MAP kinase and calcium signaling mediate fluid flow-induced human mesenchymal stem cell proliferation

Ryan C. Riddle, Amanda F. Taylor, Damian C. Genetos, and Henry J. Donahue. MAP kinase and calcium signaling mediate fluid flow-induced human mesenchymal stem cell proliferation. Am J Physiol Cell Physiol 290: C776–C784, 2006. First published November 2, 2005; doi:10.1152/ajpcell.00082.2005.—Mechanical signals are important regulators of skeletal homeostasis, and strain-induced oscillatory fluid flow is a potent mechanical stimulus. Although the mechanisms by which osteoblasts and osteocytes respond to fluid flow are being elucidated, little is known about the mechanisms by which bone marrow-derived mesenchymal stem cells respond to such stimuli. Here we show that the intracellular signaling cascades activated in human mesenchymal stem cells by fluid flow are similar to those activated in osteoblastic cells. Oscillatory fluid flow inducing shear stresses of 5, 10, and 20 dyn/cm² triggered rapid, flow rate-dependent increases in intracellular calcium that pharmacological studies suggest are inositol triphosphate mediated. The application of fluid flow also induced the phosphorylation of extracellular signal-regulated kinase-1 and -2 as well as the activation of the calcium-sensitive protein phosphatase calcineurin in mesenchymal stem cells. Activation of these signaling pathways combined to induce a robust increase in cellular proliferation. These data suggest that mechanically induced fluid flow regulates not only osteoblastic behavior but also that of mesenchymal precursors, implying that the observed osteogenic response to mechanical loading may be mediated by alterations in the cellular behavior of multiple members of the osteoblast lineage, perhaps by a common signaling pathway.

mechanotransduction; bone; marrow

MAINTENANCE OF APPROPRIATE bone mass requires the coordination of bone resorption by osteoclasts and bone deposition by osteoblasts, and it is well established that mechanical stimuli can regulate the balance between bone formation and resorption. The addition of exogenous mechanical load is believed to stimulate new bone formation through increases in osteoblastic activity and concomitant decreases in osteoclastic activity (20). Conversely, removal of mechanical load, as is the case during spaceflight and disuse, leads to decreased osteoblastic activity and loss of bone mass (42, 56).

Accumulating evidence suggests that individual bone cells, including osteocytes and osteoblasts, are responsible for perceiving and responding to mechanical signals. Such signals, which may include streaming potentials, mechanical strain, and fluid shear stress, elicit a host of biochemical responses including mobilization of second messengers such as calcium (22, 72), nitric oxide (26, 30), prostaglandins, and inositol triphosphate (IP₃) (49); activation of kinase cascades including the MAP kinase and PKC pathways (44, 50); and modulation of gene expression (5, 46, 73). Strain-induced oscillatory fluid flow has been shown by our laboratory (73) and others (45) to be a potent biophysical stimulus. In MC3T3-E1 preosteoblasts, fluid flow induces the mobilization of intracellular calcium ($Ca^{2+}$), activates ERK1/2, and increases osteopontin mRNA levels (11, 72, 73). Recent evidence has shown that exposing the osteocytic cell line MLO-Y4 to physiological levels of fluid flow induces similar responses (1, 6, 52).

Although the mechanisms by which osteoblasts and osteocytes respond to mechanical stimuli are being elucidated, little is known about how bone marrow-derived mesenchymal stem cells (MSCs) respond to mechanical signals. Capable of regenerating bone (15, 16), these cells also maintain the capacity to differentiate to multiple mesenchymal cell types including chondrocytes and adipocytes (47, 48). Emerging studies suggest that, like osteoblasts and osteocytes, MSCs are mechanoresponsive. MSCs isolated from rats after hindlimb unloading exhibit both a decreased proliferative potential and a reduced osteogenic capacity compared with loaded controls (39, 74). Specifically, cells from these animals formed fewer mineralized and alkaline phosphatase-positive colonies in vitro, two commonly used markers of osteoblastic potential. Similarly reduced osteogenic capacity was observed in MSCs isolated from mice subjected to periods of tail suspension (54). More recently, Simmons et al. (59) showed that exposing human MSCs (hMSCs) to a cyclic strain during osteoblastic differentiation enhances the degree of matrix mineralization and Li et al. (37) showed that oscillatory fluid flow stimulates hMSC proliferation in vitro.

Despite these observations, the cellular mechanisms by which MSCs respond to mechanical stimuli have yet to be defined. In these studies, we sought to investigate whether human marrow-derived progenitors respond to oscillatory fluid flow in a manner similar to that observed in more mature cells of the osteoblastic lineage. Specifically, we hypothesized that fluid flow would result in an increase in intracellular calcium concentration ($[Ca^{2+}]$) and the subsequent activation of downstream signaling proteins, such as ERK1/2 and calcineurin, which would in turn induce hMSC proliferation. $Ca^{2+}$ is a vital and ubiquitous mediator in the processes by which extracellular signals are conveyed to the cell’s interior and translated into a cellular response. Oscillations in $[Ca^{2+}]$, regulate gene expression via numerous signaling cascades (19, 57) and have been shown to provide specificity among transcriptional acti-
The serine/threonine protein phosphatase calcineurin, for instance, responds to increases in [Ca^{2+}], and calmodulin binding by dephosphorylating and activating nuclear factor of activated T cells (NFAT) transcription factors (8, 21). Likewise, ERK1/2, members of the MAP kinase family, have been shown to be key regulators in the proliferation and differentiation of numerous cell types, including hMSCs (23) and osteoblasts (28, 34, 70).

Herein we show that exposing hMSCs to oscillatory fluid flow induces a flow-rate dependent increase in [Ca^{2+}], and that these increases are mediated by the release of calcium from IP_{3}-sensitive stores. Additionally, fluid flow stimulates a robust and time-dependent increase in ERK1/2 phosphorylation that is independent of increases in [Ca^{2+}], but dependent on the activity of PLC and the binding of diacylglycerol (DAG) to PKC. Finally, we show that oscillatory fluid flow induces a proliferative response in hMSCs that is dependent on the activation of ERK1/2 and the activation of the calcium-responsive protein calcineurin.

**MATERIALS AND METHODS**

**Cell culture.** hMSCs from an 18-year-old male donor (Cambrex Biosciences) were cultured in DMEM-low glucose (DMEM-LG; Invitrogen) supplemented with 10% FBS (HyClone), 2 mM L-glutamine, and 1% penicillin-streptomycin and maintained in a humidified incubator at 37°C with 5% CO_{2}. Medium was replaced every 3–4 days. Supplements to induce the differentiation of hMSCs were not added to the culture medium at any time in these studies. At 80% confluence, cells were subcultured and seeded onto quartz slides for [Ca^{2+}] experiments or onto glass slides for ERK1/2 phosphorylation, calcineurin activity, and proliferation studies. Eighty thousand cells were seeded on quartz slides (76 × 26 × 1.6 mm) and one hundred thirty thousand cells were seeded on glass slides (75 × 38 × 1.0 mm) to attain similar levels of confluence on the day of experiments. Forty-eight hours after seeding, culture medium was replaced with flow medium (DMEM-LG containing 0.5% FBS), and cells were cultured for an additional 24 h before exposure to fluid flow.

**Application of fluid flow.** To expose cells to fluid flow, slides were positioned in parallel plate flow chambers (14) and connected to a servopneumatic materials testing device (EnduraTec), oscillating at 1 Hz, via glass Hamilton syringes and rigid wall tubing. For calcium measurements, oscillatory fluid flow was induced at a physiological frequency of 5, 10, or 20 dyn/cm^{2} for 15 min or were used as time-matched static controls. Immediately after exposure to flow, cells were washed in ice-cold PBS, and total cellular protein was isolated in 0.1% Triton X-100, 10 mM Tris, pH 8, 1 mM EDTA, 0.2 mM Na_{3}VO_{4}, supplemented with a protease inhibitor cocktail (Calbiochem). Twenty-five micrograms of protein were resolved by SDS-PAGE (GelBond and transferred onto polyvinylidene difluoride membranes (Bio-Rad). In increases in [Ca^{2+}] and time-dependent increase in ERK1/2 phosphorylation, hMSCs were exposed to oscillatory fluid flow as described for ERK1/2 experiments and immediately washed in Tris-buffered saline to avoid phosphate contamination. Cell lysates were collected in 50 mM Tris, 1 mM DTT, 100 mM EDTA, 100 mM EGTA, and 0.2% Nonidet P-40 and then incubated with the RII phosphopeptide (Asp-Leu-Asp-Val-Pro-Ile-Pro-Gly-Arg-Phe-Asp-Arg-Arg-Val-Pro-Ala-Ala-Glu) in the presence or absence of 10 mM EDTA, to inhibit calcineurin activity. Calcineurin activity was calculated by subtracting phosphatase activity in the presence of EGTA from total phosphatase activity. Results are normalized to total protein concentration (bicinchoninic acid; Pierce) and presented as picomolar phosphate liberated per milligram of protein in 30 min.

**Calcineurin activity assays.** The effect of fluid flow on cellular calcineurin activity levels was assessed using a commercially available colorimetric assay (Calbiochem) according to the manufacturer’s instructions. Briefly, cells were exposed to oscillatory fluid flow as described for ERK1/2 experiments and immediately washed in Tris-buffered saline to avoid phosphate contamination. Cell lysates were collected in 50 mM Tris, 1 mM DTT, 100 mM EDTA to inhibit calcineurin activity. Calcineurin activity was calculated by subtracting phosphatase activity in the presence of EGTA from total phosphatase activity. Results are normalized to total protein concentration (bicinchoninic acid; Pierce) and presented as picomolar phosphate liberated per milligram of protein in 30 min.

**Western immunoblotting.** Oscillatory fluid flow-induced hMSC proliferation was assessed using a FITC 5-bromo-2’-deoxyuridine (BrdU) flow kit (BD Pharmigen) according to the manufacturer’s instructions. Briefly, cells were exposed to oscillatory fluid flow with shear stresses of 5, 10, or 20 dyn/cm^{2} for 1 h or were used as static controls. Cells were postincubated for 20 h in fresh flow medium at 37°C with 5% CO_{2}. Proliferating cells were then labeled for 60 min with 10 μM BrdU, a thymidine analog incorporated into the genomic DNA of proliferating cells. Cells were subsequently washed with PBS and fixed, and the percentages of proliferating cells were quantified using fluorescence-activated cell sorting (FACS; FACScan, BD Pharmigen) with a FITC-conjugated antibody specific for BrdU.

**Pharmacological inhibitors.** To ascertain the source of fluid flow-induced [Ca^{2+}] increases and to elucidate the signaling cascades responsible for flow-induced proliferation, a series of pharmacological inhibitors of Ca^{2+} and MAP kinase signaling were used. Thapsigargin (1 μM), verapamil (5 μM), and GdCl_{3} (10 μM) (all from Sigma) were used to inhibit Ca^{2+} release, L-type voltage-sensitive calcium channel activity, and mechanosensitive cation channel activity, respectively (12, 18, 61, 69). Rynadine (1 μM; Calbiochem) was used to activate and hold open ryanodine-sensitive channels on intracellular calcium stores (41). U-73122 (10 μM; Calbiochem) was used to antagonize the activity of PLC and thus the production of IP_{3} and DAG, whereas...
U-73343, a weak PLC antagonist, was used as a negative control (3, 5). GF-109203X (1 μM; Biomol) and calphostin C (1 μM; Calbiochem) were used to antagonize PKC activity (31, 63). U-0126 (10 μM; Cell Signaling Technologies) and cyclosporin A (5 μg/ml; Calbiochem) were used to inhibit ERK1/2 activation and calcineurin activity and to examine their role in fluid flow-induced proliferation (13, 38, 59). Cells were pretreated with inhibitors or vehicle controls 30 min before and for the duration of fluid flow. For calcium imaging experiments, inhibitors were added after cells were loaded with fura-2. All pharmacological inhibitors were dissolved in DMSO, with the exception of verapamil and GdCl3. Verapamil was dissolved in ethanol, whereas GdCl3 was dissolved directly in the flow medium. Solvent concentrations in the flow medium were maintained at 0.1% (vol/vol).

Data analysis. Increases in [Ca\(^{2+}\)]\(_i\) during the static period or in response to fluid flow were determined by calculating the average [Ca\(^{2+}\)]\(_i\) and standard deviation for individual cells before exposure to flow. A calcium concentration greater than the average [Ca\(^{2+}\)]\(_i\) plus 4 standard deviations in response to flow or during the static period was defined as a response. No statistical difference was observed between vehicle controls for calcium experiments (1-way ANOVA), so all controls were combined. Because of variation between proliferation experiments, data were analyzed and expressed as the percentage of static control. All data are expressed as means ± SE. One-way ANOVA and Tukey’s multiple comparisons tests were used to compare groups. Student’s t-test was used to compare calcineurin activity at each time point examined (Prism; GraphPad Software). P < 0.05 was considered statistically significant.

RESULTS

Oscillatory fluid flow induces [Ca\(^{2+}\)]\(_i\) increases. Exposing hMSCs to oscillatory fluid flow resulted in a rapid yet transient increase in [Ca\(^{2+}\)]\(_i\), that was flow rate dependent. Calcium traces from a typical experiment in which hMSCs were exposed to a shear stress of 20 dyn/cm\(^2\) are shown in Fig. 1A. During the static period, 1.4 ± 0.6% of cells exhibited a spontaneous increase in [Ca\(^{2+}\)]\(_i\), with an amplitude of 68.9 ± 9.1 nM (Fig. 1, B and C). A significantly higher percentage of cells exhibited increases in [Ca\(^{2+}\)]\(_i\), when hMSCs were exposed to oscillatory fluid flow-inducing shear stresses of 5, 10, and 20 dyn/cm\(^2\) at 1 Hz (56 ± 2.4%, 87 ± 7.9%, and 89 ± 10.4%, respectively). Interestingly, the amplitudes of the observed [Ca\(^{2+}\)]\(_i\) increase in response to 5 and 10 dyn/cm\(^2\) (60.0 ± 10.2 and 96.8 ± 6.3 nM, respectively) were not statistically significantly different from spontaneous increases observed in static controls. In contrast, a significantly greater increase in [Ca\(^{2+}\)]\(_i\), was observed when cells were exposed to a shear stress of 20 dyn/cm\(^2\) (189 ± 12.4 nM). Results were obtained from 4 separate experiments using a total of 11 slides and at least 184 individual cells per flow rate. Unless otherwise stated, subsequent flow experiments were performed with a shear stress of 20 dyn/cm\(^2\) as it produced the most robust increase in [Ca\(^{2+}\)]\(_i\).

Source of [Ca\(^{2+}\)]\(_i\) increase. Pharmacological inhibitors were used to elucidate the source of fluid flow-induced [Ca\(^{2+}\)]\(_i\) increases. Treatment with GdCl3 or verapamil, which antagonize the activity of the mechanosensitive cation channel and the L-type calcium channel, respectively, did not have a significant effect on either the percentage of cells responding to fluid flow with an increase in [Ca\(^{2+}\)]\(_i\); (Fig. 2A) or flow-induced [Ca\(^{2+}\)]\(_i\) increases (Fig. 2B) compared with untreated flow controls. Thapsigargin (1 μM), which empties intracellular calcium stores by inhibiting the ATP-dependent Ca\(^{2+}\) pump, significantly decreased both the percentage of responding cells (15.5 ± 6.4%) and flow-induced increases (54.6 ± 8.6 nM) compared with flow controls. Flow-induced increases were similar to those observed in static controls (73.8 ± 9.9 nM). Thus we decided to further examine the mechanisms by which fluid flow induces the release of calcium from intracellular stores.

Ryano dine (1 μM), which activates and holds open ryanodine-sensitive channels present on intracellular calcium stores, did not have a significant effect on either the percentage of
Fluid flow-induced increases in 
\([Ca^{2+}]_i\), are mediated by calcium release from intracellular stores in hMSCs. Percentage of cells exhibiting an increase in 
\([Ca^{2+}]_i\) (A) and mean increase in 
\([Ca^{2+}]_i\) (B) in response to oscillatory fluid flow (20 dyn/cm², 1 Hz) in flow controls or in the presence of 5 \(\mu M\) verapamil, 10 \(\mu M\) GdCl₃, 1 \(\mu M\) thapsigargin, 1 \(\mu M\) ryanodine, 10 \(\mu M\) U-73343, or 10 \(\mu M\) U-73122 is shown. *\(P < 0.05\) compared with flow control; ***\(P < 0.001\) compared with flow control; #\(P < 0.05\) compared with U-73343; ###\(P < 0.001\) compared with U-73343.

Fluid flow induces ERK activation. Antibodies specific for ERK1/2 phosphorylated at Thr202 and Tyr204 were used to examine increases in ERK1/2 activation in response to fluid flow. Basal levels of ERK1/2 phosphorylation remained constant in static controls (Fig. 3A). In response to the onset of oscillatory fluid flow inducing a shear stress of 20 dyn/cm², we observed a time-dependent increase in ERK1/2 phosphorylation. After 15, 30, and 60 min of fluid flow, phosphorylation was markedly increased compared with time-matched static controls. However, after 120 min of fluid flow, phosphorylation levels were comparable to those of unflowed cells. To assess how quickly ERK1/2 phosphorylation levels increased, we repeated these experiments at earlier time points. Phosphorylation levels were increased after just 1 min of fluid flow, but the highest levels were achieved after 5 and 15 min (Fig. 3B). Results are typical of four independent experiments performed in duplicate.

Because several studies have noted the importance of \(Ca^{2+}\) signaling in the activation of ERK1/2 in osteoblastic cells (24, 40), we investigated the effect of fluid flow-induced \([Ca^{2+}]_i\) increases on ERK1/2 activation. Cells were treated with a series of pharmacological inhibitors 30 min before and during a 15-min exposure to oscillatory fluid flow inducing a shear stress of 20 dyn/cm². Interestingly, thapsigargin (1 \(\mu M\)), which blocked flow-induced \([Ca^{2+}]_i\) increases (Fig. 2), did not significantly affect ERK1/2 phosphorylation in response to flow (Fig. 3C). Flow-induced ERK1/2 phosphorylation was blocked by treatment with U-73122 (10 \(\mu M\)), which also inhibited \([Ca^{2+}]_i\) (Fig. 2), but was unaffected by its noninhibitory analog, U-73343. Because these data suggest a role for PKC in the flow-induced phosphorylation of ERK1/2, we treated hMSCs with 1 \(\mu M\) GF-109203X or 1 \(\mu M\) calphostin C. GF-109203X antagonizes most isoforms of PKC, but in these studies it did not affect flow-induced ERK1/2 phosphorylation. In contrast, preventing the binding of DAG to PKC by treatment with calphostin C attenuated the flow-induced phosphorylation of ERK1/2. The discrepancy between these results may be related to the isoenzyme specificity of PKC inhibitors (63, 68). Treatment with the MEK inhibitor U-0126 (10 \(\mu M\)) also abolished flow-induced increases in ERK1/2 phosphorylation and was used as a positive control. Neither GdCl₃ nor verapamil treatment affected the levels of flow-induced ERK1/2 phosphorylation, and we did not observe an effect of GF-109203X, calphostin C, or U-0126 treatment on flow-induced \([Ca^{2+}]_i\) increases (data not shown).

Because we observed a flow rate-dependent increase in \([Ca^{2+}]_i\), we examined the possibility that ERK1/2 phosphorylation might also be flow rate dependent. Our studies showed that ERK1/2 were equally phosphorylated in response to fluid flow with shear stresses of 5, 10, or 20 dyn/cm² (Fig. 3D). These data may be a result of signal amplification within the MAP kinase signal cascade or the inherent sensitivity of the pathway to extracellular signals such that all three flow rates induced the same phosphorylation response. The results are representative of three independent experiments performed in duplicate.

Calcineurin activity is increased by fluid flow. Because blocking fluid flow-induced increases in \([Ca^{2+}]_i\), did not prevent ERK1/2 phosphorylation, we examined other possible downstream targets of this response, such as activation of the protein phosphatase calcineurin. Control cells consistently exhibited low levels of calcineurin activity, ranging from 310.6 ± 117.1 pM phosphate liberated/mg protein after 1 min to 377.9 ± 76.2 pM phosphate liberated/mg protein after 15 min (Fig. 4). In response to fluid flow, calcineurin activity levels increased significantly to 901.5 ± 101.8 pM phosphate liberated/mg protein after 1 min of flow. Activity levels remained significantly elevated through 5 and 15 min of fluid flow (788.4 ± 139.8 and 766.4 ± 125.2 pM phosphate/mg protein, respectively). Results were obtained from three experiments performed in duplicate.

Fluid flow-induced hMSC proliferation is dependent on ERK1/2 and calcineurin activation. As calcium and MAP kinase signaling have both been implicated in cellular prolif-
eration, we examined the effect of oscillatory fluid flow on hMSC proliferation. hMSCs were exposed to oscillatory fluid flow for 1 h and then incubated for 20 h before labeling with 10 μM BrdU to allow cells time to synthesize the factors necessary for cell cycle progression. ERK1/2 phosphorylation levels were examined by immunoblotting. C: hMSCs were left unflowed or exposed to oscillatory fluid flow for 15 min in the presence of vehicle control, 10 μM U-0126, 1 μM thapsigargin, 10 μM U-7343, 10 μM U-73122, 1 μM GF-109203X, or 1 μM calphostin C. Densitometric analysis of these data revealed 66.3%, 81.8%, and 71.6% decreases in the ratio of phospho-ERK1/2 to total ERK1/2 with U-0126, U-73122, and calphostin C treatment, respectively. D: hMSCs were exposed to oscillatory fluid flow inducing shear stresses ranging from 5 to 20 dyn/cm² for 15 min.

We next examined the effect of fluid flow-induced ERK1/2 and calcineurin activity on flow-induced proliferation by pretreating cells with 10 μM U-0126 or 5 μg/ml cyclosporin A. Inhibition of ERK1/2 activation did not have a significant effect on basal proliferation levels but completely abolished flow-induced increases in hMSC proliferation (92.9 ± 15.3%; Fig. 5B). Treatment with cyclosporin A had a similar effect (127.8 ± 22.6%), suggesting that activation of both of these signaling molecules is an important mediator in the proliferative response of hMSCs to fluid.
flow. Our results were obtained from four independent experiments performed in duplicate.

DISCUSSION

Maintenance of skeletal homeostasis is clearly regulated by biophysical signals. Understanding the mechanisms by which individual bone cells perceive and respond to these signals may serve as a starting point for the treatment of conditions in which bone cell behavior is altered. Although the mechanisms by which osteoblasts and osteocytes perceive and respond to these signals are under scrutiny, little is known about the mechanisms by which MSCs respond to biophysical signals. Accumulating evidence suggests that marrow-derived osteoprogenitors are also mechanoresponsive (37, 39, 54, 59, 62, 74), and therefore understanding the mechanism by which MSCs respond to biophysical signals is as important as understanding that of osteoblasts or osteocytes. MSCs represent a pool of osteoprogenitors, and better insight into the factors that regulate their proliferation and differentiation may provide new therapeutic opportunities to ameliorate bone loss.

Several studies have suggested that increased bone formation in response to mechanical loading is mediated by the response of bone cells to strain-induced fluid flow (33, 64). The deformation of bone tissue during physiological levels of mechanical load (such as ambulatory motion) induces cyclical movement of interstitial fluid flow, with predicted fluid shear rates calculated to be between 8 and 30 dyn/cm² (67). Exposing osteoblasts to these shear rates in vitro induces the expression of the genes associated with bone formation (60, 72) and has been shown to be a more potent stimulus than direct application of physiological levels of mechanical strain (45, 73). In the present study, we sought to examine the effect of fluid flow on hMSC behavior. To date, all estimates of physiological levels of load-induced fluid flow in bone have been calculated with reference to osteocytic cells (67), and, because of structural differences between the cellular environment of marrow and bone, these values may not be directly applicable to MSCs. However, as cells residing in the marrow are intimately associated with bone surfaces, especially in trabecular bone, we hypothesized that MSCs are also exposed to oscillatory fluid flow. To address these issues, we selected three flow rates that encompass the predicted physiological levels of flow that MSCs might experience.

Like osteoblasts and osteocytes, hMSCs responded to fluid flow with a rapid, flow rate-dependent increase in [Ca²⁺]. Absolute increases in [Ca²⁺], in response to fluid flow with shear rates of 5 and 10 dyn/cm² were not different from spontaneous oscillations observed in hMSCs during the static period. Intriguingly, hMSCs responded to a shear stress of 20 dyn/cm² with significantly more robust [Ca²⁺] increases, on the order of 200 nM. Although the magnitude of this observed [Ca²⁺] increase is in accordance with the recent study by Li et al. (37), it is noteworthy that in previous studies conducted with osteoblastic cell models, no difference was observed between the amplitude of flow-induced increases in [Ca²⁺] and spontaneous oscillations observed in cells left unflowed (71–73). Flow-induced calcium responses were dependent on the activation of PLC because treatment with pharmacological agents that either emptied intracellular calcium stores or blocked IP₃ production attenuated flow-induced calcium increases. These data reflect previous studies that examined the mechanism by which fluid flow induces [Ca²⁺] increases in osteoblasts (5, 72). However, although several studies have implicated the L-type calcium channel and mechanosensitive cation channel in bone cell mechanotransduction (12, 36, 72), treating hMSCs with pharmacological antagonists directed

![Graph](image)

**Fig. 4. Fluid flow increases calcineurin phosphatase activity levels.** hMSCs were exposed to oscillatory fluid flow inducing a shear stress of 20 dyn/cm² at 1 Hz for 1–15 min or used as time-matched static controls. Cellular lysates were incubated with a specific phosphopeptide, and liberated phosphate was quantified. *P < 0.05 compared with static control; **P < 0.01 compared with static control.

![Graph](image)

**Fig. 5. Fluid flow induces hMSC proliferation.** A: hMSCs were exposed to oscillatory fluid flow inducing a shear stress of 5, 10, or 20 dyn/cm² for 1 h or left unflowed as static controls and then returned to culture for 20 h in flow medium, and proliferating cells were quantified using fluorescence-activated cell sorting analysis. B: hMSCs were left unflowed or exposed to oscillatory fluid flow (20 dyn/cm²) for 1 h in the presence of vehicle control, 10 μM U-0126, or 5 μg/ml cyclosporin A (CsA). Because of variation between experiments, 5-bromo-2-deoxyuridine (BrdU) results are represented as % of the static control. ***P < 0.001 compared with static control; ###P < 0.001 compared with flow control.
against these channels had no significant effect on either fluid flow-induced Ca\textsuperscript{2+} oscillations or ERK1/2 phosphorylation. These data are supported by recent electrophysiological studies suggesting that only a small fraction of hMSCs cells express functional L-type channels (29) and may mark a major difference in the mechanotransduction pathways of the osteoblast and the osteoprogenitor.

Several studies of osteoblastic cells have noted the importance of [Ca\textsuperscript{2+}]\textsubscript{i} increases in the activation of the MAP kinases ERK1/2 (24, 40). These kinases are vital regulators of osteoblastic proliferation and differentiation and have been shown to mediate flow-induced effects on proliferation (25) and gene expression (65, 72). Indeed, a recent study by Kapur et al. (28) reported the requirement of both ERK1 and ERK2 in the proliferative effect of fluid flow. Oscillatory fluid flow also induced the phosphorylation of ERK1/2 in hMSCs. However, flow-induced ERK1/2 phosphorylation in hMSCs was not dependent on increases in [Ca\textsuperscript{2+}]\textsubscript{i}, because ERK1/2 phosphorylation was blocked by PKC and PLC antagonists but not by agents that blocked only calcium release. Rather, our data imply that flow-induced calcium responses are likely to be responsible for the activation of calcineurin. Calcineurin activity levels were dramatically increased after 1 min of oscillatory fluid flow exposure and remained elevated through 15 min.

Although these data suggest that in hMSCs the calcium and MAP kinase responses are initially independent of one another, their actions appear to converge and induce a proliferative effect in response to oscillatory fluid flow. Fluid flow inducing a shear stress of 20 dyn/cm\textsuperscript{2} triggered a 126% increase in hMSC proliferation, a response that was attenuated by inhibiting both phosphorylation of ERK1/2 and activation of calcineurin with U-0126 and cyclosporin, respectively. Previous studies using nonosteoblastic cell models provide support for this signaling mechanism. MAP kinase and calcineurin signaling have both been implicated in the expression, accumulation, and activity of cyclin D, a protein necessary for the G1-to-S transition in the cell cycle (7, 27, 35). Furthermore, both signaling molecules have been implicated in cardiac hypertrophy by codependently coordinating cardiac cell growth (55). Our data suggest that ERK1/2 may have a secondary role relative to calcium signaling in the mechanism by which oscillatory fluid flow induces proliferation. The rationale for this hypothesis is that ERK1/2 was equally phosphorylated at all flow rates, but proliferation was evident only at a flow rate that caused the most robust increase in [Ca\textsuperscript{2+}]\textsubscript{i}. It should also be noted that despite its well-recognized use and efficacy as an inhibitor of calcineurin, cyclosporin A may produce nonspecific side effects via the stimulation of superoxide (32). However, at least two studies have suggested that this side effect is associated with an increase in cell proliferation and DNA synthesis (43, 66), the opposite of the data presented herein.

In contrast to the aforementioned study by Li et al. (37), which demonstrated a 57% increase in hMSC proliferation in response to fluid flow inducing a shear stress of 10 dyn/cm\textsuperscript{2}, we observed a statistically significant increase in cellular proliferation only when cells were exposed to a higher flow rate that induced a shear stress of 20 dyn/cm\textsuperscript{2}. This discrepancy is likely due to differences in experimental procedure (i.e., serum concentrations) and/or assay sensitivity (FACS vs. histological quantification). Because we observed an increase in proliferation only at the flow rate that induced the greatest increase in [Ca\textsuperscript{2+}]\textsubscript{i}, we hypothesize that a robust calcium response and the activation of downstream signaling molecules in the presence of activated MAP kinases may act as the molecular switch to induce hMSC proliferation in response to fluid flow.

Because of the oscillatory nature of the flow regime used in these studies, there exists the potential for medium moving across the hMSC monolayer to become enriched in secreted factors and metabolites. There are conflicting reports regarding whether such chemotransport may affect bone cell behavior. We previously demonstrated (11) that chemotransport contributes to the responsiveness of osteoblastic cell models to oscillatory fluid flow. However, studies using primary bone cells suggest that shear rate plays a more important role in mechanotransduction than chemotransport (2, 51). Clearly, further study is necessary to clarify the contribution of both chemotransport and fluid shear stresses in the physiological response of bone to mechanical loading.

Although these studies highlight subtle differences that exist between the signaling cascades activated in response to fluid flow in more mature cells of the osteoblastic lineage and hMSCs, together these data suggest that a common mechanotransduction pathway exists by which cells of the osteoblastic lineage respond to oscillatory fluid flow. Calcium transients were blocked by inhibiting calcium release from intracellular stores in hMSCs as is the case in osteoblastic cell lines, and flow-induced proliferation is dependent on the activation of ERK1/2 (25, 28). An intriguing possibility is that the observed effect of fluid flow on hMSCs is mediated by the release of ATP as has been proposed in osteoblasts (4, 17, 53). The expression of purinergic receptors by hMSCs (unpublished data) supports this hypothesis, and such receptors have been shown to mediate the response of osteoblastic cells to oscillatory fluid flow (71). Indeed, exogenous ATP has been shown to induce a mitogenic response in MC3T3-E1 preosteoblasts (58). Investigation of this possibility may provide further evidence for a common mechanotransduction pathway and a broader understanding of the mechanism by which fluid flow regulates hMSC activity.

In summary, this study outlines for the first time the intracellular mechanism by which oscillatory fluid flow affects human mesenchymal stem cells. We demonstrated that fluid flow induces an increase in hMSC proliferation via a mechanism that causes an increase in [Ca\textsuperscript{2+}]\textsubscript{i}, and activates ERK1/2, presumably through the action of PLC and PKC. These data suggest that fluid flow regulates not only osteoblastic behavior but also that of hMSCs and imply that a common pathway exists by which mechanical signals are translated to cellular responses. Further clarification of the intricacies of this pathway may lead to the development of novel therapeutic techniques designed to enhance the recruitment of osteoprogenitors and ameliorate bone loss.

ACKNOWLEDGMENTS

We thank Dr. Marnie Saunders of the Department of Orthopaedics and Nate Sheaffer of the Cell Science/Flow Cytometry Core Facility of the Section of Research Resources, Penn State College of Medicine, for technical expertise and assistance.

GRANTS

This work was supported by National Institute on Aging Grant AG-13087 (to H. J. Donahue) and National Aeronautics and Space Administration Predoctoral Fellowship NGT5-50366 (to D. C. Genetos).
REFERENCES


