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Chapter 12

FLUID FLOW INDUCED CALCIUM RESPONSE IN BONE CELL NETWORK*

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In vitro bone cell networks with controlled pattern and intercellular gap junctions were successfully established by using microcontact printing and self assembled monolayers technologies. The intracellular calcium responses of bone cell networks exposed to a steady fluid flow and the underlying signaling pathways were further investigated. The cell network samples were separated into eight groups for treatment with specific pharmacological agents that inhibit pathways significant in bone cell calcium signaling. The calcium transients of the network were recorded and quantitatively evaluated with a set of network parameters. The results showed that gap junction blocker, extracellular ATP pathway inhibitor, and thapsigargin (depleting intracellular calcium stores) significantly reduced the occurrence of multiple calcium peaks, which were visually obvious in the untreated group. The number of responsive peaks also decreased slightly yet significantly when either the COX-2/PGE_2 or the NOS/nitric oxide pathway was disrupted. In the absence of calcium in the culture medium, the intracellular calcium concentration decreased slowly with fluid flow without any calcium transients observed. These findings have identified important factors in the flow mediated calcium signaling of bone cells within an *in vitro* network.

1. Introduction

Osteocytes are interconnected through numerous intercellular processes, forming extensive cell networks throughout the bone tissue [1-3]. It has been shown that osteocyte density is an important physiological parameter, which decreases with age and microdamage accumulation [4]. However, most previous studies on mechanotransduction in osteocytes and osteoblasts were performed on confluent or sub-confluent uncontrolled monolayers of cells [5, 6]. In order to examine the roles of the cellular connectivity between bone cells in

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mechanotransduction, a controlled bone cell network is necessary for quantitative studies. In our previous study [7], a two-dimensional patterned bone cell network was successfully established to mimic the *in vivo* osteocyte network by using microcontact printing and self assembled monolayers (SAM) technologies. Each individual bone cell in the network was connected with four neighboring cells via functional gap junctions. Gap junctions are membrane-spanning channels, where each pair of connexons (*i.e.* hemichannels) forms a cylinder with a pore in the center through which small molecules (<1 kDa) can pass from one cell to another cell [8]. It is widely accepted that this intercellular connection plays a significant role in coordinating bone cell network activities.

Calcium signaling is essential for numerous bone cell functions, such as proliferation and differentiation [9-13]. It has been demonstrated that mechanical stimuli such as fluid flow can induce a robust intracellular calcium ($[Ca^{2+}]_i$) response in bone cells [14, 15]. The fluid flow induced elevation of cytosolic calcium comes mainly from two sources: intracellular stores (*e.g.*, endoplasmic reticulum, ER) and the extracellular environment [16, 17]. $[Ca^{2+}]_i$ signaling is mediated by various molecular pathways, *e.g.*, inositol trisphosphate (IP_3), adenosine 5'-triphosphate (ATP), prostaglandin E_2 (PGE_2), and nitric oxide (NO) (Fig. 1).

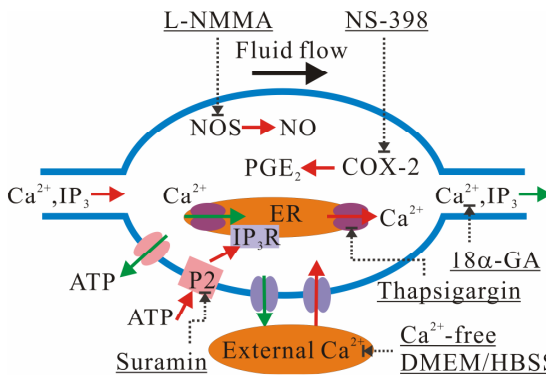

Figure 1. A schematic drawing of calcium signaling pathways in bone cells and corresponding inhibitors employed in the current study. Red arrow: influx or upregulation activity, green arrow: efflux from cytosol, dash arrow: inhibition or blocking with a specific inhibitory intervention noted and underlined.

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IP_3 can lead to a rapid release of calcium stored in the ER via binding to the ER membrane receptor [9, 11]. When a bone cell is under fluid flow induced shear stress, the activation of gap junction hemichannels (connexin 43) induces ATP efflux from the cytosol to the pericellular environment [18, 19]. Extracellular ATP can elicit a significant $[Ca^{2+}]_i$ response by binding with the purinergic membrane receptors [20]. Fluid shear stress can also prompt the induction of COX-2 protein and further PGE_2 release in MC3T3-E1 osteoblast-like cells [21]. Nitric oxide modulates $[Ca^{2+}]_i$ signaling via a cyclicguanosine monophosphate (cGMP) dependent pathway [22] or nitrosylation of proteins [23].

In the present study, *in vitro* bone cell networks with controlled pattern and functional intercellular gap junctions were built with MC3T3-E1 osteoblast-like bone cells. The cell networks were divided into 8 groups to test the effects of treatment with a battery of pharmacological agents to interrupt or inhibit $[Ca^{2+}]_i$ signaling. The spatiotemporal characteristics of the $[Ca^{2+}]_i$ responses were quantitatively characterized with a set of network parameters. The inter/intra cellular calcium signaling mechanisms and the roles of different molecular pathways in $[Ca^{2+}]_i$ signaling were further analyzed.

2. Materials and Methods

2.1. *Bone Cell Network*

Microcontact printing and SAM surface chemistry technologies were used to prepare the bone cell networks as described previously [7]. In brief, a chromium mask containing the desired square grid of connected circles was fabricated. The pattern was replicated to a master made of Shipley 1818 positive photoresist (MicroChem Corp, Newton, MA) by exposing it to UV light through the mask. The geometric parameters of the pattern adopted here were optimized for osteoblast-like cells [7]. A polydimethylsiloxane (PDMS, *Sylgard 184,* Dow Corning, Midland, MI) stamp with the desired micro-pattern was then generated by curing PDMS elastomer on the master. On the day before the fluid flow experiment, the stamp was coated with an adhesive SAM (octadecanethiol; Sigma-Aldrich Co., St. Louis, MO), and pressed onto a gold coated glass slide (150 Å Au using an e-beam evaporator, SC2000, SEMICORE Inc., Livermore, CA) for 60 seconds. The stamped glass slide was immediately immersed in a non-adhesive ethylene glycol terminated SAM solution (HS-C11-EG3; Prochimia, Sopot, Poland) for 3 hours to cover areas that were not patterned with the adhesive SAM. To facilitate cell adhesion, the patterned slide was

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further incubated in 10 μg/mL fibronectin (Sigma-Aldrich Co., St. Louis, MO) in PBS for one hour, which can only be absorbed by the adhesive SAM coated region. Osteoblast-like MC3T3-E1 cells with controlled density ($1.0x10^4$ cell/cm^2) were seeded on the patterned slide and cultured in α-MEM medium supplemented with 2% charcoal-stripped fetal bovine serum (CS-FBS, Hyclone Laboratories Inc., Logan, UT) for 24 hours before further testing. Figure 2 shows the snapshots of a typical bone cell network in the untreated group with a 70 μm separation distance.

2.2. *Steady Fluid Flow and $[Ca^{2+}]_i$ Response*

To indicate $[Ca^{2+}]_i$, patterned bone cells on the glass slide were loaded with 5 μM Fluo-4 AM (Molecular Probes, Eugene, OR) in α-MEM medium for 1 hour at 37°C. After calcium dye loading, the slide was mounted on a custom-built parallel plate flow chamber, which was further attached to an inverted fluorescence microscope (Olympus IX71, Melville, NY) with a 10X objective [16]. A magnetic gear pump was connected to the chamber to generate a steady fluid flow with a constant shear stress of 40 dyne/cm^2 on the cell surface. The $[Ca^{2+}]_i$ response of the bone cell network under fluid flow stimulation was recorded with a high-speed CCD camera (ORCA-ER-1394, Hamamatsu Photonics K.K., Hamamatsu City, Japan) for a total 10-minute period, one minute for baseline and 9 minutes after the onset of fluid flow. The intensity of $[Ca^{2+}]_i$ for each cell was later normalized by its corresponding baseline.

2.3. *Experimental Groups*

Besides the untreated group (12 slides, 130 cells analyzed), seven specific biochemical blocking agents were employed to identify the sources and the contributions of molecular pathways to the network characteristics of $[Ca^{2+}]_i$ response. *(1) Extracellular calcium depletion:* In this group, calcium-free Dulbecco's Modified Eagle Medium (DMEM, Invitrogen Corporation, Carlsbad, CA) and calcium-free Hank's Balanced Salt Solution (HBSS, Invitrogen Corporation) were introduced to replace the regular medium after Fluo-4 AM loading (8 slides, 99 cells analyzed). *(2) ER calcium store depletion*: After Fluo-4 staining, the cell-seeded slides (6 slides, 90 cells analyzed) were incubated in 1 μM Thapsigargin (TG) (Sigma-Aldrich Co.) medium for 30 min prior to flow [20]. TG depletes calcium from the ER store. *(3) PGE_2 blocking*: The patterned cells were pretreated with 10 μM NS-398 (EMD Chemicals Inc., San Diego, CA) for 24 hours before flow study. NS-398 selectively inhibits the COX-2 enzyme activity, which in turn blocks the PGE_2 release [21]. 123 cells

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on 11 slides were analyzed in this group. *(4) NO blocking*: In this group, 100 μM NG-monomethyl-L-arginine (L-NMMA; EMD Chemicals Inc.) was introduced into the cell culture medium one day before seeding the cells on slides and continuously presented in mediums afterwards. L-NMMA inhibits the production of nitric oxide via competitively inhibiting all three isoforms of nitric oxide synthase (NOS) [24]. In this group, 189 cells on 16 slides were analyzed. *(5) Gap junction blocking*: To study the role of intercellular gap junctions on calcium responses of the bone cell network, 75 μM 18α-Glycyrrhetinic acid (18α-GA, Sigma-Aldrich Co.), a reversible gap junction blocker which binds to membrane proteins and causes disassembly of gap junction plaques [25], was supplied in Fluo-4 AM loading medium and fluid flow medium [5]. In this group, 61 cells on 6 slides were studied. *(6) ATP blocking:* Suramin (EMD Chemicals Inc.), a P2 purinergic receptor blocker, was employed to investigate the effect of ATP release on the $[Ca^{2+}]_i$ response. 100 μM suramin was applied to the cells 30 minutes before exposing to shear flow [26]. 50 cells from 3 slides were analyzed. *(7) Vehicle DMSO control:* Among all the previously employed chemicals, NS-398, 18α-GA, and TG were dissolved into DMSO. Therefore vehicle control testing was performed with 0.3% v/v DMSO presented in medium (3 slides, 51 cells analyzed).

2.4. *Data analysis*

To quantitatively analyze the network characteristics of the $[Ca^{2+}]_i$ responses, a set of network parameters were defined from the time-lapse of $[Ca^{2+}]_i$ response (Fig. 2).

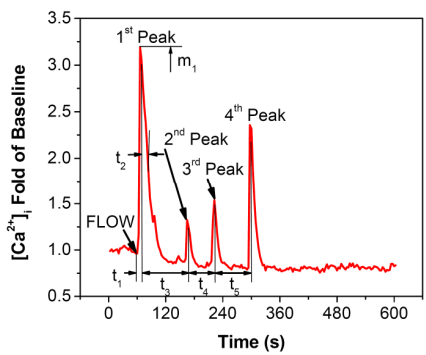

Figure 2. A typical calcium response of bone cells in an untreated network under applied steady fluid flow. The characteristic temporal parameters are also illustrated. t_1 denotes the time from flow onset to the maximum value of the first responsive peak; t_2 is the time from peak value to the 50% relaxation; t_3, t_4, and t_5 are the time intervals between different peaks. m_1 represents the normalized magnitude of the first peak.

For each individual cell, the total number of $[Ca^{2+}]_i$ responsive peaks during the flow stimulation period was counted. t_1 denoted the time between the onset point of fluid flow and the maximum value of the first responsive spike, while t_2 was the time from first peak to its 50% relaxation point. One-way analysis of variance (ANOVA) with Bonferroni's post hoc analysis was performed to determine statistical differences between mean values of different treatments, $p<0.05$. The condition of equal population variances of treatments was tested by Levene's method.

3. Results

A typical set of intracellular calcium images during the testing period are shown in Fig. 3, clearly demonstrating multiple responses of bone cells within the network under fluid flow. A set of typical $[Ca^{2+}]_i$ traces of individual cells from the untreated group and those with different treatments are shown in Fig. 4. In each group, $[Ca^{2+}]_i$ profiles of three cells from a single glass slide were plotted.

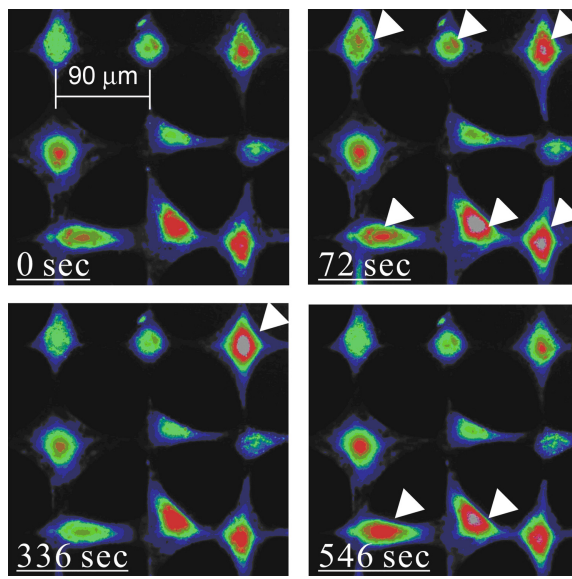

Figure 3. Pseudo-color images of typical intracellular calcium responses of a bone cell network from untreated group. Fluid flow was applied at 60 seconds. Arrowheads highlight those cells responding. Some cells were able to respond multiple times.

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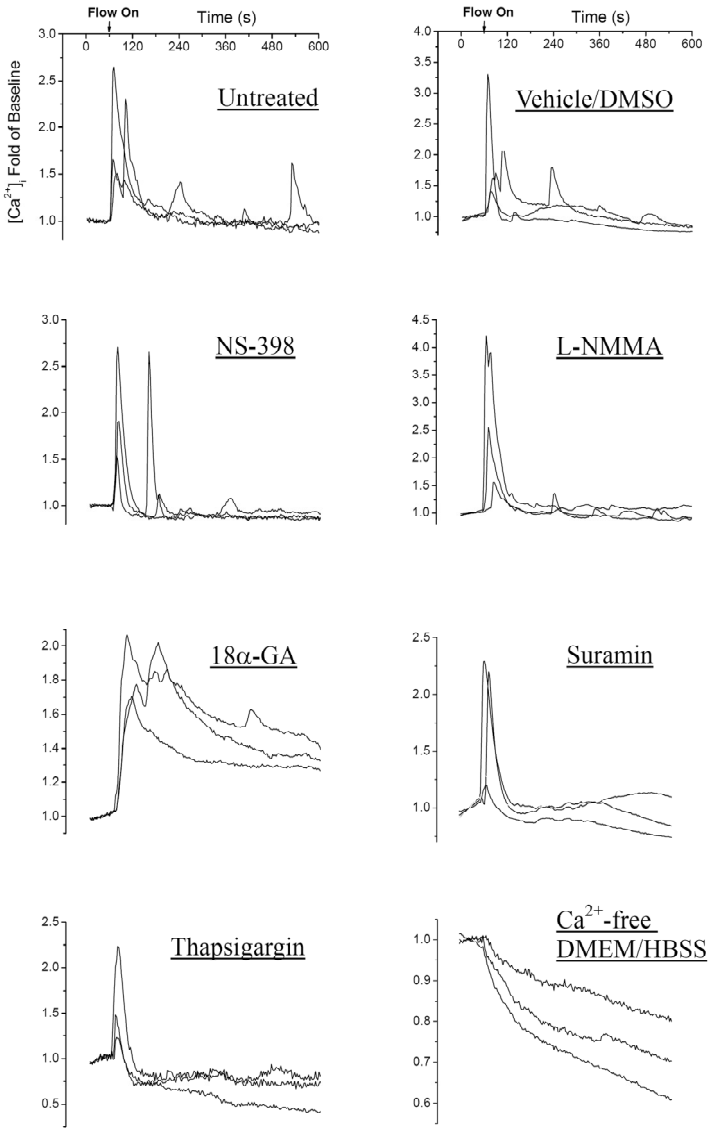

Figure 4. A set of typical intracellular calcium traces of individual cells with or without pharmacological blocker treatment. The calcium images were recorded for 10 minutes (1 minute for baseline and 9 minutes for flow stimulation). The intensity of intracellular calcium concentration shown here was normalized to the baseline.

For untreated, vehicle control, NS-398 (PGE_2 blocking), and L-NMMA (NO blocking) groups, multiple $[Ca^{2+}]_i$ responsive peaks were quite obvious even by visual inspection. However, 18α-GA (gap junction blocking) and TG (ER calcium depletion) groups tended to exhibit only a single $[Ca^{2+}]_i$ peak. In the absence of extracellular calcium, unlike the $[Ca^{2+}]_i$ response in any other group, $[Ca^{2+}]_i$ decreased slowly after the onset of fluid flow without any $[Ca^{2+}]_i$ peak.

To quantitatively and statistically analyze the calcium waves, the frequencies of multiple responding peaks are shown in Fig. 5a, while the mean value of the number of responding peaks are plotted in Fig. 5b.

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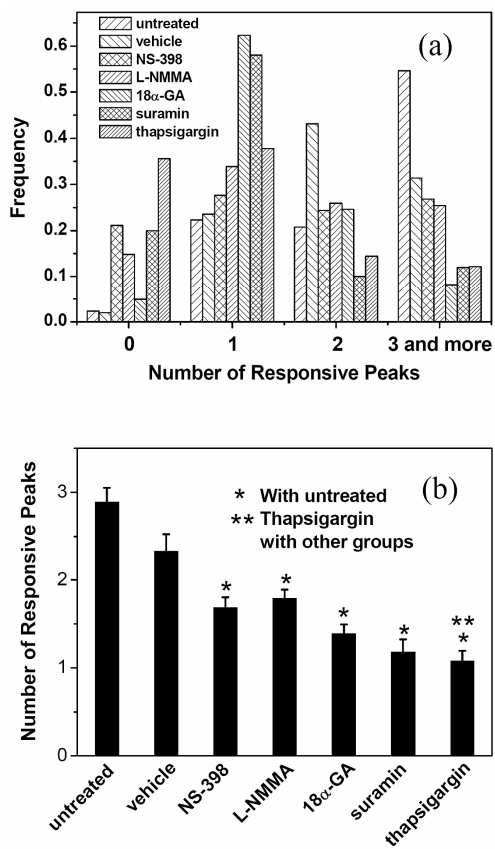

Figure 5. (a) The frequency distribution of the number of responsive peaks of cells in different groups and (b) the average of the number of responsive peaks (the error bars are standard errors of the means (SEM) while * and ** indicate statistical significance of $p<0.05$). The group of Ca^{2+} free medium had no response to fluid flow and was not included.

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In the untreated group, over 55% of cells were able to respond three or more times with an overall mean value as 2.89 ± 0.16 times of baseline. In the vehicle control group, this value was 2.33 ± 0.19 but no significant difference was detected when compared with the untreated group. Both NS-398 (PGE_2 blocking) and L-NMMA (NO blocking) resulted in fewer multiple responses, only 50% of cells exhibited two or more calcium transients. Furthermore, 15% and 22% of cells had no response to fluid flow in these two groups, respectively, which was significantly higher than those of the untreated or vehicle control group. 18α-GA (gap junction blocking) and suramin (ATP blocking) significantly reduced the number of calcium peaks, while cells with a single response comprised approximately 60% of the total population. ANOVA testing revealed no significant difference between the mean values of these two groups, but they were both significantly different from untreated and vehicle control groups. The TG-treated group (ER depleted) generated the least number of responding calcium peaks, which was significantly different from any other group. The removal of extracellular Ca^{2+} completely abolished the $[Ca^{2+}]_i$ response.

As shown in Fig. 6, cells in the gap junction blocked group took the longest time to reach the first $[Ca^{2+}]_i$ peak ($t_1 = 61 \pm 7$ seconds). Moreover, the relaxation time of this group was nearly 5 times that of all other groups ($t_2 = 222 \pm 23$ seconds). The untreated and TG groups shared a similar t_1 value (40 ± 4 *vs.* 34 ± 2 seconds, $P>0.05$). The vehicle control, NS-398, L-NMMA, and suramin groups all had significantly shorter first peak response times when compared with the unteated group, but there was no significant difference among these four groups. Aside from the 18α-GA group, all the other six groups had no significant difference in the 50% relaxation time.

4. Discussion

The network characteristics of $[Ca^{2+}]_i$ response of bone cells in a controlled cell network have been quantified, and several biochemical pathways related to $[Ca^{2+}]_i$ signaling have been examined. Notably, multiple $[Ca^{2+}]_i$ peaks, a signature of network circuits, have been observed in bone cell networks under applied steady fluid flow. Intracellular calcium signaling in bone cells involves both internal and external sources of calcium [9]. The internal calcium stores are

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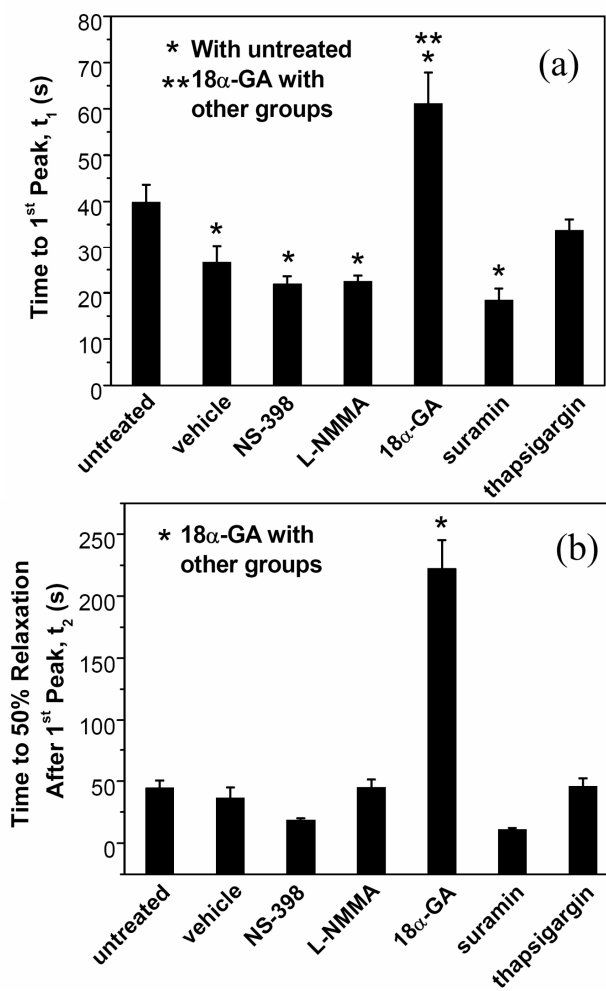

Figure 6. Network characteristic parameters of calcium response in the bone cell network under steady fluid flow from different treatment groups (the error bars are SEM while * and ** indicate statistical significance of $p<0.05$): (a) time to the first peak t_1 and (b) time to 50% relaxation after the first peak t_2. The group of Ca^{2+} free medium had no response to fluid flow and was not included.

mainly held within the membrane systems of the ER [27, 28]. Current experimental results from calcium-free media and ER depletion groups have identified the calcium source underlying the $[Ca^{2+}]_i$ responses in the bone cell networks. The cells in calcium-free media showed absolutely no $[Ca^{2+}]_i$ increase

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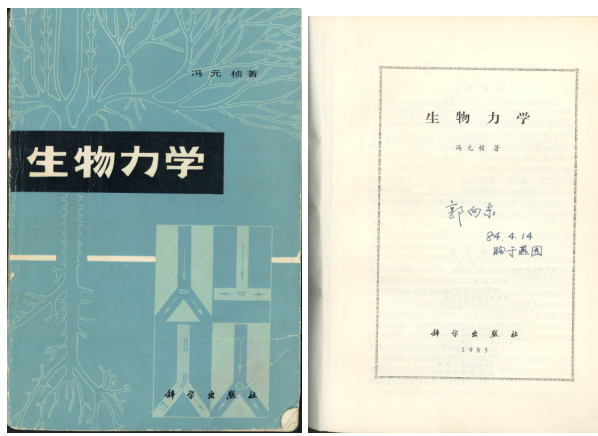
due to fluid flow, indicating the critical role of extracellular calcium. A few researchers have noted that the influx of extracellular calcium is required to evoke the opening of the calcium release channels in the ER [29, 30]. In fact, it has been shown that the gap junction dependent propagation of intercellular calcium signals in rat osteoblast cells requires influx of external calcium through L-type calcium channels [30]. Moreover, the cytosolic calcium concentration was significantly reduced in the absence of extracellular calcium, which was consistent with a previous report [20]. On the other hand when calcium was depleted from ER stores, more than 60% of cells exhibited a calcium peak under the stimulation of fluid flow, similar with those observed in randomly cultured 80-90% confluent MC3T3-E1 cells under an oscillatory fluid flow [31]. The magnitude of the first responsive peak was not significantly altered. This confirmed that extracellular calcium influx was responsible for the initial calcium peak. However, the average number of responsive $[Ca^{2+}]_i$ peaks in the cell network was significantly reduced to barely more than one. Thus ER stores appears to be the major calcium source responsible for the lower-magnitude $[Ca^{2+}]_i$ peaks following the initial calcium transient in the network of bone cells [29, 31].

The $[Ca^{2+}]_i$ responses of the networked bone cells consist of a large initial peak followed subsequently by multiple lower-magnitude peaks, which is similar to those observed in an 80-90% confluent bone cell-culture study [32]. In the untreated group, over 50% of the cells were able to generate three or more calcium peaks within 9 minutes. In the ER calcium depleted group, the ATP activation blocked group, and the gap junction blocked group, the average number of calcium peaks during 9 minutes was significantly reduced, and the number for the first two groups was very close to one (TG and suramin treated, respectively). The importance of ER calcium stores in the multiple responsive $[Ca^{2+}]_i$ peaks [29, 31] has been confirmed in the current networked bone cells. This new bone cell network study suggests the critical importance of the ATP pathway in mediating multiple $[Ca^{2+}]_i$ peaks of bone cells under steady fluid flow. This observation is consistent with literature where calcium wave propagation in bone cells induced by cell poking was influenced by the ATP pathway [30, 33, 34]. We also noted that 18α-GA (gap junction blocking) did not completely extinguish the multiple transients of bone cells, as 32% of cells responded more than once. This implied that a different pathway other than gap junction signal communication also plays a significant role in the multiple calcium peaks in the bone cell network. Indeed, inhibiting COX-2/PGE_2 or nitric oxide pathways also slightly reduced the multiple calcium peaks of individual

osteoblasts, which was similar to previous findings in other cell types [22, 23, 35, 36]. A previous study by Li et al. has shown that blocking the nitric oxide pathway can significantly reduce the refilling capability of intracellular calcium store [37], thereby decreasing the occurrence of multiple transients.

In summary, a thorough investigation of the spatiotemporal characteristics of $[Ca^{2+}]_i$ signaling of bone cells in a controlled cell network under steady fluid flow was conducted. The effects of several candidate pathways potentially involved in inter/intra cellular calcium signaling were also studied. We confirmed that the major source for the rapid initial calcium transient under mechanical stimulation was attributable to the influx of extracellular calcium. The ER calcium stores and ATP pathways are responsible for the subsequent multiple calcium peaks. The intercellular gap junctions in the cell network demonstrated a significant influence on the $[Ca^{2+}]_i$ response. Bone cells in a gap junction-blocked network exhibited a sustained high calcium concentration after the triggering from fluid flow. It was also shown that the PGE_2 and NO pathways were both involved to a lesser extent in the calcium signaling of the bone cell network.



The first Biomechanics book published in China is by Dr. Y.C. Fung and Science Press (China) in 1983, entitled “Biomechanics”. Dr. X. Edward Guo’s journey in biomechanics research started from reading this book in 1984 when Dr. Guo was a senior in Peking University. His story is not unique and many researchers currently in the field of biomedical engineering who grew up in China share the same experience.

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Dr. X. Edward Guo (left) met Dr. Y.C. Fung (center) for the first time at the Symposium of Inauguration of the Department of Biomedical Engineering at Columbia University in 2000. At right is another faculty member in the new Department of Biomedical Engineering at Columbia University, Dr. Clark T. Hung.

Dr. X. Edward Guo with Dr. Y.C. Fung at the Genomics on Biomechanics Symposium in celebration of Professor Y.C. Fung's 90th Birthday at UCSD.

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