

## LENGTHENING PRIMARY CILIA ENHANCES CELLULAR MECHANOSENSITIVITY

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### **Abstract**

The primary cilium is a mechanosensor in a variety of mammalian cell types, initiating and directing intracellular signalling cascades in response to external stimuli. When primary cilia formation is disrupted, cells have diminished mechanosensitivity and an abrogated response to mechanical stimulation. Due to this important role, we hypothesised that increasing primary cilia length would enhance the downstream response and therefore, mechanosensitivity. To test this hypothesis, we increased osteocyte primary cilia length with fenoldopam and lithium and found that cells with longer primary cilia were more mechanosensitive. Furthermore, fenoldopam treatment potentiated adenylyl cyclase activity and was able to recover primary cilia form and sensitivity in cells with impaired cilia. This work demonstrates that modulating the structure of the primary cilium directly impacts cellular mechanosensitivity. Our results implicate cilium length as a potential therapeutic target for combating numerous conditions characterised by impaired cilia function.

### **Introduction**

Mechanotransduction is a critical cellular process in a variety of tissues. Endothelial cells sense blood flow and transduce the mechanical stimuli into biochemical responses to adjust blood vessel diameter (Ku, 1997). Similarly, kidney epithelial cells in the collecting duct sense and respond to varying rates of urine flow (Liu *et al.*, 2003). Bone maintenance requires mechanical stimulation to maintain balanced formation and resorption (You *et al.*, 2008). Understanding how cells sense mechanical cues and transduce them into biochemical responses is a critical impediment for developing novel treatments for a wide variety of diseases of structural tissues.

Cell mechanotransduction has been studied through several different mechanisms. Altering cytoskeletal mechanics by disrupting actin polymerisation with cytochalasin D inhibits flow-induced osteogenic differentiation of C3H10T1/2 mesenchymal stem cells (Arnsdorf *et al.*, 2009). Similar experiments in osteoblasts treated with cytochalasin D and exposed to fluid flow demonstrated increased cellular mechanosensitivity, as measured by prostaglandin E2 release (Malone *et al.*, 2007; Norvell *et al.*, 2004). Integrins couple the extracellular matrix with the cytoskeleton through focal adhesions comprised of actin-associated proteins, such as talin and vinculin, inhibition of focal adhesion dynamics, in focal adhesion kinase knockout osteoblasts, impairs the prostaglandin E2 release indicative of mechanotransduction (Castillo *et al.*, 2012; Geiger *et al.*, 2001). Mathematical models of osteocytes *in vivo* estimate that osteocyte dendritic processes may experience higher strain than the cell body (Vaughan *et al.*, 2014). In a separate study, a transwell filter system was used to differentiate the osteocyte cell body and dendritic processes and each was separately mechanically loaded (Burra *et al.*, 2010). It was found that the glycocalyx of the dendritic processes is critical for the formation of integrin attachments that initiate a mechanotransduction pathway resulting in the opening of hemichannels on the cell body. Gap junctions between adjacent cells are thought to contribute to mechanotransduction by mediating calcium, ATP and prostaglandin E2 intercellular signalling (Plotkin *et al.*, 2015). Membrane deformations also play a significant role in cell mechanosensing where stretch-activated channels, such as polycystin-2 (PC2) and transient receptor potential vanilloid 4 (TRPV4), mediate calcium influx to initiate mechanotransduction (Lee *et al.*, 2015b; Phan *et al.*, 2009; Piperi, 2015).

In this work we focus on primary cilia, single immobile organelles extending from the surface of nearly all mammalian cells, which have been implicated as mechanosensors in a variety of cell types. Praetorius and Spring first demonstrated that kidney epithelial cells

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respond to fluid flow and specifically that this mechanical stimulation causes primary cilia deflection (Praetorius and Spring, 2001). Furthermore, fluid flow initiates an intracellular calcium increase that is diminished when cilia are removed (Praetorius and Spring, 2003). Since then, primary cilia have been identified as mechanosensing organelles in a variety of cell types, including bone (Malone *et al.*, 2007).

Osteocytes are mechanosensitive cells within bone, where the primary cilium functions as a mechanosensor (Malone *et al.*, 2007). *In vitro*, fluid flow mechanical stimulation of osteocytes enhances expression of the osteogenic genes cyclooxygenase-2, COX-2, and osteopontin, OPN. COX-2 synthesises prostaglandin E2 and OPN is a critical extracellular matrix protein. Increases in the production of both are indicative of osteogenesis (Ehrlich and Lanyon, 2002; Fujihara *et al.*, 2006; Klein-Nulend *et al.*, 1997; Raisz, 1999). When osteocyte primary cilia formation is inhibited, the cells display an abrogated osteogenic response to flow, implicating the cilium as a critical mechanosensor in osteocytes (Malone *et al.*, 2007). Furthermore, we have previously reported that adenylyl cyclases, specifically AC6, play a significant role in osteocyte mechanosensitivity (Kwon *et al.*, 2010). Adenylyl cyclases convert ATP to the ubiquitous second messenger cAMP, a process which can be specifically stimulated by forskolin.

Despite prior *in vitro* work, the role of osteocyte primary cilia *in vivo* is yet unclear. The presence of primary cilia within mineralised bone has been addressed with conflicting reports. Tonna and Lampen found that less than 4 % of osteocytes possessed primary cilia, while Uzbekov *et al.* reported more than 94 % incidence (Tonna and Lampen, 1972; Uzbekov *et al.*, 2012). Furthermore, the lacunar space within which osteocytes reside suggests that primary cilia are less than 1  $\mu\text{m}$  long, as opposed to *in vitro* lengths, which can be 4  $\mu\text{m}$  (Malone *et al.*, 2007; McNamara *et al.*, 2009). A recent fluid-structure interface model was developed to estimate how primary cilia may deform *in vivo* (Vaughan *et al.*, 2014). It was concluded that a short cilium, approximately 0.2  $\mu\text{m}$ , was not long enough to be a mechanosensor, but that an elongated cilium, spanning the full pericellular space, could be. We have previously utilised a mouse model where primary cilia were deleted from osteocytes and osteoblasts, resulting in impaired mechanotransduction and abrogated load-induced bone formation (Temiyasathit *et al.*, 2012). While *in vivo* the specific orientation and even incidence of osteocyte primary cilia remains unclear, evidence suggests that the cilium may yet play a key role in bone mechanosensing and that modifying cilium structure may alter mechanotransduction.

While several small molecules exist to increase cilia length, in this work we utilise two drugs that are both clinically approved and have distinct mechanisms of action – fenoldopam, used to treat hypertension, and lithium, a treatment for bipolar disorder – to increase primary cilia length. Fenoldopam is a dopamine D1-like receptor agonist and has previously been used to increase primary cilia length in endothelial cells and kidney epithelial cells, potentially through an adenylyl cyclase-cAMP mechanism

(Kathem *et al.*, 2014; Upadhyay *et al.*, 2014). In the context of bone, MC3T3 osteoblasts express dopamine receptors 1–5, yet the role of specific dopamine receptors and the effects of fenoldopam treatment is unknown and has never been examined in MLO-Y4 osteocytes (Lee *et al.*, 2015a). Analysis of gene expression patterns after tibial loading in adult rats reveals a wide array of upregulated genes, including dopamine D1 receptor mRNA (Mantila Roosa *et al.*, 2011). Interestingly, transgenic mice with a global deletion of this receptor have no apparent difference in bone architecture or calcification compared to controls (Drago *et al.*, 1994). Mice with a homozygous deletion of the dopamine transporter have diminished bone mass, but the role of specific dopamine receptors was not examined (Bliziotis *et al.*, 2000). Lithium is regularly used as an agonist of the Wnt signalling pathway in various cell types, including MLO-Y4 osteocytes, and has been shown to increase primary cilia length in cultured fibroblasts and neurons through a yet incompletely characterised mechanism (Bivi *et al.*, 2013; Miyoshi *et al.*, 2009). In Lrp5 knockout mice, lithium treatment restores bone metabolism and bone mass and activates Wnt signalling in isolated calvarial osteoblasts (Clément-Lacroix *et al.*, 2005). Lithium and fenoldopam have distinct mechanisms of action and both increase primary cilia length in a variety of cell types, but their effects on primary cilia-mediated mechanotransduction in osteocytes has not been studied.

Due to the significance of primary cilia in cellular mechanotransduction, we hypothesised that increasing their length would enhance mechanosensitivity. Here, we treat osteocytes with lithium and fenoldopam to increase primary cilia length and then mechanically stimulate the cells. We then examine the potential of targeting primary cilia length to recover impaired primary cilia-mediated mechanotransduction using models of impaired cilia and ciliary proteins. Our results highlight the importance of cilium length in cellular mechanosensitivity and that this is a process that can be modulated by pharmacologic intervention.

## Materials and Methods

### Cell culture and drug treatments

MLO-Y4 osteocytes were cultured on collagen I-coated dishes (Corning) in  $\alpha$ MEM (Life Technologies) supplemented with 5 % foetal bovine serum, 5 % calf serum and 1 % penicillin/streptomycin at 37 °C and 5 % CO<sub>2</sub>. Fenoldopam mesylate (Sigma) was used at 10  $\mu\text{M}$  diluted in DMSO (dimethyl sulfoxide, Sigma) and normal culture media, as previously described (Kathem *et al.*, 2014; Upadhyay *et al.*, 2014). Lithium chloride (Sigma) was used at 500  $\mu\text{M}$  diluted in normal culture media – a dose response from 50  $\mu\text{M}$  to 10 mM was examined with 500  $\mu\text{M}$  being the lowest dose to increase length significantly (data not shown). These agents, or their vehicle control, were applied to cells for 16 h prior to experimentation. MTT, (methylthiazolylidiphenyl-tetrazolium bromide) assay (Sigma) was performed according to manufacturer's protocol to assess cell viability during drug treatments. Phase contrast microscopy with an

Olympus CKX41 inverted microscope and 40 $\times$  objective was used to assess cell morphology.

### Immunocytochemistry

For primary cilia imaging and analysis, cells cultured on collagen I-coated glass were fixed in 10 % formalin and treated with anti-acetylated  $\alpha$ -tubulin primary antibody, 1:1, from a C3B9 hybridoma cell line (Sigma). Cilia were visualised with Alexa-Fluor 488 secondary antibody, 1:1000 (Life Technologies) and imaged with a 100 $\times$  oil objective on an Olympus Fluoview FV1000 confocal microscope. Nuclei were stained with DAPI (Life Technologies). Cilia length was analysed using Image J software.

### Oscillatory fluid flow

Cells were exposed to oscillatory fluid flow as a mechanical stimulus. Cells were seeded on collagen I-coated glass slides at  $\sim$  2,800 cells/cm $^2$  and cultured for 72 h before application of flow. Drug treatments were applied 16 h prior to experimentation. Slides were loaded into parallel plate flow chambers (dimensions: 75  $\times$  38  $\times$  0.28 mm) and allowed to incubate at 37 °C for 30 min prior to initiation of stimulation (Kwon *et al.*, 2010; Lee *et al.*, 2014; Malone *et al.*, 2007). Flow was applied for 1 h at 1 Hz with a peak flow rate of 18.8 mL/min, providing 1 Pa peak wall shear stress.

### mRNA expression

Immediately after flow, cells were washed with PBS and total mRNA was isolated using TriReagent (Sigma). Total mRNA was converted to cDNA by TaqMan reverse transcriptase (Applied Biosystems). Gene expression was analysed by quantitative real-time PCR using primers and probes (Life Technologies) for analysis of COX-2

(Mm00478374\_m1), OPN (Mm00436767\_m1), AC6 (Mm00475772\_m1), intraflagellar transport 88, IFT88 (Mm00493675\_m1) and GAPDH (4351309). Samples and standards were run in triplicate and gene expression was normalised to GAPDH endogenous control, as previously performed (Kwon *et al.*, 2010; Lee *et al.*, 2014).

### RNA interference

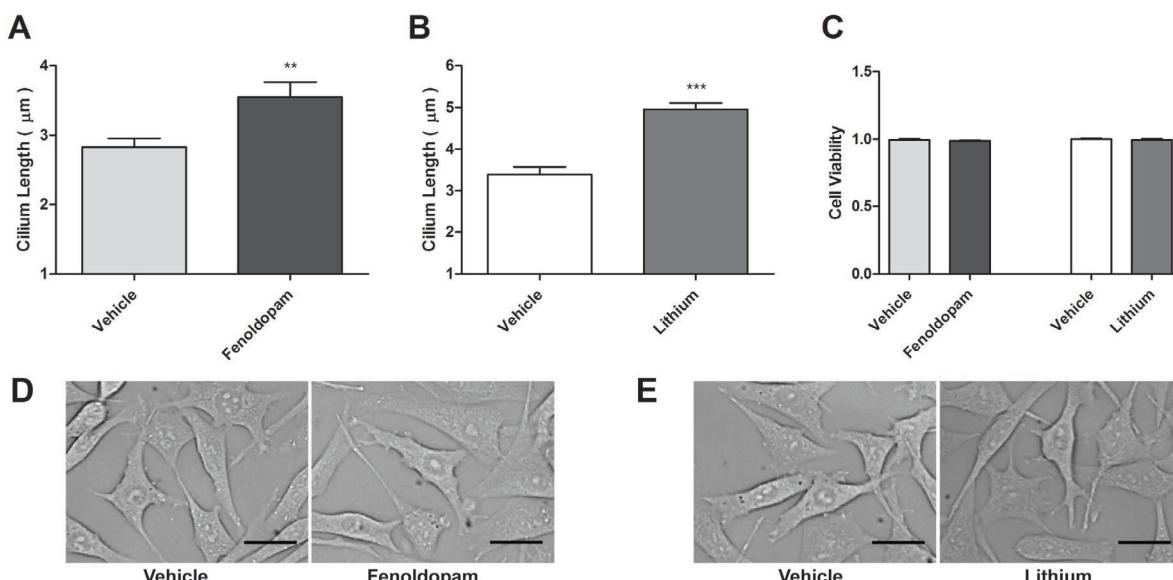
Gene silencing was performed by siRNA mediated knockdown and compared to scramble siRNA control (Life Technologies). For primary cilia disruption, cells were transfected with 20  $\mu$ M IFT88 siRNA (5'-CCAGAACAGATGAGGACGACCTT-3'), AC6 siRNA (5'-CCTGCCACCTACAACAGCTCAATT-3') or scrambled siRNA control using Lipofectamine 2000 (Life Technologies) as previously described (Kwon *et al.*, 2010).

### Adenylyl cyclase activity

Adenylyl cyclase activity was quantified by cAMP ELISA (Enzo). Cells were cultured as previously described and treated with 10  $\mu$ M fenoldopam for 16 h. Cells were stimulated with 10  $\mu$ M forskolin (Sigma) or DMSO vehicle control for 20 min prior to lysis with 0.1 M HCl. Cell lysate was analysed according to manufacturer's protocol and normalised to total protein quantified by BCA (Thermo Fisher). All samples and standards were run in duplicate.

### Analysis

All data were analysed with one-way ANOVA followed by Bonferroni *post-hoc* correction. Values were reported as mean  $\pm$  SEM, with  $p < 0.05$  considered statistically significant. Sample size,  $n$ , represents biological replicates.



**Fig. 1.** Small molecule treatments increase primary cilia length. 10  $\mu$ M fenoldopam (**A**) and 500  $\mu$ M lithium (**B**) treatment for 16 h significantly increases primary cilia length compared to vehicle control. Drug treatments elicit no change in cell viability, as assessed by MTT assay (**C**) and no gross morphological differences are exhibited (**D,E**). Mean  $\pm$  SEM;  $n > 25$  cilia for each group,  $n = 4$  for MTT assay; \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ; scale bars = 20  $\mu$ m.

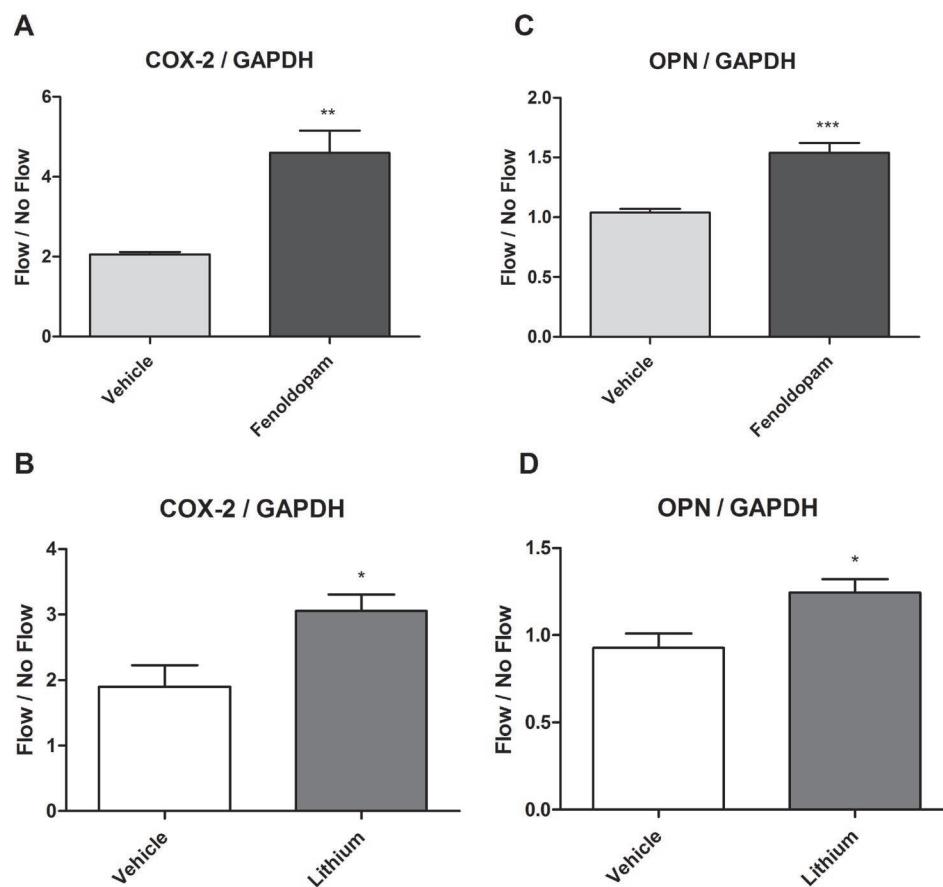
## Results

To test our hypothesis that cilium length directly affects mechanosensitivity, we first verified that we could modulate primary cilia length. We cultured MLO-Y4 osteocytes and treated them with two distinct small molecules to increase cilia length. Cells were cultured in media supplemented with fenoldopam, lithium or vehicle control for 16 h. Immunocytochemistry was used to image primary cilia and assess changes in cilia length. Both fenoldopam and lithium treatments induced significant increase in cilia length by  $26\% \pm 7\%$  and  $46\% \pm 5\%$ , respectively, compared to vehicle control (Fig. 1A, B). Cell viability was assessed with MTT assay and it found no change upon drug treatments (Fig. 1C). Additionally, no gross morphological changes resulted from fenoldopam or lithium treatment (Fig. 1D, E).

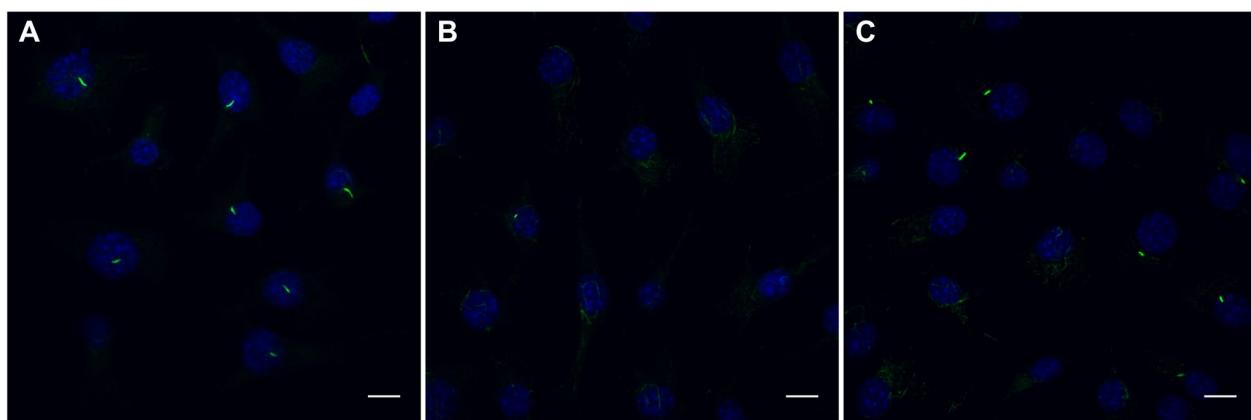
Next, we examined the effect of elongating cilia on cellular mechanosensitivity by mechanically stimulating cells with longer cilia and analysing their osteogenic response. Osteocytes were treated with fenoldopam, lithium or vehicle control, and exposed to oscillatory fluid flow for 1 h. As a control, samples were simultaneously loaded into flow chambers, but not subjected to flow. Mechanosensitivity was then quantified at the mRNA

level with analysis of COX-2 and OPN expression and presented as the fold change of flow over no flow control (Fig. 2). Cells with cilia lengthened by fenoldopam were more responsive, exhibiting elevated mRNA expression of  $124\% \pm 27\%$  and  $48\% \pm 8\%$  of COX-2 and OPN respectively compared to unlengthened controls. Lithium resulted in a more modest, but still significant increase in response of  $61\% \pm 13\%$  and  $34\% \pm 8\%$  for COX-2 and OPN. This flow-induced enhanced osteogenic response was observed in cells with elongated primary cilia, regardless of the means of lengthening, suggesting that the effect is due to lengthening and not an unanticipated effect of the agents utilised.

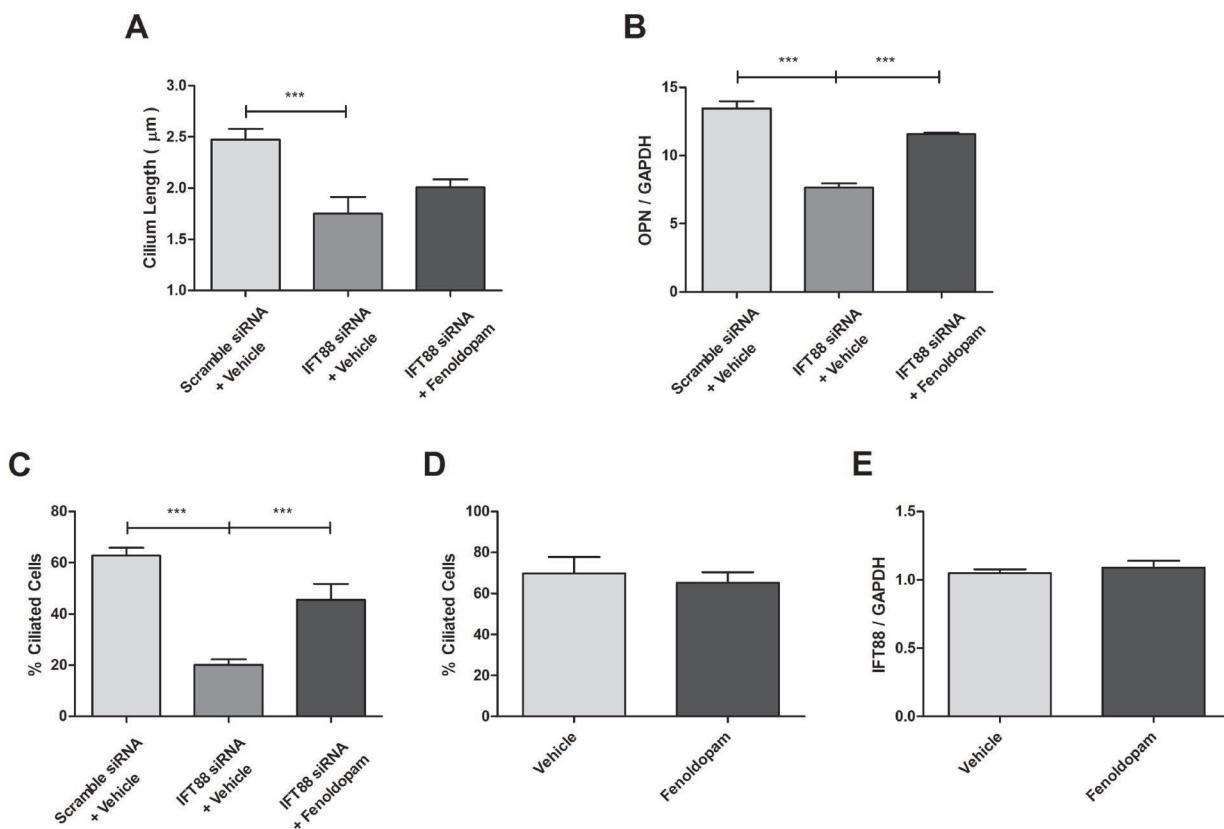
We next sought to examine the potential of targeting primary cilia length to recover impaired cilia function. IFT88 inhibition was employed as a model of dysfunctional cilia and has previously been used to mimic the effects of polycystic kidney disease (Lehman *et al.*, 2008). IFT88 is a critical component of intraflagellar transport and is necessary for proper primary cilia formation (Pazour *et al.*, 2000; Yoder *et al.*, 2002). Cells treated with IFT88 siRNA displayed decreased primary cilia length and incidence compared to scramble control (Fig. 3A, B). IFT88 siRNA treated cells were then treated with fenoldopam and cilia length and incidence were noticeably recovered (Fig.



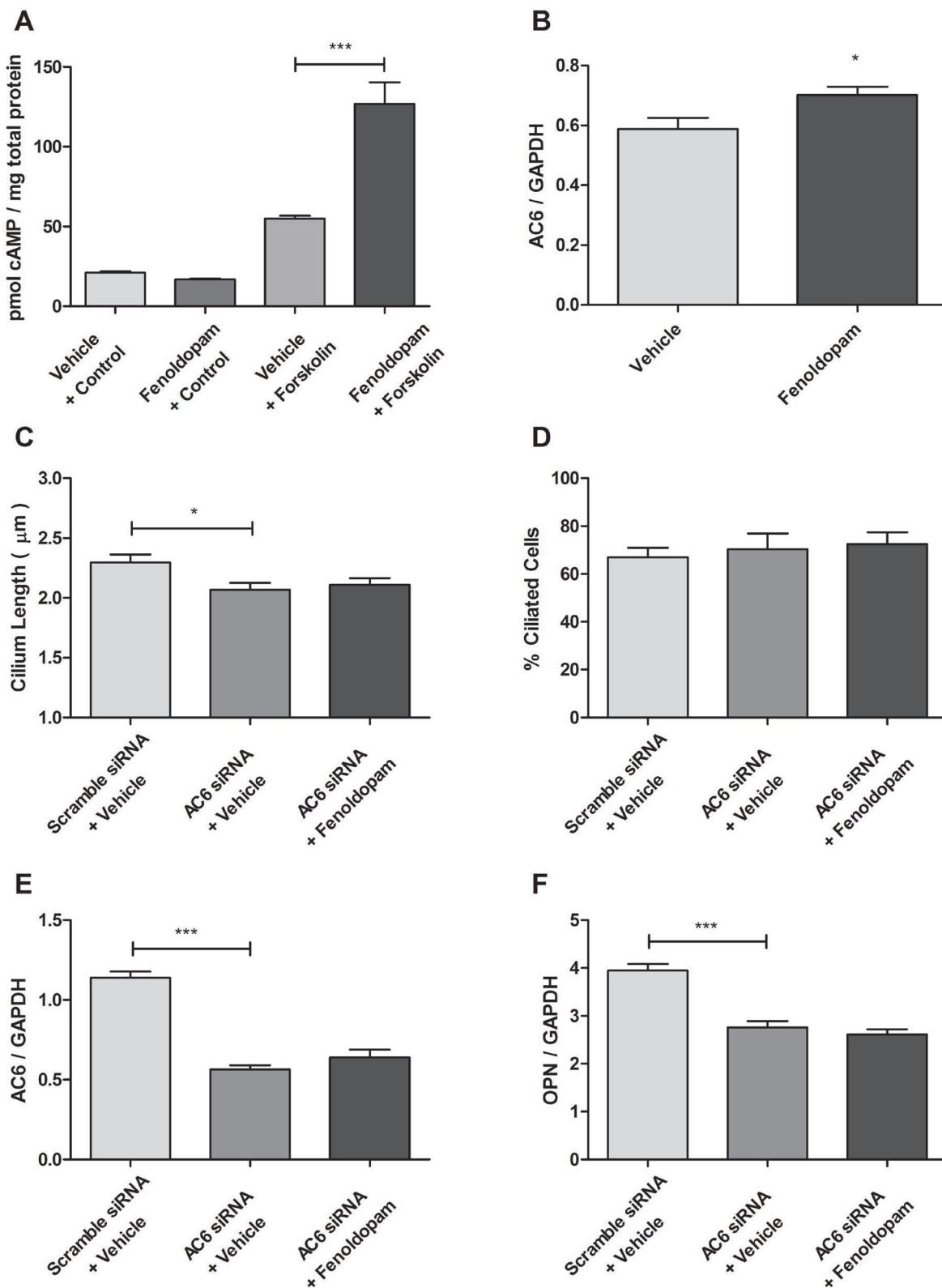
**Fig. 2.** Cells with longer cilia are more mechanosensitive. Cells were subjected to fluid flow for 1 h and the fold change of flow vs no flow control groups was compared. Cells express significant increases in COX-2 (A,B) and OPN (C,D) mRNA relative to GAPDH endogenous control when treated with either fenoldopam (A,C) or LiCl (B,D) for 16 h. Mean  $\pm$  SEM;  $n \geq 5$  for each group; \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .



**Fig. 3.** Treatment with IFT88 siRNA disrupts primary cilia formation. Overlays of primary cilia (green) and nuclei (blue) illustrates primary cilia incidence. Scramble control siRNA treatment for 48 h does not disrupt primary cilia formation (**A**). IFT88 siRNA treatment results in decreased cilia length and incidence (**B**). Fenoldopam treatment recovers primary cilia formation in IFT88 siRNA treated cells (**C**). Scale bars = 10  $\mu$ m.



**Fig. 4.** Fenoldopam rescues ciliogenesis and mechanosensing. Cells treated with IFT88 siRNA have decreased cilia length compared to scramble control, while fenoldopam treatment appears to recover cilia length ( $n \geq 25$  for scramble and fenoldopam treated,  $n = 15$  for IFT88 siRNA alone) (**A**). Oscillatory fluid flow was applied to cells treated with IFT88 or scramble control siRNA. Impaired cilia display a decreased OPN response to fluid flow, while fenoldopam treatment is able to recover flow stimulated OPN expression (**B**). Treatment with IFT88 siRNA decreases cilia incidence, but is recovered with fenoldopam treatment;  $n \geq 8$  fields of view (**C**). Fenoldopam treatment on untransfected cells has no effect on cilia incidence;  $n \geq 8$  fields of view (**D**). Fenoldopam also did not alter IFT88 mRNA expression in untransfected cells (**E**). Mean  $\pm$  SEM;  $n \geq 4$ ; \*\*\*  $p < 0.001$ .



**Fig. 5.** Fenoldopam enhances adenylyl cyclase production and activity. Cells were treated with fenoldopam or vehicle control for 16 h and then forskolin stimulated for 20 min. Fenoldopam treatment significantly increases the cAMP response to forskolin stimulation (**A**). Fenoldopam treatment significantly increases AC6 mRNA expression (**B**). AC6 knockdown decreases cilia length, but is not recovered with fenoldopam treatment (**C**). Neither AC6 siRNA nor fenoldopam alter cilia incidence;  $n \geq 8$  fields of view (**D**). Oscillatory flow applied to AC6 siRNA treated cells elicits a decrease in AC6 and OPN mRNA expression and is not recovered with fenoldopam treatment (**E,F**). Mean  $\pm$  SEM;  $n \geq 4$  for each group; \*  $p < 0.05$ , \*\*\*  $p < 0.001$ .

3C). Upon analysis, cilia of IFT88 siRNA treated cells were significantly shorter (Fig. 4A) and were present with lower incidence (Fig. 4C) than scramble control groups, with fenoldopam treatment significantly recovering cilia incidence. We then mechanically stimulated these cells to examine whether ciliogenesis recovery restored mechanosensitivity. Fluid flow was applied for 1 h and cells with impaired primary cilia formation displayed significantly decreased flow-induced OPN mRNA expression by  $43\% \pm 2\%$ , compared to scramble control (Fig. 4B). Then, fenoldopam treatment was able to recover this OPN response by  $52\% \pm 1\%$ , compared to IFT88 siRNA and vehicle treated cells. This further suggests that the length of primary cilia is critical to their function as a mechanosensor and that as cilia formation was restored, so was mechanosensitivity too. Then we confirmed that fenoldopam treatment of healthy, ciliated cells had no significant effect on cilia incidence (Fig. 4D) or IFT88 mRNA expression (Fig. 4E).

Finally, we examined a potential molecular pathway through which fenoldopam increases primary cilia length. cAMP has been previously shown to be involved in ciliogenesis and primary cilia-mediated mechanotransduction, so we quantified adenylyl cyclase activity by measuring stimulated cAMP production (Besschetnova *et al.*, 2010; Kwon *et al.*, 2010). We increased primary cilia length by fenoldopam treatment and then briefly stimulated the cells with the adenylyl cyclase agonist forskolin (Fig. 5A). Fenoldopam treatment significantly enhanced the forskolin stimulated cAMP response by  $130\% \pm 25\%$  compared to vehicle control. Additionally, fenoldopam treatment induced a  $20\% \pm 8\%$  increase in AC6 mRNA expression (Fig. 5B). Because of the significant role of AC6 in primary cilia-mediated mechanotransduction, we used inhibition of AC6 as an alternative model of impaired cell mechanosensitivity. Treatment of osteocytes with AC6 siRNA resulted in a small but significant decrease in cilia length by  $10\% \pm 3\%$  (Fig. 5C), while AC6 inhibition had no effect on cilia incidence (Fig. 5D). Fenoldopam had no effect on recovering cilia length or incidence in AC6 siRNA treated cells. When cells with diminished AC6 were mechanically stimulated, AC6 knockdown cells displayed decreased AC6 and flow-induced OPN expression by  $50\% \pm 3\%$  and  $30\% \pm 3\%$ , respectively, which was not recovered with fenoldopam treatment (Fig. 5E, F).

## Discussion

Our results demonstrated that primary cilia length plays a significant role in cell mechanosensitivity. We employed two distinct, clinically utilised, small molecules to increase cilia length and both resulted in enhanced mechanosensitivity. Cells with impaired ciliogenesis had impaired mechanosensing, but this could be recovered with fenoldopam treatment. Finally, we showed that fenoldopam modulates osteocyte mechanosensitivity through a mechanism involving AC6 and cells with diminished AC6 had shorter cilia and impaired mechanosensing.

Based on clinical and biochemical considerations,

fenoldopam was a more suitable candidate for further study than lithium and thus was only used for studying the effects of IFT88 and AC6 knockdowns. Fenoldopam is a dopamine D1-like receptor agonist clinically used as a vasodilator in cases of extreme hypertension (Murphy *et al.*, 2001; Post and Frishman, 1998). Lithium has a much less defined function and is clinically used to treat a wide range of mental disorders, including bipolar disorder (Marmol, 2008). Furthermore, lithium is an inhibitor of GSK-3 $\beta$  and can have downstream effects on various signalling pathways including Wnt and Hedgehog. While lithium has been used to increase cilia length in a variety of cell types, other GSK-3 $\beta$  inhibitors have no effect on cilia length (Jope, 2003; Ou *et al.*, 2009).

Fenoldopam treatment increased cilia length, but also plays a role in adenylyl cyclase activity. The increase in forskolin stimulated adenylyl cyclase activity with fenoldopam treatment implicates two potential mechanisms. First, it is possible that fenoldopam sensitises adenylyl cyclases, resulting in an increased cAMP response to forskolin. Alternatively, fenoldopam may increase production of adenylyl cyclases, augmenting forskolin stimulated cAMP production. This second notion is consistent with previous work indicating that fenoldopam treatment upregulates AC6, a specific adenylyl cyclase isoform, production in kidney cells (Yu *et al.*, 2014). Our results support this possible molecular pathway, demonstrating an increase in AC6 mRNA expression in response to fenoldopam stimulation. Previously, we have demonstrated that AC6 localises to the osteocyte primary cilium and is critical for primary cilia-mediated mechanotransduction (Kwon *et al.*, 2010). Besschetnova *et al.* reported that stimulating the cAMP signalling pathway results in increased cilia length (Besschetnova *et al.*, 2010). Using an siRNA-mediated knockdown, they showed that AC6 has a functional role in mediating primary cilia elongation. Together, these findings indicate that AC6-cAMP dynamics are critical to both primary cilia length and mechanotransduction and that fenoldopam treatment stimulates this pathway. Furthermore, these results suggest that fenoldopam treatment enhances ciliary protein production to promote cilium elongation.

Adenylyl cyclases and cAMP contribute to recover and elongate primary cilia by stimulating IFT particle transport. It has previously been reported that stimulation of the adenylyl cyclase-cAMP-PKA signalling pathway augments anterograde transport of IFT particles to promote cilia elongation (Besschetnova *et al.*, 2010). Because fenoldopam enhances adenylyl cyclase production, this suggests that fenoldopam treatment is potentiating adenylyl cyclase activity and IFT particle transport. Our model of impaired cilia utilised an IFT88 knockdown, not a complete knockout of the gene, so it is possible that fenoldopam was able to enhance remaining IFT88 function and promote cilia elongation and rescue cilia incidence. Furthermore, this presupposes that even though the IFT88 knockdown is satisfactory to impair cilia formation and function, sufficient IFT88 remains to elongate cilia. Our data show no change in IFT88 mRNA expression elicited by fenoldopam treatment suggesting that fenoldopam stimulated the remaining IFT88, rather than promoting

production of new IFT88. However, this does not discount the notion that fenoldopam treatment may instead prevent IFT88 knockdown driven disassembly of the cilium.

The specific means by which cells with longer cilia are more mechanosensitive remains elusive, but there are two potential mechanisms of how this may occur. Schwartz *et al.* developed one of the first models of primary cilia deflection under fluid flow and hypothesised that longer cilia would experience greater membrane strain to increase opening of stretch-activated ion channels on the ciliary membrane (Schwartz *et al.*, 1997). Alternatively, longer cilia may simply allow for the presence of more cilia-specific proteins and signalling molecules within this microdomain (Breslow *et al.*, 2013; Kee *et al.*, 2012). Increasing the total amount of ciliary protein could enhance signal transduction within the ciliary compartment, modifying primary cilia-mediated mechanosensitivity. In fact, fenoldopam treated cells exposed to fluid flow have increased ciliary influx of calcium, which has been identified as one initiator of the mechanotransduction signalling cascade (Jin *et al.*, 2014; Yuan *et al.*, 2015). It is also possible that cilium-lengthening agents actually enhance ciliary protein production and trafficking to promote cilium elongation.

The correlation between cilia length and critical ciliary proteins involved in mechanosensing was examined with AC6 siRNA treatment. The knockdown of AC6 decreased flow-induced osteogenic gene expression, yet fenoldopam treatment was not sufficient to rescue AC6 expression or OPN expression as was demonstrated in the IFT88 knockdown model. Because fenoldopam treatment was not able to recover AC6 or OPN mRNA expression in AC6 knockdown cells, this may suggest that the ability of fenoldopam to enhance AC6 activity is critical for recovering cellular mechanosensing. However, AC6 knockdown also decreased primary cilia length, which was not recovered with fenoldopam and did not alter cilia incidence. Altogether, these data suggest that both cilia length and protein production may be critical in primary cilia-mediated mechanosensing.

Cells with longer cilia are more mechanosensitive, but primary cilia cannot be elongated indefinitely. Longer cilia are exposed to greater drag force and are more likely to be sheared off (Hierck *et al.*, 2008). For example, endothelial cell primary cilia are flow sensors in regions of low shear, specifically because they are cleaved off as shear stresses increase (Van der Heiden *et al.*, 2008). Interestingly, electron microscopy has shown that primary cilia structure is not constant along the ciliary axoneme and becomes increasingly disorganised and asymmetric at the distal tip (Odor and Blandau, 1985; Yamamoto and Kataoka, 1986). This loss of microtubule symmetry reduces the bending stiffness of the cilium at the distal end, making drastically elongated cilia more susceptible to removal by fluid shear (Rydholm *et al.*, 2010).

While osteocyte primary cilia are free-standing flow sensors *in vitro*, their mechanosensing function may differ *in vivo*. It has been estimated that the lacunar space in which osteocytes reside *in vivo* allows for only a 1 µm long cilium (McNamara *et al.*, 2009; Uzbekov *et al.*, 2012). Due to the spatial limitations within the lacuna, the potential effect

of pharmacologically enhancing osteocyte cilia length *in vivo* is unclear. In fact, these spatial constraints may point to the cilium not being a free-flowing mechanosensor at all. Rather, osteocyte cilia may anchor to the lacunar wall, similarly to chondrocyte primary cilia, which form integrin attachments with the surrounding extracellular matrix, ECM (McGlashan *et al.*, 2006). A computational model by Vaughan *et al.* simulated osteocytes exposed to fluid flow within the lacunar-canalicular network (Vaughan *et al.*, 2014). The authors modelled a free-standing cilium, 0.5 µm long, within a lacuna and calculated the resulting strain at the base of the cilium. Their model suggests that a cilium in this configuration does not experience a great enough strain to function as a flow sensor, but a cilium directly attached to the ECM does. However, the authors do not account for the amount of membrane strain necessary to stimulate stretch-activated ion channels on the ciliary membrane and may have overestimated the required strain for cilium stimulation. Fenoldopam treatment not only increases length, but may also enhance mechanosensitive protein levels, such as adenylyl cyclases and ion channels, within the cilium. Regardless of cilium length, this enriched protein trafficking to the cilium would increase chemical kinetics within the ciliary microdomain to modify cellular mechanosensitivity.

Our results certainly suggest that fenoldopam treatment or similar agents, which may increase cilia length, can be a potent method to enhance cell mechanosensitivity. However, the effect of fenoldopam on whole bone has not been examined, so the potential skeletal and systemic consequences in translating this work to *in vivo* models is still unknown. Statistical analysis of patients treated with various antidepressants, such as selective serotonin reuptake inhibitors, which can block dopamine D2 receptors, revealed that these patients have increased risk of hip and femur fracture (Van Den Brand *et al.*, 2009; Smith *et al.*, 2009). Furthermore, many antidepressants and antipsychotics that block dopamine D2 receptors can cause decreased oestrogen and testosterone levels leading to significantly reduced bone mass (Phillip and Lazar, 2003). All of these studies examine treatments which may affect dopamine D2 receptors, but at present there are no examinations of the impact of dopamine D1 receptor agonists on bone formation. As such, no connection between fenoldopam treatment and skeletal health has been established. Because of this lack of direct evidence, such drugs have the potential for a wide range of unknown side effects. Further work needs to be performed before any drugs altering dopamine signalling are used to treat bone disease.

Targeting primary cilia-mediated mechanotransduction has widespread applications in preventative medicine that reach far beyond osteocytes. Numerous diseases are characterised by impaired primary cilia function. Mutations of PC2, polycystin 2, are attributed to polycystic kidney disease and skeletal deformations. Bardet-Biedl syndrome is characterised by malfunctioning BBS proteins at the base of the primary cilium, causing retinopathy, polydactyly and renal failure (Loktev *et al.*, 2008; Mochizuki *et al.*, 1996; Xiao *et al.*, 2011). Recently, primary cilia have even been implicated in tumour development. Primary

cilia help regulate Wnt signalling, changes in which have been correlated with cancer cell progression (Lancaster *et al.*, 2011). Furthermore, some cancer cell types lose their primary cilia, which potentially contributes to their insensitivity to repressive signals (Plotnikova *et al.*, 2008). Additionally, atherosclerotic plaques form in areas of reduced and disturbed arterial fluid flow, regions that interestingly have an increased incidence of primary cilia. This suggests that these cells are compensating, increasing their sensitivity to reduced fluid flow in order to promote an adequate cellular response (Van der Heiden *et al.*, 2008; Warboys *et al.*, 2011). Within bone, osteocytes utilise primary cilia to sense and respond to mechanical cues. *In vitro* and *in vivo* studies demonstrate that when these cilia are removed there is a decreased bone formation response to loading (Kwon *et al.*, 2010; Malone *et al.*, 2007; Temiyasathit *et al.*, 2012). Fenoldopam is already an FDA approved drug and our results point to it being an attractive candidate for study in numerous *in vitro* and *in vivo* applications to treat such a myriad of conditions.

### Conclusion

We have demonstrated that cells with longer primary cilia are more mechanosensitive and we present a simple, yet robust, method to enhance primary cilia-mediated mechanotransduction. Fenoldopam treatment not only increased primary cilia length, protein production and mechanosensitivity, but also rescued cilia form and function in cells with impaired cilia. Although fenoldopam and lithium are likely not ideal treatments for all cilia-related conditions, the strategy of modulating primary cilia sensitivity may aid in combating the phenotypes displayed by various ciliopathies, maintain sensitivity of cancer cells so that they respond to repressive signals, cue cellular responses to slow atherosclerotic plaque formation and stimulate load-induced bone formation.

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## Discussion with Reviewers

**Rosa Serra:** How does the author envision a clinical use for bone density disorders? Would the drug be used in combination with exercise?

**Authors:** While this work is entirely *in vitro* and at this point it is difficult to definitively make any conclusions about *in vivo* translation, these findings are suggestive of a pro-osteogenic therapeutic strategy. Our work posits that fenoldopam sensitises bone cells to mechanical stimulation, so that when a patient receives a similar treatment, exercise-induced bone adaptation would be augmented. Thus, any potential treatment regimen would likely include some exercise component in treating bone density disorders.

**Fredrick Pavalko:** How do localised changes in cAMP at the primary cilia lead to global changes in bone density?

**Authors:** Adenylyl cyclases convert ATP to cAMP, which activates PKA to regulate CREB-mediated transcriptional modification. Previous work from our group demonstrated that AC6, a specific isoform of adenylyl cyclase known to localise to cilia, plays a critical role in load-induced bone adaptation. Specifically, mice lacking AC6 display a significantly abrogated response to compressive ulnar load (Lee KL *et al.*, 2014). It is possible that primary cilia-mediated mechanotransduction is initiated in the cilium, causing a change in ciliary and cytosolic cAMP, which modifies downstream gene transcription. In osteocytes, this change in gene expression likely alters intercellular signalling to osteoblasts, osteoclasts and progenitor cells to dictate bone formation, density and overall skeletal health.

**Editor's note:** The Scientific Editor responsible for this paper was Juerg Gasser.