



## Effect of oscillating fluid flow stimulation on osteocyte mRNA expression

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### ABSTRACT

Structural adaptation of the bone tissue is mediated by loading-induced interstitial fluid flow within the bone microstructure. Within this framework, osteocytes fulfill the central mechanotransductive role in the bone remodeling process. While osteocytes have been demonstrated to be exquisitely sensitive to various forms of fluid flow stimulus *in vitro*, the effect of different oscillating fluid flow (OFF) parameters on osteocyte activity has yet to be systematically characterized. In this study, we investigate the effect of three OFF parameters on osteocyte activity *in vitro* and hypothesize that COX-2, RANKL, and OPG mRNA expression in osteocytes are sensitive to the OFF parameters: peak shear stress amplitude (0.5 Pa, 1 Pa, 2 Pa, and 5 Pa), oscillating frequency (0.5 Hz, 1 Hz, and 2 Hz), and total flow duration (1 h, 2 h, and 4 h). Our findings demonstrate that COX-2 mRNA levels are elevated in osteocytes subjected to higher peak shear stress amplitudes and longer flow durations, while RANKL/OPG mRNA levels decreased to a minimum threshold in response to higher peak shear stress amplitudes, faster oscillating frequencies, and longer flow durations. These findings suggest that dynamic fluid flow with higher peak shear stress amplitudes, faster oscillating frequencies, and longer loading durations provide the best conditions for promoting bone formation.

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### 1. Introduction

Bone is a dynamic tissue capable of loading-induced structural adaptation through the process of bone remodeling. While this concept is well accepted, the underlying mechanisms by which it is accomplished remain poorly understood. Mounting evidence suggest that (a) tissue-level strains are translated to cellular-level mechanical stimuli in the form of interstitial fluid flow within the lacunar–canalicular system, a fluid-filled network of interconnected pores permeating throughout the bone tissue (Wang et al., 2004; Fritton and Weinbaum, 2009; Kwon and Frangos, 2010; Price et al., 2011) and (b) osteocytes, terminally differentiated osteoblastic cells that reside within the lacunar–canalicular system, are able to sense and respond to mechanical stimulation (Cowin et al., 1995; Klein-Nulend et al., 1995; Jacobs et al., 1998; You et al., 2001; Han et al., 2004; Ponik et al., 2007; Huo et al., 2010; Rath et al., 2010; Cheung et al., 2011).

*In vitro* mechanical stimulation of osteocytes has been demonstrated to regulate the production of several soluble signaling molecules known to influence osteoblast and osteoclast activity including prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL), and osteoprotegerin (OPG) (Ajubi et al., 1999; Tan et al., 2007; You et al., 2008;

Kamel et al., 2010; Kitase et al., 2010). PGE<sub>2</sub> acts on osteoblasts in a paracrine fashion to promote increased bone formation while RANKL, through complexation with RANK (receptor activator of nuclear factor kappa B) receptors found on the surface of osteoclast precursors, stimulates pre-osteoclast commitment to the osteoclastic phenotype such that the total amount of bone resorption is increased. OPG acts as a decoy receptor that competes with RANK for the binding of RANKL. The relative abundance of RANKL to OPG (RANKL/OPG) is indicative of the amount of bone resorption (Hofbauer et al., 1999; Nagai and Sato, 1999; Kim et al., 2006). At the gene transcription level, physical stimulation of osteocytes has been shown to simultaneously decrease the RANKL/OPG mRNA ratio and increase the cyclooxygenase-2 (COX-2) mRNA expression. COX-2 is an essential enzyme in the synthesis of PGE<sub>2</sub> and its abundance can be directly correlated to PGE<sub>2</sub> release since PGE<sub>2</sub> is synthesized and released as needed rather than being stored by the cell. Taken together, osteocytes exposed to physiological level of mechanical stimuli produce and secrete soluble signaling molecules that regulate osteoblast and osteoclast activity in a paracrine fashion with the net effect of increasing bone formation.

The degree of cellular response elicited by osteocytes subjected to fluid flow stimulation remains largely unexplored and has been suggested to be a function of several dynamic fluid flow parameters including the applied peak shear stress amplitude, dynamic flow frequency, flow duration, and the number of loading cycles (Reich et al., 1990; Williams et al., 1994; Jacobs et al., 1998; Bacabac et al., 2004). Of these four parameters, the

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first three are independent parameters, while the number of loading cycles is dependent on both the total flow duration and the dynamic flow frequency. The relationship between these flow parameters and osteocyte activity is further complicated by the observation that osteocytes are additionally sensitive to the fluid flow stimulus profile (e.g., steady, pulsating, and oscillating) (Jacobs et al., 1998; Mullender et al., 2006; Malone et al., 2007; Ponik et al., 2007). These observations illustrate that osteocytes are exquisitely sensitive to different loading conditions and loading profiles. A thorough understanding of loading-induced bone adaptation and the etiology of some bone diseases, such as osteoporosis, requires the systematic characterization of osteocyte biochemical response under different fluid flow stimulus conditions. To date, however, there have been no systematic investigations examining the effect of different oscillating fluid flow (OFF) conditions on osteocyte activity. Herein we study the effect of OFF-induced peak shear stress amplitude, oscillating frequency, and total flow duration on osteocyte mRNA expression levels. We limit our study to the OFF profile because this flow profile is considered to be the most relevant in describing the *in vivo* loading environment experienced by osteocytes (Jacobs et al., 1998). Specifically, we investigate the effects of these conditions on the mRNA expression of RANKL, OPG, and COX-2, as the expression levels of these genes have been previously reported to be indicative of load-induced bone remodeling. We hypothesize that osteocytes differentially respond to the different OFF parameters including shear stress amplitudes, oscillating frequencies, and flow durations.

## 2. Materials and methods

### 2.1. Cell culture

MLO-Y4 osteocyte-like cells (gift from Dr. Lynda Bonewald, University of Missouri-Kansas City, Kansas City, MO, USA) were cultured on rat tail collagen I (BD Biosciences, Bedford, MA) coated polystyrene surfaces with  $\alpha$ -Modified Eagle's Medium ( $\alpha$ -MEM, GIBCO/Invitrogen, Carlsbad, CA) supplemented with 2.5% fetal bovine serum (FBS, Hyclone, Logan, UT), 2.5% calf serum (CS, Hyclone), and 1% penicillin and streptomycin (PS, GIBCO/Invitrogen). Cultured cells were maintained at 37 °C in a 5% CO<sub>2</sub> atmosphere and grown to 70% confluence.

### 2.2. Fluid flow stimulation

MLO-Y4 cells were seeded at 10,500 cells/cm<sup>2</sup> on rat tail type I collagen coated glass slides (75 mm × 38 mm × 1 mm; Corning), starved for 12 h in  $\alpha$ -MEM supplemented with 0.2% FBS and 1% PS, and placed in parallel plate flow chambers. A previously established flow system was used to apply OFF in this study (Kim et al., 2006; You et al., 2008; Cheung et al., 2011). In brief, flow was driven by a Hamilton glass syringe, which was mounted on and driven by an

electromechanical loading device. OFF stimulus parameters examined in this study are listed in Table 1. These combinations of OFF parameters were selected with the intention to examine the independent effect of peak shear stress amplitude, frequency, and duration. Combinations of frequencies and flow durations were also selected such that comparisons can be made between flow conditions with a constant number of loading cycles. Osteocytes were exposed to peak wall shear stress values of 0.5, 1.0, 2.0, or 5.0 Pa at frequencies of 0.5, 1.0, or 2.0 Hz for 1, 2, or 4 h at 37 °C and 5% CO<sub>2</sub>. These peak shear stress values encompass the predicted *in vivo* physiological shear stress ranges experienced by osteocytes within the lacuna–canalicular system (Weinbaum et al., 1994; Bacabac et al., 2004). The OFF frequencies were selected to reflect normal walking and running frequencies, and the total flow durations were selected based on the flow durations used in previous studies (Kim et al., 2006; You et al., 2008). Flow durations longer than 4 h were not included in this study due to the concern of cell viability under hypoxic conditions. The desired fluid flow conditions were achieved by adjusting the syringe stroke length, syringe volume, and the cyclic stroke period. MLO-Y4 cells placed in parallel plate flow chambers for 1, 2, or 4 h and subjected to no flow served as experimental controls. Each flow experiment was run in parallel alongside a control experiment of the same duration. All experiments were performed at 37 °C in a 5% CO<sub>2</sub> atmosphere.

### 2.3. mRNA quantification

Following flow, RNA was extracted from the MLO-Y4 cells ( $n=4$ ) using an RNeasy Mini Kit (Qiagen, USA), treated with DNase I (Fermentas, USA) and reverse-transcribed using SuperScript™ III RT (Invitrogen, USA) to synthesize cDNA. Quantitative PCR was used to amplify and quantify the amount of cDNA in each sample using gene-specific primers (Table 2; Operon, USA) and SYBR Green I (Roche, USA). The gene copy number for each experimental group was normalized to 18S (housekeeping gene).

### 2.4. Data analysis

All experiments were repeated in duplicate, with 4 replicates per condition. The COX-2/18s and RANKL/OPG mRNA levels for each OFF experimental condition were normalized to the COX-2/18s and RANKL/OPG mRNA values obtained from their corresponding no flow control experiments. This enables comparison of COX-2/18s and RANKL/OPG levels between different experimental conditions and also allows for comparison between mRNA levels in OFF conditions and no flow conditions (which have a normalized value of 1). Separate student's *t*-tests (two-tailed, unequal variance) were used to test significance between each OFF and no-flow control groups. A total of 40 student's *t*-tests were performed. For all two-hour time points, in which both stress amplitude and frequency are varied, two-way ANOVA was conducted to examine the effects of peak stress amplitude and loading frequency on both COX-2 and RANKL/OPG mRNA expression. A separate one-way ANOVA analysis was conducted for each comparison group in which only a single condition was varied. The Bonferroni test was used as a post-hoc test. Homogeneity of variance between groups was assessed using Levene's test for equality of error variances. A significance level of 0.05 was employed for all statistical analyses. Results presented in Figs. 1–3 are reported as a mean  $\pm$  SD.

## 3. Results

### 3.1. Effect of oscillating fluid flow

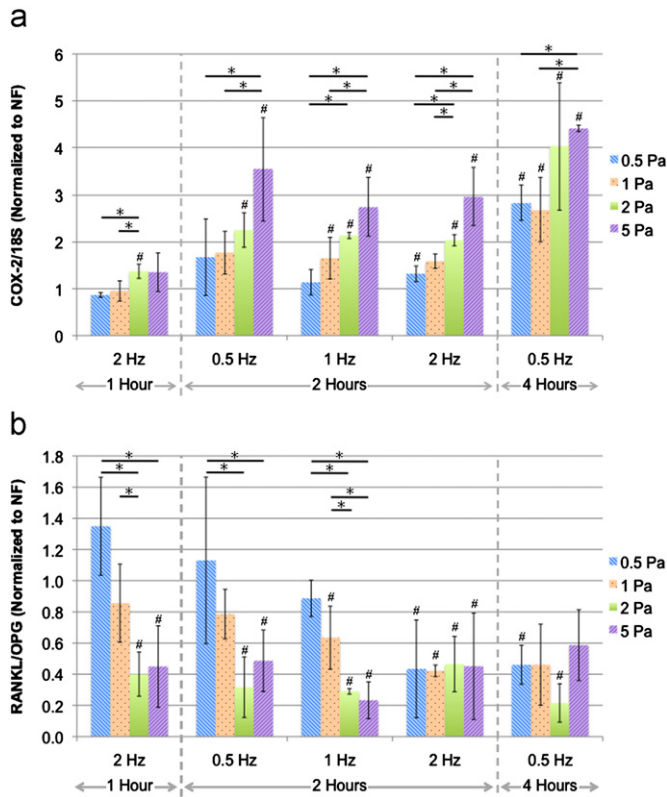
Fig. 1 shows the normalized COX-2 and RANKL/OPG mRNA levels for four different shear stress amplitudes (0.5, 1, 2 and 5 Pa) while all other fluid flow parameters are held constant. Osteocytes subjected to OFF generally exhibited elevated COX-2 mRNA levels (Fig. 1a) and decreased RANKL/OPG mRNA levels (Fig. 1b) as compared to no-flow controls, which have a normalized value of 1. The significance of these differences are dependent on the magnitude of each of the three OFF parameters such that statistically significant differences

**Table 1**  
Experimental oscillating fluid flow conditions.

Shear Stress	Oscillating frequency		
	0.5 Hz	1 Hz	2 Hz
0.5 Pa	2, 4 h	2 h	1, 2 h
1.0 Pa	2, 4 h	2 h	1, 2 h
2.0 Pa	2, 4 h	2 h	1, 2 h
5.0 Pa	2, 4 h	2 h	1, 2 h

**Table 2**  
RT-PCR primer sequence.

Gene	5'-Forward-3'	5'-Reverse-3'	Product size (bp)
COX-2	TCCTCCTGGAACATGGACTC	CCCCAAGATAGCATCTGGA	173
RANKL	CAGCATCGCTCTGTTCCTGTA	CTGCGTTTTTCATGGAGTCTCA	107
OPG	GGGCGTTACCTGGAGATCG	GAGAAGAACCCATCTGGACATTT	125
18S	GAGAAACGGCTACCACATCC	CCTCCAATGGATCCTCGTTA	158

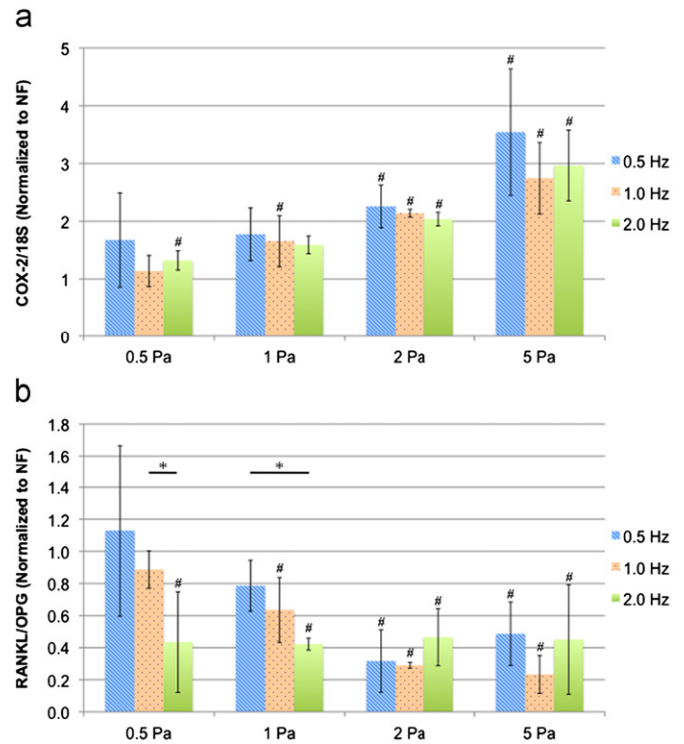


**Fig. 1.** (a) Cox-2 mRNA levels and (b) RANKL/OPG mRNA levels in osteocytes subjected to oscillatory fluid flow stimulus. Experimental groups are grouped with increasing peak shear stress amplitudes for constant oscillating frequency and flow duration. Significant difference between individual flow experiments as compared to their respective no-flow control groups is indicated with a #. Significant difference between two flow conditions is indicated with a \* over a horizontal bar. (\* $p < 0.05$ ; # $p < 0.05$ ;  $n = 4$ ). Vertical bars represent mean  $\pm$  SD.

( $p < 0.05$ ) in both COX-2 and RANKL/OPG mRNA levels, as compared to no-flow controls, are not observed for flow conditions with lower shear stress amplitudes (Fig. 1), oscillating frequencies (Fig. 2), and flow durations (Fig. 3), but become significant at higher parameter values.

### 3.2. Effect of stress amplitude and frequency on COX-2 mRNA expression

COX-2 mRNA levels in osteocytes subjected to OFF stimulus was found to be dose-dependent on the peak shear stress amplitude (Fig. 1a), but not oscillating frequency (Fig. 2a). For the 2 h flow conditions, no statistically significant interaction was observed between the effects of shear stress amplitude and frequency on COX-2 mRNA expression ( $F = 0.424$ ,  $P = 0.858$ ). Simple main effects analysis on all 2h OFF conditions shows that higher peak stress levels result in significantly higher COX-2 mRNA expression at all loading frequencies ( $P < 0.0005$  for 0.5, 1, and 2 Hz conditions, respectively). In addition, no statistical differences in COX-2 mRNA expression were observed between the different frequency levels at each loading magnitude ( $P = 0.307$ ,  $0.929$ ,  $0.790$  and  $0.113$  for 0.5, 1, 2, and 5 Pa). Thus, osteocytes subjected to larger peak shear stress amplitudes exhibited higher COX-2 mRNA levels, irrespective of the applied loading frequency, with the highest COX-2 levels corresponding to flow conditions with a 5 Pa peak shear stress amplitude. This trend was maintained across all experimental groups with different flow frequencies and flow durations, including both 1 and 4 h time points (Figs. 1a and 2a), and suggests that COX-2 mRNA



**Fig. 2.** (a) Cox-2 mRNA levels and (b) RANKL/OPG mRNA levels in osteocytes subjected to oscillatory fluid flow stimulus. Experimental groups are grouped with increasing oscillating frequency for constant shear stress magnitude and flow duration (2 hours for all experimental groups). Significant difference between individual flow experiments as compared to their respective no-flow control groups is indicated with a #. Significant difference between two flow conditions is indicated with a \* over a horizontal bar. (\* $p < 0.05$ ; # $p < 0.05$ ;  $n = 4$ ). Vertical bars represent mean  $\pm$  SD.

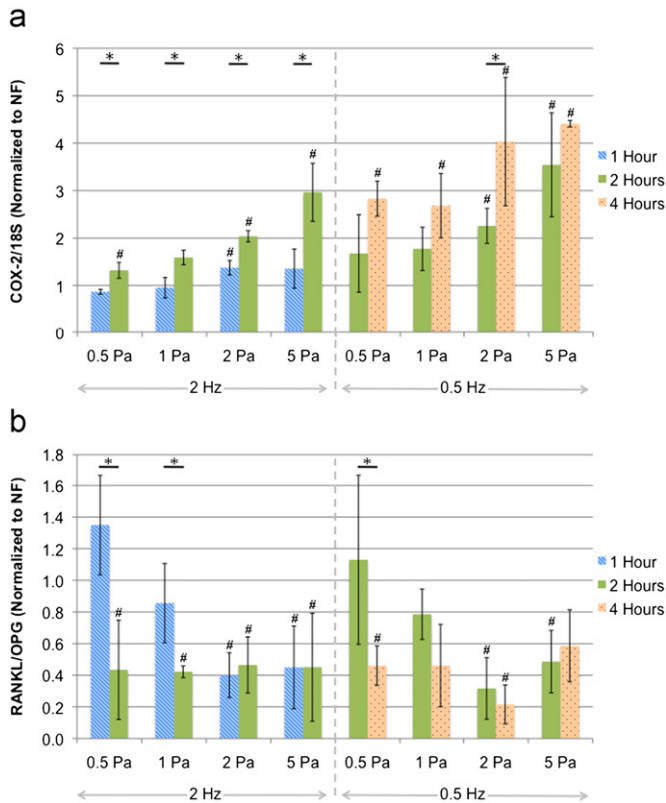
expression in osteocytes is insensitive to both the loading frequency and the number of loading cycles.

### 3.3. Effect of stress amplitude and frequency on RANKL/OPG mRNA expression

A decreasing dose-dependent response, with respect to both peak amplitude and oscillating frequency, was observed for RANKL/OPG mRNA levels (Figs. 1b and 2b) at lower peak amplitudes and frequencies. Amongst the 2h flow conditions, there was a significant interaction between the effects of stress amplitude and frequency on RANKL/OPG mRNA expression ( $F = 2.991$ ,  $P = 0.017$ ). Simple main effects analysis showed that higher stress levels resulted in significantly lower RANKL/OPG mRNA expression at loading frequencies of 0.5 and 1 Hz ( $P < 0.0005$  and  $0.001$ , respectively), but not at 2 Hz ( $P = 0.997$ ). In addition, statistically significant differences in RANKL/OPG mRNA expression were observed between the different frequency levels at 0.5 and 1 Pa loading magnitudes ( $P = 0.001$  and  $0.019$ , respectively), but not at 2 or 5 Pa ( $P = 0.501$  and  $0.291$ , respectively). Thus, as the peak stress amplitude and/or oscillating frequency increases from 0.5 to 5 Pa and 0.5 to 2 Hz, respectively, the RANKL/OPG ratio decreases from a value of 1 (the normalized control value) to a minimum threshold value. These results suggest that RANKL/OPG transcription is influenced by either loading frequency and/or the number of loading cycles.

### 3.4. Effect of flow duration

Fig. 3 presents the normalized COX-2 and RANKL/OPG mRNA levels for osteocytes subjected to different OFF durations.



**Fig. 3.** (a) Cox-2 mRNA levels and (b) RANKL/OPG mRNA levels in osteocytes subjected to oscillatory fluid flow stimulus. Experimental groups are grouped with increasing flow duration for constant peak shear stress amplitude and flow frequency. Significant difference between individual flow experiments as compared to their respective no-flow control groups is indicated with a #. Significant difference between two flow conditions is indicated with a \* over a horizontal bar. (\* $p < 0.05$ ; # $p < 0.05$ ;  $n = 4$ ). Vertical bars represent mean  $\pm$  SD.

COX-2 mRNA levels were found to be consistently elevated in osteocytes subjected to longer flow durations (Fig. 3a). RANKL/OPG mRNA ratio was found to decrease for longer flow durations at 0.5 and 1 Pa, but not at 2 and 5 Pa, shear stress conditions (Fig. 3b).

#### 4. Discussion

We investigated the separate effects of three independent OFF parameters (shear stress amplitude, frequency, and duration) on osteocyte response *in vitro*. Our results demonstrate that osteocytes subjected to different OFF conditions exhibited several interesting behaviors. In general, the application of OFF stimulation simultaneously up-regulated the COX-2 mRNA expression and down-regulated the RANKL/OPG mRNA levels. Given that increased COX-2 mRNA expression promotes bone formation by osteoblasts via the release of PGE<sub>2</sub>, and a higher RANKL/OPG ratio favors increased bone resorption through osteoclastogenesis, our findings suggest that OFF stimulation of osteocytes tips the balance between osteoblast and osteoclast activity in favor of net increased bone formation.

More notable, however, is the observation that osteocytes responded to changes in all three OFF parameters. This reinforces the idea that osteocytes are exquisitely sensitive to mechanical stimulation. The dose-dependent response on shear stress amplitude, loading frequency, and flow duration has also been observed by other groups studying osteoblast morphological response to increasing fluid shear stresses (Liu et al., 2010), nitric oxide production by osteoblasts subjected to different shear stress rates

(Bacabac et al., 2004), osteocyte displacement in the presence of different fluid flow frequencies (Kwon and Jacobs, 2007), flow-induced calcium oscillations in osteocytes (Jacobs et al., 1998; Donahue et al., 2001), and RANKL and OPG production in osteocytes (You et al., 2008) and osteoblasts (Kim et al., 2006). It should be noted that osteocytes subjected to fluid flow conditions with lower parameter values (e.g., 0.5 Pa or 1 Pa peak shear stress amplitudes, 0.5 Hz oscillating frequency, and/or 1 h flow duration) generally did not exhibit statistically significant differences in COX-2 or RANKL/OPG mRNA levels when compared to their respective no-flow control experiments due to insufficient stimulation provided by these flow conditions. This is a reasonable observation given the dose-responsive behavior of osteocyte towards the various fluid flow stimulus parameters. While the above-mentioned trends are generally well supported by our findings, careful examination of our results reveals several interesting exceptions that allow us to speculate on the mechanisms involved in osteocyte mechanotransduction.

Osteocyte COX-2 and RANKL/OPG mRNA levels exhibited qualitatively different responses to the same fluid flow parameters. This is exemplified by the fact that while the RANKL/OPG mRNA ratio changed as a result of varying fluid flow frequency, the COX-2 mRNA expression levels showed no significant sensitivity towards this parameter ( $p > 0.05$ ; Fig. 2). A more subtle demonstration of this point can be noted when comparing the dose-response regulation of COX-2 and RANKL/OPG mRNA levels to increasing shear stress magnitudes in which RANKL/OPG mRNA levels did not continue to decrease when subjected to higher shear stresses beyond 2 Pa, while COX-2 mRNA levels continued to change even for 5 Pa peak shear stress (Fig. 1). This would seem to suggest that loading-induced COX-2 and RANKL/OPG gene transcription are regulated via different transduction mechanisms.

Another interesting observation is that the effect of the different OFF parameters on osteocyte COX-2 and RANKL/OPG mRNA expression appears to be additive. For instance, while both higher shear stress magnitudes and longer flow durations independently increased osteocyte COX-2 mRNA levels, the combination of both elevated shear stress magnitude and longer flow durations resulted in a substantially higher COX-2 mRNA level as compared to cases where either of these parameters acted alone (Fig. 1a). Therefore, the highest measured COX-2 mRNA levels were observed when osteocytes were subjected to OFF parameters with the highest shear stress (5 Pa) and longest flow duration (4 h). A similar situation can be observed for RANKL/OPG mRNA levels in which both higher shear stress magnitudes and larger cycle numbers (via either frequency or duration) separately decreased this ratio. Yet when combined, the decrease in RANKL/OPG mRNA levels down to the apparent lower limit occurs at lower shear stress amplitudes (2 Pa for 0.5 Hz, 2 h as compared to 0.5 Pa for either the 2 Hz, 2 h flow condition or the 0.5 Hz, 4 h flow condition). This observation suggests that osteocytes respond to each of the three fluid flow parameters in a synergistic manner.

The experimental methods employed in this study present several inherent limitations, which should be carefully considered when interpreting our results. Experimental conditions with flow durations longer than 4 h were not pursued in this study due to concern regarding osteocyte viability due to poor nutrient exchange and oxygen supply levels within the flow chambers. For the purposes of this study, however, the differences in nutrient supply and oxygen levels for different flow durations were accounted for by subjecting both flow and no-flow control groups to the same conditions. Further, while cellular mRNA levels are generally indicative of protein expression, we recognize that the two quantities may not be directly correlated as post-translational

modifications may also contribute significantly to the final protein levels. Therefore, work currently being pursued in our lab includes the characterization of soluble protein products secreted from the mechanically stimulated osteocytes.

In conclusion, we subjected osteocytes to OFF conditions *in vitro* and examined the effects of three different fluid flow parameters (peak shear stress amplitude, oscillating frequency, and total flow duration) on COX-2 and RANKL/OPG mRNA expression. COX-2 mRNA levels were shown to be sensitive to shear stress amplitude and total flow duration, but not oscillating frequency. RANKL/OPG mRNA levels were sensitive to variation in all three fluid flow parameters, however a minimum limit appeared to exist for this ratio. A significant interaction between shear stress amplitude and loading frequency was observed in the regulation of RANKL/OPG, but not COX-2, mRNA expression. Results from this study suggest that (1) osteocytes exhibit distinctly different responses to each of the three independent OFF parameters: peak shear stress amplitude, oscillating frequency, and total flow duration, (2) different mechanotransduction mechanisms likely exist for regulating osteocyte COX-2 and RANKL/OPG mRNA expression, (3) the effects of each OFF parameter appear to work together in a cumulative manner in regulating osteocyte activity, and (4) loading conditions with higher peak shear stress amplitudes, higher oscillating frequency, and longer loading durations provide the best stimulus for promoting bone formation.

### Conflict of interest statement

The authors report no conflicts of interest.

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