Calcium response in bone cells at different osteogenic stages under unidirectional or oscillatory flow

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Shurong Wang,1 Shuna Li,1 Man Hu,2 and Bo Huo1,a)

Affiliations
1Biomechanics Lab, Department of Mechanics, School of Aerospace Engineering, Beijing Institute of Technology, Beijing 100081, People’s Republic of China
2Institute of Mechanics, Chinese Academy of Sciences, Beijing 100080, People’s Republic of China

a)Author to whom correspondence should be addressed: huobo@bit.edu.cn. Tel: 8610-68915760.

Abstract
It was found that preosteoblast MC3T3-E1 cells were less responsive in calcium signaling than mature osteocyte MLO-Y4 cells when a steady fluid flow was exerted on a micropatterned cell network. However, the effect of fluid flow on the calcium response in preosteocyte MLO-A5 was seldom investigated. In the present study, MLO-A5 as well as MC3T3-E1 and MLO-Y4 cells were cultured on a regular substrate with high or low density under unidirectional or oscillatory fluid flow. The results showed that calcium oscillation in the cells during late osteogenesis was significantly stronger than during early osteogenesis regardless of the fluid flow type or the presence of a physical cell-cell connection. Calcium oscillation produced by the oscillatory flow in the three types of cells was stronger than that produced by the unidirectional flow, but MC3T3-E1 and MLO-A5 cells exhibited limited potential for calcium oscillation compared with MLO-Y4 cells. After suramin was used to block the binding of extracellular adenosine triphosphate (ATP) to the membrane P2 receptor, the calcium oscillation in the three types of bone cells with or without physical connections was significantly suppressed as a single responsive peak under unidirectional flow. For the ATP-blocking group of low-density cells under oscillatory flow, the number of oscillation peaks in three types of cells was still more than two. It indicates that besides the ATP pathway, other mechanosensitive calcium pathways may exist under oscillatory flow. The present study provided further evidence for the osteogenic stage-dependent calcium response of bone cells under unidirectional or oscillatory fluid flow.

Introduction
Osteogenesis is the process of bone tissue formation and is initiated by osteoblasts derived from mesenchymal stem cells. Active osteoblasts synthesize the organic matrix of bone, including collagen, osteocalcin, and osteopontin, and produce calcium and phosphate to precipitate the mineral hydroxyapatite. Mature osteoblasts are gradually embedded into the mineralized matrix and finally differentiate into osteocytes. At different osteogenic stages, the bone cells exhibit different phenotypes, such as expression of osteogenetic markers and cellular morphology. Active preosteoblasts on the bone surface display plump or cuboidal morphology and can express peristin. The postosteoblasts or preosteocytes embedded in the osteoid have long processes with dendritic projections and contain abundant alkaline phosphatase (ALP). The mature osteocytes in the mineralized matrix express osteocalcin and are in contact with the neighboring osteocytes through the dendritic process. In vitro studies on bone cells usually involve representative cell lines, such as preosteoblast MC3T3-E1,2 preosteocyte MLO-A5,1 and mature osteocyte MLO-Y4.3,4 Mechanical stimulation enhances the osteogenic differentiation of bone cells.5–8 As an adaptive system, the bone incessantly remodels its structure in response to external chemical and physical stimuli,9 and fluid flow is regarded as an essential mechanical stimulant for bone cells.10 Three types of fluid flow are typically used in in vitro experiments of osteoblasts and osteocytes, that is, steady, pulsating, and oscillatory fluid flow.11–13 However, a systematic study involving the above mentioned three cell lines at different osteogenic stages...
under unidirectional and oscillatory fluid flow has not yet been conducted.

Calcium is an important second messenger within a cell. The fluctuation of intracellular calcium concentration ([Ca^{2+}]) is usually called calcium response, which plays a key role in osteogenesis.14–16 Previous studies found that fluid flow could induce calcium response and intercellular calcium transfer in bone cells.17–22 The two possible pathways responsible for intercellular calcium transfer are gap junction and adenosine triphosphate (ATP). Osteoblasts on the bone surface form a cell monolayer connected with gap junctions, whereas osteocytes in the dispersed lacunae build a cell network with their numerous dendritic and long processes connected by gap junctions.24–26 Gap junctions primarily regulate the mechanical stimulation-induced intercellular calcium transfer.27 ATP molecules are released from mechanically stimulated osteoblasts or osteocytes,27,28 diffused in the extracellular solution, and bound with the P2 receptor of the neighboring cells to activate the intracellular calcium response.29 Some studies showed that the intercellular calcium transfer through gap junction only appears in osteoblasts of long-term culture of 1–4 months, and the ATP pathway dominates the transient calcium response in osteoblasts or osteocytes.31,32

In our previous studies, we used microcontact printing to establish cell networks of MC3T3-E1,22,34,35 or MLO-Y4,36,37 with controlled spacing and functional intercellular gap junctions. The chemical reagent 18α-GA was used to block the intercellular calcium transfer through the gap junction. However, some researchers demonstrated that 18α-GA interferes with the regular release of ATP from the cytoplasm to the pericellular environment through hemichannels.39 We further established a micropatterned cell network without intercellular connection.39 But it is still difficult to avoid abnormal effects of the micropatterned substrate on the biological behavior of cells. Therefore, we investigated the effect of gap junctions on intercellular calcium transfer by freely seeding the cells at high and low density. We determined in our previous study that the mature osteocyte MLO-Y4 network is more sensitive and dynamic than the preosteoblast MC3T3-E1 network, particularly under low-level mechanical stimulations.36 However, the mechanisms underlying the way MLO-A5 cells, which represent postosteoblasts or preosteocytes, respond to mechanical stimulations remain unknown.

In the present study, we established cell networks with or without physical gap junctions by controlling the growth density of the three types of cells, namely, MC3T3-E1, MLO-A5, and MLO-Y4, which are at different osteogenic stages. We examined how these bone cells responded to unidirectional and oscillatory fluid flow and whether intercellular calcium transfer for the cell network occurred without physical connections. We also investigated whether blocking the ATP pathway suppressed calcium oscillation completely.
A 20 min resting period was necessary to allow the cells to recover to a stable state for repetitive calcium responses. The calcium response of the cells under unidirectional or oscillatory flow stimulation was recorded with a high-speed charge-coupled device camera (ANDOR, UK) for 10 min, i.e., 1 min for baseline followed by 9 min for flow stimulation. The peristaltic pump was connected to the chamber to run the fresh working medium, including the culture medium or blocking reagent, through the chamber with a desired flow rate to maintain the FSS level. The fluorescence intensity of each cell was subtracted from the background values and normalized by their corresponding baseline. Suramin (MedChemExpress, USA) was used to block the binding of ATP with a membrane P2 purinergic receptor to investigate the roles of the ATP pathway in calcium response and propagation. In this study, 100 μM suramin in the culture medium was applied to the cells 30 min before exposure to fluid flow. More than 50 cells from at least three slides for each group were analyzed.

Data analysis

The percentage of responsive cells in each group was evaluated. A cell was considered responsive to flow stimulation if it successfully released a calcium spike with a magnitude four times higher than its fluctuations during baseline measurement. Therefore, the subsequent calcium response conforming to this definition was regarded as a responsive peak although it might be weaker than the first peak. The number of [Ca^{2+}] peaks during the stimulation period was counted for all responsive cells and is
shown in Figs. 5 and 6. Several parameters of responsive peaks were defined [Fig. 1(c)] to quantitatively evaluate the properties of flow-induced calcium response. The magnitude of the first \( [\text{Ca}^{2+}]_i \) peak was denoted as \( m_1 \). The time \( t_1 \) from the onset of fluid flow to the maximum value of the first responsive spike was determined to evaluate the cells’ speed of response to mechanical stimulation. Relaxation time \( t_2 \) is the time for the first peak dropping 50% of its magnitude. \( t_3 \), \( t_4 \), \( t_5 \), \( t_6 \), \( t_7 \), and \( t_8 \) are the time intervals between the successive peaks.

One-way analysis of variance was used to determine the significant difference of each parameter between various types of cell lines, between high- and low-density groups, between control and suramin-treated groups, or between unidirectional and oscillatory fluid flow. Data are shown as mean ± standard deviation (SD). The mean values between different groups were significantly different at \( p < 0.05 \). The condition of equal population variances of groups was tested by Bonferroni’s post hoc analysis.

RESULTS

Unidirectional flow induces strong calcium oscillation in cells during late osteogenesis

The pseudocolor fluorescent images of MC3T3-E1, MLO-A5, and MLO-Y4 cells with high or low density after unidirectional fluid flow stimulation are shown in Fig. 3(a). The mean intensity of each cell in the time-lapsed images relative to the corresponding baseline value before fluid flow was calculated to indicate the change of \( [\text{Ca}^{2+}]_i \). Some typical \( [\text{Ca}^{2+}]_i \) traces of unidirectional fluid flow are shown in Figs. 3(b)–3(e). The untreated MLO-Y4 or MLO-A5 cells revealed repetitive, spikelike \( [\text{Ca}^{2+}]_i \) peaks, and some cells responded more than 10 times during the 9 min flow stimulation [Figs. 3(b) and 3(c)]. In addition, the \( [\text{Ca}^{2+}]_i \) peaks of MLO-Y4 or MLO-A5 cells appeared had a sharp profile. Most of the MC3T3-E1 cells showed a relatively strong peak at the initial stage of fluid flow followed by a few weak peaks.

Under unidirectional flow stimulation, MC3T3-E1, MLO-A5, and MLO-Y4 cells in untreated groups with high density had the responsive percentages of 45%, 69%, and 99%, respectively [Fig. 5(a)]. The average numbers of \( [\text{Ca}^{2+}]_i \) peaks were 1.6, 2.8, and 4.5, respectively [Fig. 5(c)]. The mean values of the two above-mentioned parameters were significantly different for the three types of bone cells, and the cells at the late osteogenic stage had a higher responsive percentage and stronger calcium oscillation than those of the cells at the early osteogenic stage. The parameters of the first peak reflected the sensitivity of a cell to fluid flow stimulation. The average values of \( t_1 \) for MLO-A5 and MLO-Y4 cells were 23 and 12 s, which were significantly shorter than the 59 s of MC3T3-E1 cells [Fig. 6(a)]. Thus, the bone cells at the late stage of osteogenesis are sensitive to fluid stimulation. \( t_2 \) and \( m_1 \) of the first peak did not differ significantly for the three types of bone cells. Thus, the responsive level of cytosolic calcium or its recovery ability is similar for bone cells during osteogenesis.
FIG. 3. Effects of cell density and ATP pathway on the flow-induced calcium response in bone cells under unidirectional flow. (a) Time-lapsed pseudo-color fluorescent images and (b)–(e) typical calcium responsive curves of MC3T3-E1, MLO-A5, and MLO-Y4 cells with different cell density and with suramin treatment or not. The digits on the cells indicate the number of calcium responsive peaks during the 9 min of flow stimulation. Scale bar, 50 μm.
Oscillatory flow produces calcium oscillation levels in bone cells higher than that produced by unidirectional flow

The pseudocolor fluorescent images of MC3T3-E1, MLO-A5, and MLO-Y4 cells with high or low density after oscillatory flow stimulation are shown in Fig. 4(a). Figures 4(b)–4(e) show the typical \([\text{Ca}^{2+}]\) traces of MC3T3-E1, MLO-A5, and MLO-Y4 cells under oscillatory flow. Similar to the cells under unidirectional flow, the untreated MLO-Y4 or MLO-A5 cells revealed repetitive, spike-like \([\text{Ca}^{2+}]\) peaks during the 9 min flow stimulation [Figs. 4(a) and 4(b)]. However, the untreated MC3T3-E1 cells mostly exhibited two to three spike-like \([\text{Ca}^{2+}]\) peaks compared with the unidirectional flow groups and significantly less than those of MLO-A5 and MLO-Y4 cells.

Under oscillatory flow stimulation, the responsive percentages of MC3T3-E1, MLO-A5, and MLO-Y4 cells in untreated groups with high density were 92%, 97%, and 100%, respectively [Fig. 5(a)], and the average numbers of \([\text{Ca}^{2+}]\) peaks were 2.2, 2.8, and 5.6, respectively [Fig. 5(c)]. Compared with the unidirectional flow, the responsive percentages of MC3T3-E1 and MLO-A5 cells were significantly increased in oscillatory flow and almost close to those of the MLO-Y4 cells. In addition, the numbers of \([\text{Ca}^{2+}]\) peaks of MLO-Y4 cells under oscillatory flow were significantly higher than those under unidirectional flow, whereas MLO-A5 and MC3T3-E1 cells did not significantly differ under both types of fluid flow. Thus, oscillating flow improves flow-induced calcium response in bone cells and promotes flow-induced calcium oscillation in the cells during late osteogenesis compared with unidirectional flow.

Similar to the unidirectional flow group, the average value of \(t_1\) for MLO-Y4 cells was 11 s, which was significantly shorter than 19 s for MC3T3-E1 cells and 24 s for MLO-Y5 under oscillatory flow [Fig. 6(a)]. In addition, the average value of \(t_1\) for MC3T3-E1 cells under oscillatory fluid flow significantly decreased from 59 s to 19 s, thereby indicating that the MC3T3-E1 cells are more sensitive than unidirectional flow under oscillatory fluid flow. The \(t_2\) of MLO-Y4 cells under oscillatory flow was shorter than those of MC3T3-E1 and MLO-A5 cells and significantly lower than that of MLO-A5 cells [Fig. 6(c)]. Moreover, \(m_1\) of the first peak of MLO-Y4 cells was significantly greater than those of MC3T3-E1 and MLO-A5 cells [Fig. 6(e)]. The responsive level of cytosolic calcium or its recovery ability significantly increased along with osteogenesis under oscillatory flow compared with unidirectional flow.

Loss of physical cell–cell connection reduces calcium oscillation under flow stimulations

The bone cells were cultured with low density, and cells far from each other and without visible cell–cell connections were analyzed to clarify the mechanism underlying flow-induced calcium oscillation. Under unidirectional flow stimulation, approximately 80% of MC3T3-E1, MLO-A5, or MLO-Y4 cells with low density were responsive to fluid flow [Fig. 5(b)]. Compared with the cells with high density, MC3T3-E1 cells with low density had a significantly high response percentage. In addition, calcium oscillation occurred even without intercellular connections [Fig. 5(b)]. The average numbers of \([\text{Ca}^{2+}]\), peaks of responding cells were significantly different for three types of bone cells, and their values were 1.6, 1.8, and 3.1, respectively [Fig. 5(d)]. The average numbers of \([\text{Ca}^{2+}]\), peaks of MLO-A5 or MLO-Y4 cells were significantly decreased compared with those of high-density groups. The \(t_1\) and \(m_1\) of the first peaks were not significantly different among the three types of bone cells [Figs. 6(b) and 6(f)], but \(t_2\) of MC3T3-E1 was significantly larger than the other two types of cells [Fig. 6(d)]. This result suggests that the time needed by pre-osteoblasts to recover their baseline level of \([\text{Ca}^{2+}]\) is longer than that needed by the bone cells at the late stage of osteogenesis at low density.

Under oscillatory flow stimulation for MC3T3-E1, MLO-A5, or MLO-Y4 cells with low density, their response percentages were 83%, 88%, and 97%, respectively [Fig. 5(b)], and the average numbers of \([\text{Ca}^{2+}]\), peaks were 2.1, 1.8, and 4.4, respectively [Fig. 5(d)]. The responsive percentage of MLO-Y4 cells was significantly higher than that of MLO-A5 cells. Moreover, the average numbers of \([\text{Ca}^{2+}]\), peaks of MLO-Y4 cells were significantly greater than MC3T3-E1 and MLO-A5 cells. In addition, compared with high-density groups, the average numbers of \([\text{Ca}^{2+}]\), peaks of MLO-A5 and MLO-Y4 cells significantly decreased. The \(t_1\) was not significantly different for the three types of bone cells [Fig. 6(b)], but \(t_2\) of MLO-A5 was significantly larger than that of the two other types of cells [Fig. 6(d)]. The \(m_1\) of the first peaks of MLO-Y4 cells was significantly greater than that of MLO-A5 cells [Fig. 6(f)]. Compared with unidirectional flow, the \(t_2\) of MLO-A5 and MLO-Y4 cells under oscillatory flow significantly increased. Thus, the time needed by bone cells under oscillatory flow to recover their baseline level of \([\text{Ca}^{2+}]\) is longer than that of bone cells under unidirectional flow at low density.

ATP pathway regulates flow-induced calcium oscillation

The ATP pathway is another mechanism that regulates flow-induced calcium oscillation. When suramin inhibited the binding of ATP with the P2 receptor, only one \([\text{Ca}^{2+}]\) peak was observed in the cells with or without physical connections under unidirectional flow [Figs. 3(d) and 3(e)]. The responsive percentage ranged from 48% to 77%, but no significant difference was detected for different types of bone cells and different cell densities [Figs. 5(a) and 5(b)]. Compared with the untreated groups, inhibition of the ATP pathway significantly influenced the responsiveness of only MLO-Y4 cells with high density and MC3T3-E1 cells with low density. In addition, ATP blocking significantly decreased the magnitude of the first responsive peak for bone cells during the late osteogenic stage, that is, MLO-A5 and MLO-Y4 with high density or MLO-Y4 with low density [Figs. 6(e) and 6(f)]. The \(t_1\) of MLO-Y4 cells was significantly shorter than that of MLO-A5 cells with high density [Fig. 6(a)]. The \(t_2\) of MLO-Y4 cells was significantly shorter than that of MLO-A5 cells regardless of cell density [Figs. 6(c) and 6(d)] and was shorter than that of MC3T3-E1 cells with high density [Fig. 6(c)]. The ATP pathway mainly regulated calcium oscillation but did not influence the first responsive peak.

Under oscillatory flow stimulation, when suramin inhibited the binding of ATP with the P2 receptor, more than two \([\text{Ca}^{2+}]\),
FIG. 4. Effects of cell density and ATP pathway on the flow-induced calcium response in bone cells under oscillatory flow. (a) Time-lapsed pseudocolor fluorescent images and (b)–(e) typical calcium responsive curves of MC3T3-E1, MLO-A5, and MLO-Y4 cells with different cell density and with suramin treatment or not. The digits on the cells indicate the number of calcium responsive peaks during the 9 min flow stimulation. Scale bar, 50 μm.
peaks were observed in the cells with or without physical connections [Figs. 4(d) and 4(e)]. The responsive percentage of the blocking groups ranged from 90% to 100%, and the difference among bone cells or cell density groups was not significant [Figs. 5(a) and 5(b)]. However, compared with the unidirectional flow, the responsive percentage of MLO-A5 or MLO-Y4 cells with high density and MC3T3-E1 cells with low density under oscillatory flow was significantly high. The average numbers of $[\text{Ca}^{2+}]_i$ peaks of blocking groups were 2.1, 1.8, and 3.6 for the cells with high density, respectively, and 2.0, 1.5, and 3 for those with low density [Figs. 5(c) and 5(d)]. In comparison with the untreated groups, the MLO-A5 and MLO-Y4 cells in high-density groups or MLO-Y4 cells in low-density groups express long dendritic processes and produce small amounts of alkaline phosphatase (ALP) and type I collagen, large amounts of osteocalcin, and very large amounts of connexin43, a gap junction protein. MLO-A5 was obtained from a long mouse bone and it expresses ALP, type I collagen, parathyroid hormone/parathyroid hormone-related peptide (PTH/PTHrP), bone sialoprotein (BSP), and osteocalcin. MLO-A5 also expressed periostin, the osteoblast-specific factor 2, suggesting that these cells still possess osteoblast properties. In contrast to osteoblast progenitor cells, mineralization by MLO-A5 was rapid. MLO-A5 most likely represents a much later stage of differentiation, that of postosteoblasts, preosteocyte capable of triggering mineralization of osteoid. The present work was the first to study the mechanically stimulated calcium response in MLO-A5 cells and to compare it with that of

**DISCUSSION**

The cytosolic calcium response of the three cell lines, namely, MC3T3-E1 (osteoblasts), MLO-A5 (preosteocytes), and MLO-Y4 (osteocytes) cells, at different osteogenic stages under the stimulation of unidirectional or oscillatory fluid flow was systematically investigated and compared. MC3T3-E1 was established from normal mouse calvarias, in which the osteoblast-specific factor 2 (also called as periostin) is expressed. The cell line MLO-Y4 has the characteristics of mature osteocytes and is established from a long mouse bone. Compared with osteoblasts, MLO-Y4 cells express long dendritic processes and produce small amounts of alkaline phosphatase (ALP) and type I collagen, large amounts of osteocalcin, and very large amounts of connexin43, a gap junction protein. MLO-A5 was obtained from a long mouse bone and it expresses ALP, type I collagen, parathyroid hormone/parathyroid hormone-related peptide (PTH/PTHrP), bone sialoprotein (BSP), and osteocalcin. MLO-A5 also expressed periostin, the osteoblast-specific factor 2, suggesting that these cells still possess osteoblast properties. In contrast to osteoblast progenitor cells, mineralization by MLO-A5 was rapid. MLO-A5 most likely represents a much later stage of differentiation, that of postosteoblasts, preosteocyte capable of triggering mineralization of osteoid. The present work was the first to study the mechanically stimulated calcium response in MLO-A5 cells and to compare it with that of...
osteoblasts and osteocytes. The bone cells had a higher responsive percentage and stronger calcium oscillation during the late osteogenic stage than during the early osteogenic stage regardless of the type of flow stimulation. This result is consistent with our previous results about osteoblasts and osteocytes. Therefore, MLO-A5 cells embedded in the osteoid could enhance their ability to sense mechanical stimulation compared with MC3T3-E1. When the cells were completely trapped within the mineral matrix and finally become mature osteocytes (MLO-Y4), they became mostly sensitive to mechanical stimulation within the bone.

The study results demonstrated that the responsive percentages of MC3T3-E1 and MLO-A5 and the number of [Ca\textsuperscript{2+}], peaks of MLO-Y4 cells under oscillatory flow close to the in vivo physiological environment around cells were significantly higher than those under unidirectional flow. Weinbaum’s theoretical model predicted that the intrinsic sensitivity of bone cells decreases with increasing frequency, because the rapid switching of flow direction reduces or counteracts the effect of increasing FSS. A previous study showed that the in vitro oscillatory flow was far less stimulating on osteoblasts than the unidirectional flow at 0.5–2 Hz and 2 Pa, thereby
decreasing responsiveness with increasing frequency.\textsuperscript{13} The oscillatory flow with the low frequency of 0.1–0.8 Hz was more stimulating than steady flow, and responsiveness decreased with increasing frequency from 0.2 Hz to 0.8 Hz.\textsuperscript{42} In the present study, we adopted the relatively low frequency of 0.25 Hz of fluid flow and did not investigate the effect of oscillating frequency on calcium response.

We established a cell monolayer with or without a gap junction by controlling cell density to study the role of intercellular signaling pathways in bone cells at different stages of osteogenesis. The loss of gap junction reduced the number of $[\text{Ca}^{2+}]_i$ peaks and the responsive ability of the cells, although no effect was observed on the first peak. In addition, the effect of oscillatory flow on calcium response in the cells was higher than that of unidirectional flow. In contrast to the osteogenic stage-dependent responsive percentage of the cells with high density under unidirectional flow, the three types of bone cells with low density had similar responsive percentages, i.e., approximately 80\% [Fig. 5(b)]. MC3T3-E1 cells with low density were significantly more responsive than those with high density. Our previous numerical simulation showed that the hydrodynamic parameters, such as FSS or strain rate, around the micropatterned cells were obviously higher than those on a blank substrate because of the direct exposure of micropatterned cells to fluid flow.\textsuperscript{13} Considering the low height of the high-density cells compared with low-density cells, the enhanced FSS for low-density groups may activate more MC3T3-E1 cells than the high-density groups. In our previous studies,\textsuperscript{22,34–37} we seeded the cells on micropatterned islands with 300–400 $\mu$m$^2$ area, which was smaller than approximately 1000 $\mu$m$^2$ for the osteoblasts or osteocytes cultured on a blank surface. These micropatterned bone cells with constrained spreading areas revealed an FSS dose-dependent phenomenon, and nearly all of the MC3T3-E1 or MLO-Y4 cells responded to 2 Pa FSS.\textsuperscript{36}

Regardless of cell density and cell types in unidirectional flow, the numbers of $[\text{Ca}^{2+}]_i$ peaks decreased from two to five in control groups to only one in the ATP-blocking groups [Figs. 5(c) and 5(d)]. Thus, the ATP pathway was critical to calcium oscillation. This result was consistent with our previous studies on micropatterned MC3T3-E1 or MLO-Y4 cell networks, in which the blocked ATP pathway significantly reduced the flow-induced calcium oscillation into a single peak.\textsuperscript{24,25} The calcium oscillation of MLO-A5 and MLO-Y4 cells with low density was significantly lower than those with high density [Figs. 5(c) and 5(d)], which may have been caused by the long diffusion distance for ATP in low-density groups. FSS could induce ATP efflux from the cytosol to the pericellular environment.\textsuperscript{44,45} Although FSS around the cells without physical connection may be higher than the cell monolayer at high density, ATP molecules released from the mechanically stimulated cells diffused across a long distance to bind to P2 receptors of the neighboring cells and further activated their intracellular calcium response. Given that calcium oscillation in cells with physical cell-cell connections at high density was not observed after blocking the ATP pathway, the $[\text{Ca}^{2+}]_i$ transfer through the gap junction among cells may not contribute to the subsequent response peaks after the first peak (Fig. 7).

Oscillatory flow might significantly stimulate the cells with high or low density in ATP-blocking groups compared with unidirectional flow. Under oscillatory flow, the number of $[\text{Ca}^{2+}]_i$ oscillation peaks was two in the ATP-blocked cells during the early and intermediate osteogenesis and three in the cells during the late osteogenesis, regardless of cell density (Fig. 7). However, only one peak in the...
ATP-blocking group was observed under unidirectional flow. Considering that the first \([\text{Ca}^{2+}]\) oscillation peak in cells is usually produced by calcium outflow from the intracellular calcium pool, the oscillatory flow might activate calcium response in bone cells through other mechanosensitive channels. A previous study found that blocking the stretch-activated membrane ion channel with gadolinium chloride did not influence the calcium response in MC3T3-E1 cells under 1 Hz oscillatory fluid flow, whereas the L-type voltage-operated calcium channel did.\(^6\) Another study found that the tension-activated sensitive channel, that is, transient receptor potential cation channel, subfamily M, and member 7, played an important role in calcium response in osteoblasts under 0.2 Hz oscillatory flow.\(^5\) Therefore, multiple mechanotransduction pathways in bone cells might exist, and these pathways may have complicated regulatory processes for calcium response in bone cells in response to the magnitude, frequency, and type of flow stimulation.

In conclusion, the flow-induced calcium response in bone cells depends on osteogenic stages, i.e., calcium oscillation in the cells during late osteogenesis is evidently stronger than the cells at early osteogenesis. Low-frequency oscillatory flow leads to calcium oscillation in bone cells at different osteogenic stages that is stronger than that during unidirectional flow. In addition, the ATP pathway dominantly regulates the flow-induced calcium oscillation in bone cells with or without physical cell–cell connections. For low-frequency oscillatory flow, other mechanosensitive ion channels might be responsible for calcium response or oscillation. These results may provide further insights into the mechanism underlying the mechanical regulation of bone remodeling.

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