 2 µg RNA and 2 µL cDNA were used for RT-PCR. Fold changes in expression relative to untreated control were determined using 2-(Ct(target gene)-Ct(GAPDH)). Taqman gene expression probes (Applied Biosystems, Thermo Fisher Scientific) were used to analyze the following genes: RANKL, OPG, FGF23, SOST, DKK1, and DMP1. All primers used for RT-PCR are summarized in Supplementary Table 1.

2.9. ELISA

The effluent culture medium was collected over a period of 24 h after the PTH treatment as mentioned. The effluent was concentrated 7 times by protein concentration filtration (Millipore). Sclerostin, RANKL, and OPG expression were tested using the ELISA kit (R&D systems).

2.10. Immunofluorescence

The 3D culture samples were removed from the culture chambers and fixed with 4% PFA. The fixed samples were paraffin-embedded, cut into 10-µm thick histological sections, and

stained with hematoxylin and eosin (H&E, Sigma-Aldrich). 20 cells from 3 separate histology images were randomly selected at the center of 3D tissue. The nucleus-to-nucleus distance was measured from the selected cells with the nearest interstitial space between beads. Slides were deparaffinized and permeabilized using 0.1% (v/v) triton x-100 for 10 min. To block unspecific antibody binding, cells were incubated in 3% (w/v) BSA made in PBS for 1 h at room temperature. The cells were then further incubated overnight at 4 C with a rabbit anti-human sclerostin antibody (Abcam) followed by secondary staining with a TRITC-conjugated antibody (goat anti-rabbit TRITC, Abcam). Cells were counterstained with DAPI (Sigma-Aldrich) and mounted. Slides stained with secondary antibody only were used as negative control. The stained slides were observed under a fluorescence microscope (Eclipse Ti-E, Nikon).

To determine the numbers of sclerostin positive cells upon PTH treatment and mechanical loading compared to control culture, 3 separate images per condition were selected and sclerostin expression was quantified in 30 to 50 cells in the center of 3D tissue.

2.11. Statistics

Statistical variance was expressed as mean $\pm$ standard error. Statistical significance between control (untreated) and mechanical loading was evaluated using Student's t-test (Excel, 2010). The statistical significance between control and PTH-treated groups in 2D or 3D conditions were evaluated using one-way ANOVA, followed by Tukey's multiple comparison test (GraphPad Prism, v.7.0). PTH treatment with MLO-A5 were performed in triplicate chambers and reproduced in 3 separate experiments. PTH treatment with primary human 3D-networked osteocytes was performed in triplicate chambers and reproduced in 2 separate experiments. Ca^{2+} oscillations with loading was performed in triplicate chambers and reproduced in 2 experiments. Mechanical loading on both MLO-A5 and primary human 3D-networked osteocytes were performed in triplicate chambers and reproduced in 2 separate experiments.

3. Results

3.1. 3D tissue construction with 3D-networked osteocytes

Human primary osteoblasts and BCP microbeads were assembled and cultured in the perfusion device for 14 days to form a mechanically integrated 3D tissue structure (Fig. 1C). The histology image with H&E staining (Fig. 1D) showed that cells resided in the interstitial space between microbeads, and became interconnected through process formation and extension. Also, cells were distributed with an average of cell-to-cell distance of 18.5 ± 0.7 μm by measuring the nucleus-to-nucleus distance between cells in the neighboring interstitial spaces with 20 randomly picked cells in Fig. 1D.

3.2. PTH treatment effects on gene and protein expressions of osteocytes

After the PTH treatment for 48 h, the gene expressions of human primary cells and MLO-A5 cells in the 3D bone tissue samples were evaluated. For 3D-cultured human primary cells (Fig. 3), the PTH treatment significantly decreased SOST (16 times) and OPG. Also, the treatment increased FGF23 and decreased RANKL, but without statistical significance. In

comparison, these trends were difficult to observe and resolve in 2D culture, since SOST expression was very low and FGF23 expression was not detected (CT values were shown in Supplementary table 2). Fig. 4 is a representative image showing that the number of sclerostin positive cells decreased upon PTH treatment. About 18% of the cells in the PTH group could be counted as sclerostin-positive cells vs. 29% in the control group (Fig. 4). An image of negative control is shown in Supplementary Fig. 1. Overall gene expression trends observed for MLO-A5 cells cultured in 3D and 2D were similar to those observed with primary bone cells (Supplementary Fig. 2). ELISA measurements of the effluent from MLO-A5 and human primary cells 3D culture medium showed that the PTH treatment: (1) decreased sclerostin production by about 1.5 times in MLO-A5, but was not detectable in human primary cells, (2) increased RANKL in MLO-A5, and (3) decreased OPG in both cell types (Supplementary Fig. 3). CT values from PCR measurement are shown in Supplementary table 2.

3.3. Cyclic compressive loading effects on gene and protein expressions of osteocytes

Fig. 5 shows that cyclic compressive loading: (1) decreased the SOST expression of human primary osteocytes in the 3D bone tissue by 30 times, (2) decreased DKK1 by 4 times, and (3) did not significantly influence DMP1. Immunostaining data in Fig. 6 show that the intensity of sclerostin staining was much lower with mechanical loading. From the images in Fig. 6, we determined that the percentage of sclerostin-positive cells decreased from 48% in the control group to 20% in the loading group. Similar gene expression trends were observed with MLO-A5 (Supplementary Fig. 4). All the CT values in PCR measurement were shown in Supplementary table 2.

3.4. Ca²⁺ oscillation using cyclic pressurizing method vs. cyclic flow method

Ca²⁺ staining (Fluo-8 AM) was performed with 3D-constructed MLO-A5 cells to confirm that the cells actually sense the cyclic compressive loading applied to the bone tissue sample. As shown in Figs. 7A–C, the fluorescence intensity increased by 2.5 times as soon as the loading was applied. Also, the intensity fluctuated during the loading period with 13 peaks in 81 seconds at an average frequency of 0.17 Hz. This frequency matched with the frequency of flow rate fluctuations (0.17 Hz) controlled by the syringe pumps (Fig. 2C). In contrast, Figs. 7D–F showed that, in the samples that were not pressurized, fluorescence intensity did not exhibit any periodic fluctuation.

4. Discussion

The 3D bone tissue was constructed: (1) using MLO-A5 cells and primary osteoblastic cells isolated from human bone samples and proliferated in vitro and (2) via biomimetic assembly with BCP microbeads and subsequent perfusion culture in the microfluidic device. The bone tissue consisted of 3D-networked and non-proliferating osteocytes with an average cell-to-cell distance of 18.5 μm (Fig. 1D). These results were close to the distance of the nearest-neighbor osteocytes observed in vivo⁴⁰. Also these results were consistent with our previous findings obtained with primary murine and human bone cells as well as MLO-A5 cells.^{28,35–37} In addition, the 3D-networked cells developed the significant gene expressions of mature osteocytes (SOST and FGF23) in comparison to those cultured in 2D (Fig. 3).

These strong expressions of SOST and FGF23 have been difficult to produce in other in vitro 3D tissue model studies,^{31,40} as they were not able to recapitulate cell-to-cell distance and spatial cell density in bone tissues. These geometrical features are known to be important for influencing cell-cell signaling pathways associated with osteocyte process growth and the 3D cellular network's sensitivity for mechanotransduction. Of note, other in vitro studies were not able to show a clear relationship between mechanical loading and SOST gene expression in primary osteocytes^{30,42}.

In addition cyclic compression loading decreased the SOST and Dkk1 expressions of the 3D-networked osteocytes (Fig. 5) and decreased sclerostin production (Fig. 6). These results are consistent with those from in vivo studies^{11,42} that found decreased SOST and FGF23 expressions when bones were cyclically compressed, e.g., 360 loading cycles per day at a loading frequency of 2 Hz over 2 days.¹⁹ The down-regulated expression of SOST was correlated to new bone deposition in murine model studies.^{19,44} Note that our results indicated that the expression of DMP1 was not significantly affected by the cyclic loading, whereas an increase in DMP1 was observed with mechanical stimuli in other studies.^{42,45} Taken together, we have, for the first time to our knowledge, show the effect of cyclic compression on the SOST expression of primary human osteocytes.

Our tissue compression approach, i.e., cyclically pressurizing the tissue sample (Fig. 2C), provides a physiologically relevant means of applying fluid-induced shear stress to 3D-networked cells. In order to discern this critical point, we labeled MLO-A5 with Fluo-8 AM to monitor Ca^{2+} concentration and flux in cells during the cyclic loading. It is well known^{46,47} that the intracellular concentration of Ca^{2+} increases instantly when cells sense shear stress. As a result, the fluorescence intensity increases. This method has been used to test cell response to fluid-induced shear stress by other investigators.^{39,48}

Due to the damage of human primary cells during Fluo-8 AM staining, MLO-A5 cells were used to further demonstrate that cells responded to our loading method using direct cell imaging in combination with our system. Our results in Fig. 7 clearly show that the Ca^{2+} fluorescence intensity of MLO-A5 cells increased immediately with the cyclic pressurization cycles, but not when the culture chamber and tissue sample were not cyclically pressurized. Also, importantly, the measured frequency of the intensity fluctuations during the loading period and the programmed frequency of flow rate fluctuations matched at 0.17 Hz. Although the 3D tissue is not mechanically strong after 14 days of culture, it is sufficiently dense with the formation of extracellular matrix for the medium to uniformly perfuse through. Therefore, unless the tissue samples are pressurized, the medium is expected to flow through physical gaps and channels present within the control tissue samples and not generate shear stresses on cell bodies and processes within the tissue samples.

In this investigation, we also replicated the effects of the PTH treatment effect on the gene expression of 3D-constructed primary human osteocytes. It is known that PTH, depending on how it is dosed, can induce: (1) osteoblastogenesis via decreasing the SOST expression of osteocytes or (2) osteoclastogenesis by increasing the RANKL/OPG ratio.⁴⁹ We continuously treated cells with 10 nM PTH for 48 h to cause the downregulation of SOST and upregulation of RANKL/OPG in both MLO-A5 and human primary osteocytes. The

ELISA results and immunostaining showed the same trend that sclerostin expression decreased with PTH treatment. These results were similar to those observed from previous in vivo and in vitro studies.^{50,51} For example, continuous PTH treatment suppressed the expressions of SOST and sclerostin in murine models.^{52,53} And, during continuous PTH infusion in rats, decreased OPG and increased RANKL expressions were observed.⁵⁴ With mouse primary bone cells in vitro, continuous PTH treatment was found to decrease SOST and increase RANKL.⁵¹

Taken together, the results from this study showed that 3D osteocytes assembled and cultured with BCP microbeads can replicate osteocytes' functions associated with PTH treatment and mechanotransduction. In future research studies, we will perform numerical simulations using COMSOL to estimate the magnitude of shear stresses.

Our biomimetic approach of constructing the ex vivo human 3D bone tissue is expected to provide a clinically relevant means of developing high-throughput in vitro bone tissue models. These 3D tissue models can be used for: (1) evaluating the efficacy of drugs targeting osteocytes in bone diseases, (2) study the interaction between osteocytes and tumor cells, and (3) reducing current reliance on animal models.

5. Conclusions

A human bone tissue consisting of 3D-networked primary osteocytes was constructed ex vivo using: (1) the biomimetic assembly of 20–25 µm microbeads and MLO-A5 or primary cells isolated from human bone samples of one donor and (2) subsequent perfusion culture in the microfluidic device. The pressurization of the culture chambers was used to: (1) apply cyclic compression to the 3D bone tissue at the frequency of 0.17 Hz and (2) replicate the in vivo effects of cyclic compression on down-regulated SOST and DKK1 expressions. As an example of using our ex vivo model to evaluate therapeutic agents, we confirmed previously reported findings that PTH decreases SOST, increases FGF23, and increases the gene expression ratio of RANKL and OPG.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Research in this publication was supported by grants from: (1) the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Number 1R21AR065032 to WYL and JZ and (2) the National Science Foundation (DMR 1409779) to WYL and JZ. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health and the National Science Foundation. We would like to thank Professor Lynda Bonewald at Indiana University, Indianapolis for kindly providing us with the MLO-A5 cells. We thank: (1) Dr. Genevieve Brown and Professor Edward Guo at Columbia University, New York for their help with the Ca²⁺ staining procedures and (2) Luke Fritzky at the Histology Core Facility at Rutgers-New Jersey Medical School for paraffin embedding and sectioning.

References

1. Dallas SL, Prideaux M, Bonewald LF. The Osteocyte: An Endocrine Cell and More. *Endocr Rev.* 2013; 34:658–90. [PubMed: 23612223]
2. Bonewald LF. The Amazing Osteocyte. *J Bone Min Res.* 2011; 26:229–238.
3. Loisel AE, Jiang JX, DH. Gap junction and hemichannel functions in osteocytes. *Bone.* 2013; 54:205–12. [PubMed: 23069374]
4. Franz-odendaal TA, Hall BK, Witten PE. Buried Alive: How Osteoblasts Become Osteocytes. *Dev Dyn.* 2006; :176–190. DOI: 10.1002/dvdy.20603 [PubMed: 16258960]
5. Kurata K, Heino TJ, Higaki H, VH. Bone marrow cell differentiation induced by mechanically damaged osteocytes in 3D gel-embedded culture. *J Bone Min Res.* 2006; 21:616–25.
6. Sottnik JL, Dai J, Zhang H, Campbell B. Tumor-induced pressure in the bone microenvironment causes osteocytes to promote the growth of prostate cancer bone metastases. *Cancer Res.* 2015; 75:2151–2158. [PubMed: 25855383]
7. Delgado-Calle, Jesus, Anderson, Judith, Cregor, Meloney D., Hiasa, Masahiro, Chirgwin, John M., Carlesso, Nadia, Yoneda, Toshiyuki, Mohammad, Khalid S., Plotkin, Lilian I., David Roodman, G., TBS. Bidirectional Notch Signaling and Osteocyte-Derived Factors in the Bone Marrow Microenvironment Promote Tumor Cell Proliferation and Bone Destruction in Multiple Myeloma. *Cancer Res.* 2016; 76:1089–1100. [PubMed: 26833121]
8. Delgado-Calle J, Bellido T, RG. Role of osteocytes in multiple myeloma bone disease. *Curr Opin Support Palliat Care.* 2014; 8:407–13. [PubMed: 25289928]
9. Baron R, Kneissel M. WNT signaling in bone homeostasis and disease: from human mutations to treatments. *Nat Med.* 2013; 19:179–193. [PubMed: 23389618]
10. Johnson LFB, ML. Osteocytes, Mechaosensing and Wnt Signaling. *Bone.* 2009; 42:606–615.
11. Gillet JP, Varma S, GM. The clinical relevance of cancer cell lines. *J Natl Cancer Inst.* 2013; 105:452–8. [PubMed: 23434901]
12. Li A, et al. Genomic Changes and Gene Expression Profiles Reveal That Established Glioma Cell Lines Are Poorly Representative of Primary Human Gliomas. *Mol Cancer Res.* 2008; 6:21–30. [PubMed: 18184972]
13. Lorsch BJR, Collins FS, Lippincott-schwartz J. Fixing problems with Cell lines. *Science* (80-). 2014
14. Rosaa, Natacha, Simoesb, Ricardo, Magalhãesd, Fernão D., ATM. From mechanical stimulus to bone formation: A review. *Med Eng Phys J.* 2015; doi: 10.1016/j.medengphy.2015.05.015
15. Burra S, et al. Dendritic processes of osteocytes are mechanotransducers that induce the opening of hemichannels. *PNAS.* 2010; 107
16. Bacabac RG, Bakker AD. Group, M. B. & City, C. MECHANICAL LOADING AND HOW IT AFFECTS BONE CELLS: THE ROLE OF THE OSTEOCYTE CYTOSKELETON IN MAINTAINING OUR SKELETON. *Eur Cells Mater.* 2012; 24:278–291.
17. Crockett JC, et al. Bone Remodelling at a Glance. *J Cell Sci.* 2011; 2011:991–998.
18. Mechanism M, et al. Sclerostin Is a Locally Acting Regulator of Late-Osteoblast/Preosteocyte Differentiation and Regulates Mineralization Through a MEPE-ASARM-Dependent Mechanism. *JBMR.* 2011; 26:1425–1436.
19. Robling AG, et al. Mechanical Stimulation of Bone in Vivo Reduces Osteocyte Expression of Sost/ Sclerostin. *J Biol Chem.* 2008; 283:5866–5875. [PubMed: 18089564]
20. Papapoulos NMASE, Romosozumab ÁBÁ. Sclerostin Inhibition in the Management of Osteoporosis. *Calcif Tissue Int.* 2016; 98:370–380. [PubMed: 27016922]
21. Larson SR, et al. Characterization of Osteoblastic and Osteolytic Proteins in Prostate Cancer Bone Metastases. *Prostate.* 2013; 940:932–940.
22. Bodine PV, Vernon SK, KB. Establishment and hormonal regulation of a conditionally transformed preosteocytic cell line from adult human bone. *Endocrinology.* 1996; 137:4592–604. [PubMed: 8895322]
23. Kato Y, Boskey A, Spevak L, Dallas M, Hori M, BL. Establishment of an osteoid preosteocyte-like cell MLO-A5 that spontaneously mineralizes in culture. *J Bone Min Res.* 2001; 16:1622–23.

24. Rosser J, Bonewald LF. Studying osteocyte function using the cell lines MLO-Y4 and MLO-A5. *Methods Mol Biol.* 2012; 816:67–81. [PubMed: 22130923]
25. Yang W, et al. Gene expression signatures of a fibroblastoid preosteoblast and cuboidal osteoblast cell model compared to the MLO-Y4 osteocyte cell model ☆. *Bone.* 2009; 44:32–45. [PubMed: 18973839]
26. Papanicolaou SE, Phipps RJ, Fyhrie DP, GD. Modulation of sclerostin expression by mechanical loading and bone morphogenetic proteins in osteogenic cells. *Biorheology.* 2009; 46:389–99. [PubMed: 19940355]
27. Torreggiani E, Matthews BG, Pejda S, Matic I, Horowitz MC, Grcevic D, KI. Preosteocytes/Osteocytes Have the Potential to Dedifferentiate Becoming a Source of Osteoblasts. *PLoS One.* 2013; 8
28. Sun Q, et al. Ex vivo 3D osteocyte network construction with primary murine bone cells. *Bone Res.* 2015; 3:15026. [PubMed: 26421212]
29. Pajevic PD. Review Article Recent Progress in Osteocyte Research. *Endocrinol Metab.* 2013;255–261.
30. Vazquez M, et al. A new method to investigate how mechanical loading of osteocytes controls osteoblasts. *Front Endocrinol.* 2014; 5:1–18.
31. Boukhechba F, Balaguer T, Ackermann K, Quincey D. Human Primary Osteocyte Differentiation in a 3D Culture System. *J Bone Min Res.* 2009; 24:1927–1935.
32. Pajevic, Paola Divieti, Spatz, Jordan M., Garr, Jenna, Adamson, Chris, LM. Osteocyte biology and space flight. *Curr Biotechnol.* 2014; 2:179–183.
33. Feng S, Wang J, Zhang Y, Creighton CJ. FGF23 promotes prostate cancer progression. *Oncotarget.* 2015; 6
34. Suvannasankha A, et al. FGF23 is elevated in multiple myeloma and increases heparanase expression by tumor cells. *Oncotarget.* 2015; 6:19647–60. [PubMed: 25944690]
35. Sun, Qiaoling, Choudhary, Saba, Mannion, Ciaran, Kissin, Yair, Zilberberg, Jenny, WYL. Ex Vivo Construction of Human Primary 3D-Networked Osteocytes. *Bone.* 2017; 105:245–252. [PubMed: 28942121]
36. Gu Y, Zhang W, Sun Q, Hao Y. Microbead-guided reconstruction of the 3D osteocyte network during microfluidic perfusion culture. *J Mater Chem B.* 2015; 3:3625–3633.
37. Choudhary, Saba, Sun, Qiaoling, Mannion, Ciaran, Kissin, Yair, Zilberberg, Jenny, Lee, WY. Hypoxic Three-Dimensional Cellular Network Construction Replicates Ex Vivo the Phenotype of Primary Human Osteocytes. *Tissue Eng Part A.* 2017
38. Donahue SW, Jacobs CR, Donahue HJ. Flow-induced calcium oscillations in rat osteoblasts are age, loading frequency, and shear stress dependent. 2001; 1295:1635–1641.
39. Jing D, et al. In situ intracellular calcium oscillations in osteocytes in intact mouse long bones under dynamic mechanical loading. *FASEB J.* 2017; 28:1582–1592.
40. Hannah KM, Thomas CDL, Clement JG, Carlo F, De Peele AG. Bimodal distribution of osteocyte lacunar size in the human femoral cortex as revealed by micro-CT. *Bone.* 2010; 47:866–871. [PubMed: 20691298]
41. Bouet G, et al. VALIDATION OF AN IN VITRO 3D BONE CULTURE MODEL WITH PERFUSED AND MECHANICALLY STRESSED CERAMIC SCAFFOLD. *Eur Cells Mater.* 2015; 29:250–67.
42. Spatz JM, et al. The Wnt-inhibitor Sclerostin is Up-regulated by Mechanical Unloading in Osteocytes in-vitro. *J Biol Chem.* 2015; doi: 10.1074/jbc.M114.628313
43. Rochefort GY. The osteocyte as a therapeutic target in the treatment of osteoporosis. *Ther Adv Musculoskelet Dis.* 2014; :79–91. DOI: 10.1177/1759720X14523500 [PubMed: 24891879]
44. Li X, et al. Targeted Deletion of the Sclerostin Gene in Mice Results in Increased Bone Formation and Bone Strength. *JBMR.* 2008; 23:860–869.
45. Harris SE, Gluhak-Heinrich J, Harris MA, Yang W, Bonewald LF, Riha D, Rowe PSN, Robling AG, Turner CH, Feng JQ, McKee MD, Nicollala D. DMP1 and MEPE expression are elevated in osteocytes after mechanical loading in vivo: Theoretical role in controlling mineral quality in the perilacunar matrix. *J Musculoskelet Neuronal Interact.* 2007; 7:313–15. [PubMed: 18094489]

46. Peppiatt-wildman CM, et al. Calcium signalling - An overview. CELL Dev Biol. 2001; doi: 10.1006/scdb.2000.0211
47. Han S, Wouters W, Clark A, Herzog W. Mechanically Induced Calcium Signaling in Chondrocytes In Situ. J Orthop Res. Mar.2012 :475–481. DOI: 10.1002/jor.21536 [PubMed: 21882238]
48. Lucas Lu X, Huo Bo, Chiang Victor, XEG. Osteocytic Network Is More Responsive in Calcium Signaling than Osteoblast Network under Fluid Flow. J Bone Min Res. 2013; 27:563–574.
49. Qin L, Raggatt LJ, Partridge NC. Parathyroid hormone: a double-edged sword for bone metabolism. TRENDS Endocrinol Metab. 2004; 15
50. Bellido T, Saini V, Divieti P. Effects of PTH on osteocyte function. Bone. 2013; 54:250–257. [PubMed: 23017659]
51. Rhee Y, et al. Parathyroid hormone receptor signaling in osteocytes increases the expression of fibroblast growth factor-23 in vitro and in vivo. Bone. 2011; 49:636–643. [PubMed: 21726676]
52. Bellido T, et al. Chronic Elevation of Parathyroid Hormone in Mice Reduces Expression of Sclerostin by Osteocytes: A Novel Mechanism for Hormonal Control of Osteoblastogenesis. Endocrinology. 2005; 146:4577–4583. [PubMed: 16081646]
53. Keller H, Kneissel M. SOST is a target gene for PTH in bone. Bone. 2005; 37:148–58. [PubMed: 15946907]
54. Ma YL, et al. Vivo Is Associated with Sustained Stimulation of RANKL and Inhibition of Osteoprotegerin and Gene-Associated Bone Formation. Endocrinology. 2001; 142:4047–4054. [PubMed: 11517184]

Highlights

- Human bone tissue model, mimicking 3D-networked primary osteocytes
- Replicated the cell density, phenotype, and functions of primary human osteocytes in vitro

Fig. 1.

3D human bone tissue construction consisting of 3D-networked primary osteocytes: A. microfluidic perfusion culture device; B. woven mesh and membrane used to support tissue reconstruction in the culture chamber; C. 3D bone tissue constructed by the biomimetic assembly of primary human osteoblastic cells and BCP microbeads after 14 days of culture; and D. histology image, showing the formation of 3D-networked human primary osteocytes.

Fig. 2. Schematic illustrations of microfluidic perfusion culture device: A. device fabrication, B. device design, and C. cyclic compression loadings via programmed flow rate changes of culture medium with the medium outlet closed for chamber pressurization and de-pressurization.

Fig. 3.

Effects of PTH treatment on gene expressions of 3D-constructed primary human bone cells or 2D-cultured. One-way ANOVA followed by Tukey's multiple comparison test was used to determine statistical significance between control and PTH conditions and between the same condition in 2D vs. 3D culture. * $P < 0.05$ within 3D culture, # $P < 0.05$ compared to the same condition group in 2D. The data presented in this figure were representative of experiments repeated in 2 separate experiments with triplicated wells each.

Fig. 4.
Effect of PTH treatment on sclerostin expression of 3D-constructed primary human bone cells, characterized by immunofluorescence. Control: regular culture without PTH. Sclerostin (red), nuclei (DAPI staining, Blue). White arrows, pointing to microbeads. Scale bar: 20 μ m.

Fig. 5.

Effects of cyclic mechanical loading effect on gene expressions of 3D-constructed human primary bone cells. Control: culture without PTH. Student t-test was used to compare control vs. loaded conditions. *P<0.05. The data presented in this figure were representative of 2 separate experiments with triplicate wells each.

Fig. 6. Effect of cyclic mechanical loading on sclerostin expression of 3D-constructed primary human bone cells, characterized by immunofluorescence. Sclerostin (red), nuclei (DAPI staining, blue). White arrows, pointing to microbeads. Scale bar: 20 μ m.

Fig. 7. Effect of cyclic mechanical loading on the fluorescent intensity changes due to Ca^{2+} oscillations in MLO-A5 cells: A, B, and C with cyclic compression and D, E, and F without cyclic loading as control, the data presented in this figure were representative of 6 chambers.