The primary cilium functions as a mechanical and calcium signaling nexus

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Abstract

Background: The primary cilium is an antenna-like, nonmotile structure that extends from the surface of most mammalian cell types and is critical for chemosensing and mechanosensing in a variety of tissues including cartilage, bone, and kidney. Flow-induced intracellular calcium ion (Ca²⁺) increases in kidney epithelia depend on primary cilia and primary cilium-localized Ca²⁺-permeable channels polycystin-2 (PC2) and transient receptor potential vanilloid 4 (TRPV4). While primary cilia have been implicated in osteocyte mechanotransduction, the molecular mechanism that mediates this process is not fully understood. We directed a fluorescence resonance energy transfer (FRET)-based Ca²⁺ biosensor to the cilium by fusing the biosensor sequence to the sequence of the primary cilium-specific protein Arl13b. Using this tool, we investigated the role of several Ca²⁺-permeable channels that may mediate flow-induced Ca²⁺ entry: PC2, TRPV4, and PIEZO1.

Results: Here, we report the first measurements of Ca²⁺ signaling within osteocyte primary cilia using a FRET-based biosensor fused to ARL13B. We show that fluid flow induces Ca²⁺ increases in osteocyte primary cilia which depend on both intracellular Ca²⁺ release and extracellular Ca²⁺ entry. Using siRNA-mediated knockdowns, we demonstrate that TRPV4, but not PC2 or PIEZO1, mediates flow-induced ciliary Ca²⁺ increases and loading-induced Cox-2 mRNA increases, an osteogenic response.

Conclusions: In this study, we show that the primary cilium forms a Ca²⁺ microdomain dependent on Ca²⁺ entry through TRPV4. These results demonstrate that the mechanism of mechanotransduction mediated by primary cilia varies in different tissue contexts. Additionally, we anticipate that this work is a starting point for more studies investigating the role of TRPV4 in mechanotransduction.

Keywords: Mechanotransduction, Primary cilium, Calcium signaling, Osteocyte, Kidney epithelia, Biosensor

Background

Mechanotransduction is a process by which cells sense and convert mechanical signals into biochemical and transcriptional changes. The calcium ion (Ca²⁺) is a ubiquitous second messenger that regulates numerous signaling pathways. Despite how universal Ca²⁺ is, discrete intracellular signaling mechanisms occur because Ca²⁺ gradients are spatiotemporal and do not comprise one general pool that changes uniformly. For example, there are discrete microdomain Ca²⁺ signals including “Ca²⁺ sparks,” “Ca²⁺ sparklets,” and “scraps” that modulate constriction and relaxation in vascular smooth muscle cells [1,2]. Mechanical loading generates rapid and temporal intracellular Ca²⁺ increases in many cell types including osteocytes, osteoblasts, neurons, and kidney cells. Ca²⁺ mobilization is required for flow-induced prostaglandin E2 (PGE₂) release and flow-induced osteopontin gene regulation in osteocytes, demonstrating that Ca²⁺ is upstream of mechanotransduction activities and paracrine signaling [3,4].

The primary cilium is an antenna-like, nonmotile structure that extends from the surface of most mammalian cell types into the extracellular space [5,6]. While initially considered a vestigial structure, in the past decade, several labs have demonstrated that primary cilia are critical for chemosensing and mechanosensing in a variety of tissues including cartilage, bone, and kidney.
The osteocyte primary cilium deflects with mechanical stimulation and mediates mechanotransduction at the transcriptional level; however, previous experiments have not resolved Ca$^{2+}$ within the primary cilium from the cytosol [14].

Recent advances in monitoring ciliary Ca$^{2+}$ mobilization have improved our understanding of the primary cilium-mediated mechanism of mechanotransduction in kidney epithelia. In the past, traditional diffusive BAPTA-based fluorescent indicator dyes were used to measure intracellular Ca$^{2+}$ levels but did not target specific subcellular domains. In some pivotal studies, Praetorious and Spring and Nauli et al. demonstrated that primary cilia are required for mechanically induced Ca$^{2+}$ increases in kidney epithelial cells [12,13,15]. The dependence of flow-induced Ca$^{2+}$ increases on kidney epithelia primary cilium and the presence of mechanosensitive Ca$^{2+}$-permeable channels on the ciliary membrane suggest that mechanical loading opens stretch-activated ion channels on the primary cilium that mediate Ca$^{2+}$ entry. In the last couple of years, Delling et al., Su et al., and Jin et al. directed genetically encoded single fluorescence Ca$^{2+}$ biosensors to the primary cilium using a variety of ciliary targeting sequences in human retina pigmented epithelia and kidney epithelial cells [16-18]. Su et al. and Jin et al. exposed kidney epithelial cells to fluid flow, which bent primary cilium and increased ciliary and cytosolic Ca$^{2+}$ levels [17,18]. The Ca$^{2+}$-permeable channel polycystin-2 (PC2) associates with the mechanosensitive protein polycystin-1 and localizes to the primary cilium. Jin et al. reported that flow-induced Ca$^{2+}$ elevations occur first in the primary cilium and are followed by cytosolic Ca$^{2+}$ mobilization. Both ciliary and cytosolic Ca$^{2+}$ increases were dependent on PC2 [18]. Furthermore, blocking ryanodine receptors inhibited cytosolic Ca$^{2+}$ increases without affecting the flow-induced ciliary Ca$^{2+}$ response [18,19]. Collectively, these recent flow studies on kidney epithelia primary cilium demonstrate that fluid flow activates PC2 through which extracellular Ca$^{2+}$ enters and triggers ryanodine receptors in Ca$^{2+}$-induced Ca$^{2+}$ release.

Current knowledge of the osteocyte primary cilium-mediated mechanism of mechanotransduction is relatively poor compared with recent progress in kidney epithelia primary cilium mechanotransduction research. Our group previously used the fluorescent dye Fura 2-AM to demonstrate that flow-induced Ca$^{2+}$ increases in MLO-Y4 osteocyte-like cells are independent of primary cilium and stretch-activated channels, which is different from kidney cells [14]. While these results suggest that the osteocyte primary cilium-regulated mechanism of mechanotransduction is not linked to intracellular Ca$^{2+}$ levels, it is unknown if the local primary cilium Ca$^{2+}$ environment is distinct from the cytosol. We hypothesized that the osteocyte primary cilium mediates mechanotransduction by forming a distinct Ca$^{2+}$ microdomain. Therefore, the objective of this study was to monitor flow-induced ciliary Ca$^{2+}$ levels and elucidate the intricate role of the osteocyte primary cilium as a biochemical and mechanical signaling nexus.

In this study, we directed a fluorescence resonance energy transfer (FRET)-based Ca$^{2+}$ biosensor to the primary cilium by fusing a biosensor sequence to the sequence of the primary cilium-specific protein Arl13b. The modified YC3.6 Ca$^{2+}$-sensitive FRET-based biosensor with the ECFP-YPet donor-acceptor pair contains a calmodulin (CaM) region with four Ca$^{2+}$-binding domains [20]. Binding of Ca$^{2+}$ results in a conformational change that increases FRET signal, which is characterized by decreased ECFP and increased YPet fluorescence [21]. YPet as an acceptor produces the largest FRET dynamic range in live mammalian cells compared to Citrine, Venus, or cpVenus [20]. Using a targeted version of a FRET-based Ca$^{2+}$ biosensor containing ECFP and YPet and the diffusive Ca$^{2+}$ indicator dye Fura Red, we detected ciliary and cytosolic Ca$^{2+}$ increases within individual MLO-Y4 cells exposed to fluid flow stimulation. Additionally, we examined the role of several Ca$^{2+}$-permeable channels on the primary cilium: PC2, transient receptor potential vanilloid 4 (TRPV4), and PIEZO1. Coste et al. recently characterized mechanically activated current in neuroblastoma cells and proposed that the multipass transmembrane ion channels PIEZO1 and PIEZO2 mediate mechanically activated cation activity, leading us to explore the role of PIEZO channels in osteocyte mechanotransduction [22]. Our data demonstrate that TRPV4, but not PC2 or PIEZO1, mediates flow-induced ciliary Ca$^{2+}$ increases and a loading-induced osteogenic response at the transcriptional level. Collectively, our study demonstrates that the osteocyte primary cilium microdomain is distinct from the cytosol and that sources of loading-induced ciliary Ca$^{2+}$ mobilization are different in kidney epithelia and osteocytes. These are the first measurements of Ca$^{2+}$ signaling within the osteocyte primary cilium, and we anticipate this work is a starting point for more studies investigating the role of TRPV4 in osteocyte mechanotransduction [23].

Methods
Plasmid construction
Drs. Yingxiao Peter Wang and Mingxing Ouyang previously developed a calcium-sensitive FRET-based biosensor composed of an ECFP donor, calmodulin region, M13 calmodulin-binding region, and YPet acceptor (CaB). Drs. Kenji Kontani and Kristen Verhey generously shared with us the Arl13b gene. We fused Arl13b to the N terminus of CaB using a 15-amino-acid-long flexible linker to form Arl13b-L-CaB (ALC) [24]. Deletions of Trp$^3$ and Phe$^{17}$ in the M13 region were performed to block changes in FRET during Ca$^{2+}$ increases, serving as
negative controls of CaB and ALC (mutCaB and mutALC) [25].

Cell culture and transfection
MLO-Y4 osteocyte-like cells (a gift from Dr. Lynda Bonewald of the University of Missouri-Kansas City) were cultured in MEM alpha (Life Technologies) with 5% FBS, 5% CS, and 1% PS at 37°C in 5% CO2. Using the BTX 360 with a 300-V, 100-Ω, 1,000-μF pulse, 1.25 million cells were transfected with 10 μg plasmid by electroporation. Cells were co-transfected with the ALC plasmid and 0.5 nmol siRNA. siRNA sequences included Pkd2 (5′-CCUCUUGGCAUUUCAGCCUGUAAA-3′), Trpv4 (5′-GAUGGACUCUCUCUUCUU GUUGA-3′), and Piezo1 (5′-CACCAGCAUCUAGCUAAUA-3′) [22]. Transfected MLO-Y4 cells were seeded onto collagen I-coated glass slides (#1.5 glass, Warner Instruments) at a density of 4,000 cells/cm² and cultured for 3 days in reduced serum containing 2.5% FBS and 2.5% CS. IMCD cells (purchased from ATCC®, CRL-2123™) were cultured in DMEM (Life Technologies) with 10% FBS and 1% PS. Transfected IMCD cells were seeded onto fibronectin-coated glass slides at a density of 8,000 cells/cm² and cultured for 3 days in 1% FBS. Prior to imaging, cells were incubated in 20 μM Fura Red-AM (Life Technologies, F-3021) with 0.1% Pluronic® F-127 (20% solution in DMSO) (Life Technologies, A11031), goat anti-rabbit IgG-HRP antibodies (Santa Cruz, sc-2004), and HRP goat anti-mouse Ig (BD Technologies, A11008), Alexa Fluor 568 goat anti-mouse IgG (Abcam, ab11003). We used the following secondary antibodies: rabbit anti-α-tubulin (Abcam, ab24610), and mouse anti-actin (Abcam, ab11003). We used the following primary antibodies: rabbit anti-polyclastin-2 (Santa Cruz, sc-25749), rabbit anti-sera to TRPV4 (generously provided by Heller group), rabbit anti-PIEZO1 (Novus NP1-78537 for immunostaining and NBP2-10504 for Western blot), mouse anti-acetylated alpha tubulin (Abcam, ab24610), and mouse anti-actin (Abcam, ab11003).

Imaging flow chamber
Slides were placed in the RC-30 Confocal Imaging Chamber (Warner Instruments) and attached to a syringe containing phenol red-free alpha MEM with 1% FBS and 1% CS for MLO-Y4 cells or phenol red-free DMEM with 1% FBS and 1% PS for IMCD cells. MLO-Y4 cells were stimulated with oscillatory fluid flow resulting in a 10-dyn/cm² shear stress, and IMCD cells were stimulated with steady fluid flow resulting in a 1-dyn/cm² shear stress, both within physiologic range for each cell type. Thapsigargin was added to the imaging media at a final concentration of 10 μM for appropriate samples for a 20-min rest period and maintained at the same concentration during flow. A separate syringe with 10 μM ionomycin was connected to the chamber to verify cell viability after the initial flow stimulus.

Ca²⁺ imaging in cilia and cytosol
One fluorescing cell per slide was selected at random for imaging. The selected cilium was focused on, with the cell body captured within the field of view on an Olympus IX71 inverted epifluorescence microscope with a 1.30 N.A. ×40 oil immersion objective. Donor excitation was achieved on a xenon lamp using a 430/24-nm filter while images were collected simultaneously using a Quad-View system (QV2, Photometrics) with emission filters of 470/28, 530/30, and 641/75 nm. Cells were left to rest for 20 min prior to imaging. For calibration studies, we added ionomycin at a final concentration of 5 μM and CaCl₂ at a final concentration of 0.1, 0.25, and 0.5 mM. Baseline signal was recorded for 30 s followed by 5 min of flow. Images were taken at 4 Hz with a 150-ms exposure.

Image analysis
Images of each sample were trimmed to regions of interest that tightly enclosed the primary cilium or cell during imaging. Using MATLAB, images were corrected for background and bleedthrough and a pixel-by-pixel basis, resulting in signal only in the cilium and cytosol. Specifically, using MetaMorph, the same background region for ECFP and YPet images was selected from the untrimmed image far from the sample within the field of view, and bleedthrough coefficients were calculated prior to performing experiments [26]. To aid in peak detection, the average intensity data of a region of interest over time was smoothed using a 1D Savitsky-Golay filter with a window width of 31, and a fourth-degree smoothing polynomial. Oscillation amplitude was identified by finding local maxima with MATLAB. A baseline value for each sample was determined by averaging the baseline signal collected 30 s prior to flow. The maximum baseline oscillation amplitude was defined as maximum oscillation amplitude that occurred during the recording of the baseline signal, 30 s prior to flow. The Ca²⁺ peak height was defined as the peak amplitude that occurred during flow exposure. Multiple Ca²⁺ peaks may occur during the 5-min period of flow. A sample was considered responsive if a Ca²⁺ peak height was equal to or greater than 1.5 times the maximum baseline oscillation amplitude [27,28]. Not all samples were analyzed due to movement of the sample out of plane.

Antibodies
We used the following primary antibodies: rabbit anti-polyclastin-2 (Santa Cruz, sc-25749), rabbit anti-sera to TRPV4 (generously provided by Heller group), rabbit anti-PIEZO1 (Novus NP1-78537 for immunostaining and NBP2-10504 for Western blot), mouse anti-acetylated alpha tubulin (Abcam, ab24610), and mouse anti-actin (Abcam, ab11003). We used the following secondary antibodies: Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies, A11008), Alexa Fluor 568 goat anti-mouse IgG (Life Technologies, A11031), goat anti-rabbit IgG-HRP (Santa Cruz, sc-2004), and HRP goat anti-mouse Ig (BD Biosciences, 554002).

Immunocytochemistry and confocal microscopy
MLO-Y4 and IMCD cells were seeded on 35-mm glass-bottom dishes at approximately 1,000 and 2,000 cells/
Western blot

Cells transfected with Pkd2-, Trpv4-, Piezo1-, and MedGC-scrambled siRNA (Life Technologies) were lysed in RIPA buffer (Santa Cruz, sc-24948) supplemented with sodium orthovanadate, PMSF, and protease inhibitor cocktail 3 days post electroporation. Five micrograms of each protein sample was run through NuPAGE® Novex® 4%–12% Bis-Tris gels (Life Technologies). After electrophoresis, proteins were transferred to InvitroLink™ PVDF membranes (Life Technologies). The membranes were cut in half to separately label actin bands. Membranes for PC2, PIEZO1, and actin were blocked with 5% BSA (Sigma-Aldrich), and membranes for TRPV4 were blocked with 5% nonfat milk. HRP-conjugated antibodies were detected with chemiluminescence (Clarity Western ECL Substrate, Bio-Rad) on a Fujifilm LAS-4000 biomolecular imager.

Flow chamber

Transfected MLO-Y4 cells were seeded onto collagen I-coated glass slides at approximately 5,000 cells/cm² and cultured for 3 days. Slides were placed in larger, custom-made parallel-plate flow chambers (56 × 24 × 0.28 mm) so that sufficient amounts of RNA are isolated for gene expression analysis. Chambers with slides were incubated for 30 min and then exposed to 5 min of oscillatory fluid flow (OFF) at 1 Hz with a peak shear stress of 10 dyn/cm². Slides were removed from chambers after 55 min, and RNA was isolated immediately.

Quantitative real-time RT-PCR

RNA was extracted from cells using TriReagent (Sigma-Aldrich) and isolated, followed by cDNA synthesis using TaqMan reverse transcriptase (Applied Biosystems). cDNA samples were amplified with Trpv4 (Mm00499025_m1), Pkd2 (Mm00435829_m1), Piezo1 (Mm01241570_g1), Piezo2 (Mm01262433_m1), Cox-2 (Mm00478374_m1), and Gapdh (4352339E) primers and probes (Applied Biosystems) by quantitative real-time RT-PCR using the ABI PRISM 7900 detection system (Applied Biosystems). Samples and standards were run in triplicate and were normalized to the endogenous Gapdh expression. Relative gene levels between samples were determined using the relative standard curve method (ABI Prism 7700 User Bulletin 2; Applied Biosystems).

Statistical analysis

Results are shown as mean ± SEM. Unpaired t-tests (two-tailed) were used to analyze differences between treated and untreated groups. Comparisons of multiple groups were performed using one-way ANOVA followed by Dunnett’s multiple comparison post hoc test or Bonferroni’s multiple comparison post hoc test for Cox-2 mRNA level comparisons. For all tests, p < 0.05 was considered significant.

Results

Arl13b-linker-Ca²⁺ biosensor detects ciliary Ca²⁺

For this study, we developed a novel primary cilium-localized, fully ratiometric biosensor using the modified YC3.6 Ca²⁺-sensitive FRET-based biosensor (CaB) containing ECFP and YPet fused to ARL13B. First, we established that ARL13B localizes to primary cilia in MLO-Y4 osteocyte-like cells and verified ARL13B localization to IMCD primary cilia (Additional file 1: Figure S1A and B) [29]. Our biosensor design consisted of Arl13b at the N terminus followed by a 15-amino-acid-long flexible linker, ECFP, calmodulin, M13 calmodulin-binding region, and YPet at the C terminus (Figure 1A) [20,24]. Addition of Ca²⁺ leads to increased FRET signal, and transfecting Arl13b-linker-CaB (ALC) enabled us to detect Ca²⁺ levels within the primary cilium separate from the cytosol. The addition of 5 μM ionomycin, a Ca²⁺ ionophore, in media containing 1.8 mM Ca²⁺ led to detectable increases in FRET (represented by the emission ratio of YPet:ECFP fluorescence intensity) (Figure 1B, Additional file 2: Video S1). Furthermore, the FRET signal increased at a slower rate and with delay to smaller concentrations of calcium chloride (CaCl₂) added with ionomycin in Ca²⁺-free media compared with higher concentrations of CaCl₂ (Figure 1C). As expected with a Kd = 250 nM, the biosensor activity eventually reaches saturation for all CaCl₂ concentrations. Thus, we determined that ALC is sensitive to different levels of ciliary Ca²⁺.

Mechanical stimulation of MLO-Y4 cells leads to ciliary and cytosolic Ca²⁺ mobilization

We preferentially imaged cells with primary cilia having a vertical component prior to flow which typically bent in the direction of flow (Figure 1D). We loaded the Ca²⁺ indicator dye Fura Red into cells transfected with ALC to monitor cytosolic Ca²⁺ levels. Use of a Photometrics Quad-View beam splitter allowed us to collect images through several emission filters, and we monitored...
Figure 1 (See legend on next page.)
We examined three Ca\(^{2+}\)-permeable channels localized to the osteocyte primary cilium: PC2, TRPV4, and PIEZO1. PC2 and TRPV4, among other channels, localize to primary cilia and mediate flow-induced Ca\(^{2+}\) increases in kidney epithelia [15,31]. Coste et al. reported that mechanically activated ion channels PIEZO1 and PIEZO2 are expressed in several murine tissues including kidney and have relatively lower expression in brain and heart tissues [22]. We observed Piezo1 mRNA expression in MLO-Y4 and IMCD cells and Piezo2 mRNA expression only in IMCDs (Additional file 4: Figure S3). We immunostained MLO-Y4 cells for PC2, TRPV4, and PIEZO1 and found that all three channels are present in both the primary cilium and plasma membrane (Figure 2A–C). siRNA transfection reduced protein and mRNA levels compared with scrambled siRNA controls (all groups (n = 4–10) vs. control mRNA levels (n = 10), p < 0.005)(Figure 2D, E).

TRPV4 mediates flow-induced ciliary Ca\(^{2+}\) increases in osteocytes

PC2 mediates mechanically induced ciliary and cytosolic Ca\(^{2+}\) signaling in kidney epithelia, and here, our data suggest that osteocyte mechanotransduction is independent of PC2. Following co-transfection of ALC and either Pkd2, TRPV4, or Piezo1 siRNA, MLO-Y4 cells were loaded with Fura Red and exposed to oscillatory fluid stimulation (n = 14–16). Upon an initial general analysis of primary cilia from all viable cells (ionomycin-responsive), neither thapsigargin nor any of the siRNA-mediated knockdowns of Pkd2, TRPV4, or Piezo1 exhibited a significant difference in flow-induced Ca\(^{2+}\) peak amplitude relative to untreated samples. Similarly, the only significant difference in peak amplitude relative to untreated samples occurred in the cytosol of cells treated with thapsigargin (Additional file 5: Figure S4). Using the spike threshold of 1.5× baseline, there was no significant difference in the percentage of cells exhibiting a Ca\(^{2+}\) response in the primary cilium and cytosol with Pkd2, TRPV4, and Piezo1 knockdown. To illustrate the role of candidate mechanically activated channels in regulating Ca\(^{2+}\) entry in a different way, we plotted the percent of viable cells exhibiting flow-induced Ca\(^{2+}\) responses as a function of peak amplitude (a multiple of the sample's maximum baseline oscillation amplitude) for all treatment groups. Thus, a flow-induced peak was considered a response if its amplitude was 1.5× greater than the maximum baseline oscillation amplitude, and we demonstrated that the percentage of cells responding decreased as a peak was considered a larger multiple of maximum baseline oscillation. Out of all the candidate Ca\(^{2+}\)-permeable channels, only the loss of TRPV4 clearly impaired the percent of responding cells (Figure 3A). TRPV4 deficiency did not lower the percent of cells exhibiting cytosolic Ca\(^{2+}\) peaks (Figure 3B). Additionally, only thapsigargin treatment affected the percent of cells exhibiting flow-induced cytosolic Ca\(^{2+}\) increases (Figure 3B, n = 6). We analyzed the response of TRPV4 siRNA-treated cells further and found that 75% of untreated cells had an
intense response, a Ca\(^{2+}\) peak height at least five times the maximum baseline oscillation amplitude, compared with only 30% of Trpv4 siRNA-treated cells that had intense ciliary Ca\(^{2+}\) peaks. Pkd2 and Piezo1 deficiencies did not inhibit the percent of ciliary or cytosolic Ca\(^{2+}\) peaks. Interestingly, Piezo1 knockdown sensitized the cytosolic Ca\(^{2+}\) response to flow, perhaps because of a different Ca\(^{2+}\) permeable channel.

As mentioned earlier, there is a delay in peak FRET signal with lower Ca\(^{2+}\) concentrations relative to higher Ca\(^{2+}\) concentrations. Compared with untreated samples, ciliary and cytosolic Ca\(^{2+}\) peaks were delayed in MLO-Y4 cells treated with thapsigargin or deficient in Trpv4 and Piezo1 (Figure 3C, D). These differences were not statistically significant in cells lacking Piezo1. On an individual cell basis, there was no significant change in the percent of peaks occurring in the primary cilium prior to the cytosol. 57 ± 5% of peaks first occurred in the primary cilium in untreated cells, compared with 75 ± 13% in thapsigargin-treated cells, compared with 54 ± 5% in Pkd2 siRNA-treated cells, 43 ± 5% in Trpv4 siRNA-treated cells, and 55 ± 6% in Piezo1 siRNA-treated cells. Thus, in cells deficient in Trpv4, there was a decrease in the percent of Ca\(^{2+}\) peaks occurring first in the primary cilium in cells deficient in Trpv4, although this difference was not significant. Collectively, these results suggest that fluid flow mechanically activates TRPV4 channels on the primary cilium. Separately, fluid flow also activates intracellular Ca\(^{2+}\) release, which diffuses from the cytosol into the primary cilium microdomain.

**TRPV4 is required for osteocyte mechanotransduction**

Next, we were interested in determining if TRPV4 also mediates osteocyte mechanotransduction at the transcriptional level in addition to regulating early ciliary Ca\(^{2+}\) mobilization. The Cox-2 gene encodes an enzyme which catalyzes formation of PGE\(_2\), an osteogenic cytokine released by osteoblasts and osteocytes in response to mechanical stimulation, and Cox-2 expression increases correspond to more PGE\(_2\) release [14]. After 5 min of OFF stimulation (identical to the imaging experiments) and a rest period of 55 min, we isolated and
quantified Cox-2 mRNA expression relative to the endogenous control Gapdh (n = 9–11). Scrambled siRNA-transfected cells demonstrated a flow-induced increase in Cox-2 mRNA expression levels, and this flow-induced response was blocked in cells lacking Trpv4 (1.3 ± 0.3-fold (control) vs. 1.0 ± 0.1-fold (Trpv4) increase) (Figure 3E). MLO-Y4 cells transfected with scrambled control siRNA and Trpv4 siRNA expressed similar levels of Cox-2 mRNA expression at baseline. Interestingly, Piezo1 deficiency did not affect downstream flow-induced changes in Cox-2 mRNA. MLO-Y4 cells transfected with Piezo1 siRNA demonstrated a 1.4 ± 0.2-fold flow-induced increase in Cox-2 mRNA expression which was not significantly different from controls, although they exhibited lower baseline Cox-2 mRNA expression levels (Figure 3F). We did not examine the role of PC2 in flow-induced transcriptional changes due to the absence of ciliary and cytosolic Ca\textsuperscript{2+} peak amplitude or timing changes with Pkd2 deficiency. Taken together, these data suggest that loss of Trpv4 alters osteocyte mechanotransduction at the molecular and transcriptional levels.

**Primary cilium-regulated mechanisms of mechanotransduction are different in kidney epithelia and osteocytes**

With the suggestion that the osteocyte primary cilium-mediated mechanism of mechanotransduction depends on TRPV4 and not PC2, we conducted similar flow studies with kidney epithelial cells to verify that this difference was due to cell type and not the experimental approach. We immunostained IMCD cells for PC2 and found similar localization relative to MLO-Y4 cells (Figure 4A). As expected and previously reported, Pkd2 knockdown (11.85 ± 0.03% relative to IMCD control mRNA levels, p < 0.005 (Figure 4B, C, n = 4)) blocked steady flow-induced ciliary and cytosolic Ca\textsuperscript{2+} increases in IMCDs normalized to...
baseline value, and these differences were statistically significant (Figure 4D–G) [18].

Discussion
In this study, we directed a FRET-based Ca\(_{2+}\) biosensor to the primary cilium and loaded cells with a nontargeted fluorescent Ca\(_{2+}\) indicator dye to resolve the local Ca\(_{2+}\) environment in the osteocyte primary cilium from the cytosol. We monitored ciliary and cytosolic Ca\(_{2+}\) levels using an epifluorescence microscopy system and observed flow-induced Ca\(_{2+}\) peaks in both domains. Trpv4 deficiency reduced flow-induced ciliary Ca\(_{2+}\) peaks but did not impair flow-induced cytosolic Ca\(_{2+}\) mobilization, illustrating that the primary cilium microdomain is distinct from the cytosol. Thapsigargin treatment impaired flow-induced ciliary and cytosolic Ca\(_{2+}\) peaks, demonstrating that intracellular Ca\(_{2+}\) release and separate Ca\(_{2+}\) entry through TRPV4 are both components of ciliary Ca\(_{2+}\) mobilization. In contrast, knockdown of Pkd2 and Piezo1 did not affect ciliary or cytosolic Ca\(_{2+}\) peaks. Last, we linked the role of TRPV4 in regulating flow-induced ciliary Ca\(_{2+}\) mobilization with a downstream osteogenic response at the transcriptional level by determining that flow-induced changes in Cox-2 expression depend on TRPV4. Collectively, our study demonstrates that the loading-induced ciliary Ca\(_{2+}\) mechanism is different between kidney epithelia and osteocytes.

After observing flow-induced Ca\(_{2+}\) peaks in both the osteocyte primary cilium and cytosol, we were motivated to identify the source of the ciliary Ca\(_{2+}\) peak. To deplete intracellular Ca\(_{2+}\) stores, we treated cells with thapsigargin and continued to observe ciliary Ca\(_{2+}\) peaks, suggesting that Ca\(_{2+}\)-permeable channels on the primary cilium have a role in mediating flow-induced Ca\(_{2+}\) entry. However, thapsigargin treatment did lower flow-induced ciliary and cytosolic Ca\(_{2+}\) peak magnitudes and responsiveness compared with untreated cells, indicating that intracellular Ca\(_{2+}\) release is a component of flow-induced ciliary Ca\(_{2+}\) mobilization in osteocytes. Furthermore, a statistically significant delay in ciliary and cytosolic Ca\(_{2+}\) peaks occurred in thapsigargin-treated cells compared with controls. Thus, our data demonstrate that intracellular Ca\(_{2+}\) release contributes, in part, to the local primary cilia Ca\(_{2+}\) environment and suggests that the primary cilium serves as an important signal integrator.

It is important to note that a general ensemble analysis of our data did not reveal an effect of PC2, TRPV4, or PIEZO1 on flow-induced ciliary Ca\(_{2+}\) mobilization. In fact, the only significant reduction in peak Ca\(_{2+}\) mobilization was demonstrated in the cytosol with thapsigargin treatment. On the one hand, although the effect of thapsigargin on flow-induced Ca\(_{2+}\) mobilization was clear, using such a potent agent provides limited details. As expected, extracting the finer details of the role of Ca\(_{2+}\)
channels in targeted Ca\(^{2+}\) channel blocking experiments required more selective analysis, which included categorizing cells into responders and nonresponders. While selecting a discriminating threshold can be confounding to the analysis, we found no significant difference in average peak ciliary Ca\(^{2+}\) mobilization with any treatment, suggesting that the results of this approach are not sensitive to the magnitude of the threshold (Additional file 5: Figure S4).

In this study, we present evidence that fluid flow activates TRPV4 on the primary cilium membrane and mediates Ca\(^{2+}\) influx. Using immunocytochemistry techniques, we determined that the stretch-activated Ca\(^{2+}\)-permeable channel TRPV4 localizes to the primary cilium and plasma membrane. Interestingly, TRPV4 (and PC2 and PIEZO1) appears throughout the cell, which is consistent with TRPV4 and PC2 immunostaining in the literature [32-35]. While the distribution of the channels appears higher on the cell membrane relative to the primary cilium and may mediate a larger Ca\(^{2+}\) flux compared with the flux into the primary cilium, it is likely that the machinery and spatiotemporal molecular pathways unique to the primary cilium play a role in downstream mechanotransduction. Our flow experiments revealed that Trpv4 knockdown lowered flow-induced ciliary Ca\(^{2+}\) peaks but did not impair cytosolic Ca\(^{2+}\) peaks. Unlike kidney epithelia, where Ca\(^{2+}\) reportedly enters the primary cilium through TRPV4 entry through stretch-activated channels, Malone et al. [2015] 4:7 reported that the removal of primary cilia did not affect flow-induced cytosolic Ca\(^{2+}\) flux in osteocytes. The results in this study, here, are consistent with the previous paper and provide additional insight to the understanding of osteocyte mechanotransduction. Using advanced imaging techniques that provide enhanced resolution, our data demonstrate that flow-induced cytosolic Ca\(^{2+}\) flux is independent of extracellular Ca\(^{2+}\) entry through stretch-activated channels; furthermore, flow-induced ciliary Ca\(^{2+}\) flux is dependent on the Ca\(^{2+}\)-permeable, stretch-activated TRPV4 channel. Thus, our data suggest that Ca\(^{2+}\) mobilization occurs differently in the cytosol versus the primary cilium during osteocyte mechanotransduction.

Our understanding of primary cilium bending mechanics and mechanical forces on the plasma membrane covering the primary cilium is essential to elucidating the molecular mechanism of mechanotransduction mediated by the primary cilium. Recently, Young et al. studied the tension force distribution along a primary cilium under flow and suggested that stretch-activated ion channels are likely to be activated and open near the base of the primary cilium where tension force is the highest [37]. While primary cilia bending is one potential physical event, it is possible that primary cilium deflection is not physiologic and that mechanical loading of the primary cilium occurs in other ways. For example, \(\beta\)-integrins are localized to MDCK primary cilium, and \(\beta\)-integrins have been implicated in mediating osteocyte mechanotransduction and loading-induced bone formation [38-40]. Thus, increased membrane tension is not limited to primary cilium bending and may involve primary cilium integrin-extracellular matrix interactions.

We anticipate that TRPV4 will be an attractive pharmacologic target for treating disuse-induced bone loss due to its role mediating osteocyte mechanotransduction and its sensitivity to existing biochemical agents (agonists: , GS1016790A, and RN1747 and antagonist: RN1734) [41-43]. Thus, treatment with TRPV4 agonists and therapies that elongate osteocyte primary cilia (lithium chloride, hydrogen sulfide, interleukin-1) to enhance mechanical strain levels may amplify osteogenic responses and prevent disuse-induced bone loss in patients restricted to bed rest [8,44,45]. Furthermore, O’Conor et al. have shown that TRPV4 plays a role as a physical transducer in chondrocytes, which may provide insight into functional cartilage tissue engineering approaches [46].

Other groups have suggested that TRPV4 is a candidate therapeutic target for bone loss disease. Mizoguchi et al. and Masuyama et al. have examined TRPV4 deficiency in unloading-induced bone formation, and they determined that Trpv4 knockout reduces unloading-induced bone loss due to suppressed ostecostal numbers and impaired bone resorption [47,48]. Interestingly,
Mizoguchi et al. do not exclude the possibility that Trpv4 is expressed by osteocytes. Osteocytes are mechanosensing cells in bone that regulate osteoclasts’ resorption activities, and it will be important to understand the role of TRPV4 in the population of cells involved in mechanotransduction. The effect of an osteocyte-specific Trpv4 deletion in loading-induced bone formation in vivo would provide evidence indicating whether TRPV4 is involved in the mechanotransduction process versus restricted to mediating osteoclast numbers and bone resorption. Additionally, it is important to recognize that primary cilia play important roles in development; however, in this study, we have not examined how TRPV4 plays a role in developmental ciliary pathways such as Hedgehog and Wnt signaling pathways.

While Jin et al. reported that flow-induced Ca\(^{2+}\) mobilization occurs in primary cilia before cytosolic Ca\(^{2+}\) increases in kidney epithelial cells, our flow studies do not demonstrate a clear difference in the timing between ciliary and cytosolic Ca\(^{2+}\) peaks in osteocytes [18]. While the amount of ciliary Ca\(^{2+}\) peaks occurring before cytosolic Ca\(^{2+}\) peaks is numerically higher than the number of ciliary Ca\(^{2+}\) peaks occurring after cytosolic Ca\(^{2+}\) peaks, it is unclear if ciliary Ca\(^{2+}\) triggers cytosolic Ca\(^{2+}\) increases. Unlike the side-dimension imaging method leveraged by Jin et al. to capture calcium signaling along the length of the primary cilium, our imaging method collected signal from only a part of the primary cilium that was in plane. Thus, we are unable to characterize a relationship between ciliary and cytosolic Ca\(^{2+}\) peak timing.

Several other groups have demonstrated that flow-induced ciliary Ca\(^{2+}\) mobilization is dependent on PC2 in kidney epithelia [15,18,31]. Our data suggest that flow-induced ciliary Ca\(^{2+}\) mobilization is dependent on TRPV4, and not PC2, in osteocytes. We conducted similar flow studies with kidney epithelial cells to verify that this difference was due to cell type and not the experimental approach. Our studies with IMCD cells also showed that flow-induced ciliary and cytosolic Ca\(^{2+}\) increases depend on PC2. The consistency in our results suggests that the mechanism of mechanotransduction mediated by primary cilium varies across different tissue contexts. Another difference between the MLO-Y4 and IMCD flow studies was the type of flow regime, consisting of OFF resulting in a 10-dyn/cm\(^2\) shear stress for MLO-Y4 cells and steady flow resulting in a 5-dyn/cm\(^2\) shear stress for IMCD cells. Previously, Malone et al. determined that flow-induced Ca\(^{2+}\) flux differences in MC3T3-E1 osteoblasts and MDCK kidney cells did not depend on these specific flow regimes, which suggests that primary cilium-mediated mechanosensation in osteoblasts and kidney cells is indeed different [14]. Thus, the application of different but physiologically relevant mechanical loads was appropriate for elucidating intricacies in the mechanotransduction mechanism in IMCD and MLO-Y4 cells.

Interestingly, in addition to being well-established in kidney epithelial mechanotransduction [15,18,31], PC2 has recently been implicated in osteocyte mechanotransduction [49]. The authors found siRNA-mediated knockdown of PC2 inhibited downstream flow-induced nitric oxide production and inducible nitric oxide synthase. While fluid flow-induced shear stress is known to activate the nitric oxide pathway, this activation occurs over a much longer time scale of hours compared to Ca\(^{2+}\) flux of seconds and minutes. Here, we studied the early signaling response to flow occurring within seconds after exposure to flow. Thus, our finding that PC2 was independent of mechanically induced ciliary and cytosolic Ca\(^{2+}\) signaling in osteocytes does not contradict the findings by Xu et al. It is possible that PC2 is involved in the later downstream signaling response to flow. While we found that PC2 may not be involved in the early Ca\(^{2+}\) response to flow, it is nonetheless an important channel in bone. Pkd2 mutations have been implicated in skeletal development [50,51], and mutations in Pkd1, encoding the other subunit of the polycystin complex, have impaired both skeletal development and adaptation [52].

Conclusions

In conclusion, this study highlights the specialization of primary cilium mechanisms across different tissue contexts. Here, we demonstrate that mechanically stimulated ciliary Ca\(^{2+}\) mobilization is different between kidney epithelia and osteocytes. Strikingly, the osteocyte primary cilium forms a distinct microdomain from the cytosol during mechanical loading. The primary cilium microdomain may help maintain spatiotemporal organization within the cell which allows numerous molecular mechanisms to occur with just a limited number of signaling molecules. We expect that the Ca\(^{2+}\) \(^*\)-permeable channel TRPV4 will be an attractive therapeutic target for bone loss disease because of its location in an area of high membrane strain on the primary cilium, demonstrated role as a physical transducer in osteocytes in this study and chondrocytes in a recent study by O’Connor et al., and sensitivity to existing biochemical agents [46]. Furthermore, we anticipate that other flow studies on second messenger-mediated pathways in the primary cilium microdomain and loading-induced bone formation studies using mice with an osteocyte-targeted deletion of Trpv4 will elucidate how TRPV4-mediated Ca\(^{2+}\) entry in the primary cilium microdomain regulates osteogenic responses to mechanical loads.
Additional files

**Additional file 1:** Figure S1. ARL13B localizes to primary cilium in MLO-Y4 and IMCD cells. (A-B) IMCD and MLO-Y4 cells fixed and stained for acetylated alpha tubulin and ARL13B, demonstrating localization of ARL13B to the primary cilium.

**Additional file 2:** Video S1. Ciliary Ca\(^{2+}\) increases detected by ALC. Images of an individual primary cilium in a time-lapse video. MLO-Y4 cells were transfected with ALC, cultured in 1.8 mM Ca\(^{2+}\)-containing media, and treated with ionomycin which permeabilizes the plasma membrane to Ca\(^{2+}\). Images were captured at 10-s intervals. Display rate is three frames per second.

**Additional file 3:** Figure S2. Mutant CaB and ALC do not exhibit flow-induced Ca\(^{2+}\) peaks. Deletions of Trp5 and Phe\(^{178}\) in the M13 region inhibit FRET changes during Ca\(^{2+}\) increases. (A-B) In initial testing and prior to targeting the mutated biosensor to the primary cilium, MutCaB failed to detect flow-induced Ca\(^{2+}\) increase with the addition of 10 \(\mu\)M Ca\(^{2+}\) (n = 3) and steady flow (5 dyn/cm\(^2\)) (n = 2). (C) MutALC failed to detect flow-induced Ca\(^{2+}\) increases occurring with oscillatory fluid flow (10 dyn/cm\(^2\)) (n = 4).

**Additional file 4:** Figure S3. MLO-Y4 cells express Piezo1 and not Piezo2. (A) Piezo1 and (B) Piezo2 mRNA levels in MLO-Y4 cells, IMCD cells, and adult murine (C57BL/6) brain and heart relative to GAPDH mRNA expression (n = 4–5). Piezo1 = 1.6 ± 0.2 and Piezo2 = 0.003 ± 0.001 in MLO-Y4 cells. Error bars show ±SEM.

**Additional file 5:** Figure S4. FRET ratios of all viable MLO-Y4 cells exposed to fluid flow. (A) Mean FRET ratios over time, where solid lines indicate the mean background-corrected FRET ratios (no threshold applied) while the dashed lines indicate SEM. (B) Flow-induced Ca\(^{2+}\) peak amplitude averaged from all viable cells (no threshold applied). Error bars show ±SEM.

Abbreviations

Ca\(^{2+}\): Cyclooxygenase 2; IMCD: Inner medulary collecting duct; PC-2: Polycystin-2; TRPV4: Transient receptor potential vanilloid 4; CaB: Calcium biosensor; ALC: Arl13b-linker-calcium biosensor.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

KLL and CRJ conceived the study, designed the experiments, and wrote the paper. YW contributed molecular tools. KLL and MCC built the ALC plasmid. KLL performed Western blotting and qRT-PCR. MDG carried out immunostaining and imaging. KLL, MCC, and MDG performed the imaging experiments and analyzed data. KLL, AMN, and CRJ contributed to interpretation of the data. All authors reviewed and critically revised the paper and approved the final manuscript.

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