


OCY454 Osteocytes as an in Vitro Cell Model for Bone Remodeling Under Mechanical Loading

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ABSTRACT: Osteocytes' mechano-regulation of bone formation and resorption is key to maintaining appropriate bone health. Although extensive in vitro studies have explored osteocyte mechanobiology using the well-established MLO-Y4 cell model, the low amount of sclerostin secreted by this cell line renders it inadequate for studying cross-talk between osteocytes and osteoblasts under mechanical loading. Here, we investigated the potential of the sclerostin-expressing OCY454 osteocyte cell model in fulfilling this role. Fully differentiated OCY454 cells were tested for mechano-sensitivity by measuring changes in protein secretion, total adenosine triphosphate (ATP) content, and intracellular calcium in response to oscillatory fluid flow. Increases in sclerostin expression and total ATP content were observed. However, very low levels of receptor activator of the nuclear factor κ -B ligand were detected, and there was a great inconsistency in calcium response. Conditioned medium (CM) from OCY454 cells was then used to culture osteoblast and osteoclast precursors. Osteoblast activity was quantified with alkaline phosphatase (ALP) and Alizarin Red S stain, while osteoclast differentiation was quantified with tartrate-resistant acid phosphatase (TRAP) staining. We demonstrated that mechanically stimulated OCY454 cells released soluble factors that increased osteoblasts' ALP activity ($p < 0.05$) and calcium deposition ($p < 0.05$). There was also a significant decrease of large-sized TRAP-positive osteoclasts when osteoclast precursors were treated with CM from flow-stimulated OCY454 cells ($p < 0.05$). Results from this study suggest that OCY454 cells respond to mechanical loading with the release of key factors such as sclerostin to regulate downstream bone cells, thus demonstrating its potential as a novel cell model for in vitro osteocyte mechanobiology studies. © 2019 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. *J Orthop Res*

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Bone remodeling is essential for bone health and fracture healing. A balance between bone formation by osteoblasts and bone resorption by osteoclasts enables continuous bone remodeling, which re-shapes the bone corresponding to a key regulatory force—mechanical loading.¹ Earlier studies on bone remodeling focused on osteoclasts and osteoblasts.² Recently, osteocytes have been found to play a major role in regulating the bone remodeling process by sensing the mechanical load on the bone and signaling to osteoblasts and osteoclasts through mechanotransduction.^{3,4} Studies have shown that osteocytes can respond to mechanical loading with secreted soluble factors, which in turn regulate the function of other cell populations.^{5,6} Some key soluble factors for osteocyte mechanotransduction include the receptor activator of the nuclear factor κ -B ligand (RANKL),^{7,8} osteoprotegerin (OPG),^{8,9} prostaglandin E2 (PGE2),¹⁰ and sclerostin.¹¹ These factors have been extensively studied and abnormal expression levels of these signaling factors have been linked with bone diseases.^{12–15} Therefore, understanding the mechanism underlying osteocyte mechanotransduction and regulation of other cell populations is crucial in finding effective therapeutic strategies for various bone diseases.

Current in vitro experimentations on osteocyte regulation of bone remodeling rely on the MLO-Y4 osteocyte-like cell-line, established by Dr. Bonewald in 1997.¹⁶ Isolated from mouse long bones, these osteocytes were selected based on their dendritic phenotype. MLO-Y4 cells have been shown to be sensitive to mechanical loading by changes in the secretion level of many key soluble factors.^{17,18} Among all the mechanical stimuli that osteocytes are exposed to in vivo, loading-induced fluid flow is well-accepted to be important and therefore was used in many in vitro studies.^{19–21} Using MLO-Y4 cells, researchers have characterized the mechano-regulation of osteoclasts via osteocytes^{22,23} and established a solid foundation for understanding this process. Furthermore, MLO-Y4 cells have been used in many studies of osteocyte intracellular calcium response, setting it as a major marker for measuring osteocyte mechano-sensitivity.^{24–26} Mechanical regulation of Wnt signaling²⁶ in osteocytes, as well as the roles of PGE2,^{10,27} have also been widely explored. Recently, a novel study demonstrated the release of extracellular vesicles by osteocytes using MLO-Y4 cells.²⁸ These in vitro experiments often used the osteocyte conditioned medium (CM), which comprises osteocytes' secretory response, to study the indirect effect of fluid stimulation on effector cells via osteocytes.

However, there is a major limitation of using MLO-Y4 cells as an osteocyte cell model. In vivo studies have shown that osteoblasts are regulated by an important molecule released by osteocytes—sclerostin.¹⁰ It has been revealed that sclerostin inhibits osteoblast

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activity and bone formation through the Wnt/ β -catenin pathway,^{29,30} and that mechanical loading on the bone leads to a reduction in sclerostin levels in vivo.³¹ These demonstrated sclerostin's critical role in mechanotransduction studies between osteocytes and osteoblasts. Unfortunately, this essential signaling molecule was undetectable from soluble factors released by the MLO-Y4 cells, giving the MLO-Y4 cell model a great disadvantage in studying osteocyte regulation of osteoblast function under mechanical loading.

Recently, a new cell line for osteocytes had been developed by Dr. Pajevic (Boston University), the OCY454 osteocytes.³² Also derived from mouse long bones, these cells have been shown to secrete significant levels of soluble sclerostin and dentin matrix acidic phosphoprotein 1 (DMP1) after differentiation. They were also shown to be mechano-sensitive, significantly increasing their expression of sclerostin in response to microgravity in vitro. Using both two- and three-dimensional cultures of OCY454 cells, the authors demonstrated that messenger RNA expression of several important signaling molecules, such as sclerostin, DMP1, and OPG, were significantly affected by uni-directional laminar fluid flow with a shear stress of 0.2 Pa. These promising results suggest that OCY454 cells have the potential of serving as a new cell model for studying osteocyte mechano-regulation of osteoblasts. Nevertheless, there has yet to be any study on how mechanically stimulated OCY454 osteocytes affect downstream bone cells. It is crucial to understand whether this new cell line is capable of regulating osteoblast differentiation and activities, since it has the potential to initiate a considerable number of new intercellular communication studies looking at the bone remodeling process.

In this study, we aimed to investigate OCY454 osteocyte's mechano-sensitivity by measuring known markers of osteocyte mechanotransduction, both immediately and 24 h after oscillatory fluid flow (OFF). We also examined the response of osteoblasts and osteoclasts to CM from flow-stimulated OCY454 osteocytes. These results provided a good indication whether this new cell line could be used as an in vitro model for studying osteocyte mechano-regulation of other bone cells.

METHODS

Cell Culture

OCY454 osteocytes were grown on collagen-coated (0.15 mg/ml type I collagen; Corning, MA) dishes with α -minimum essential medium (MEM) (Wisent, QC, Canada) containing 10% fetal bovine serum (FBS) (Gibco, ON, Canada) and 1% antibiotic-antimycotic (Gibco, NY). OCY454 cells were seeded at 10^5 cells per 100 mm diameter dish (Corning, NY), and allowed to reach 80% confluency at the permissive temperature (33°C) for 3 days before passaging. The cells were differentiated at the semi-permissive temperature (37°C) for 10 days before stimulation with flow.

MLO-Y4 osteocytes were grown on collagen-coated dishes with α -MEM containing 2.5% calf serum (Gibco, ON, Canada), 2.5% FBS, and 1% penicillin-streptomycin (Gibco, NY). MC3T3-E1 osteoblast precursors were grown in α -MEM containing 10% FBS and 1% penicillin-streptomycin. MC3T3-E1s were seeded at 5×10^3 cells per well in a 24-well plate for the osteoblast conditioning experiment. RAW264.7 osteoclast precursors were grown in D-MEM media (Wisent) containing 10% FBS, 2% L-glutamine (Sigma-Aldrich, MO), and 1% penicillin-streptomycin. RAW264.7 were seeded at 2.5×10^3 cells per well in a 24-well plate for the osteoclast conditioning experiment.

Parallel Plate Flow Chamber Experiments

OCY454 osteocytes were seeded on collagen-coated glass slides at 2×10^5 cells per slide and incubated at 37°C overnight. Slides were then inserted into custom-made flow chambers³³ for OFF stimulation with a peak shear stress of 1.2 Pa for 2 h in the incubator. Static control cells were also inserted into flow chambers without OFF stimulation. After flow stimulation, slides were incubated in 10 ml media for 24 h at 37°C. CM was then extracted and used for osteoblast and osteoclast conditioning experiment.

Sclerostin and RANKL/OPG Enzyme-Linked Immunosorbent Assay (ELISA)

Both sclerostin and the RANKL/OPG ratio were quantified using ELISA on CM collected 24 h after the osteocytes were exposed to fluid stimulation. Briefly, 96-well plates were coated with capture antibodies (R&D Systems, MN) overnight and blocked with 10% bovine serum albumin (Bioshop, ON, Canada) in Dulbecco's phosphate-buffered saline (DPBS). CM was added and incubated for 2 h, after which detection antibodies (R&D Systems) and secondary horseradish peroxidase-labeled antibodies (R&D Systems) were added for colorimetric detection using a Spectromax i3 Multimode Detection Platform (Molecular Devices, CA).

Intracellular Calcium Response Quantification

Cells were seeded into flow channels (ibidi, Bavaria, Germany) at a density of 2×10^5 per channel and incubated overnight. The cells were stained with Fura-2 AM (Thermo Fisher Scientific, MA) for 45 min before imaging. Using a custom syringe pump,³⁴ the cells were stimulated with OFF at 4 Pa peak shear stress and 1 Hz frequency for up to 4 min. Static control cells were seeded into ibidi channels without applying OFF. Fluorescent signals were read, and a final ratio of 340/380 nm was used to generate the calcium response curves. A calcium response was identified as having two-times fold-change or greater compared to baseline fluctuations.

Fluid Flow and Quantification of Adenosine Triphosphate (ATP) Synthesis

Differentiated OCY454 cells were seeded into flow channels (ibidi). After overnight incubation, cell numbers

were quantified with bright field imaging. The cells in the devices were then stimulated with OFF (2 Pa, 1 Hz) for 20 min. Cell lysate and collected media were combined for the quantification of total ATP synthesis, as mentioned previously.³⁵ ATP levels were measured using a luciferin-luciferase reaction (Abcam, Cambridge, UK).

Osteoblast Conditioning Experiment

CM from a OCY454 parallel plate flow chamber experiment was used on MC3T3-E1 osteoblasts seeded in 24-well plates. CM was aliquoted and stored at -20°C , and individually thawed during each media change. The media in sample wells consisted of 50% CM and 50% MC3T3-E1 media, and were replaced on day 4, 7, 10, and 14 with the same formulation. Media extracted from these time points were used for measurements of extracellular ALP activity, after which the cells were used for Alizarin Red S (ARS) quantification on day 14.

Quantification of Extracellular ALP activity

Extracellular ALP activity levels of cultured MC3T3-E1 were quantified using a colorimetric assay. Briefly, medium collected (40 μl) from an osteoblast conditioning experiment was added to 40 μl of deionized water and 50 μl of 5 mM pNPP (Sigma-Aldrich), followed by 60 min incubation at room temperature. Finally, 20 μl of NaOH (Sigma-Aldrich) was added into the mixture to stop the reaction. The resultant absorbance reading at 450 nm was detected using the Spectromax i3 Multimode Detection Platform (Molecular Devices).

Calcium Deposit Quantification

MC3T3-E1 osteoblasts cultured for 14 days in CM were fixed with 4% formaldehyde (Sigma-Aldrich) before staining with 40 mM ARS (ScienCell, CA) for 30 min. After extensive washing with DPBS, the cells were incubated in 10% acetic acid (ScienCell) for 30 min before being heated at 85°C for 10 min. The samples were then centrifuged at 16,000g for 20 min, after which 10% ammonium hydroxide was added to neutralize the acid. The supernatant was read at 405 nm using the Spectromax i3 Multimode Detection Platform (Molecular Devices).

Osteoclast Conditioning Experiment and Tartrate-Resistant Acid Phosphatase (TRAP) Staining

RAW264.7 osteoclasts seeded in 24-well plates were supplied with media consisting of 50% CM and 50% RAW264.7 media supplemented with 10 ng/ml recombinant RANKL (R&D Systems). CM from osteocyte flow experiments was aliquoted and stored at -20°C and individually thawed during each media change. The same media composition described above was used for media replacement on days 4, 5, and 6. Osteoclasts were visualized using TRAP staining on day 7, as previously described.³³ Briefly, cells were fixed and stained with the leukocyte acid phosphatase kit

(Sigma-Aldrich). TRAP-positive cells were counted and grouped into small (2–4 nucleus) and large (5 or more nucleus) osteoclasts.

Statistical Analysis

A minimum of three individual experimental trials were run with a minimum of two samples per experimental group, where a sample is one independent slide of cells inside the flow chamber, or one independent channel within an ibidi device. Student's *t*-test (two-tail, non-paired) was used on the combined data of all trials to test the significance between the flow and no flow groups ($\alpha=0.05$). The *n* value represents the total number of samples from all experimental trials.

RESULTS

Sclerostin Expression Under OFF

To establish the OCY454 cell line as an appropriate model for studying osteocyte intercellular signaling to osteoblasts in vitro, there was a need to demonstrate its mechano-sensitivity under physiological flow conditions (0.8–3 Pa, 1–9 Hz^{33,36,37}). OFF (1.2 Pa, 1 Hz) was applied to stimulate differentiated OCY454 cells, and soluble protein levels were measured 24 h after flow. We observed that sclerostin expression by OCY454 cells decreased after exposure to flow (Fig. 1A), but no decrease in RANKL/OPG ratio was measured (Fig. 1B). However, the RANKL secretion was significantly lower than those previously reported from MLO-Y4 cells under the same condition.¹⁸

Intracellular Calcium Response After Fluid Flow

It is well established that MLO-Y4 cells respond to fluid flow with an instantaneous intracellular calcium response,²¹ which is one of the major early events in the mechanotransduction cascade. Outcome variables such as responding cell percentage, response magnitude, and number of responding peaks have been used as indicators of the response to flow to evaluate osteocyte mechano-sensitivity. We here applied OFF at 4 Pa peak shear stress to differentiated OCY454 cells and showed that OCY454 cells had a calcium response to this level of flow (Fig. 2). However, compared to the calcium response of MLO-Y4 cells stimulated under the same flow conditions, OCY454 cells had a significantly lower percentage of responding cells (Fig. 2A). Furthermore, the OCY454 osteocytes sometimes had a much higher magnitude of response, unseen in MLO-Y4 cells (Fig. 2B and D). The response magnitudes also varied considerably between different experiments, while those from MLO-Y4 cells were known to be more stable and precise. Multiple peak responses were rarely seen in the OCY454 cells (Fig. 2C), but occasionally observed in the MLO-Y4 cells (Fig. 2D). Furthermore, OCY454 cells typically had longer response durations than the MLO-Y4 cells, as can be seen in the long response curve in Figure 2D, in contrast to the sharp curve observed for MLO-Y4 cells.

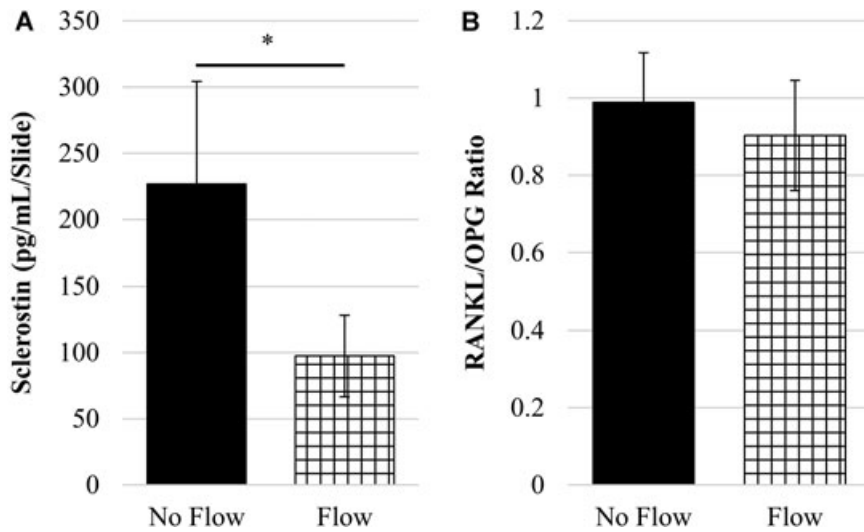


Figure 1. Sclerostin and RANKL/OPG. Extracellular sclerostin and RANKL/OPG concentration in media from OCY454 cells were quantified 24 h after flow stimulation. (A) Sclerostin levels measured from the flow-stimulated OCY454 cells were significantly lower compared to the no flow control group ($p < 0.05$, $n = 6$). (B) Both RANKL and OPG secretion levels were also measured. No significant difference in RANKL/OPG ratio was observed between flow and no flow groups ($p = 0.39$, $n = 12$). OPG, osteoprotegerin; RANKL, receptor activator of the nuclear factor κ -B ligand.

Total ATP Content After Fluid Flow

One of the important markers of osteocyte mechanosensitivity is the immediate production of ATP upon flow stimulation. After 20 min of OFF stimulation (2 Pa, 1 Hz), both media and cell lysates were extracted

from the ibidi chambers to measure for total ATP contents. The results showed a significant increase in the total ATP content of OCY454 cells stimulated with OFF (Fig. 3). This agreed with the previously observed response from MLO-Y4 osteocytes in vitro.²¹

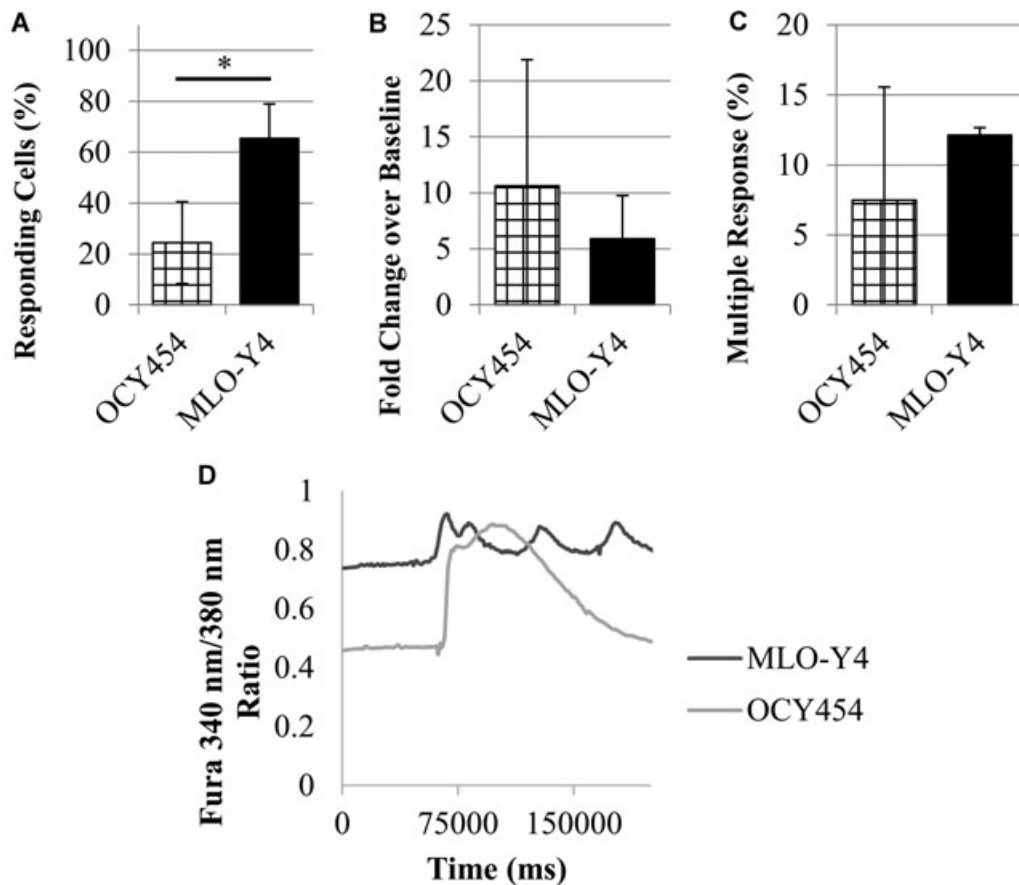


Figure 2. Calcium response. Immediate intracellular calcium response was measured at the onset of oscillatory fluid flow (OFF) (4 Pa, 1 Hz) stimulation. (A) Significant difference in percentage of responding cells was observed ($p < 0.05$, $n = 6$), however, no statistically significant difference observed for (B) average magnitude of response and (C) percentage of cells exhibiting multiple responses. (D) A graph showing the typical calcium response curve (flow stimulation started at 1 min) observed from the two different osteocyte cell lines, with the multi-peak response shown in the MLO-Y4 curve.

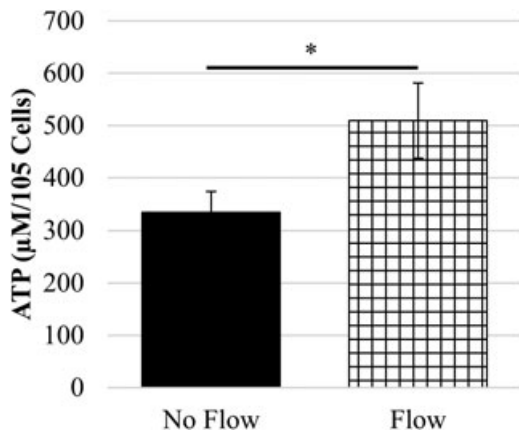


Figure 3. Total ATP level. Total ATP contents were measured from OCY454 cells immediately after a 20-min stimulation with OFF. Cell lysate and media were combined to test for total ATP. There was a significant difference observed between cells exposed to fluid flow versus no flow ($p < 0.05$, $n = 6$). ATP, adenosine triphosphate; OFF, oscillatory fluid flow.

Flow Stimulated OCY454 Affects Osteoblast Activity

Cross-talk between mechano-sensing osteocytes and osteoblasts is essential to the loading-regulated metabolic bone remodeling process. To investigate this regulation, CM from OCY454 cells 24 h after OFF stimulation was used to culture MC3T3-E1 osteoblasts over a period of 14 days. ALP activity was measured on day 4, 7, 10, and 14 using media collected from conditioned osteoblasts (Fig. 4A). There was a noticeable increase in ALP activity of osteoblasts cultured in CM from flow-stimulated osteocytes in comparison to CM from static osteocytes in the first three time points (Fig. 4A). These differences diminished by day 14, where the ALP activity stabilized to similar levels between osteoblasts conditioned with flow and static osteocyte CM.

In addition to intracellular ALP, calcium deposition is often used as an important indicator of osteoblast activity in vitro.³⁸ Calcium deposition levels were also measured at the end of day 14 using the ARS stain. Although ALP levels started to stabilize, there was a significant increase in the final calcium deposition from osteoblasts conditioned in flow osteocyte CM (Fig. 4B). This showed a similar trend as those observed for ALP activity measurements from day 4 to 10 (Fig. 4B). These results demonstrated that flow-stimulated OCY454 cells increased bone formation activities.

OCY454 Affects Osteoclast Differentiation

Similarly, cross-talk between osteocytes and osteoclasts is critical in the regulation of bone resorption. CM from mechanically stimulated OCY454 osteocytes was used to culture RAW264.7 osteoclast precursors supplemented with RANKL for 7 days. Although no significant differences were observed between the overall numbers of differentiated osteoclasts in the flow and no flow groups (Fig. 5A), there was a significantly higher

number of large osteoclasts with more than five nuclei in the no flow group (Fig. 5B). This demonstrated that flow experienced by OCY454 cells stimulated a change in secreted factors that altered osteoclast activity, even though the initial ELISA results showed no differences in the RANKL/OPG ratio.

DISCUSSION

To address one of the major challenges in the osteocyte mechanobiology field—the lack of secreted sclerostin from MLO-Y4 cells, we here investigated the potential of OCY454 cells as a new osteocyte model. We showed that OCY454 cells responded to physiologically relevant OFF with both immediate ATP synthesis as well as long-term sclerostin secretion. Furthermore, the results from our conditioning experiments confirmed that the factors secreted by OCY454 cells were capable of regulating osteoblast activities. This is a significant finding, since, to the best of our knowledge, there have been very limited in vitro studies on osteocyte–osteoblast intercellular communication, and one of the significant factors contributing to this was the absence of sclerostin secretion from MLO-Y4 cells.^{39,40} Our

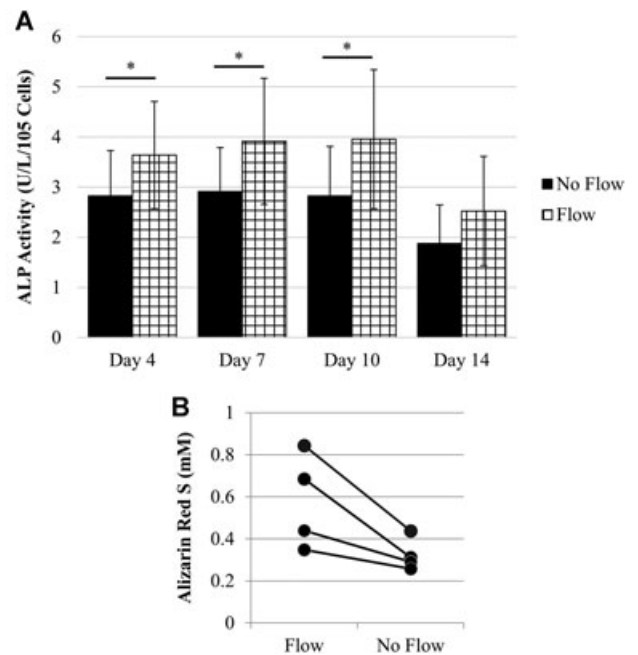


Figure 4. Osteoblast activity. Intracellular ALP activity and ARS stain for calcium deposition in osteoblasts conditioned in CM were measured from static or flow-stimulated OCY454 cells. (A) A significant difference in ALP activity was observed between flow and no flow groups on days 4 ($p < 0.05$, $n = 11$), 7 ($p < 0.05$, $n = 10$) and 10 ($p < 0.05$, $n = 10$). However, this difference decreased by day 14 ($p > 0.05$, $n = 7$). (B) A significant difference between flow and no flow groups was observed in calcium deposition at day 14 (each line represents a separate trial, $p < 0.05$, $n = 9$). This verified that the observed differences in ALP activity affected osteoblast functions. ALP, alkaline phosphatase; ARS, Alizarin Red S; CM, conditioned medium.

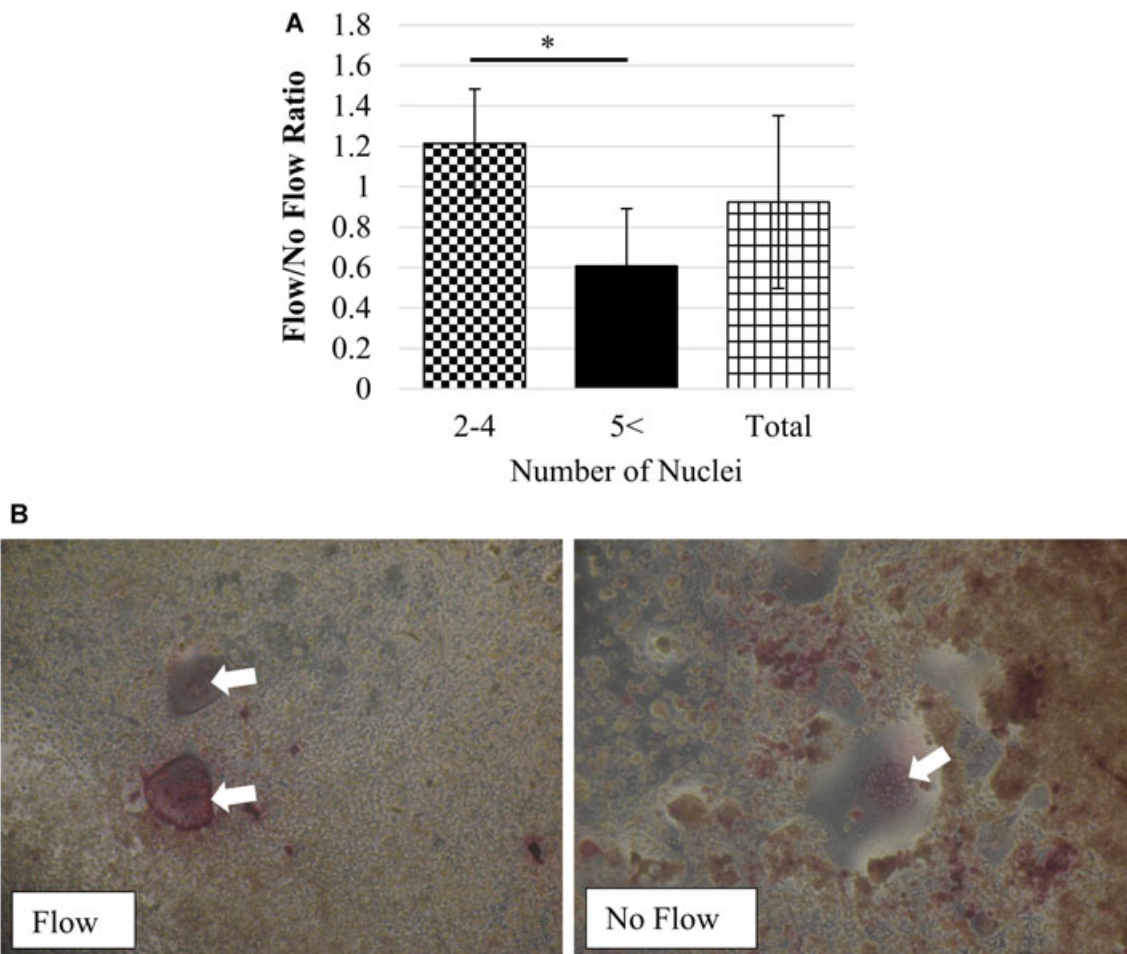


Figure 5. Osteoclast activity. Osteoclast activity was measured using TRAP stain on osteoclasts cultured in CM from OCY454 cells for 7 days. (A) There was no difference in the total number of TRAP-positive osteoclasts. However, there was a significant decrease in the number of large osteoclasts with five or greater nuclei when conditioned in media from the flow-stimulated OCY454 cells ($p < 0.05$, $n = 8$). (B) Sample images with arrow showing osteoclast nucleus, where no flow group has a significantly higher number of nuclei per osteoclast. CM, conditioned medium; TRAP, tartrate-resistant acid phosphatase. [Color figure can be viewed at wileyonlinelibrary.com]

results suggested that OCY454 cells have the potential to fill this gap in knowledge.

In osteoblast conditioning experiments, the ALP activity of MC3T3-E1 osteoblasts conditioned with flow-stimulated osteocyte CM was consistently higher than that of the no flow group at 4, 7, and 10 days after the start of conditioning. However, the ALP activity slightly decreased by day 14, and there was no more significant difference between the flow and no flow groups. We believed this might be due to our experimental setup. As we were interested in the time-lapse change of osteoblast ALP activity in response to the same group of osteocytes, all CM for a single well of osteoblasts came from one specific glass slide of osteocytes. During the conditioning experiment, vials of CM were thawed from -20°C during media changes. Due to the length of storage, media used on Day 10 might have significantly less protein content compared to when it was first collected. This would affect the ALP activity measured on Day 14, and could explain why there was an overall reduction in ALP activity

measured in both the flow and no flow groups. Although fresh CM could be generated from new flow experiments for each media change, this would result in large variations between the CM due to differences, such as cell passages, making it difficult to isolate the effect of the osteocyte mechano-regulation of osteoblasts. However, the final ARS staining stood as a more assuring proof to the overall effect of the 14-day conditioning of osteoblasts using OCY454 CM. ARS stain from the flow group was consistently higher than that from the no flow groups, demonstrating the lasting effect of the mechano-regulation of osteoblasts via osteocytes. The difference observed between osteoblasts conditioned in CM from static and flow-stimulated OCY454 cells was likely due to the flow-induced downregulation of sclerostin expression, though more molecular experiments would be needed to confirm this proposition.

Results from osteoclast conditioning experiments also demonstrated a difference between the flow and no flow groups. This was unexpected as the initial

ELISA quantification of the RANKL/OPG ratio showed no major change when the OCY454 osteocytes were exposed to flow. Though it had been suggested that sclerostin might have a catabolic mechanism to increase osteoclast activity through a RANKL-dependant pathway,⁴¹ RANKL levels measured from OCY454 cells using standard ELISA were often very low, similar to the results obtained by the original developers of the cell line,³² indicating other pathways were at play. There was also a possibility that the decrease in osteoclast differentiation in the flow group could be due to β -catenin signals. It had been proposed in the literature that β -catenin signals play a role in osteoclast differentiation,⁴² and, as an inhibitor of the Wnt pathway, a decrease in sclerostin might also decrease osteoclast differentiation. However, this phenomenon is still widely debated in the literature,^{43,44} and OCY454 osteocytes may be an ideal candidate to explore this pathway in vitro.

We have also tested the immediate response of OCY454 osteocytes to flow, in an attempt to verify and quantify its mechano-sensitivity. Flow-induced ATP release is a well-known indicator of mechanotransduction for osteocytes, often coupled with autophagy³⁵ and transient calcium response.^{21,45} Similar to the results observed for MLO-Y4 cells,⁴⁶ the new OCY454 osteocytes also exhibited a significant increase in total ATP expression when stimulated with flow. Interestingly, the calcium response measured from the OCY454 cells was very different compared to the MLO-Y4 cells. Under elevated flow condition of 4 Pa shear stress, MLO-Y4 cells exhibited consistency in the percentage of responding cells, mean magnitude of response, and percentage of multiple responses. But when the same flow conditions were applied to the OCY454 cells, large variations in response were observed between different experiments. Overall, significantly less OCY454 cells responded to flow. Furthermore, when applied with a standard in vitro fluid flow condition of 2 Pa shear stress, almost no calcium response was observed from the OCY454 cells (data not shown). We speculated that the large variations in response could have been due to the heterogeneity of the cell line. Since it was a relatively new cell line, the OCY454 cells we had been working with were yet to be isolated to a homogeneous population. As further development of the cell line occurs, there should be a significant reduction in the variation of the response. However, this did not explain the observation that there was little to no response to the normal physiological flow condition of 2 Pa shear stress. A previous study had elicited measurable calcium responses at 0.4 and 1.6 Pa shear stress,⁴⁷ using omnidirectional laminar flow,⁴⁸ which is very different from the physiological OFF used in this study. Previous studies have shown different cell responses under different flow profiles,⁴⁹ prompting the speculation that OCY454 cells might also have different calcium response profiles when exposed to different flow patterns.

There is a distinct difference between the culturing methods for the OCY454 osteocytes compared to MLO-

Y4 cells. The overall differentiation period of the new cell line was 14 days, which resulted in an extremely confluent layer of cells at the end of differentiation. This was very different from MLO-Y4 cells, which required passaging before reaching full confluence. It is possible that traditional cell culturing techniques are not ideal for OCY454 cells, and novel methods that encompass a steady supply of nutrients during the differentiation stage should be explored. Furthermore, the original authors had noted avoiding passaging the cells during the differentiation period, making it difficult to culture to the full length of the differentiation period without a substantial amount of cell death. As osteocytes in the bone are situated in sparse networks without direct contact between cell bodies, it is reasonable to question whether this condition is truly representative of the in vivo osteocytes. Future experimentation with the OCY454 cells should investigate whether there is a more optimal protocol for culturing these cells that can instigate a similar morphology to in vivo osteocytes.

Based on our data herein, we believe that the OCY454 cell line is an ideal candidate for studying mechanically stimulated osteocytes and their effects on osteoblast function. Their low RANKL expression also makes them interesting candidates for studying RANKL-independent osteocyte mechanoregulation of osteoclasts. However, the large variations in calcium response and the low level of RANKL expression highlights the limitation of this cell line in terms of studying the intracellular calcium dependent mechanotransduction pathway and RANKL/OPG-dependent osteoclast regulation, which are the strengths of the MLO-Y4 cell line. Further studies will be required to better understand the characteristics of OCY454 cells before they can replace the currently established MLO-Y4 cells, particularly for intracellular calcium studies. The scientific question yet to be answered will dictate which cell model is most appropriate.

Due to the lack of sclerostin expression by the MLO-Y4 cells, in vitro studies on osteocytes and many bone diseases had been limited as there was a lack of a physiologically accurate cell line to characterize the diseases. Animal studies and clinical data were often used, but without an appropriate understanding of their pathways in vitro, it was extremely difficult to uncover clinical solutions to these diseases. We suggest that, by demonstrating their capability to regulate osteoblast and osteoclast activities, OCY454 cells can be used in vitro to study diseases such as bone dysplasia and van Buchem disease.⁵⁰ The incorporation of OCY454 cells will provide insight into the imbalances between bone resorption and bone formation, leading to potential clinical solutions to disorders such as osteoporosis.

CONCLUSION


In summary, we showed that OCY454 osteocytes responded to flow with changes in intracellular calcium concentration, ATP contents, and sclerostin release. We

also showed that soluble factors released from fluid flow-stimulated OCY454 cells could further regulate osteoblasts and osteoclasts. Therefore, our findings suggested that OCY454 is a promising new cell model for in vitro osteocyte mechanotransduction studies. We hope that, by demonstrating these traits, OCY454 cells will be recognized as a new cell line capable of tackling intercellular signaling studies between bone cells and unveil molecular targets that are key to future clinical solutions.

AUTHORS' CONTRIBUTION

LHX and LY conceived the project. LX developed the methods with support from YM, LHX, and HS carried out the experiments. LHX wrote the manuscript with support from YM and LY supervised the project.

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REFERENCES

- Crockett JC, Rogers MJ, Coxon FP, et al. 2011. Bone remodelling at a glance. *J Cell Sci* 124(7):991–998.
- Tanaka Y, Nakayamada S, Okada Y. 2005. Osteoblasts and osteoclasts in bone remodeling and inflammation. *Curr Drug Targets Inflamm Allergy* 4(3):325–328.
- Chen H, Senda T, Kubo K. 2015. The osteocyte plays multiple roles in bone remodeling and mineral homeostasis. *Med Mol Morphol* 48(2):61–68.
- Bellido T. 2014. Osteocyte-driven bone remodeling. *Calcif Tissue Int* 94(1):25–34.
- Klein-Nulend J, Bacabac RG, Bakker AD. 2012. Mechanical loading and how it affects bone homeostasis: the role of the osteocyte cytoskeleton in maintaining our skeleton. *Eur Cell Mater* 24: 278–291.
- Schaffler MB, Kennedy OD. 2012. Osteocyte signaling in bone. *Curr Osteoporos Rep* 10(2):118–125.
- Nakashima T, Hayashi M, Fukunaga T, et al. 2011. Evidence for osteocyte regulation of bone homeostasis through RANKL expression. *Nat Med* 17(10):1231–1234.
- Xiong J, Onal M, Jilka RL, et al. 2011. Matrix-embedded cells control osteoclast formation. *Nat Med* 17(10):1235–1241.
- Ogasawara T, Yoshimine Y, Kiyoshima T, et al. 2004. In situ expression of RANKL, RANK, osteoprotegerin and cytokines in osteoclasts of rat periodontal tissue. *J Periodontol Res* 39(1):42–49.
- Riquelme MA, Burra S, Kar R, et al. 2015. Mitogen-activated protein kinase (MAPK) activated by prostaglandin E₂ phosphorylates connexin 43 and closes osteocytic hemichannels in response to continuous flow shear stress. *J Biol Chem* 290(47):28321–28328.
- van Bezooijen RL, Roelen BAJ, Visser A, et al. 2004. Sclerostin is an osteocyte-expressed negative regulator of bone formation, but not a classical BMP antagonist. *J Exp Med* 199(6):805–814.
- Balemans W, Patel N, Ebeling M, et al. 2002. Identification of a 52 kb deletion downstream of the SOST gene in patients with van Buchem disease. *J Med Genet* 39(2):91–97.
- Verde ME, Bermejo D, Gruppi A, Grenón M. 2015. Effect of bisphosphonates on the levels of Rankl and Opg in gingival crevicular fluid of patients with periodontal disease and post-menopausal osteoporosis. *Acta Odontol Latinoam* 28(3): 215–221.
- Delion M, Braux J, Jourdain M-L, et al. 2016. Overexpression of RANKL in osteoblasts: a possible mechanism of susceptibility to bone disease in cystic fibrosis. *J Pathol* 240(1):50–60.
- Campbell GM, Ominsky MS, Boyd SK. 2011. Bone quality is partially recovered after the discontinuation of RANKL administration in rats by increased bone mass on existing trabeculae: an in vivo micro-CT study. *Osteoporos Int* 22(3): 931–942.
- Kato Y, Windle JJ, Koop BA, et al. 2010. Establishment of an osteocyte-like cell line, MLO-Y4. *J Bone Miner Res* 12(12): 2014–2023.
- Bonewald LF. 1999. Establishment and characterization of an osteocyte-like cell line, MLO-Y4. *J Bone Miner Metab* 17(1):61–65.
- Zhao S, Kato Y, Zhang Y, et al. 2002. MLO-Y4 osteocyte-like cells support osteoclast formation and activation. *J Bone Miner Res* 17(11):2068–2079.
- Verbruggen SW, Vaughan TJ, McNamara LM. 2014. Fluid flow in the osteocyte mechanical environment: a fluid-structure interaction approach. *Biomech Model Mechanobiol* 13(1):85–97.
- Li J, Rose E, Frances D, et al. 2012. Effect of oscillating fluid flow stimulation on osteocyte mRNA expression. *J Biomech* 45(2):247–251.
- Lu XL, Huo B, Park M, Guo XE. 2012. Calcium response in osteocytic networks under steady and oscillatory fluid flow. *Bone* 51(3):466–473.
- You L, Temiyasathit S, Lee P, et al. 2008. Osteocytes as mechanosensors in the inhibition of bone resorption due to mechanical loading. *Bone* 42(1):172–179.
- Tan SD, de Vries TJ, Kuijpers-Jagtman AM, et al. 2007. Osteocytes subjected to fluid flow inhibit osteoclast formation and bone resorption. *Bone* 41(5):745–751.
- Lu XL, Huo B, Chiang V, Guo XE. 2012. Osteocytic network is more responsive in calcium signaling than osteoblastic network under fluid flow. *J Bone Miner Res* 27(3):563–574.
- Gu Y, Preston MR, Magnay J, et al. 2001. Hormonally-regulated expression of voltage-operated Ca²⁺ channels in osteocytic (MLO-Y4) cells. *Biochem Biophys Res Commun* 282(2):536–542.
- Jing D, Lu XL, Luo E, et al. 2013. Spatiotemporal properties of intracellular calcium signaling in osteocytic and osteoblastic cell networks under fluid flow. *Bone* 53(2):531–540.
- Kitase Y, Barragan L, Qing H, et al. 2010. Mechanical induction of PGE₂ in osteocytes blocks glucocorticoid-induced apoptosis through both the β -catenin and PKA pathways. *J Bone Miner Res* 25(12):2657–2668.
- Morrell AE, Brown GN, Robinson ST, et al. 2018. Mechanically induced Ca²⁺ oscillations in osteocytes release extracellular vesicles and enhance bone formation. *Bone Res* 6(1):6.
- Li X, Zhang Y, Kang H, et al. 2005. Sclerostin binds to LRP5/6 and antagonizes canonical wnt signaling. *J Biol Chem* 280(20):19883–19887.
- van Dinther M, Zhang J, Weidauer SE, et al. 2013. Anti-sclerostin antibody inhibits internalization of sclerostin and sclerostin-mediated antagonism of Wnt/LRP6 signaling. *PLoS ONE* 8(4):e62295.
- Robling AG, Niziolek PJ, Baldrige LA, et al. 2008. Mechanical stimulation of bone in vivo reduces osteocyte expression of sost/sclerostin. *J Biol Chem* 283(9):5866–5875.
- Spatz JM, Wein MN, Gooi JH, et al. 2015. The Wnt inhibitor sclerostin is up-regulated by mechanical unloading in osteocytes in vitro. *J Biol Chem* 290(27):16744–16758.
- Lara-Castillo N, Kim-Werooha NA, Kamel MA, et al. 2015. In vivo mechanical loading rapidly activates β -catenin signaling

- in osteocytes through a prostaglandin mediated mechanism. *Bone* 76:58–66.
34. Middleton K, Kondiboyina A, Borrett M, et al. 2017. Microfluidics approach to investigate the role of dynamic similitude in osteocyte mechanobiology. *J Orthop Res* 36(2):663–671.
 35. Zhang B, Hou R, Zou Z, et al. 2018. Mechanically induced autophagy is associated with ATP metabolism and cellular viability in osteocytes in vitro. *Redox Biol* 14:492–498.
 36. Han Y, Cowin SC, Schaffler MB, Weinbaum S. 2004. Mechanotransduction and strain amplification in osteocyte cell processes. *Proc Natl Acad Sci USA* 101(47):16689–16694.
 37. Bacabac RG, Smit TH, Mullender MG, et al. 2004. Nitric oxide production by bone cells is fluid shear stress rate dependent. *Biochem Biophys Res Commun* 315(4):823–829.
 38. Kim E-J, Bu S-Y, Sung M-K, Choi M-K. 2013. Effects of silicon on osteoblast activity and bone mineralization of MC3T3-E1 cells. *Biol Trace Elem Res* 152(1):105–112.
 39. Bakker AD, Kulkarni RN, Klein-Nulend J, Lems WF. 2014. IL-6 alters osteocyte signaling toward osteoblasts but not osteoclasts. *J Dent Res* 93(4):394–399.
 40. Vezeridis PS, Semeins CM, Chen Q, Klein-Nulend J. 2006. Osteocytes subjected to pulsating fluid flow regulate osteoblast proliferation and differentiation. *Biochem Biophys Res Commun* 348(3):1082–1088.
 41. Wijenayaka AR, Kogawa M, Lim HP, et al. 2011. Sclerostin stimulates osteocyte support of osteoclast activity by a RANKL-dependent pathway. *PLoS ONE* 6(10):e25900.
 42. Wei W, Zeve D, Suh JM, et al. 2011. Biphasic and dosage-dependent regulation of osteoclastogenesis by β -catenin. *Mol Cell Biol* 31(23):4706–4719.
 43. Maeda K, Kobayashi Y, Udagawa N, et al. 2012. Wnt5a-Ror2 signaling between osteoblast-lineage cells and osteoclast precursors enhances osteoclastogenesis. *Nat Med* 18(3):405–412.
 44. Hayashi S, Witte PL, Shultz LD, et al. 1988. Lymphohemopoiesis in culture is prevented by interaction with adherent bone marrow cells from mutant viable motheaten mice. *J Immunol* 140(7):2139–2147.
 45. Huo B, Lu XL, Costa KD, et al. 2010. An ATP-dependent mechanism mediates intercellular calcium signaling in bone cell network under single cell nanoindentation. *Cell Calcium* 47(3):234–241.
 46. Seref-Ferlengez Z, Maung S, Schaffler MB, et al. 2016. P2X7R-Panx1 complex impairs bone mechanosignaling under high glucose levels associated with type-1 diabetes. *PLoS ONE* 11(5):e0155107.
 47. Lyons JS, Joca HC, Law RA, et al. 2017. Microtubules tune mechanotransduction through NOX2 and TRPV4 to decrease sclerostin abundance in osteocytes. *Sci Signal* 10(506):eaan5748.
 48. Lyons JS, Iyer SR, Lovering RM, et al. 2016. Novel multi-functional fluid flow device for studying cellular mechanotransduction. *J Biomech* 49(16):4173–4179.
 49. Ponik SM, Triplett JW, Pavalko FM. 2007. Osteoblasts and osteocytes respond differently to oscillatory and unidirectional fluid flow profiles. *J Cell Biochem* 100(3):794–807.
 50. van Bezooijen RL, Bronckers AL, Gortzak RA, et al. 2009. Sclerostin in mineralized matrices and van buchem disease. *J Dent Res* 88(6):569–574.