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Osteoblasts and Osteocytes Respond Differently to Oscillatory and Unidirectional Fluid Flow Profiles

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Abstract Bone cells subjected to mechanical loading by fluid shear stress undergo significant architectural and biochemical changes. The models of shear stress used to analyze the effects of loading bone cells in vitro include both oscillatory and unidirectional fluid shear profiles. Although the fluid flow profile experienced by cells within bone is most likely oscillatory in nature, to date there have been few direct comparisons of how bone cells respond to these two fluid flow profiles. In this study we evaluated morphologic and biochemical responses to a time course of unidirectional and oscillatory fluid flow in two commonly used bone cell lines, MC3T3-E1 osteoblasts and MLO-Y4 osteocytes. We determined that stress fibers formed and aligned within osteoblasts after 1 h of unidirectional fluid flow, but this response was not observed until greater than 5 h of oscillatory fluid flow. Despite the delay in stress fiber formation, oscillatory and unidirectional fluid flow profiles elicited similar temporal effects on the induction of both cyclooxygenase-2 (Cox-2) and osteopontin protein expression in osteoblasts. Interestingly, MLO-Y4 osteocytes formed organized stress fibers after exposure to 24 h of unidirectional shear stress, while the number of dendritic processes per cell increased along with Cox-2 protein levels after 24 h of oscillatory shear stress. Despite these differences, both flow profiles significantly altered osteopontin levels in MLO-Y4 osteocytes. Together these results demonstrate that the profile of fluid shear can induce significantly different responses from osteoblasts and osteocytes. J. Cell. Biochem. 100: 794–807, 2007.

Key words: shear stress; stress fibers; morphology; cyclooxygenase-2; osteopontin

Mechanical loading of bone is a potent stimulus for new bone formation. At the cellular level, osteocytes have been described as the primary mechanosensors within bone [Cowin et al., 1991; Lanyon, 1993]. However, both osteoblasts and osteocytes are responsive to mechanical loading and both cell types are commonly used to study mechanically induced signaling in bone. In vivo mechanical loading of bone generates fluid shear stress which is thought to be dynamic in nature. Therefore, oscillatory fluid flow may be a more physiologic fluid flow profile than unidirectional fluid flow. To study mechanotransduction in bone cells both unidirectional (including steady and pulsatile) and oscillatory fluid flow profiles have been widely used. However, only a few studies have directly compared the differences in bone cell responses to variations in unidirectional fluid flow [McAllister and Frangos, 1999; Jiang and Cheng, 2001; Bacabac et al., 2004] and, to the best of our knowledge, Jacobs et al. [1998] have made the only direct comparison between oscillatory and unidirectional fluid flow profiles.

Subjecting osteoblasts and osteocytes to fluid flow has lead to many important advances in the field of mechanotransduction. One of the most well-characterized signaling pathways which is required for mechanically induced bone formation is the metabolism of prostaglandins [Forwood, 1996]. Specifically, it has been shown that both osteoblasts and osteocytes increase expression of cyclooxygenase-2 (Cox-2) and release of prostaglandin-E_2 (PGE_2) in response to either unidirectional or oscillatory fluid flow [Reich and Frangos, 1991; Klein-Nulend et al.,

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1995, 1997; Jiang and Cheng, 2001; Norvell et al., 2004]. However, not all fluid flow responses have been evaluated using both osteoblasts and osteocytes. For example, the actin cytoskeleton, which is hypothesized to be an important mechanosensory component [Ingber et al., 1994; Alenghat and Ingber, 2002; Juliano, 2002], and osteopontin, an abundant bone matrix protein involved in the mechanical regulation of matrix mineralization [Terai et al., 1999], have been evaluated in response to fluid flow primarily in osteoblasts. Fluid shear stress has been shown to regulate osteopontin mRNA levels in osteoblasts [You et al., 2001a; Batra et al., 2005]. Actin stress fiber formation in response to unidirectional fluid flow has been previously demonstrated in osteoblasts by our lab [Pavalko et al., 1998; Norvell et al., 2004]. However, neither osteopontin regulation nor actin reorganization in response to both oscillatory and unidirectional fluid flow has been evaluated in osteocytes.

In this study we completed a side-by-side comparison of the response of bone cells to unidirectional and oscillatory fluid flow profiles for 1, 5, and 24 h. We analyzed changes actin reorganization, cell morphology, and signal transduction in both MLO-Y4 osteocytes and MC3T3-E1 osteoblasts. Our results demonstrate that different types of bone cells respond differently to oscillatory and unidirectional fluid flow profiles. In MC3T3-E1 osteoblasts stress fiber organization in response to oscillatory fluid flow was delayed compared to unidirectional fluid flow. However, temporal induction of Cox-2 and osteopontin in osteoblasts was similar in response to both fluid shear profiles. In contrast, induction of Cox-2 protein, actin organization, and overall cell morphology was drastically different in MLO-Y4 osteocytes subjected to oscillatory fluid flow compared to unidirectional fluid flow. These findings provide an important comparison of the response of commonly used bone cell lines to different fluid shear profiles, thereby giving new insight into the process of mechanotransduction in bone cells.

METHODS

Cell Culture

MC3T3-E1 osteoblasts (subclone 14) were grown in α-MEM media purchased from Invitrogen Corporation (Carlsbad, CA) containing 10% fetal calf serum (FCS) from Atlantic Biologicals (Miami, FL). *MLO-Y4 osteocytes* were cultured on (5 μg/cm^2) collagen-coated dishes in α-MEM media supplemented with 5% FCS and 5% calf serum. Collagen was purchased from BD Biosciences (San Jose, CA) and calf serum was purchased from Sigma-Aldrich (St. Louis, MO). Human umbilical cord endothelial cells (HUVEC) were obtained from Clonetics (East Rutherford, NJ) and grown in endothelial growth media (EGM) (Clonetics). EGM contains 2% fetal bovine serum, bovine brain extract (12 μg/ml), human epidermal growth factor (1 μg/ml), hydrocortisone (1 μg/ml), getamicin, and amphotericin B.

For comparison of flow experiments all cell types were grown on glass slides in the presence of the appropriate serum for 48 h prior to the start of static or flow conditions. MC3T3-E1 osteoblasts and HUVECs were 95% confluent at the start of the flow experiment while MLO-Y4 osteocytes were only 80% confluent (MLO-Y4 osteocytes were kept subconfluent to maintain their normal phenotype as described in Bonewald et al. [Bonewald, 1999]. For experiments different serum starving conditions were determined for each cell type to maximize changes to the cytoskeleton induced by fluid flow while eliminating cell death due to serum deprivation. Prior to short flow experiments (1 and 5 h) MC3T3-E1 osteoblasts were serum starved in α-MEM containing 0.5% FCS for 12 h and then subjected to static or fluid flow in 30 ml of α-MEM containing 0.1% FCS. MC3T3-E1 osteoblasts subjected to 24 h of fluid shear stress under these low serum conditions resulted in significant cell death (data not shown). Therefore, for 24 h static and flow experiments MC3T3-E1 cells were not serum starved but placed in 30 ml of α-MEM containing 1.0% FCS at the start of flow or static conditions. For all experimental time points (1, 5, and 24 h) MLO-Y4 osteocytes and HUVECs were switched to 30 ml of α-MEM containing 1.0% FCS at the start of flow or static conditions.

Fluid Flow

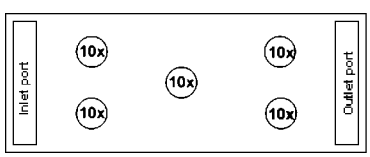
Unidirectional fluid flow was performed in parallel plate flow chambers at 37°C with the media subjected to a stream of 5% CO_2 using the flow loop system designed by Frangos et al. [1985] and marketed by Cytodyne (San Diego, CA). This system subjects the cells to unidirectional fluid flow at a shear stress of 8 dynes/cm^2.

Oscillatory fluid flow was performed in parallel plate flow chambers at 37°C using a PHD2000 series programmable syringe pump (Harvard Apparatus, Holliston, MA). Hard-walled tubing was used to connect the pump to the chamber inlet, and a reservoir was attached to the outlet to allow for movement of fluid and exchange of 5% CO_2. This system subjects the cells to oscillating fluid flow at a frequency of 0.5 Hz resulting in a peak shear stress of 11 dynes/cm^2 and the root mean square (RMS) value of 5.5 dynes/cm^2.

Static controls were held in cell culture dishes at 37°C with 5% CO_2.

Fluorescence Microscopy

After 1, 5, or 24 h of oscillatory or unidirectional fluid shear or static conditions, cells for fluorescence microscopy were fixed in 4% paraformaldehyde, permeabilized in 0.2% triton, and F-actin was visualized using rhodamine-phalloidin (Molecular Probes, Eugene, OR). Images were recorded using an Olympus digital camera attached to a Nikon Diaphot 200 inverted epifluorescent microscope using Plan 10× and PlanApo 60× Nikon objectives. Morphometric image analysis was performed on a Dell Dimension 4550 computer using the UTHSCSA *ImageTool* program (developed at the University of Texas Health Science Center at San Antonio, Texas and available from Internet). Five 10× fields were taken from controlled geographic locations on each of four slides per treatment condition. The cells,



including cellular processes, in these 20 fields were manually outlined, then the images were used for the analysis of long axis orientation and elongation using ImageTool software. Elongation was determined by dividing the long axis length by the short axis length. Cells with an elongation factor $\geq$2 were considered to be elongated. Long axis orientation was evaluated by measuring the long axis angle relative to the direction of fluid flow within a possible 180° surrounding the direction of flow. Cells with a long axis angle falling $\pm$30° from the direction of flow were considered to be orientated in the direction of flow.

Immunoblot Analysis and Antibodies

For immunoblot analysis, cells subjected to static, unidirectional, or oscillatory fluid flow conditions were harvested in SDS sample buffer and protein concentrations were determined using the amide black method [Sheffield et al., 1987]. Equal cellular protein (25 μg) was loaded onto SDS–PAGE gels for separation and transferred to nitrocellulose for Cox-2 and vinculin immunoblotting or to PVDF for osteopontin immunoblots. COX-2 polyclonal antibody was purchased from Cayman Chemical (Ann Arbor, MI), osteopontin polyclonal antibody from Santa Cruz Biotechnology (Santa Cruz, CA), and vinculin monoclonal antibody, VIN 11-5, was purchased from Sigma-Aldrich. The appropriate secondary antibodies were purchased from Jackson Immunoresearch (West Grove, PA) and the antibody signal was detected and analyzed using a Fuji chemiluminescence imager (Fuji, Edison, NJ) following reaction in a luminescence solution described by Norvell and Green [1998]. The experiments were carried out in triplicate.

Enzyme Immunometric Assays

For detection of released osteopontin 1 ml media samples were taken after 24 h of fluid flow or static treatment. The media samples were centrifuged at 14,000*g* for 1 min to pellet any particulates. The supernatants were transferred to a new tube and the levels of osteopontin present in samples were determined according to manufacturer's instructions using an enzyme immunoassay kit from Assay Designs, Inc. (Ann Arbor, MI).

Statistical Analysis

Statistical analysis was performed using the statistical package Statview©, version 5.0.1. Differences between static, unidirectional, and oscillatory flow were tested using Student's *t*-test for unpaired variants (*P*-values $\leq$0.05 were considered significant).

RESULTS

Stress Fiber Formation in Bone Cells Differs in Response to Oscillatory and Unidirectional Fluid Shear Profiles

To better evaluate the response of the actin cytoskeleton to mechanical loading we analyzed stress fiber formation in MC3T3-E1 osteoblasts

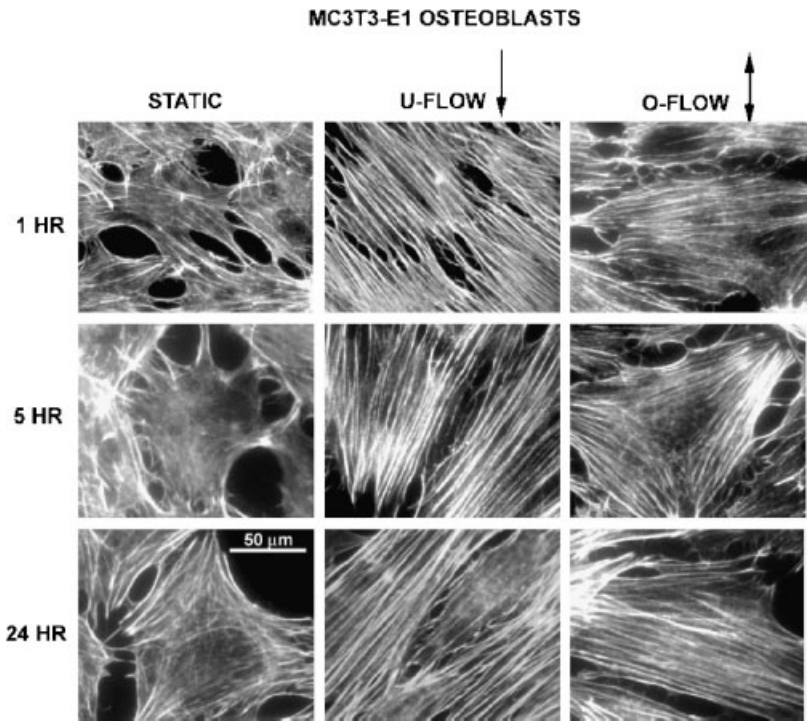


Fig. 1. Stress fiber organization is delayed in MC3T3-E1 osteoblasts subjected to oscillatory fluid flow compared to unidirectional fluid flow. MC3T3-E1 osteoblasts subjected to static or flow conditions for 1, 5, or 24 h were fixed and F-actin was visualized using rhodamine-phalloidin. Few stress fibers form in cells held in static culture. Unidirectional fluid flow induces organized stress fiber formation at all time points. Actin reorganizes into a few stress fibers in response to 1 and 5 h of oscillatory fluid flow. After 24 h of oscillatory fluid flow organized stress fibers form in MC3T3-E1 osteoblasts. Arrows indicate the direction of fluid flow relative to the images.

and MLO-Y4 osteocytes. As noted previously, MC3T3-E1 osteoblasts held in static culture had very few stress fibers (Fig. 1) [Pavalko et al., 1998]. In contrast, a dramatic reorganization of actin into aligned stress fibers occurred in osteoblasts subjected to unidirectional fluid flow for as little as 1 h and after 5 h aligned stress fibers continued to be present (Fig. 1). At these same time points, MC3T3-E1 osteoblasts that were subjected to oscillatory fluid flow formed fewer stress fibers which were not aligned within the cells (Fig. 1). However, after 24 h stress fibers formed and aligned similarly in response to both fluid flow profiles (Fig. 1).

MLO-Y4 osteocytes, in contrast to osteoblasts, did not show any noticeable change in actin organization when subjected to 1 or 5 h of either fluid flow profile compared to static culture (Fig. 2). Interestingly, comparison of actin organization after 24 h of unidirectional and oscillatory fluid flow resulted in dramatic differences. Osteocytes maintained a normal dendritic morphology in response to 24 h of oscillatory fluid flow (Fig. 2). In striking contrast, osteocytes subjected to unidirectional fluid flow for 24 h formed robust, organized stress fibers (Fig. 2). Together, these results reveal temporal differences in stress fiber organization in MC3T3-E1 osteoblasts subjected to different fluid flow profiles, while in MLO-Y4 osteocytes overall actin reorganization differs dramatically in response to oscillatory and unidirectional fluid flow profiles.

Alterations in Cell Morphology in Response to Fluid Flow Profiles Differs Between Osteoblasts and Osteocytes

In addition to actin stress fiber formation we also analyzed overall changes in bone cell elongation and orientation relative to the direction of flow for both fluid flow profiles. Endothelial cell realignment parallel to the direction of flow has been clearly demonstrated following 24 h of fluid flow [Dewey et al., 1981; Levesque and Nerem, 1985]. Thus, HUVECs were used for comparison of cell elongation and alignment. Based on our results along with previously published data for HUVECs, 24 h was chosen as the time point for analysis (1 and 5 h experiments were completed without any change in

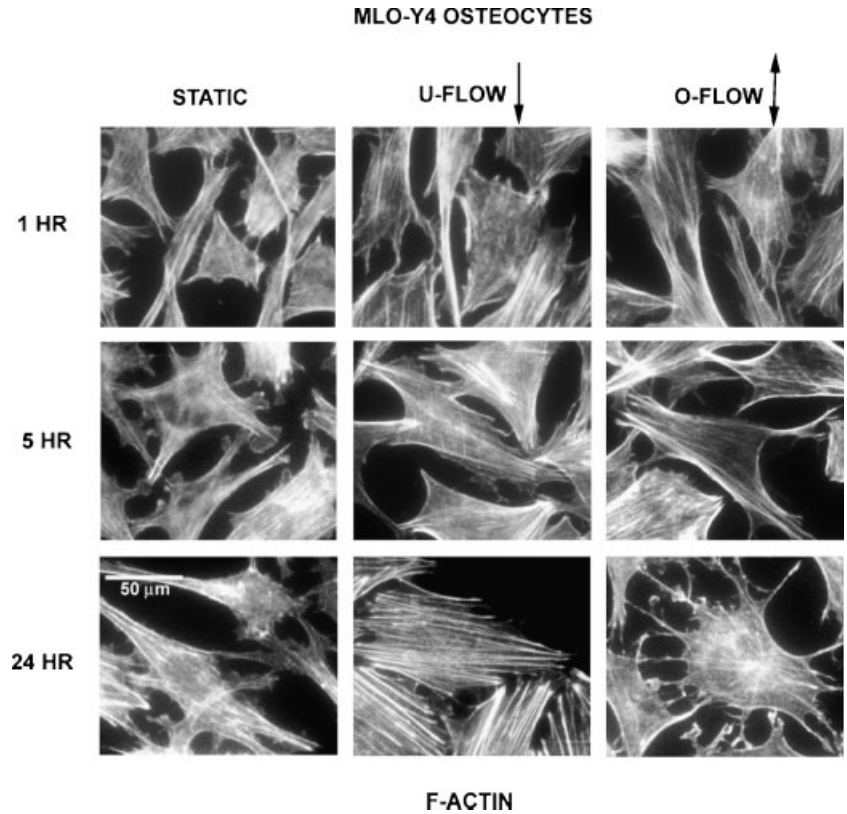


Fig. 2. MLO-Y4 osteocytes subjected to 24 h of oscillatory fluid flow have a pronounced dendritic morphology. MLO-Y4 osteocytes subjected to static or flow conditions for 1, 5, or 24 h were fixed and F-actin was visualized using rhodamine-phalloidin. No visible change in F-Actin organization was visualized after 1 or 5 h of static, unidirectional or oscillatory flow. Robust, organized stress fibers form and align within the cell in response to 24 h of unidirectional flow. A normal osteocyte-like dendritic morphology is observed after 24 h of oscillatory fluid flow. Arrows indicate the direction of fluid flow relative to the images.

cell elongation or long axis alignment in any of the cell types analyzed, data not shown).

First, we measured changes in cell elongation in response to unidirectional and oscillatory fluid flow. In response to 24 h of fluid flow, HUVECs showed an increase of 24% and 27% of the cells with an elongation factor ≥ 2 when subjected to either oscillatory or unidirectional fluid flow, respectively, compared to static controls (Fig. 3). Similarly, when subjected to 24 h of oscillatory fluid flow, the proportion of elongated MC3T3-E1 osteoblasts increased by 21% compared to static controls. In response to 24 h of unidirectional fluid flow the proportion of elongated MC3T3-E1 osteoblasts increased by even greater amounts (51%) compared to static controls as well as (30%) compared to oscillatory flow (Fig. 3). The enhanced proportion of MC3T3-E1 osteoblasts with an elongation factor ≥ 2 after 24 h of unidirectional fluid flow may result from the earlier onset of organized stress fiber formation in response to unidirectional fluid flow compared to oscillatory fluid flow. Interestingly, the proportion of elongated MLO-Y4 osteocytes was not different in response to oscillatory fluid flow compared to static controls. However, when subjected to unidirectional fluid flow the proportion of elongated MLO-Y4 osteocytes significantly decreased by 11% compared to static controls (Fig. 3). The decrease in cell elongation in response to unidirectional flow correlates with the loss of the osteocyte dendritic morphology and the formation of stress fibers. Further analysis of MLO-Y4 osteocyte morphology was compared between cells held in static conditions and those subjected to unidirectional or oscillatory fluid flow. Specifically, the number of dendritic processes per cell was evaluated and the percentage of osteocytes with zero dendrites (cuboidal morphology), one to two dendrites (elongated morphology), three dendrites (triangular morphology), and greater than three dendrites (dendritic morphology) was determined (Fig. 4). Strikingly, the majority

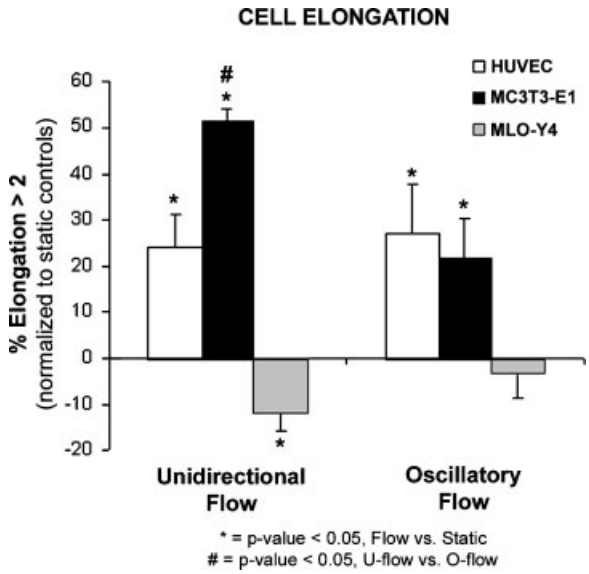


Fig. 3. Osteoblasts but not osteocytes elongate when subjected to fluid flow. Cells subjected to 24 h of static, unidirectional, and oscillatory flow were fixed and F-actin was visualized by fluorescence microscopy using rhodamine-phalloidin. Cell elongation (major axis length/minor axis length including all cellular processes) was determined using ImageTool software. Elongation for each cell type was reported as percentage of cells subjected to flow with elongation factor ≥ 2 divided by the average percentage of static controls with elongation ≥ 2. MC3T3-E1 osteoblasts subjected to either flow profile elongated compared to static controls. The proportion of osteoblasts elongated in response to unidirectional flow was also significantly increased compared to oscillatory flow. MLO-Y4 osteocytes subjected to oscillatory flow had no change in elongation compared to static controls. MLO-Y4 osteocytes subjected to 24 h of unidirectional flow significantly decreased in elongation compared to static controls.

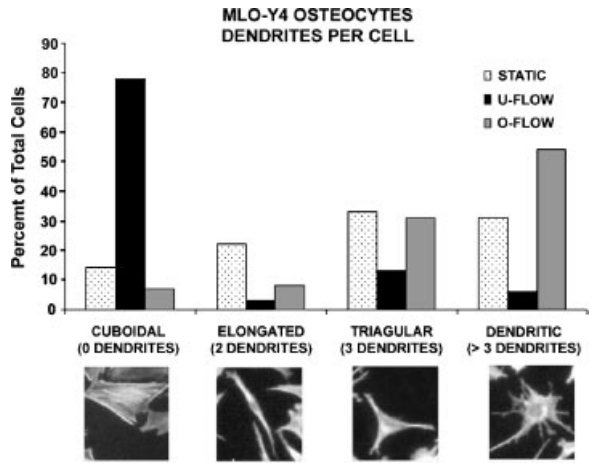


Fig. 4. The number of dendritic processes per cell increases in MLO-Y4 osteocytes subjected to oscillatory fluid flow. From images used for elongation analysis (Fig. 3) MLO-Y4 osteocytes were manually categorized into four groups: cuboidal (zero dendrites/cell), elongated (two dendrites/cell), triangular (three dendrites/cell), dendritic (>three dendrites per cell). Osteocytes maintained in static culture had a fairly even distribution among categories. Fifty-four percent of osteocytes subjected to oscillatory flow had more than three dendrites per cell. However, when subjected to unidirectional flow the majority of osteocytes (78%) were categorized as cuboidal.

(54%) of MLO-Y4 osteocytes subjected to oscillatory flow had a dendritic morphology compared to only 31% of the cells held in static conditions having greater than three dendrites per cell (Fig. 4). Osteocytes subjected to unidirectional flow had a dramatic shift in cell morphology compared to static cells as 78% of the cells had no dendrites and only 6% had greater than three dendrites (Fig. 4).

Second, we analyzed cell orientation relative to the direction of unidirectional or oscillatory fluid flow. In theory, random cell orientation would result in one-third of the cells being oriented with their long axes $\pm 30^\circ$ from any random line drawn through the field of cells. When cells held in static culture were analyzed in this way, we did in fact find that 35%, 34%, and 31% of HUVECs, MC3T3-E1 osteoblasts, and MLO-Y4 osteocytes, respectively, were oriented with their long axes (including all cellular processes) $\pm 30^\circ$ from an arbitrarily selected line (Fig. 5a–c). Interestingly, no significant realignment relative to the direction of either fluid flow profile in MC3T3-E1 osteoblasts or MLO-Y4 osteocytes was observed in our study (Fig. 5b,c). This is in stark contrast to what has long been observed and to the results we calculated in endothelial cells exposed to unidirectional fluid flow [Dewey et al., 1981; Levesque and Nerem, 1985]. We found 75% of HUVECs had a major axis angle that had realigned $\pm 30^\circ$ from the direction of unidirectional fluid flow compared to 38% which aligned parallel to the direction of oscillatory fluid flow, respectively (Fig. 5a).

Similar Temporal Induction of Cox-2 Protein Results From Either Fluid Flow Profile in Osteoblasts, While in Osteocytes Increased Cox-2 Protein Expression Only Results From Oscillatory Fluid Flow

Next, we evaluated potential differences in the ability of unidirectional and oscillatory fluid flow profiles to activate signaling pathways that are involved in mechanically induced bone formation. To this end, Cox-2 protein levels in MC3T3-E1 osteoblasts and MLO-Y4 osteocytes subjected to both fluid flow profiles were analyzed by immunoblot. In MC3T3-E1

osteoblasts we found that after 1 h a statistically significant twofold increase in Cox-2 protein expression occurred in response to both unidirectional and oscillatory fluid flow compared to static controls (Fig. 6). Similarly, both fluid flow profiles increased Cox-2 protein levels in MC3T3-E1 osteoblasts by approximately four- and sixfold above static controls at 5 and 24 h, respectively (Fig. 6). These results demonstrate that in MC3T3-E1 osteoblasts, despite delayed stress fiber organization in response to oscillatory fluid flow, both fluid flow profiles

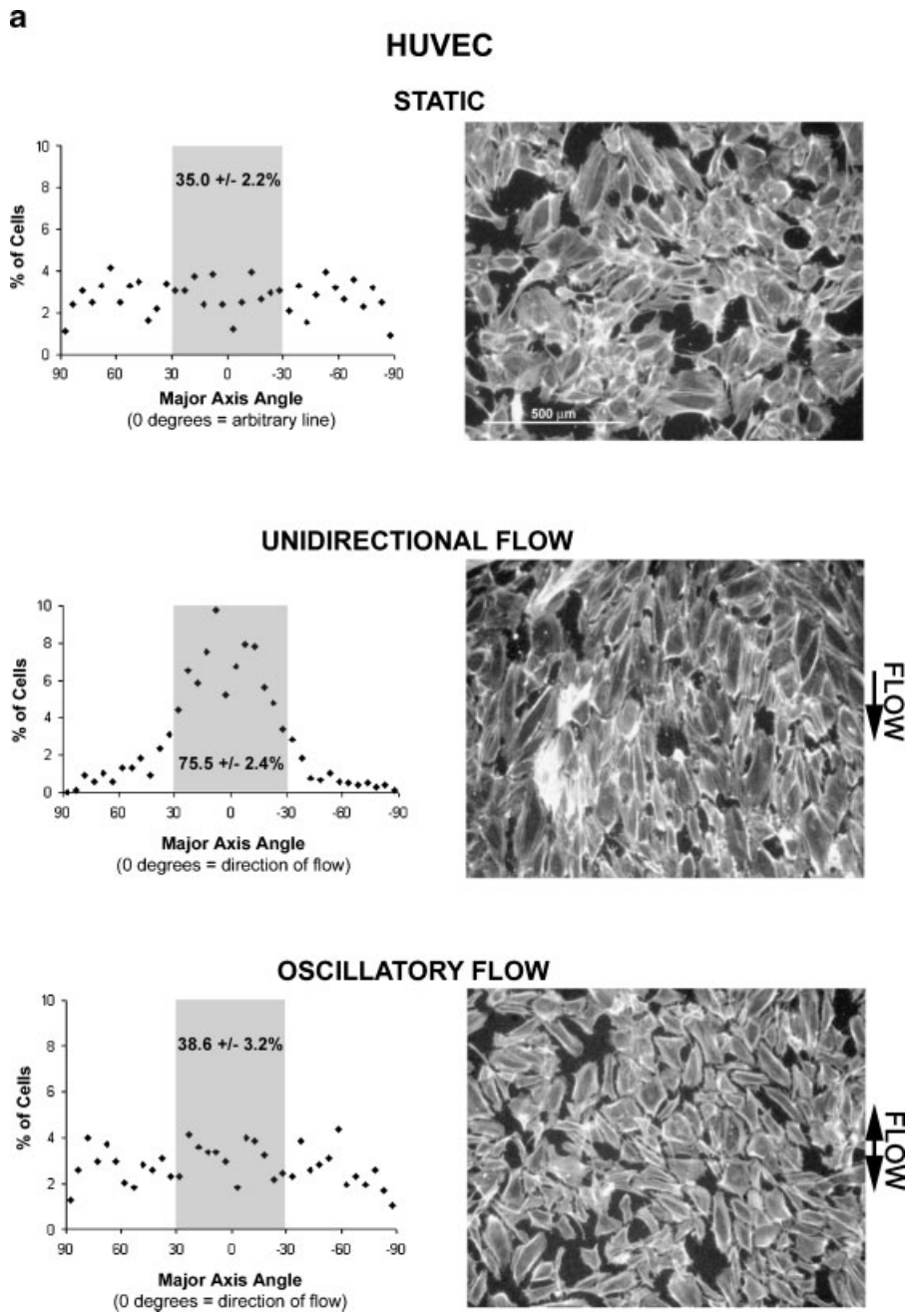


Fig. 5. Osteoblasts and osteocytes, unlike endothelial cells, do not align in the direction of flow. Cells subjected to 24 h of static, unidirectional, and oscillatory flow were fixed and F-actin was visualized by fluorescence microscopy using rhodamine-phalloidin. A representative 10× image is shown next to each graph, representing major cell axis angle in degrees versus percentage of cells. As a control, HUVEC were used to demonstrate significant long axis alignment to unidirectional but not oscillatory fluid flow (**a**). After 24 h of either unidirectional or oscillatory fluid flow neither MC3T3-E1 osteoblasts (**b**) nor MLO-Y4 osteocytes (**c**) align in direction fluid flow. Arrows next to the image indicate the direction of fluid flow.

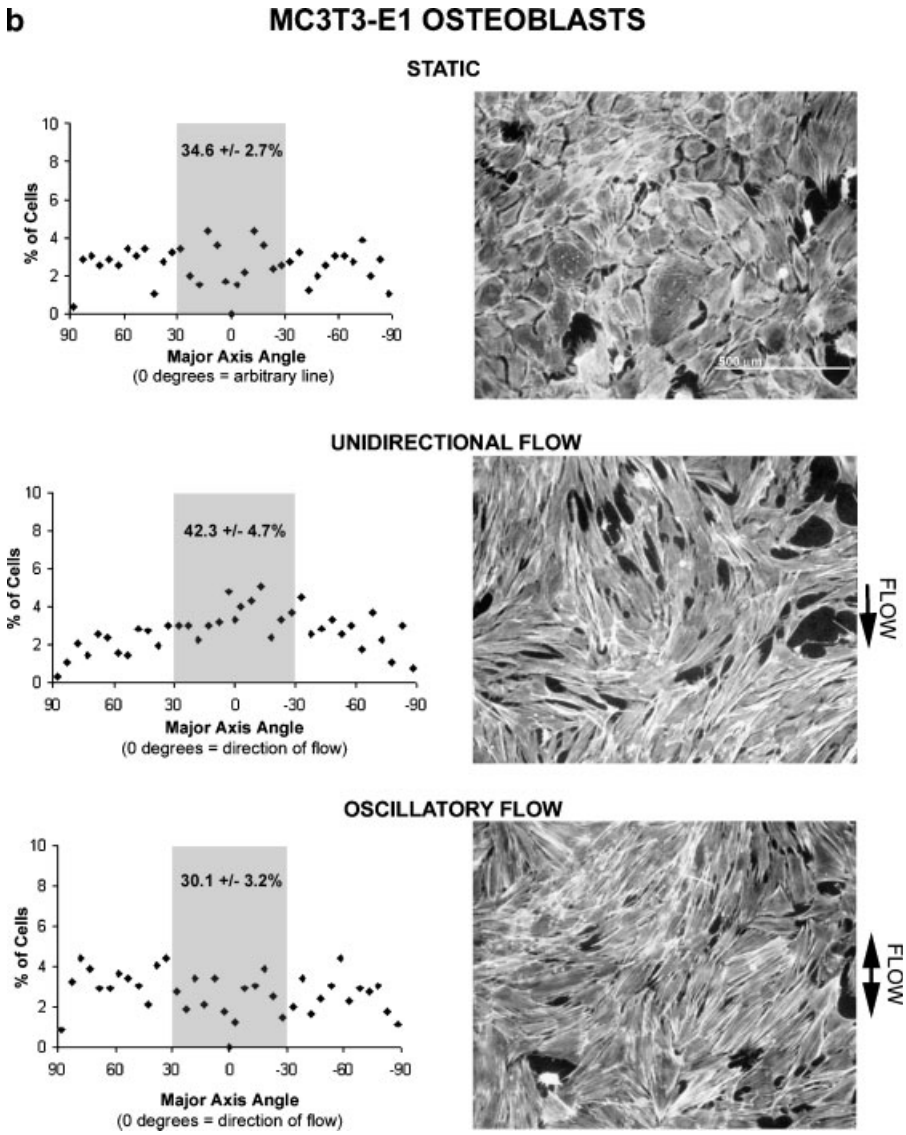


Fig. 5. (*Continued*)

elicit similar temporal increases in Cox-2 protein expression.

Results using MLO-Y4 osteocytes were notably different from MC3T3-E1 osteoblasts. Cox-2 protein levels were not altered in osteocytes subjected either 1 or 5 h of unidirectional or oscillatory fluid flow compared to static controls (Fig. 6). After 24 h, Cox-2 protein levels significantly increased above static controls in MLO-Y4 osteocytes subjected to oscillatory, but not unidirectional, fluid flow (Fig. 6). These results suggest that fluid flow profile rather than the resulting stress fiber formation is the critical determinant for signaling leading to increased Cox-2 protein expression in MLO-Y4 osteocytes.

Both Fluid Flow Profiles Regulate Osteopontin Levels in MC3T3-E1 Osteoblasts and MLO-Y4 Osteocytes

To further elucidate the biochemical response of bone cells to different fluid flow profiles we evaluated fluid flow-induced regulation of osteopontin. Immunoblot analysis of osteopontin revealed no change in osteopontin protein levels in MC3T3-E1 osteoblasts or MLO-Y4 osteocytes subjected to unidirectional or oscillatory fluid flow for 1 or 5 h compared to static controls (data not shown). However, in response to 24 h of unidirectional and oscillatory fluid flow osteopontin levels in MC3T3-E1 osteoblasts significantly increased by 1.8- and 2.5-fold,

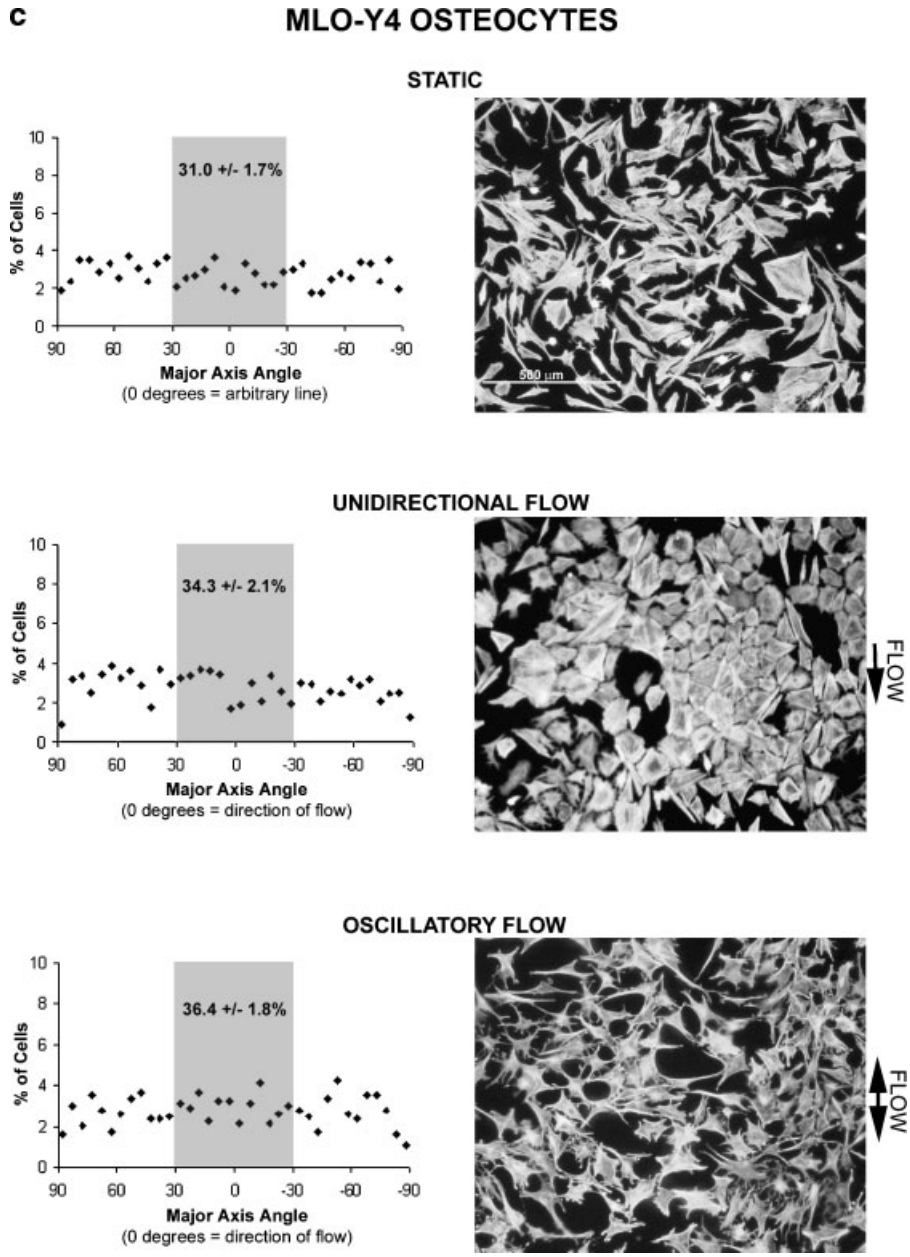


Fig. 5. (*Continued*)

respectively, compared to static controls (Fig. 7a). Surprisingly, we observed a decrease in osteopontin protein expression in MLO-Y4 osteocytes subjected to 24 h of either fluid flow profile compared to static controls (Fig. 7a). The decrease in osteopontin protein expression in MLO-Y4 osteocytes was more pronounced in response to oscillatory fluid flow compared to unidirectional fluid flow (Fig. 7a).

To more thoroughly investigate the regulation of osteopontin by fluid flow we measured osteopontin released from osteoblasts and osteocytes in response to both fluid flow profiles. Opposite of our immunoblot detection, osteopontin released from the MC3T3-E1 osteoblasts in response to unidirectional and oscillatory fluid flow decreased compared to static controls (Fig. 7b). The decrease in released osteopontin was significant only in MC3T3-E1 osteoblasts subject to oscillatory fluid flow; however, a similar trend was observed in response to unidirectional fluid flow. Interestingly, we found that MLO-Y4 osteocytes increased the release osteopontin into the media in response

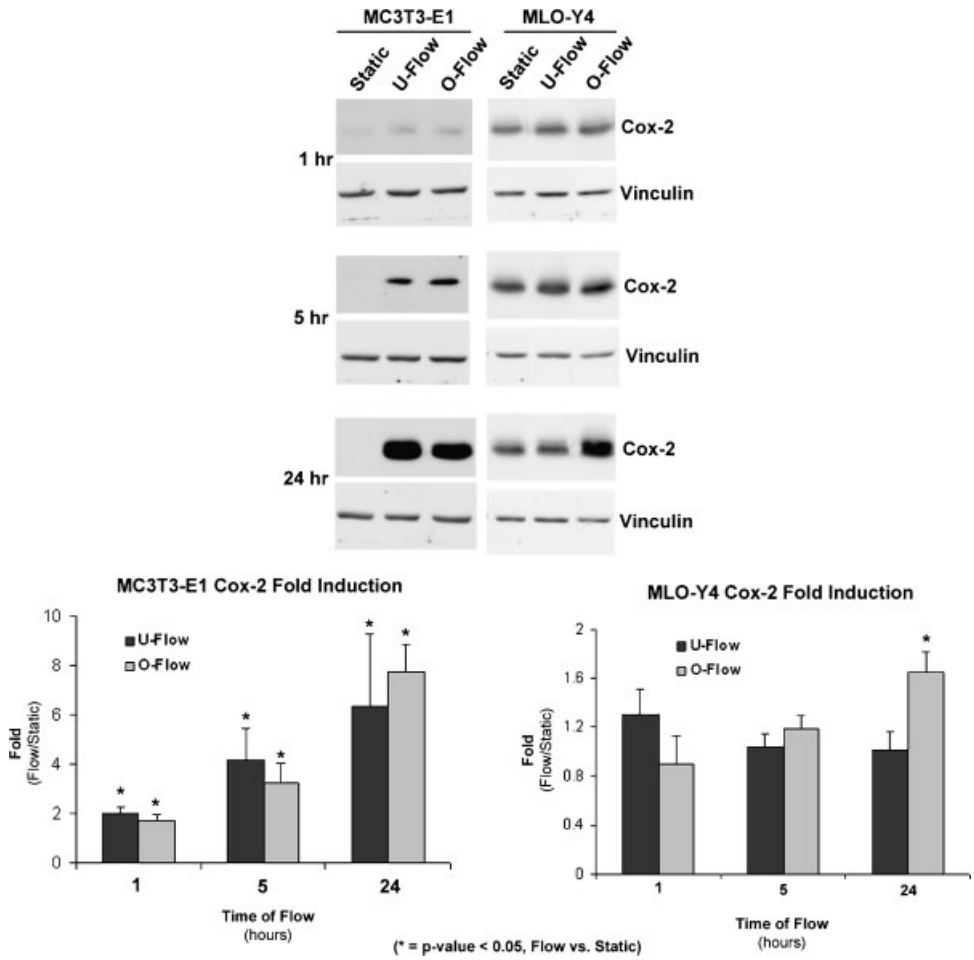


Fig. 6. Cox-2 protein levels increase in response to both fluid flow profiles in MC3T3-E1 osteoblasts but in MLO-Y4 osteocytes Cox-2 only increases in response to oscillatory fluid flow. Cells were subjected to static conditions or either fluid flow profile for 1, 5, and 24 h and Cox-2 protein levels were analyzed by Western blot (25 μg of protein was loaded per lane and vinculin was used as a loading control) (n = 4). Cox-2 levels were first standardized to vinculin then fold increase (flow/static) was calculated. Cox-2 levels in MC3T3-E1 osteoblasts significantly increased in cells subjected to both oscillatory and unidirectional fluid flow compared to static controls by two-, four-, and sixfold at 1, 5, and 24 h, respectively. Cox-2 levels remain unchanged in MLO-Y4 osteocytes subjected to 1 or 5 h of unidirectional or oscillatory fluid flow. Twenty-four hours of oscillatory, but not unidirectional, fluid flow induced a significant increase in Cox-2 protein compared to osteocytes maintained in static conditions.

to unidirectional and oscillatory fluid flow (Fig. 7b). Like osteoblasts, the release measurement from osteocytes is also opposite of our immunoblot data of osteopontin levels. Together these results demonstrate that both fluid flow profiles regulate osteopontin, but the regulation is cell type specific.

DISCUSSION

The goal of this study was to directly compare the effects of unidirectional and oscillatory fluid flow profiles on important mechanotransduction pathways in both osteoblasts and osteocytes. While these two fluid flow profiles are inherently different, our systems for delivering unidirectional and oscillatory fluid flow were set up to have as many similarities as possible. Anderson et al. [2006] have shown that comparison of fluid shear stress is difficult to accomplish when different chambers are used due to the variability in the number of cells exposed to flow. Therefore, the same chambers were used to subject cells to unidirectional and oscillatory fluid flow. Our systems were also set to deliver a similar peak shear of approximately 10 dynes/cm^2. The actual shear rates of 8 dynes/cm^2 for unidirectional flow and 11 dynes/cm^2 (peak shear) for oscillatory flow were the average peak shear rates measured directly with our flow systems. These rates are within the predicted physiologic range (8–30 dynes/cm^2)

occurring within bone in vivo [Weinbaum et al., 1994]. We cannot rule out the possibility that the small difference in peak shear (3 dynes/cm^2) may account for differences in our results comparing oscillatory and unidirectional fluid flow. However, we believe that changes in cellular response to unidirectional versus oscillatory fluid flow are due to the fundamental differences in the properties of the flow profile. The main difference in these two fluid flow profiles is the amount of chemotransport the cells are exposed to. Unidirectional flow results in a net fluid transport across the cells, while oscillatory fluid flow results in no net fluid transport. In fact, the study by Jacobs et al. [1998] comparing increased intracellular calcium levels in osteoblasts subjected to oscillatory flow or steady flow, found oscillatory flow to be a less potent stimulus than pulsatile or steady flow and these authors attribute the difference in response to the exposure of net fluid transport. The difference in chemotransport between the two fluid flow profiles may elicit fundamentally different mechanisms of mechanotransduction in osteoblasts and osteocytes and account for the differences we see in response to unidirectional and oscillatory fluid flow.

In this study we have shown for the first time that MLO-Y4 osteocytes have a more dendritic morphology in response to oscillatory fluid flow; however, in response to 24 h of unidirectional fluid flow, these osteocyte-like cell lines form robust, highly organized stress fibers. Alternatively, MC3T3-E1 osteoblasts subjected to both unidirectional and oscillatory fluid flow elongate and form stress fibers. Second, we found that signal transduction leading to altered gene expression was dependent on the type of bone cell analyzed as well as the type of fluid shear profile used to stimulate the cells. In MC3T3-E1 osteoblasts both fluid flow profiles induced the temporal activation of signal transduction similarly, as measured by Cox-2 and osteopontin upregulation. However, MLO-Y4 osteocytes

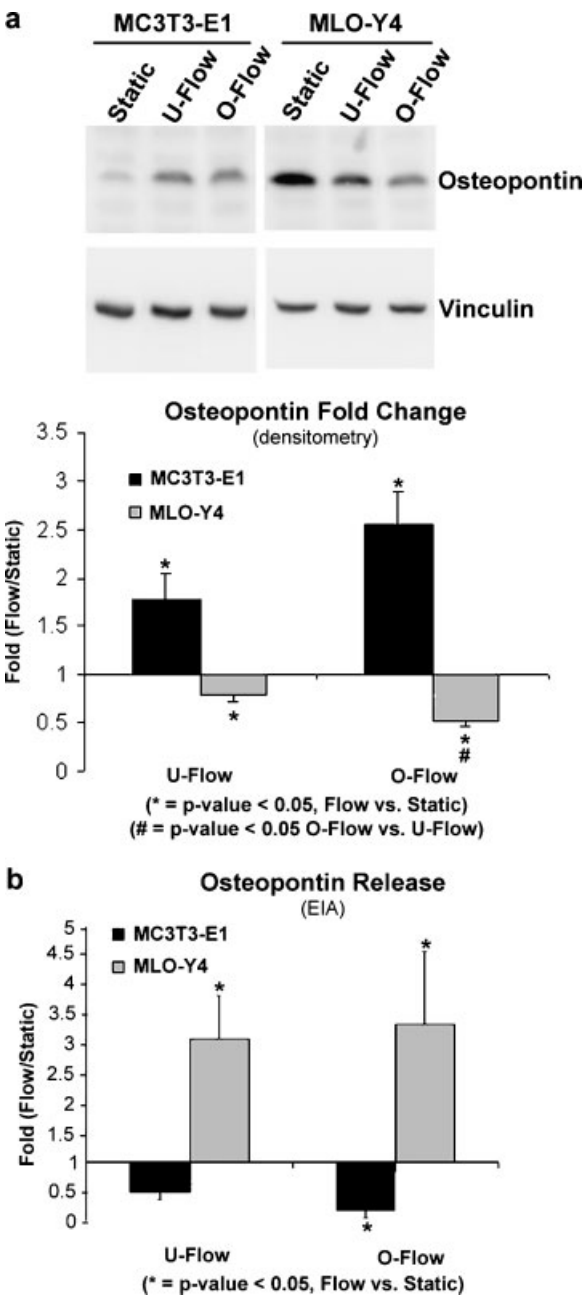


Fig. 7. Fluid flow-induced alterations in osteopontin protein levels in osteoblasts and osteocytes. **a**: Cells were subjected to static conditions or either fluid flow profile for 24 h and osteopontin protein levels were analyzed by Western blot (25 μg of protein was loaded per lane and vinculin was used as a loading control, n = 4 for each condition and cell type). Osteopontin levels were first standardized to vinculin then fold increase (flow/static) was calculated. At early time points (1 and 5 h) there was no change in the level of osteopontin in MC3T3-E1 osteoblasts or MLO-Y4 osteocytes subjected to either fluid flow profile (data not shown). MC3T3-E1 osteoblasts significantly increase osteopontin in response to 24 h of both unidirectional and oscillatory flow. In contrast, both flow profiles decrease osteopontin levels in MLO-Y4 osteocytes compared to static controls. Additionally, osteopontin levels in osteocytes subjected to 24 h of oscillatory fluid flow are significantly reduced compared to cells subjected to unidirectional flow. **b**: Osteopontin release into the media was measured using EIA. Opposite of protein levels detected by Western blot, osteopontin released into the media decreased in MC3T3-E1 osteoblasts subjected to either fluid flow profile, while release of osteopontin into the media increased in MLO-Y4 osteocytes subjected to either fluid flow profile.

were more responsive to oscillatory fluid flow compared to unidirectional fluid flow, as measured by Cox-2 upregulation and osteopontin release. Lastly, morphometric analysis allowed us to show that neither osteoblasts nor osteocytes realigned their long axis parallel to the direction of flow, contrary to what has been published for endothelial cells [Galbraith et al., 1998; Noria et al., 2004].

The actin cytoskeleton, along with the other filament networks, has been hypothesized to play an important role in mechanotransduction in bone cells [Ingber et al., 1994; Alenghat and Ingber, 2002]. The reorganization of actin into stress fibers in response to unidirectional fluid flow is well characterized in osteoblasts, as well as endothelial cells [Franke et al., 1984; Pavalko et al., 1998]. In endothelial cells fluid flow-induced stress fiber formation drives cellular long axis alignment parallel to the direction of unidirectional flow after 24 h [Galbraith et al., 1998; Noria et al., 2004]. This response is thought to be a mechanism by which endothelial cells adapt to their mechanical environment [Dewey et al., 1981]. However, we did not detect any cellular long axis realignment with respect to either fluid flow profile in MC3T3-E1 osteoblasts or MLO-Y4 osteocytes despite the observed reorganization of actin. Unlike endothelial cells, these data support a hypothesis that cellular realignment in the direction of flow is not a response of bone cells to mechanical stimulation.

The role of the actin cytoskeleton in mechanotransduction with respect to signal transduction remains controversial. Our results show that Cox-2 protein levels increase in MC3T3-E1 osteoblasts subjected to unidirectional and oscillatory fluid flow by two- and fourfold after 1 and 5 h, respectively. At these same time points stress fibers are aligned within the cell in response to unidirectional but not oscillatory fluid flow, suggesting that stress fiber formation is not an absolute requirement for flow-induced Cox-2 upregulation. Along these lines, our lab and others have previously shown that disruption of the actin network in osteoblasts does not block the increase in prostaglandin release [McGarry et al., 2005] nor Cox-2 protein expression in response to fluid shear stress [Norvell et al., 2004]. However, other mechanosensory components, such as focal adhesions, have been shown to be required for fluid flow-induced prostaglandin metabolism in osteoblasts [Ponik and Pavalko, 2004; McGarry et al., 2005]. Taken together, we conclude that osteoblasts increase Cox-2 protein expression independent of actin reorganization, in response to either unidirectional or oscillatory fluid flow profiles.

In osteocytes, actin localized to the dendritic cell processes, and not in stress fibers, may play a critical role in mechanotransduction. The dendritic processes of osteocytes extend into the lacuno-canalicular network and are exposed to fluid shear stress when bone is strained [Kufahl and Saha, 1990; Turner et al., 1994; Knothe Tate et al., 1998; Burger and Klein-Nulend, 1999]. As reviewed by You et al. [2001b] and Han et al. [2004] fluid flow through the lacuno-canalicular network results in strain amplification to actin filaments within the osteocyte cell processes. Additionally, unlike osteoblasts, disruption of the actin cytoskeleton blocks fluid flow-induced prostaglandin release in osteocytes [Ajubi et al., 1996; McGarry et al., 2005]. Interestingly, our results demonstrate that under oscillatory fluid flow conditions, which enhance dendrite formation, Cox-2 protein levels increase in MLO-Y4 osteocytes. In contrast, MLO-Y4 osteocytes formed organized stress fibers after 24 h of unidirectional fluid flow, but under these conditions Cox-2 protein levels were not different from static controls. It is possible that the two fluid shear profiles elicit differences in chemotransport, an important component of fluid shear-induced signaling in osteoblasts, which may account for the increase in Cox-2 protein in osteocytes subjected to oscillatory but not unidirectional fluid flow. However, our results suggest that increased number of dendritic processes in osteocytes subjected to oscillatory fluid flow may also be important for the normal mechanotransduction response of increased Cox-2 protein expression.

Another important endpoint of mechanotransduction in bone cells is the regulation of the extracellular matrix protein osteopontin. Osteoblasts are known to contribute to the remodeling process by increasing extracellular matrix deposition in response to fluid flow [Sikavitsas et al., 2003; Ponik and Pavalko, 2004]. Our results demonstrate that MC3T3-E1 osteoblasts increased osteopontin protein expression as measured by immunoblot, which includes both cellular and matrix proteins, while released osteopontin levels decrease in response to both fluid flow profiles compared to

static controls. Therefore, we hypothesize that osteoblasts either increase osteopontin protein expression or alter post-translational modification of osteopontin in response to fluid flow, which results in osteopontin retained in the cell or incorporation into the matrix. In contrast to the results in osteoblasts, MLO-Y4 osteocytes express high cellular and matrix levels of osteopontin under static conditions, which decrease in response to fluid flow. In osteocytes, decreased cellular levels of osteopontin correlated with increased osteopontin release into the media. Osteopontin, has been shown to play a signaling role in the regulation of bone remodeling [Denhardt and Noda, 1998; Giachelli and Steitz, 2000]. Therefore, the osteopontin released from osteocytes in response to fluid flow could potentially function as a signaling molecule to activate bone remodeling. Further studies examining the deposition of osteopontin into the matrix by osteoblasts and the signaling properties of soluble osteopontin are needed to directly test these hypotheses.

In summary, our results provide novel information for the analysis of mechanotransduction by directly comparing commonly used cell types and fluid flow profiles. Specifically, we have demonstrated significant differences in the effects of unidirectional and oscillatory fluid flow on MC3T3-E1 osteoblasts and MLO-Y4 osteocytes. The differences are most dramatically illustrated in MLO-Y4 osteocytes by increasing the number of dendritic processes per cell coupled with pronounced changes in Cox-2 protein expression and osteopontin levels in response to oscillatory fluid flow compared to unidirectional fluid flow. While understanding the effects of fluid flow on bone cells in vitro remains important, based on these results it is necessary to note that not all bone cell types respond the same to all fluid flow profiles; and, not all fluid flow profiles elicit the same response in a given bone cell type.

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