Pharmacologically Increasing Primary Cilia Length Enhances Osteocyte Mechanotransduction

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Introduction: Bone cells continually respond to mechanical stimuli via molecular signaling pathways which mediate bone formation [1]. The primary cilium is a single immotile organelle protruding from the surface of most mammalian cells, and is a mechanosensor in a variety of different cell and tissue contexts including kidney, endothelia, and bone [2, 3, 4]. Recent work suggests that osteocytes are mechanosensing cells within bone and utilize primary cilia to respond to fluid flow by upregulating osteogenic markers such as cyclooxygenase-2 (COX-2) and osteopontin (OPN) gene expression [4, 5]. Furthermore, disruption of osteocyte primary cilia by siRNA knockdown of IFT88, a subunit of an intraflagellar transport protein necessary for cilia formation, abrogates this flow-induced osteocyte mechanotransduction [4]. Membrane-bound stretch-activated ion channels, such as TRPV4 and PC2, localize to the cilium and may be stimulated by cilia deflection [6]. Several agents, such as fenoldopam and lithium chloride (LiCl) have been used to increase primary cilia length in endothelial and fibroblast cells [3, 7]. We hypothesize that increasing osteocyte primary cilia length enhances their mechanosensitivity and augments flow-induced osteogenic signaling. In this study we increased cilia length by two independent small molecules and measured the cellular response to mechanical stimulation.

Methods: Cell Culture. MLO-Y4 osteocyte-like cells were cultured on collagen-coated dishes in MEMα (Life Tech) with 5% FBS, 5% CS, and 1% P/S. Cilia Imaging. Cells were seeded onto collagen-coated glass-bottom dishes. Once the cells reached 80% confluence, they were treated with 10 μM fenoldopam (Sigma), 500 μM LiCl (Sigma), or vehicle control (DMSO and normal culture media, respectively) for 16 hours. Cilia were labeled with an antibody against acetylated alpha tubulin, visualized with Alexa Fluor 488, and imaged by confocal microscopy. Cilia lengths were measured using ImageJ.

Fluid Flow. Cells were seeded on collagen-coated glass slides 72 hours before flow. Cells were treated with fenoldopam, LiCl, or vehicle control for 16 hours prior to experimentation. Oscillatory fluid flow was applied for 1 hr at 1Hz, providing 1 Pa wall shear stress. Slides were also left in static conditions as a no flow control.

Gene Expression. Immediately after flow, cells were washed with PBS and total RNA was isolated using TriReagent. Real time RT-PCR was used to quantify mRNA expression using primers and probes for COX-2, OPN, and GAPDH. All gene expression is reported relative to GAPDH endogenous control.

RNA Interference. Primary cilia formation was disrupted using siRNA mediated knockdown of IFT88, while scramble siRNA was used as a control.

Statistics. All values reported as mean ± SEM. To compare cilia lengths and flow over no flow mRNA expression, unpaired t-test was used. To compare siRNA and fenoldopam treated cells, a one-way ANOVA with Bonferroni correction was used.

Results: Cells treated with either fenoldopam or LiCl for 16 hours have elongated cilia (Fig. 1). Treatment with both drugs significantly increased cilia length compared to vehicle controls, without any
distinguishable changes in cell morphology. Cells with longer cilia elicited significant increases in the fold change of osteogenic markers, COX-2 and OPN, in response to fluid flow compared to vehicle control (Fig. 2). Treatment with IFT88 siRNA significantly decreased the length of primary cilia, while fenoldopam moderately restored cilia length (Fig. 3). IFT88 siRNA treatment decreased OPN expression in response to flow, yet fenoldopam partially rescued this impaired osteogenic signaling.

**Discussion:** In this study, we increased the length of osteocyte primary cilia by two separate strategies. Our results suggest that increasing primary cilia length with either fenoldopam or LiCl enhances osteocyte mechanotransduction. While fenoldopam treatment did not significantly increase cilia length in cells disrupted by IFT88 siRNA, we still observed a modest increase in cilia length and an increase in the incidence of primary cilia. Furthermore, fenoldopam was able to restore the flow-induced increase in osteogenic signaling that is impaired with disrupted primary cilia. Together, this suggests that fenoldopam enhances primary-cilia mediated mechanotransduction, potentially by promoting intraflagellar transport to stimulate cilia elongation. Longer cilia experience greater membrane tension with deflection under fluid flow, which may further stimulate stretch-activated ion channels to promote increased primary cilia-mediated mechanotransduction. Collectively these data suggest that primary cilia length is an important contributor to osteocyte mechanosensitivity. We anticipate that increasing cilia length in vivo will augment the bone formation response to loading.

**Significance:** Pharmacologically increasing the length of primary cilia can be a potent strategy to enhance osteocyte mechanotransduction. Subsequently enhancing osteocyte primary cilia-mediated mechanotransduction has the potential to stimulate bone formation and combat bone loss diseases.

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**Figure 1.** (A) Treatment with fenoldopam (Fen) vehicle control for 16 hours. (B) Treatment with LiCl or vehicle control for 16 hours. Both agents significantly increase cilia length compared to vehicle controls (***p<0.01, ****p<0.001).
Figure 2. (A/B) Fenoldopam and LiCl treatment led to higher flow-induced COX-2 mRNA increases compared to vehicle control. (C/D) These drugs also increase flow-induced OPN mRNA expression (*p<.05, **p<.01, ***p<.001).
Figure 3. (A) Knockdown of IFT88 significantly decreases cilia length compared to scramble control. Fenoldopam restores cilia length in IFT88 siRNA treated cells. (B) IFT88 siRNA decreases flow-induced OPN expression compared to scramble control, but this is rescued with fenoldopam treatment (**p<0.001).