DEVELOPMENTAL BIOLOGY. For the article “Primary cilia mediate mechanosensing in bone cells by a calcium-independent mechanism,” by Amanda M. D. Malone, Charles T. Anderson, Padmaja Tummala, Ronald Y. Kwon, Tyler R. Johnston, Tim Stearns, and Christopher R. Jacobs, which appeared in issue 33, August 14, 2007, of Proc Natl Acad Sci USA (104:13325–13330; first published August 2, 2007; 10.1073/pnas.0700636104), the authors wish to note the following. “The authors have realized that the concentration of BAPTA used in the experiment shown in Fig. 4C is insufficient to fully chelate extracellular calcium. Thus, our conclusion that extracellular calcium entry is not required for calcium flux in MC3T3-E1 cells is invalid. However, our results demonstrating a lack of correlation between primary cilia and calcium flux, calcium flux in cells lacking primary cilia, and calcium flux after treatment with gadolinium chloride support our conclusion that mechanosensation via the primary cilium is independent of calcium flux and polycystin 2 in bone cells. The authors regret any confusion this error has caused.”

MEDICAL SCIENCES. For the article “Mechanism of and requirement for estrogen-regulated MYB expression in estrogen-receptor-positive breast cancer cells,” by Yvette Drabsch, Honor Hugo, Rui Zhang, Dennis H. Dowhan, Yu Rebecca Miao, Alan M. Gewirtz, Simon C. Barry, Robert G. Ramsay, and Thomas J. Gonda, which appeared in issue 34, August 21, 2007, of Proc Natl Acad Sci USA (104:13762–13767; first published August 9, 2007; 10.1073/pnas.0700104104), the authors note that on page 13767, left column, in Myb Knockdown by RNA Interference, the first sequence in the second sentence, “5’-GAACCUCUUAC-AUUUUCAGAAAACCUUUCAUGAGAAGUGUUCUGC-AUUGUGUAAGG GUUCUU-3’,” should instead read: “5’-GAACCUCUUAGAA UUUGCAGAAAACCUUUCAUGAG-AAGUGUUCUGCAA AUUCUAAGG GUUCUU-3’.” This error does not affect the conclusions of the article.

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Primary cilia mediate mechanosensing in bone cells by a calcium-independent mechanism


*Palo Alto Veterans Administration Medical Center, Palo Alto, CA 94304; Departments of †Mechanical Engineering, ‡Bioengineering, and §Biological Sciences, Stanford University, Stanford, CA 94305; and ¶Department of Genetics, Stanford University Medical School, Stanford, CA 94305

Edited by Robert J. Lefkowitz, Duke University Medical Center, Durham, NC, and approved June 15, 2007 (received for review January 23, 2007)

Primary cilia are sensory organelles that translate extracellular chemical and mechanical cues into cellular responses. Bone is an exquisitely mechanosensitive organ, and its homeostasis depends on the ability of bone cells to sense and respond to mechanical stimuli. One such stimulus is dynamic fluid flow, which triggers biochemical and transcriptional changes in bone cells by an unknown mechanism. Here we report that bone cells possess primary cilia that project from the cell surface and deflect during fluid flow and that these primary cilia are required for osteogenic and bone resorptive responses to dynamic fluid flow. We also show that, unlike in kidney cells, primary cilia in bone translate fluid flow into cellular responses in bone cells independently of Ca²⁺ flux and stretch-activated ion channels. These results suggest that primary cilia might regulate homeostasis in diverse tissues by allowing mechanical signals to alter cellular activity via tissue-specific pathways. Our identification of a mechanism for mechanotransduction in bone could lead to therapeutic approaches for combating bone loss due to osteoporosis and disuse.

Results

Primary Cilia Extend from the Surface of Bone Cells and Deflect During Fluid Flow. To investigate whether primary cilia might play a role in mechanotransduction in bone, we sought to determine whether primary cilia extend from bone cells into the extracellular environment and, if so, whether bone cell primary cilia are deflected by fluid flow. Xiao et al. (17) found cilium-like structures in MC3T3-E1 osteoblasts and MLO-Y4 osteocytes to establish that these structures extended from the centrosome and were in fact primary cilia, these cells were stained for acetylated α-tubulin, which is enriched in ciliary microtubules (33), and the centrosomal component CEP135 (34). Both MC3T3-E1 and MLO-Y4 cells possess acetylated α-tubulin-positive primary cilia extending from the centrosome (Fig. 1A and B and Table 1). To determine whether primary cilia protrude from the surface of bone cells (35), MC3T3-E1 cells were stained for acetylated α-tubulin and 5-chloromethylfluorescein diacetate (CMFDA) to label the cell surface, and confocal image...
A siRNA: polaris actin control polaris

Fig. 2. RNAi of polaris prevents primary cilium formation in cultured bone cells. (A) Western blot showing reduced levels of protein in MC3T3-E1 osteoblasts transfected with polaris siRNA (right lane) relative to cells transfected with control siRNA (left lane). Western blot for actin is a loading control. (B) Control MC3T3-E1 osteoblasts showing polaris (green) at the primary cilium (arrowhead) marked by acetylated α-tubulin (red). DNA is stained with DAPI (blue). (C) Polaris siRNA-treated MC3T3-E1 osteoblasts showing reduced polaris staining (green) and the absence of a primary cilium extending from the centrioles (arrowhead). Stable cytoplasmic microtubules are marked by acetylated α-tubulin (red) and DNA is stained with DAPI (blue). (Scale bars: 10 μm.)

prevented primary cilium formation by siRNA-mediated depletion of the IFT component polaris, which is required for primary cilium biogenesis and function (37). Depletion of polaris was confirmed by Western blotting and immunofluorescence (Fig. 2) and caused ~50% reduction in the fraction of cells with primary cilia (Table 1). siRNA transfection efficiencies of 82.7 ± 4.5% of cells were obtained, as assayed by Cy3-labeled siRNA, indicating that some transfected cells still possessed levels of polaris sufficient to grow ciliary axonemes.

Primary Cilia Are Required for Osteogenic and Bone Resorptive Responses to Dynamic Fluid Flow. Osteoblasts and osteocytes respond to dynamic fluid flow with characteristic increases in gene expression and cytokine release. For example, bone-forming osteoblasts up-regulate the expression of osteopontin (OPN), a bone matrix protein, in response to flow (14). We measured the levels of OPN mRNA in MC3T3-E1 osteoblasts after exposure to oscillatory fluid flow. In untreated cells, exposure to flow resulted in a 3-fold increase in OPN mRNA levels ($P < 0.01$), whereas in cells treated with chloral hydrate, exposure to flow did not result in increased levels of OPN mRNA (Fig. 3A). OPN mRNA levels increased 4.4-fold in chloral hydrate-treated cells exposed to 10 nM vitamin D (Sigma, St. Louis, MO) (38), demonstrating that chloral hydrate does not inhibit OPN gene expression nonspecifically (data not shown). In cells treated with control siRNA, exposure to flow resulted in a 3-fold increase in OPN mRNA ($P < 0.005$), whereas in cells treated with siRNA targeting polaris, exposure to flow did not result in increased OPN mRNA levels and in fact caused a 50% reduction in OPN mRNA ($P < 0.05$) (Fig. 3A).

The cytokine prostaglandin $E_2$ (PGE$_2$) is produced by osteoblasts and osteocytes after mechanical loading in vivo and regulates bone metabolism (39). Because PGE$_2$ is released by cultured osteoblasts and osteocytes in response to dynamic fluid flow (40, 41), we asked whether flow-induced PGE$_2$ release requires primary cilia. In untreated MC3T3-E1 cells, exposure to flow resulted in a 3.7-fold increase in PGE$_2$ release ($P < 0.001$),

Table 1. Percentage of cells with primary cilia under various conditions

<table>
<thead>
<tr>
<th>Condition</th>
<th>MC3T3-E1</th>
<th>MLO-Y4</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>60.8 ± 8.4</td>
<td>62.0 ± 15.2</td>
</tr>
<tr>
<td>Chloral hydrate</td>
<td>10.2 ± 6.1</td>
<td>6.8 ± 2.2</td>
</tr>
<tr>
<td>Control siRNA</td>
<td>58.3 ± 7.9</td>
<td>47.8 ± 2.1</td>
</tr>
<tr>
<td>Polaris siRNA</td>
<td>35.6 ± 13.9</td>
<td>25.5 ± 8.0</td>
</tr>
</tbody>
</table>

Total cells for each condition: $n = 400$. ± represents SD from at least four experiments.
whereas in cells treated with chloral hydrate, exposure to flow resulted in no change in PGE2 release (Fig. 3B). As a control, MC3T3-E1 and MLO-Y4 cells were treated with chloral hydrate, and they displayed 13-fold and 3.3-fold increases in PGE2 release, respectively, after 40-min exposure to 200 μM A23187 calcimycin (Sigma), a calcium ionophore known to induce PGE2 release (42) (data not shown). In cells treated with control siRNA, exposure to flow resulted in a 2.2-fold increase in PGE2 release (Fig. 3B). In MLO-Y4 cells, increases in PGE2 release with flow were also abrogated by treatment with chloral hydrate or polaris siRNA (data not shown).

We next examined whether primary cilia mediate flow-induced changes in cyclooxygenase 2 (COX2) gene expression. COX2 converts arachidonic acid to prostaglandin H2, which is subsequently converted into PGE2 in bone cells (43). In untreated cells, exposure to flow resulted in a 2-fold increase in COX2 mRNA levels (P < 0.005), whereas in cells treated with chloral hydrate, exposure to flow did not result in increased COX2 mRNA levels (Fig. 3C). In cells treated with control siRNA, exposure to flow resulted in a 1.9-fold increase in COX2 mRNA (P < 0.005), but this increase was eliminated in cells treated with siRNA targeting polaris (Fig. 3C). This increase was also observed in MC3T3-E1 cells and was lost with chloral hydrate or polaris siRNA treatment (data not shown).

We hypothesized that primary cilia might also play a role in bone resorption. The osteoprotegerin (OPG)/receptor activator of NF-κB ligand (RANKL) signaling system regulates the formation rate of bone-resorbing osteoclasts (12, 44, 45). RANKL binds to receptor activator of NF-κB (RANK) on preosteoclasts and promotes osteoclast differentiation, which is inhibited by OPG, a soluble decoy receptor of RANKL. Both osteocytes and osteoblasts produce OPG and RANKL, but MLO-Y4 cells can support osteoclast formation, whereas MC3T3-E1 cells cannot (46). Thus, we examined OPG/RANKL ratios in MLO-Y4 cells. In untreated cells, exposure to flow resulted in a 1.6-fold increase in the ratio of OPG/RANKL mRNA (P < 0.005), whereas in cells treated with chloral hydrate, exposure to flow did not result in significant changes in the ratio of OPG/RANKL mRNA (Fig. 3D). In cells treated with control siRNA, exposure to flow resulted in a 2.2-fold increase in the ratio of OPG/RANKL mRNA levels (P < 0.02), and this increase was eliminated in cells treated with siRNA targeting polaris (Fig. 3D).

Ca2+ Flux in Bone Cells During Flow Is Independent of Primary Cilia and Polycystin 2. Deflecting primary cilia in kidney cells causes Ca2+ influx through the stretch-sensitive Ca2+ channel polycystin 2, triggering intracellular Ca2+ release (24, 27, 47). We and others (14, 48) have shown that bone cells respond to fluid flow with increases in intracellular Ca2+. Thus, we sought to determine whether flow-induced Ca2+ flux in bone cells was mediated by primary cilia. On the basis of the observation that ~50% of MC3T3-E1 cells exhibit Ca2+ flux during flow (14) and that ~60% of these cells possess primary cilia (Table 1), we hypothesized that only cells possessing primary cilia display Ca2+ flux during flow. To test this hypothesis, Ca2+ flux was measured during flow in individual MC3T3-E1 osteoblasts (Fig. 4A). The same cells were then stained for acetylated α-tubulin and scored for primary cilia (Fig. 4A’). Unexpectedly, 64 of 172 cells that lacked primary cilia displayed Ca2+ flux during flow, and there was not a significant correlation (P = 0.11, χ2 test) between possession of a primary cilium and Ca2+ flux. Also, many cells (114/207) that possessed primary cilia did not exhibit Ca2+ flux during flow. Similarly, no correlation between primary cilia and Ca2+ flux was found in MLO-Y4 osteocytes, with 73/150 cells without cilia exhibiting Ca2+ flux (P = 0.44, χ2 test) and 99/178 cells with cilia not displaying Ca2+ flux during flow.
In kidney cells, primary cilium-mediated Ca\textsuperscript{2+} flux propagates between cells via gap junctions (24). Thus, cells without primary cilium might exhibit Ca\textsuperscript{2+} flux during flow because of propagation of Ca\textsuperscript{2+} from neighboring cells, rather than in direct response to flow. To eliminate this confounding effect, cells were scored for Ca\textsuperscript{2+} flux and the presence of primary cilium while gap junctions were blocked using 30 \( \mu \)M 18a-glycyrhetinic acid (Sigma) (49). Cells that lacked cilia still responded to flow (102/159 cells) after gap junction inhibition, and there was no significant correlation between the presence of primary cilia and Ca\textsuperscript{2+} flux (\( P = 0.11, \chi^2 \) test), indicating that cell–cell communication could not account for the observed lack of correlation.

To test whether Ca\textsuperscript{2+} flux during flow can occur independently of primary cilia in bone cells, MC3T3-E1 osteoblasts were treated with either chloral hydrate to remove primary cilia or siRNA targeting polars to prevent cilium formation and then assayed for Ca\textsuperscript{2+} flux during flow. Neither treatment significantly altered the fraction of cells exhibiting Ca\textsuperscript{2+} flux relative to control cells (Fig. 4B). Previous work (24, 26) establishing the role of the primary cilia in flow-induced Ca\textsuperscript{2+} flux used kidney cells exposed to constant flow. Our finding that Ca\textsuperscript{2+} flux in response to dynamic flow is independent of primary cilia in bone cells suggests either that kidney and bone cells differ in their primary cilium-mediated responses to fluid flow or that steady and dynamic flow cause different cellular responses. To distinguish between these possibilities, constant flow was applied to MC3T3-E1 cells after chloral hydrate treatment. As was the case for untreated cells, a high percentage of these cells exhibited Ca\textsuperscript{2+} flux (data not shown). Also, a greatly reduced percentage of MDCK kidney cells treated with chloral hydrate displayed Ca\textsuperscript{2+} flux during dynamic flow (Fig. 4B), similar to experiments using steady flow (26). These data suggest that the mechanisms responsible for cilia-mediated mechanosensation in MC3T3-E1 bone cells and MDCK kidney cells are indeed distinct.

We next tested whether polycystin 2 is involved in Ca\textsuperscript{2+} response in bone cells. In kidney cells, removal of extracellular Ca\textsuperscript{2+} or treatment with the stretch-activated ion channel inhibitor gadolinium chloride (25, 50) eliminates flow-induced Ca\textsuperscript{2+} flux. In contrast, we found that treating MC3T3-E1 osteoblasts with 10 \( \mu \)M gadolinium chloride (Sigma) for 30 min or removing extracellular Ca\textsuperscript{2+} by pretreating flow medium with 90 \( \mu \)M BAPTA (Sigma) did not eliminate flow-induced Ca\textsuperscript{2+} flux (Fig. 4C). Thus, extracellular Ca\textsuperscript{2+} entry and polycystin 2 apparently do not play a role in flow-induced Ca\textsuperscript{2+} response in bone cells. These data, in combination with our findings that bone cells lacking primary cilia exhibit Ca\textsuperscript{2+} flux during flow, indicate that primary cilia act as flow sensors in bone cells by a mechanism that is different from that of kidney cells.

**Discussion**

Many tissues are sensitive to mechanical stimuli, and this sensitivity is essential for their physiological functioning. However, the mechanisms by which cells sense and respond to mechanical stimuli are understood in remarkably few cases. In bone, this question is compelling because the deformations due to loading are small, yet loading is required in order to maintain the structure and function of the skeleton. In this study, we present evidence that primary cilia act as mechanosensors in bone cells, providing a potential mechanism by which mechanical stimuli are translated into osteogenic and bone resorative responses. We demonstrate that cilia-mediated mechanotransduction in bone differs from that described in other tissues, particularly kidney epithelium, with respect to the molecular events that initiate intracellular signaling. We also show that that primary cilia sense dynamic flow, in addition to the constant flow that has been examined in the context of the kidney. Our evidence that the primary cilium is a mechanosensor in bone highlights it as a potential therapeutic target for efforts to prevent bone loss during disease and disuse.

We first show that primary cilia protrude from the apical surface of bone cells and are deflected during fluid flow. These results, in combination with scanning electron microscopy data showing that primary cilium-like structures are external in MLO-Y4 osteocytes (17), indicate that bone cell primary cilia possess physical properties consistent with a flow-sensing function. However, it is important to note that these in vitro experiments do not reproduce the complex 3D environment of osteocytes and osteoblasts. It is possible that bone cell primary cilia interact with extracellular matrix proteins, as has been shown in cartilage (51), and if this were the case, integrins on the primary cilium could translate deformations of the extracellular matrix into intracellular signals, amplifying mechanical stimuli. Further experiments will be required in order to characterize the structural and molecular environment of the primary cilium in bone tissue.

It should be noted that the methods we used to abrogate primary cilia have important limitations. Chloral hydrate removes primary cilia, possibly by disrupting the junction of the cilium and the basal body (52), but is also known to disrupt mitosis (53). Although chloral hydrate could thus have nonspecific effects on cell physiology, it is currently one of the only chemical methods for removing primary cilia from vertebrate cells. We used a protocol (26) in which chloral hydrate was removed for 24 h before experiments were performed and found that 24 h after drug removal, cells possessed normal morphology and cytoplasmic microtubules but low numbers of primary cilia (Table 1). Primary cilium did eventually recover, returning to pretreatment numbers 72 h after drug removal (data not shown). After polaris siRNA treatment, only 50% fewer cells had primary cilia than control cells (Table 1), but a complete loss of flow response was observed in these cells (Fig. 3). A possible explanation for this discrepancy is that the efficiency of IFT might decrease upon reduction of polaris protein levels, preventing delivery of functional ciliary components to some fraction of the remaining cilia. Indeed, ciliary localization of polycystin 2 is lost after moderate RNAi of another IFT component, IFT20, even though the ciliary axonomes appear normal (54), suggesting that axoneme growth is not sufficient for ciliary function. However, the consistency of the results we obtained by using these two independent methods to abrogate primary cilia, combined with control experiments (see Results), allowed us to confidently assess the role of primary cilia in sensing flow in bone cells.

We also show that primary cilia are required for bone-specific cellular responses to dynamic fluid flow. We found that primary cilia are required for flow-induced increases in OPN mRNA levels in MC3T3-E1 cells and for increases in PGE\textsubscript{2} release and COX2 mRNA levels in both MLO-Y4 and MC3T3-E1 cells, indicating that primary cilia play a role in osteogenic responses to flow. Primary cilia are also required in order to increase the ratio of OPG/RANKL mRNA after fluid flow in MLO-Y4 cells, suggesting that primary cilia may play an antiresorptive role in osteocytes. Although these results do not allow us to conclude whether primary cilia mediate bone resorption in vivo, they show that flow-induced increases in the OPG/RANKL mRNA ratio are dependent on primary cilia. Our results suggest that primary cilia may contribute to the balance between bone formation and resorption that is necessary for bone homeostasis. A complication in studying the role of primary cilia in bone homeostasis is that primary cilia have been implicated in the developmental and patterning steps that precede the formation of mineralized bone (17, 32). Thus, in vivo experiments to determine whether primary cilia play a role in mechanosensation and homeostasis in bone will require the ability to manipulate the presence of primary cilia after bone has fully developed.
We have shown that in bone cells, primary cilia are responsible for changes in cellular activity after mechanical stimulation, similar to the case of kidney cells (7). However, unlike kidney cells, in which primary cilia mediate polycystin 2-dependent Ca²⁺ entry and intracellular Ca²⁺ release in response to fluid flow, bone cells that lack primary cilia exhibit Ca²⁺ flux in response to fluid flow. This response persists after inhibition of stretch-sensitive ion channels or chelation of extracellular Ca²⁺, indicating that Ca²⁺ entry is not required for intracellular Ca²⁺ flux in bone cells. Thus, our results suggest that a Ca²⁺ entry- and polycystin 2-independent signaling mechanism links primary cilia to downstream cellular responses in bone cells. This is consistent with previous evidence (40) that some osteogenic responses to mechanical stimulation are Ca²⁺-independent. In cholangiocytes, primary cilia mediate changes in cAMP levels during flow (55), and in kidney cells, proteolysis of polycystin 1 is involved in transcriptional responses to flow (7). Further experiments will be required in order to determine whether these or other signaling pathways could connect flow-induced binding of the primary cilium to the cilium-dependent cellular responses we have observed in bone cells.

It is becoming increasingly clear that primary cilia act in diverse cell and tissue types to detect a range of chemical and physical signals. One unanswered question is to what extent primary cilia act in concert with other cellular sensory systems. Our data indicate that flow-induced Ca²⁺ flux in bone cells is independent of primary cilia, but both Ca²⁺ flux and MAPK phosphorylation have been shown to be required for flow-induced increases in OPN gene expression (14), although it is not clear whether these two signals are part of the same signaling pathway. We show here that primary cilia are required for flow-induced increases in OPN mRNA levels but are not required for Ca²⁺ flux, which we previously demonstrated is required for the OPN response to flow. This would suggest that multiple mechanosensitive pathways act in concert to contribute to changes in OPN expression. Our results also raise the possibility that, as is the case for PDGF (20), flow might induce MAPK phosphorylation by means of the primary cilium. Furthermore, there is evidence that focal adhesions play a role in mechanosensation in bone cells (56, 57), and primary cilium-mediated signaling could be integrated with focal adhesion-mediated signaling to allow cells to incorporate multiple mechanical signals into the appropriate cellular response. In their role as mechanosensors, primary cilia could thus be part of a comprehensive array of sensory tools that allow bone cells to maintain bone homeostasis.

Methods

Cell Culture. MC3T3-E1 and MLO-Y4 cells were cultured as described in ref. 58; MDCK kidney cells were cultured in DMEM (Invitrogen, Carlsbad, CA) with 10% FBS and 1% penicillin/streptomycin. Cells were maintained at 37°C and 5% CO₂. For Ca²⁺ imaging experiments, cells were grown on tissue culture treated polyester membranes (Cornwell, WA). For PGE₂ and gene expression experiments, cells were grown on 1.6-mm UV-transparent quartz slides (Quartz Scientific, Vancouver, WA). For PGE₂ and gene expression experiments, each group had ≥4 slides, and for gene expression and PGE₂ experiments, each group had ≥6 slides. All experiments were repeated over multiple days, and for each experimental set of slides, a control set was tested in parallel to ensure that any differences detected were not due to performing flow experiments and response assays at distinct times. To compare no-flow vs. flow responses, a two-sample Student t test was used, in which sample variance was not assumed to be equal. To compare observations from more than two groups, a one-way ANOVA was used followed by a Bonferroni selected-pairs multiple comparisons test.

Immunofluorescence. Cells were fixed in −20°C methanol and processed for immunofluorescence as in ref. 59, using 6-11B-1 anti-acetylated α-tubulin (1:2,000, Sigma), rabbit anti-CEP135 (1:500; a gift from R. Kuriyama, University of Minnesota, Minneapolis, MN), or rabbit anti-polaris (1:500; a gift from B. Yoder, University of Alabama at Birmingham, Birmingham, AL) primary antibodies, followed by incubation with goat anti-mouse Alexa 594 and goat anti-rabbit Alexa 488 secondary antibodies (1:200; Molecular Probes, Eugene, OR). DNA was stained with DAPI (1:100,000 of a 5 mg/ml stock; Sigma).

Confocal Imaging of Primary Cilia. Cells were stained for acetylated α-tubulin and the cell body was stained using 5 μM CMFDA (CellTracker Green; Molecular Probes). Cells were imaged on a Nikon (Florham Park, NJ) C-1 confocal microscope using a 1.4 numerical aperture ×60 violet corrected objective. Image stacks were deconvolved by using a point-spread function free adaptive algorithm (Media Cybernetics, Silver Spring, MD).

Visualization of Primary Cilia in Side View. MC3T3-E1 cells were grown on tissue culture treated polyester membranes (Corning, Corning, NY). Membranes were excised, folded in half cell-side out, and positioned in a small flow chamber so that the folded edge was parallel to the direction of flow and aligned with the chamber edge. Cells were imaged for 20 sec (5 sec before and after flow) at 10 frames per second under ×40 magnification using Hoffman modulation contrast and exposed to a 10-sec pulse of ~0.03 Pa steady flow. Images were contrast-enhanced using Image J software (National Institutes of Health, Bethesda, MD).

Abrogation of Primary Cilia. To prevent cilium formation by knockdown of polaris, 20 μM siRNA targeting polaris (sequence: 5′-CCAGAAACAGATGAGGACGCCTT-3′) or 20 μM scrambled control siRNA (Invitrogen) was transfected into cells by using XtremeGene (Roche Molecular Systems, Alameda, CA). Medium was changed 24 h after transfection, and flow experiments were performed 48 h after transfection. Neither the scrambled siRNA nor the polaris siRNA had any significant effect on overall cellular morphology. To remove primary cilia, 4 mM aqueous choleular hydrate (Sigma) was added to cells for 72 h, cells were washed three times with PBS, and fresh medium was added for 24 h before flow experiments (26).

Western Blotting. Total cellular protein was isolated in RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA), and total protein quantity was determined by BCA assay (Pierce, Rockford, IL). Samples were separated by electrophoresis in 4–12% precast NuPAGE Bis-Tris gels (Invitrogen), transferred onto nitrocellulose membranes (Invitrogen), and probed with rabbit anti-polaris (1:5,000), anti-actin (1:1,000; Santa Cruz Bio-technology) antibodies. Bound primary antibodies were detected by chemiluminescent detection of HRP-conjugated goat anti-rabbit antibodies.

Fluid Flow. A previously described fluid flow device (14) was used to deliver oscillatory laminar fluid flow with a period of 1 Hz and a cell surface shear stress of 1 Pa. Shear stress was generated using a peak flow rate of 9 ml/min in the small chamber (38 × 10 × 0.28 mm) used for Ca²⁺ experiments and 18.8 ml/min in the large chamber (56 × 24 × 0.28 mm) used for all other experiments. For steady flow experiments, a syringe pump (KD Scientific, New Hope, PA) was used to generate flow at 9 ml/min. For calcium experiments, each group had ≥4 slides, and for gene expression and PGE₂ experiments, each group had ≥6 slides. All experiments were repeated over multiple days, and for each experimental set of slides, a control set was tested in parallel to ensure that any differences detected were not due to performing flow experiments and response assays at distinct times. To compare no-flow vs. flow responses, a two-sample Student t test was used, in which sample variance was not assumed to be equal. To compare observations from more than two groups, a one-way ANOVA was used followed by a Bonferroni selected-pairs multiple comparisons test.

PGE₂ Assay. After 1 h of flow, slides were removed from flow chambers, placed in dishes with 1 ml of medium, and incubated for 1 h. Medium PGE₂ levels were measured by using an enzyme immunoassay kit (Amersham, Piscataway, NJ). PGE₂ levels were
normalized to total protein as determined by BCA assay (Pierce). All samples and standards were run in duplicate.

**Real-Time RT-PCR.** Real-time quantitative RT-PCR was performed using the ABI Prism 7900 detection system. Primers and probes for 18S, OPN, COX2, OPG, RANKL, and GAPDH were obtained from Applied Biosystems (Foster City, CA). Immediately after 1-h flow, total RNA was extracted with TriReagent (Invitrogen), and cDNA was synthesized using the TaqMan reverse transcription kit (Applied Biosystems). cDNA samples were then amplified by real-time PCR. Amplification curves for control and experimental genes were recorded, and relative gene levels between samples were quantified by using the relative standard curve method (ABI Prism 7700 User Bulletin 2; Applied Biosystems). All samples were normalized to endogenous control 18S rRNA. All samples were run in triplicate. GAPDH levels were measured as a control to verify that global gene expression levels did not increase after flow (data not shown).

**Ca2+ Imaging.** Intracellular Ca2+ was quantified by using ratio-metric imaging (14). Before exposure to flow, cells were incubated with 10 μM Fura-2-AM (Molecular Probes) for 30 min. Slides were then mounted in a small chamber and placed on a Nikon Eclipse TE-300. Flow medium consisted of MEM-α and 2% FBS. Fluorescence intensity was recorded (Metafluor; Universal Imaging, Downingtown, PA) every 2 sec for 3 min before flow and for 3 min during flow. A response was defined as a transient increase in total cellular fluorescence intensity of at least four times the maximum intensity increase recorded during the 3 min before flow.

**Ca2+ Response/Cilium Correlation.** Slide backs were marked with a diamond scribe, and Ca2+ response of cells at a mark was quantified during flow as described above. After flow, slides were fixed in −20°C methanol and stained for primary cilia, again as described above. The field of cells analyzed for Ca2+ response was found and scored for the presence of primary cilia. Cells were scored blindly for Ca2+ response (Y/N) and primary cilia (Y/N), and correlation significance was tested by χ2 analysis.

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