Targeting primary cilia-mediated mechanotransduction to promote whole bone formation

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ABSTRACT

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Osteoporosis is a devastating condition characterized by decreased bone mass, and affects over 50% of the population over 50 years old. Progression of osteoporosis results in significantly heightened risk of fracture leading to loss of mobility, prolonged rehabilitation, and even mortality due to extended hospitalization. Current therapeutic options exist to combat low bone mass, but these treatments are being met with increasing concern as reports emerge of atypical fractures and necrosis. Thus, new therapeutic strategies are required.

Bone is highly dynamic, and it has long been known that physical load is a potent stimulus of bone formation. Despite this, none of the current treatments for bone disease leverage the inherent mechanosensitivity of bone – the ability of bone cells to sense and respond to mechanical forces such as exercise. One potential therapeutic target is the primary cilium. Primary cilia are solitary antenna-like organelles, and over the last 20 years have been identified as a critical cellular mechanosensor. Primary cilia and cell mechanotransduction are critical to the function of numerous cells and tissues. Thus, understanding primary cilia-mediated mechanotransduction has potential applications in treating kidney and liver disease, atherosclerosis, osteoarthritis, and even certain cancers. Previous work from our group has
demonstrated that disruption of the cilium impairs bone cell mechanosensitivity, resulting in abrogated whole bone adaptation in response to physical load.

In this thesis we examine the potential of targeting the primary cilium to enhance bone cell mechanosensitivity and promote whole bone formation. First, we demonstrate the pharmacologically increasing primary cilia length significantly enhances cell mechanotransduction. Next, we expand our list of candidate compounds to manipulate ciliogenesis through the use of high-throughput drug screening. We developed an automated platform for culturing, staining, imaging, and analyzing nearly 7000 small molecules with known biologic activity, and classify them based on mechanism of action. One of these compounds is then used in a co-culture model to study the effects of manipulating osteocyte primary cilia-mediated mechanosensing on pro-osteogenic paracrine signaling to promote the activity of bone-forming osteoblasts and osteogenic differentiation of mesenchymal stem cells. Finally, we translate our in vitro findings into an in vivo model of load-induced bone formation using the same compound to enhance cell mechanotransduction. We demonstrate that we can sensitize bones to mechanical stimulation to enhance load-induced bone formation in healthy and osteoporotic animals, with minimal adverse effects. Together, this work demonstrates the therapeutic potential and viability of targeting primary cilia-mediated mechanotransduction for treating bone diseases.
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Dedicated to:

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Chapter 1

1. Introduction

1.1. Osteoporosis

Medical advances are resulting in humans living longer than ever; however, these patients are now increasingly being met with skeletal deficiencies and abnormalities. Osteoporosis is the most prevalent of these conditions, and is marked by a decrease in bone mass resulting in heightened risk of fracture. In fact, over 50% of the US population over 50 years old are affected by osteoporosis impacting the lives of over 50 million men and women in the US, and over 200 million women alone worldwide \(^1\). It is expected that the number of Americans over 65 is expected to double by 2060, and the incidence of hip fractures in men and women is estimated to rise 310% and 240%, respectively, between 1990 and 2050 \(^2\). Hip fractures represent the most devastating result of osteoporosis, with 20% patient mortality within the first year following fracture \(^3,4\). Surviving patients are still left with dramatically impaired quality of life, with 60% requiring extra assistance, and 33% entirely reliant on caretakers, leaving less than 7% reaching full recovery \(^5,6\). Direct medical costs of osteoporotic fractures in the US alone are estimated to be over $20 billion per year, and are expected to rise to $50 billion by 2050 \(^7\). The World Health Organization considers osteoporosis to be the second greatest health concern following cardiovascular disease, and the disability caused by osteoporosis to been classified as more significant than that produced by cancers and chronic non-transferable pathologies \(^7,8\). While the majority of the burden of bone diseases is due to conditions marked by low bone mass,
several rare bone diseases, such as osteopetrosis (1 in 100-500k people) and melorheostosis (1 in 1.1 million people), present with excessive bone formation\textsuperscript{9,10}.

It has long been known that mechanical stimulation is a potent anabolic stimulus of bone formation. Julius Wolff first described that bone actively responds to mechanical load, becoming stronger to prevent fracture from that sort of loading\textsuperscript{11}. This is phenomenon is clearly witnessed in active versus sedentary people, and is most notable in tennis and baseball players, whose dominant arms exhibit drastically greater bone density compared to non-dominant arms\textsuperscript{12}. Conversely, absence of load results in reduced bone mass, heightening susceptibility to fracture as experienced with astronauts and sedentary patients, as well as less mobile elderly patients\textsuperscript{13}.

1.2. Bone and current therapeutics

Bone is a dynamic organ system, constantly responding to external stimuli. Chemical stimuli such as parathyroid hormone signaling, vitamin D, calcitonin, or calcium signaling can elicit formation or resorption of bone tissue\textsuperscript{14}. The work in this thesis, however, focuses on the mechanosensitivity of bone.

Within bone, the three predominant cell types are osteoclasts, osteoblasts, and osteocytes. Osteoclasts are the bone resorbing cells. These are multinucleated cells which secrete digestive enzymes to break down mineralized bone tissue. Osteoblasts are the primary bone forming cells, producing matrix proteins such as collagen I and osteopontin, as well as mineral apatite as the foundation of new bone\textsuperscript{14}. Osteocytes are considered the signaling cells within bone. As osteoblasts age and become more embedded in cortical bone, they become osteocytes. These cells reside in fluid-filled pockets within bone called lacunae, connected via canaliculi. The
lacunar-canalicul network allows for osteocytes to sense chemical and physical stimuli, and efficiently communicate with each other and other bone cells. Thus, the osteocyte is viewed as a nexus coordinating signaling between osteoblasts and osteoclasts to regulate bone formation, resorption, and overall skeletal maintenance. For example, osteocytes secrete RANKL (receptor activator of nuclear factor kappa-B ligand) to stimulate osteoclast activity, and OPG (osteoprotegerin) to prevent RANKL binding and inhibit osteoclast activation \(^{14}\). Osteoblast activity can be regulated by osteocyte secretion of \(\text{PGE}_2\) (prostaglandin \(\text{E}_2\)), \(\text{ATP}\) (adenosine triphosphate), and \(\text{NO}\) (nitric oxide). Osteocytes also secrete sclerostin, which acts as a negative regulator of osteoblastic bone formation \(^{15}\). Many bone diseases such as osteoporosis result from an imbalance of the normal signaling between osteocytes, osteoblasts, and osteoclasts.

Though existing first-line therapeutics are available and have demonstrated reasonable success in mitigating the effects of osteoporosis, they present significant limitations. Bisphosphonates are the current gold standard and prevent osteoclasts from resorbing bone material, resulting in increased accumulation of microdamage within bone \(^{16}\). Clinically, bisphosphonates have been met with increased risk of atypical fracture and mandibular necrosis \(^{17,18}\), leading to a drastic change in patient sentiment to that of fear of medical intervention. Media reports of atypical jaw and long bone fractures have in large part caused the 50% decrease in bisphosphonate prescriptions between 2008 and 2012 \(^{19}\). Due to the current patient discomfort with current treatment options, it is estimated that after one year only 40% of patients will continue with their prescribed treatment, and after two years only 20% will continue treatment, demonstrating a need for new therapeutic options \(^{20,21}\). RANKL inhibitors comprise the next in line treatment option, but similarly inhibit osteoclast activity, and can result in similar adverse
effects as bisphosphonates. Amgen is in phase III trials with an antibody against sclerostin, preventing normal inhibition of osteoblast activity. However, reports suggest this may not be a feasible long-term strategy and may result in certain cancers. Due to these concerns with existing therapeutics, there is a significant need for a new class of bone therapeutic.

1.3. Methods of mechanotransduction

Mechanotransduction is the process by which cells sense and respond to their mechanical environment and external physical forces. Mechanical stresses direct and dictate cell and tissue functions throughout the entire lifespan of an organism. Growing work, in a number of different cell and tissue types, as well as organisms, has helped uncover the mechanisms of cellular mechanotransduction. Within the cell, several different mechanosensing mechanisms exist, often working in concert producing a range of responses – from short-term changes such as cytoskeletal reorganization, to long-term gene expression and epigenetic modifications. These mechanosensing mechanisms can include the cytoskeleton, cell membrane mechanics, the glycocalyx, focal adhesions, adherens junctions, caveolae, deformation of the nucleus, and primary cilia. Together these mechanotransduction pathways allow cells to adapt to changing mechanical environments, modulating whole tissue function, morphology, and remodeling.
Figure 1.1: Mechanisms of cellular mechanotransduction

Cells experience several different types of mechanical stimulation. This can include, but is not limited to, fluid shear across the apical surface of the cell, as well as compression and tension generated by fluid pressure and extracellular matrix/tissue deformation. These forces may be sensed by any combination of adherens junctions (a), the glycocalyx (b), membrane mechanics (c), the cytoskeleton (d), focal adhesions (e), deformation of the nucleus (f), caveolae (g), and primary cilia (h) 23.

The three basic mechanical stresses acting on cells are tension, compression, and shear, Fig. 1.1. For example, tendon and ligaments sustain large tensile stresses, arteries are stretched and sheared from the pulsatile blood flow, and bones resist compressive and tensile stresses. But the predominant stress is not always the most important stimulus for cells. In bone, the strains induced by physiological compression are very small (on the order of 2,000 microstrain) yet bone cells in vitro respond to strain on the order of 30,000 microstrain, suggesting that whole bone strain is amplified at the cellular level 2. Indeed, the current paradigm is that small strains squeeze interstitial fluid flow through a network of small channels, the canaliculi, much as a sponge does when a load is applied. The moving fluid leads to shear stress, and the magnitudes of shear stress estimated to occur physiologically in these channels have been shown to be potent stimuli for bone cells in vitro 3,4. Additionally, flow through the canalicular network in the
absence of strain has been shown to induce bone formation. It is therefore crucial to understand the environment of the cell to know which type of stress is acting on the cell and which temporal patterns and magnitudes of stimulation elicit a cellular response.

1.4. Primary cilia

While cells utilize numerous different mechanisms to sense and respond to mechanical stimulation, the work in this thesis focuses on primary cilia-mediated mechanotransduction.

*Primary cilia structure*

The primary cilium is a single immotile organelle protruding from the apical surface of nearly all mammalian cells. Primary cilia share similar structural characteristics with motile cilia, both consisting of 9 microtubule doublets forming the ciliary axoneme. Motile cilia, however, also contain a central microtubule doublet, radial spokes, and a nexin ring, allowing for cellular functions, such as propulsion or clearing of mucus and debris, not experienced in primary cilia. While primary cilia were identified in mammalian cells over 100 years ago, they were long considered vestigial structures. Recently improved molecular and biochemical techniques have enabled keen insights into this once forgotten organelle. Today, primary cilia are understood to play a critical role in the transduction of chemical and mechanical cues. In addition, primary cilia have been implicated as a nexus for numerous signaling pathways critical to cell function, such as Wnt and Hedgehog.
Primary cilia have three major zones, the axoneme, the transition zone and the basal body. The axoneme is comprised of 9 microtubule doublets and is surrounded by the ciliary membrane, while the basal body contains 9 microtubule triplets. Anterograde IFT (intraflagellar transport) allows delivery of cargo towards the distal tip through kinesin-2, while IFT-dynein is responsible for retrograde transport. The microtubules within the cilium are heavily acetylated, contributing to their bending stiffness. Furthermore, the cilium forms a microdomain distinct from the rest of the cell to which specific proteins are trafficked. This is done through a diffusion barrier, excluding large (80-100 kDa) proteins from freely diffusing in or out of the cilium, thought to exist either in the transition zone or between the transition zone and basal body. This figure was adapted from Nachury 2014, and Ishikawa+ 2011 26,27.

**Ciliopathies**

Impairment of primary cilia function and ciliary associated proteins has been identified as the cause of numerous different conditions known as ciliopathies. Polycystic kidney disease can result from genetic mutations in *Pkd1* and *Pkd2*, coding for the PC1/2 mechanosensitive ion channel complex 28. Bardet-Biedl Syndrome (BBS) is characterized by renal failure, polydactyly,
and retinitis pigmentosis, and is caused by mutations in ciliary associated BBS proteins. Retinal and skeletal conditions, such as Joubert and Meckel-Grouber Syndromes, are also attributed to cilia dysfunction. Primary cilia have also been suggested to have a potential role in cystic and fibrotic liver disease, atherosclerosis, bone maintenance, and osteoarthritis, as described in the following section. The growing list of ciliopathies and cilia-associated conditions has stimulated further research into the vast array of roles of the primary cilium.

While primary cilia do serve as chemosensors, this thesis focuses on their mechanosensory capabilities. Mechanical loading may result in a variety of physicochemical stimuli, such as changes in osmolarity, hydrostatic pressure, and pH, which may be sensed by the primary cilium. The prevailing theory is that mechanical stimulation, such as fluid flow or tissue deformation, causes primary cilia deflection to initiate downstream mechanotransduction signaling cascades.

**Ciliary protein localization**

Numerous proteins, such as PC1, PC2, TRPV4, and AC6, localize to the primary cilium and have been identified as critical for mechanotransduction. The localization of stretch-activated ion channels such as PC2 and TRPV4 suggest that their activation in response to fluid shear induces changes in ion concentrations within the cilium that initiate mechanotransduction signaling cascades. This signaling cascade is continued through proteins converting these ion changes into second messengers, with one hypothesis being that AC6 is inhibited by calcium increases to decrease cAMP levels, altering downstream PKA signaling and CREB-mediated transcriptional regulation.
**Intracilliary calcium signaling**

It is believed that deflection of the primary cilium opens the stretch-activated ion channels localized to the ciliary membrane, initiating calcium flux into the ciliary microdomain, resulting in downstream signaling cascades to the rest of the cell. Recent advances in molecular techniques have enabled several groups to develop fluorescently encoded calcium biosensors that specifically localize to the primary cilium. First, Work by Su et al. (2013) demonstrated that fusing the 5HT6 cilia-targeted protein to a genetically encoded calcium indicator (circularly permuted GFP located between M13, light chain kinase, and CaM, calmodulin, domains) allows for examination of calcium transients within the ciliary microdomain. This allowed measurements of fluid flow-induced calcium increases specifically within the cilia of kidney epithelial cells. Jin et al. (2014) then demonstrated similar results using a distinct ciliary-targeted calcium biosensor. These results were coupled with cytoplasmic calcium measurements, recording ciliary calcium changes that precede those in the cytoplasm, suggesting that ciliary calcium entry could initiate mechanotransduction events. Our group recently utilized a FRET-based biosensor (fluorescence resonance energy transfer) targeted to the cilium using Arl13b in both osteocytes and kidney epithelial cells. Again, measured ciliary calcium transients preceded cytoplasmic calcium changes.
1.5. Primary cilia-mediated mechanotransduction in various cell and tissue types

While it is known that bone actively responds to mechanical stimulation, it is incompletely understood how cells sense and respond to these applied physical forces – mechanotransduction. Much of our understanding of primary cilia-mediated mechanosensing in bone is derived from studying other cell and tissue types. The primary cilium is involved in mechanosensing in a wide range of cell and tissue types. These various tissues all require mechanical stimulation for proper function and homeostasis and rely on primary cilia-mediated mechanotransduction.

*Embryonic node*

Primary cilia play a critical role in embryonic development. In fact, global deletion of proteins required for formation of primary cilia, such as IFT88 (intraflagellar transport 88; required for transport of cargo within the cilium) or Kif3a (kinesin family member 3A; a motor protein subunit required for cilia formation), is embryonic lethal. During development, motile cilia within the embryonic node actually begin to move and generate directional flow of extracellular fluid, which is sensed by non-motile primary cilia in the surrounding crown cells. These cilia working in tandem results in proper left-right differentiation, and impairing either primary or motile ciliary function disrupts embryogenesis. Primary and motile cilia have distinct mechanical and functional characteristics; but play critical roles in directing cell and tissue function.
Kidney

Much of the early work describing primary cilia mechanotransduction was conducted in kidney cells \(^{43,44}\). Proper kidney function is dependent on regulated fluid flow through the nephrons and collecting ducts to control glomerular filtration rate, and this flow is sensed by kidney epithelial cell primary cilia \(^{45,46}\). Deflection of the primary cilium, by both fluid flow and micropipette manipulation, induces an increase in intracellular calcium, in a primary cilia-dependent manner \(^{44,46,47}\). This calcium influx is also mediated by the PC1/2 ion channel complex \(^{46}\). PC1 and PC2 both localize to the primary cilium, where PC1 is a mechanosensitive membrane protein regulating the opening of the PC2 channel \(^{48}\). In addition to calcium signaling, PC1 has been implicated in direct activation of STAT (signal transducer and activator of transcription) to regulate gene expression, demonstrating the potential for mechanotransduction pathways distinct from calcium \(^{49–51}\). In renal cells, functional defects of either portion of this ion channel complex, along with complete disruption of cilia formation, result in polycystic kidney disease \(^{52}\).

Endothelia

Endothelial cells sense blood flow and regulate vessel diameter to maintain blood pressure \(^{53}\). The shear stress stimulates endothelial nitric oxide production, an important vasodilator, and endothelial primary cilia have been implicated as important mechanosensors that regulate nitric oxide production \(^{54,55}\). Furthermore, PC1/2 has been identified as a critical component for this mechanotransduction response to occur, with cells lacking this ion channel complex exhibiting an attenuated response to fluid flow \(^{54}\). Mice lacking PC1 display decreased
serum nitric oxide leading to a hypertensive phenotype. Similarly to kidney epithelial cells, chemical removal of primary cilia, using chloral hydrate, in endothelial cells abrogates the response to flow. Interestingly, in vivo, endothelial cilia are only present at areas of low shear stress, potentially as a means to sensitize cells to lower amounts of mechanical stimulation. Furthermore, flow sensing primary cilia appear to be more prevalent at atherosclerotic predilection sites where shear stresses are low and disturbed. Removal of endothelial primary cilia impairs nitric oxide signaling resulting in increased atherosclerotic plaque formation.

Liver

Similarly to endothelial and renal epithelial cells, cholangiocyte primary cilia detect fluid flow in the bile duct. Fluid flow through isolated intrahepatic bile duct units results in a similar calcium influx as in renal cells, as well as a decrease in intracellular cAMP – responses which are cilium dependent. Cilia-localized PC1 and PC2 are necessary for this flow induced calcium increase and cAMP decrease. Furthermore, adenylyl cyclase 6 (AC6), which converts ATP into the second messenger cAMP, is calcium inhibited and localizes to cholangiocyte primary cilia. Mutations to these proteins can impair mechanotransduction, resulting in several cholangio-ciliopathies such as cystic and fibrotic liver disease. It has also been identified that ciliogenesis is heterogenous between larger and smaller bile ducts; however it is not yet clear if this is due to spatial limitations or to optimize primary cilia-mediated mechanosensing to varying fluid flow conditions.
**Cartilage**

Chondrocytes actively respond to cartilage compression and deformation, and moderate loading may slow the progression of osteoarthritis. Chondrocyte cilia, however, may have a distinct structure from previously mentioned flow sensors. Extracellular matrix binding proteins and integrins both localize to chondrocyte cilia, suggesting that these cilia may not be flow sensors, but rather directly sense local tissue deformation. Mechanical stimulation of chondrocytes initiates an up-regulation of proteoglycan and aggrecan synthesis, but this response is abrogated in cells lacking primary cilia. In addition, chondrocytes display a significant calcium influx in response to hypoosmotic loading, a response that is lost with chemical removal of primary cilia by chloral hydrate treatment. Interestingly, chondrocytes lacking primary cilia still display mechanically induced ATP release to initiate intercellular calcium signaling, a response that is lost in cilia-deficient epithelial cells – suggesting the potential for multiple and distinct mechanotransduction processes in different cell types. Compressive loading increases chondrocyte matrix deposition, in a response that is mimicked with treatment with an agonist (GSK1016790A) of the ciliary ion channel, TRPV4. These works demonstrate that chondrocyte cilia are not exclusively flow sensors, but may also respond to compressive and osmotic loading conditions. Chondrocyte primary cilia may also play a role in organizing cell polarity. In the growth plate, chondrocytes organize in columnar stacks that become completely disorganized with primary cilia disruption by Kif3a knockout. This cell and cilia organization may optimize the cells to sense mechanical stimulation, in a manner similar to tenocytes within tendon; however, the specific mechanosignaling function of primary cilia in tendon has not yet been characterized to the same degree as the previously mentioned cell types.
Cancer

Recently, primary cilia mechanosensing has been associated with tumor progression. Increased interstitial pressure and fluid flow have been described in a number of cancers, but how these biophysical effects are sensed and transduced to regulate tumor progression are incompletely understood \(^{69}\). Primary cilia facilitate Hedgehog (Hh) and Wnt signaling pathways which help direct cell proliferation \(^{24}\). Mutations or irregular stimulation of Hh and Wnt signaling are instrumental in the progression of numerous cancers \(^{70,71}\). Furthermore, both Hh and Wnt signaling can be modulated by the presence or absence of fluid flow mechanical stimulation, as well as alterations in cilia structure \(^{72-74}\). Breast, prostate, and liver cancer cells experience a decrease in primary cilia incidence through tumor progression, and recovery of cilia incidence by HDAC6 inhibition has been shown to slow progression of cholangiocarcinoma \(^{75,76}\). In contrast, basal cell carcinomas are actually frequently ciliated and removal of primary cilia can inhibit tumor formation, demonstrating multiple roles of cilia in cancer progression \(^{77}\). Primary cilia formation is related to, but exclusive from, cell proliferation and senescence, as ciliogenesis occurs upon entry of the cell into the G0 phase of the cell cycle \(^{78}\). Thus, manipulating ciliogenesis provides potential to regulate tumor cell growth. Many tumors are also characterized by an increase in tissue stiffness, with associated mechanotransduction events causing translocation to the nucleus of YAP (Yes-associated protein) and TWIST transcription factors \(^{79,80}\). Altering matrix rigidity, however, can slow growth and metastasis demonstrating that cancerous cells are indeed mechanosensitive, yet it is not completely clear what role primary cilia have in direct sensing of the tumor mechanical environment. While it is incompletely understood how ciliogenesis dictates tumor mechanosensing and growth, evidence is mounting
that targeting primary cilia-mediated signal transduction may be a therapeutic strategy to slow tumorigenesis.

1.6. Primary cilia in bone

Osteocytes are considered the primary mechanosensing cells within bone, detecting fluid flow through the lacunar-canalicular network \(^\text{81}\). Within these cells, the primary cilium has been identified as a critical mechanosensor, both \textit{in vitro} and \textit{in vivo} \(^\text{82,83}\). Mice with an osteoblast and osteocyte specific deletion of primary cilia, Kif3a\(^{-/-}\) (a motor protein subunit required for cilia formation), exhibit a reduction in load-induced bone formation \(^\text{83}\). It has also been revealed that PC1 plays a significant role in osteocyte mechanosensing. Xiao \textit{et al.} (2011) utilized a Dmp1-driven Cre mouse model to conditionally disrupt Pkd1 in osteocytes \(^\text{84}\). When subjected to mechanical loading, the Pkd1 deficient mice displayed reduced load-induced bone formation, suggesting that PC1 plays an important role in bone cell mechanotransduction. Mutations to another cilia-localized mechanosensitive ion channel, TRPV4, results in a wide range of skeletal dysplasias \(^\text{85}\). As in cholangiocytes, AC6 is also found in the osteocyte primary cilium, and fluid flow induces an increase in intracellular calcium and a decrease in cAMP, in a process mediated by AC6 \(^\text{36}\). \textit{In vivo}, AC6 knockout mice display no phenotypic difference in bone microarchitecture compared to wildtype mice, but when subjected to mechanical load, the AC6\(^{-/-}\) mice have an attenuated bone formation response \(^\text{86}\). Primary cilia-mediated mechanotransduction has even been implicated in healing around a bone implant, with mice lacking primary cilia within bone displaying impaired cell mechanosensitivity and osteogenesis in the peri-implant space \(^\text{87}\).
1.7. Overall goal of this thesis

The overall goal of this thesis is to demonstrate primary cilia as a viable target for sensitizing cells to mechanical stimulation, in an effort to guide the development of novel osteoporosis therapeutics. The overall hypothesis is that by increasing primary cilia length cells will become more mechanosensitive, resulting in an enhanced osteogenic response to external physical stimuli. The rationale for this is that increasing primary cilia length will enhance production of ciliary mechanosensing proteins, and also increase ciliary bending in response to mechanical stimulation to further activate ciliary ion channels. Together, these biochemical and structural changes will potentiate mechanotransduction events.

**Figure 1.3: Increasing primary cilia length will potentiate cellular mechanosensing**

Increasing primary cilia length is hypothesized to have two effects. 1) increasing length will enhance production of ciliary mechanosensitive proteins such as AC6. 2) increased cilia length could allow increased bending of the cilium to further stimulate stretch activated ion channels localized to the ciliary membrane.
Chapter 2

2. Lengthening primary cilia enhances cell mechanotransduction

2.1. Abstract

The primary cilium is a mechanosensor in a variety of mammalian cell types, initiating and directing intracellular signaling 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 hypothesized 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 are 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 characterized by impaired cilia function.
2.2. Introduction

Bone cell mechanotransduction has been studied through several different mechanisms. Altering cytoskeletal mechanics by disruption of actin polymerization with cytochalasin D inhibits flow-induced osteogenic differentiation of C3H10T1/2 mesenchymal stem cells. Similar experiments in osteoblasts treated with cytochalasin D and exposed to fluid flow demonstrated increased cellular mechanosensitivity, as measured by prostaglandin E2 release. Integrins couple the extracellular matrix with the cytoskeleton through focal adhesions comprised of actin-associated proteins such as talin and vinculin, and inhibition of focal adhesion dynamics impairs mechanosensitivity in response to fluid shear. Mathematical models of osteocytes in vivo estimate that osteocyte dendritic processes may experience higher strain than the cell body. In a separate study, a transwell filter system was used to differentiate the osteocyte cell body and dendritic processes, and each were mechanically loaded separately. It was found that the glycocalyx of the dendritic processes is critical in forming integrin attachments that initiate a mechanotransduction pathway that results 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 signaling. 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.

Osteocytes are mechanosensitive cells within bone, and previously, we have demonstrated that the primary cilium functions as a mechanosensor in this context. Fluid flow mechanical stimulation of osteocytes in vitro enhances expression of the osteogenic genes.
cyclooxygenase-2, COX-2, and osteopontin, OPN. COX-2 synthesizes prostaglandin E2, and OPN is a critical extracellular matrix protein. Increases in the production of both are indicative of osteogenesis. 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. Furthermore, we have previously reported that adenylyl cyclases, specifically AC6, play a significant role in osteocyte mechanosensitivity. 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 mineralized 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 greater than 94% incidence. Furthermore, the lacunar space within which osteocytes reside suggests that primary cilia are less than 1 µm long, as opposed to *in vitro* lengths which can be 4 µm. A recent fluid-structure interface model was developed to estimate how primary cilia may deform *in vivo*. It was concluded that a short cilium, approximately 0.2 µm, was not long enough to be a mechanosensor, but that an elongated cilium spanning the full pericellular space could be. We have previously utilized a mouse model where primary cilia were deleted from osteocytes and osteoblasts, resulting in impaired mechanotransduction and abrogated load-induced bone formation. While the specific orientation, and even incidence, of osteocyte primary cilia *in vivo* 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 utilize 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. 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 MLOY4 osteocytes. Analysis of gene expression patterns after tibial loading in adult rats reveals a wide array of upregulated genes, including dopamine D1 receptor mRNA. Interestingly, transgenic mice with a global deletion of this receptor have no apparent difference in bone architecture or calcification compared to controls. Mice with a homozygous deletion of the dopamine transporter have diminished bone mass, but the role of specific dopamine receptors was not examined. Lithium is regularly used as an agonist of the Wnt signaling 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 characterized mechanism. In Lrp5 knockout mice, lithium treatment restores bone metabolism and bone mass, and activates Wnt signaling in isolated calvarial osteoblasts. 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 hypothesized 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 cillum length in cellular mechanosensitivity, and that this is a process that can be modulated by pharmacologic intervention.

2.3. Materials and Methods

2.3.1. Summary

In this Chapter, we study how pharmacologic manipulation of primary cilia length impacts cellular mechanosensitivity. Osteocytes are treated with two distinct small molecules and exposed to oscillatory fluid flow as a mechanical stimulus, and resulting changes in mRNA expression are quantified. siRNA-mediated knockdown is also used to discern potential signaling pathways through which these changes in mechanosensitivity may occur.

2.3.2. Cell culture and drug treatment

MLO-Y4 osteocytes were cultured on collagen I-coated (Corning) dishes in MEMα (Life Technologies) supplemented with 5% fetal bovine serum (FBS), 5% calf serum (CS), and 1% penicillin/streptomycin (P/S) at 37°C and 5% CO₂. Fenoldopam mesylate (Sigma) was used at 10 µM diluted in DMSO, dimethyl sulfoxide, (Sigma) and normal culture media, as previously described 56,102. Lithium chloride (Sigma) was used at 500 µM diluted in normal culture media – a dose response from 50 µM to 10 mM was examined with 500 µM being the lowest dose to increase length significantly, data not shown. These agents, or their vehicle control, were applied to cells for 16 hours prior to experimentation. MTT, methylthiazolyl diphenyl-tetrazolium
bromide, assay (Sigma) was performed according to manufacturer’s protocol to assess cell viability with drug treatments. Phase contrast microscopy with an Olympus CKX41 inverted microscope and 40X objective was used to assess cell morphology. MLO-Y4 osteocytes used throughout this thesis were graciously donated by Dr. Lynda Bonewald.

2.3.3. Immunocytochemistry

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

2.3.4. Oscillatory fluid flow

Cells were exposed to oscillatory fluid flow as a mechanical stimulus. Cells were seeded on collagen I-coated glass slides at ~ 2800 cells/cm² and cultured for 72 hours before application of flow. Drug treatments were applied 16 hours prior to experimentation. Slides were loaded into parallel plate flow chambers (dimensions: 75 x 38 x 0.28 mm) that we have previously described and allowed to incubate at 37°C for 30 minutes prior to initiation of stimulation. Flow was applied for 1 hour at 1 Hz with a peak flow rate of 18.8 ml/min, providing 1 Pa peak wall shear stress.

2.3.5. 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
Gene expression was analyzed by quantitative real-time PCR using primers and probes (Life Technologies) for analysis of cyclooxygenase-2, \( COX-2 \) (Mm00478374_m1); osteopontin, \( OPN \) (Mm00436767_m1); adenylyl cyclase 6, \( AC6 \) (Mm00475772_m1); intraflagellar transport 88, \( IFT88 \) (Mm00493675_m1); and \( GAPDH \) (4351309). Samples and standards were run in triplicate, and all gene expression was normalized to \( GAPDH \) endogenous control, as previously performed\(^{36,86}\).

### 2.3.6. 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 \( \mu \text{M} \) \( IFT88 \) siRNA (5′-CCAGAAACAGATGAGGACGACCTT-3′), \( AC6 \) siRNA (5′-CCTGCCACCTACAACAGCTCAATTA-3′), or scrambled siRNA control using Lipofectamine 2000 (Life Technologies) as previously described\(^{36}\).

### 2.3.7. Adenylyl cyclase activity

Adenylyl cyclase activity was quantified by cAMP ELISA (Enzo). Cells were cultured as previously described and treated with 10 \( \mu \text{M} \) fenoldopam for 16 hours. Cells were stimulated by 10 \( \mu \text{M} \) forskolin (Sigma) or DMSO vehicle control for 20 minutes prior to lysis with 0.1 M HCl. Cell lysate was analyzed according to manufacturer’s protocol, and normalized to total protein quantified by BCA (Thermo Fisher). All samples and standards were run in duplicate.
Figure 2.1: Forskolin stimulates cAMP production by adenylyl cyclases
Treatment with 10 µM Forskolin for 20 minutes stimulates adenylyl cyclases to convert ATP (adenosine triphosphate) to cAMP (cyclic adenosine monophosphate), a critical second messenger within the cell.

2.3.8. Analysis
All data were analyzed with one-way ANOVA followed by Bonferroni post-hoc correction. Values are reported as mean ± SEM, with p < 0.05 considered statistically significant. Sample size, n, represents biological replicates.

2.4. Results
2.4.1. Increased cilia length with Fenoldopam and Lithium
To test our hypothesis that cilia 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 hours. Immunocytochemistry was used to image primary cilia and assess changes in cilia length. Both fenoldopam and lithium treatments induced significant increases in cilia length by 26% ± 7% and 46% ± 5%, respectively, compared to vehicle control (Fig. 2.2A, B). Cell viability was assessed
with MTT assay and found no change effect of the drug treatments (Fig. 2.2C). Additionally, no gross morphological changes resulted from fenoldopam or lithium treatment (Fig. 2.2D, E).

![Figure 2.2: Small molecule treatments increase primary cilia length](image)

10 μM fenoldopam (A) and 500 μM Lithium (B) treatment for 16 hours significantly increases primary cilia length compared to vehicle control, with no changes in cellular morphology. Drug treatments elicited no change in cell viability, as assessed by MTT assay (C), and no gross morphological differences were exhibited (D, E). Mean ± SEM; n > 25 cilia for each group, n = 4 for MTT assay; **p < 0.01, ***p < 0.001; scale bars = 20 μm.

2.4.2. Increased cilia length enhances cell mechanosensitivity

Next, we examined the effect of elongating cilia on cellular mechanosensitivity by mechanically stimulating cells with longer cilia and analyzing their osteogenic response. Osteocytes were treated with fenoldopam, lithium, or vehicle control, and exposed to oscillatory fluid flow for 1 hr. 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. 2.3). 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 utilized.
**Figure 2.3: Cells with longer cilia are more mechanosensitive**

Cells were subjected to fluid flow for 1 hour, and the fold change of flow vs no flow control groups was compared. Cells expressed 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 hours. Mean ± SEM; n ≥ 5 for each group; *p < 0.05, **p < 0.01, ***p < 0.001.

2.4.3. Recovery of cilia form and function

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. IFT88 is a critical component of intraflagellar transport and is necessary for proper primary cilia formation.

**Figure 2.4: Treatment with IFT88 siRNA disrupts cilia formation**

Overlays of primary cilia (green) and nuclei (blue) illustrate primary cilia incidence. Scramble control siRNA treatment for 48 hours 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 µm.

Cells treated with IFT88 siRNA displayed decreased primary cilia length and incidence compared to scramble control (Fig. 2.4A, B). IFT88 siRNA treated cells were then treated with
fenoldopam, and cilia length and incidence were noticeably recovered (Fig. 2.4C). Upon analysis, cilia of IFT88 siRNA treated cells were significantly shorter (Fig. 2.5A) and were present with lower incidence (Fig. 2.5C) 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 hr and cells with impaired primary cilia formation displayed significantly decreased flow-induced OPN mRNA expression by 43% ± 2%, compared to scramble control (Fig. 2.5B). Fenoldopam treatment was then able to recover this OPN response by 52% ± 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 too was mechanosensitivity. We then confirmed that fenoldopam treatment of healthy, ciliated cells had no significant effect on cilia incidence (Fig. 2.5D) or IFT88 mRNA expression (Fig. 2.5E).
Figure 2.5: Fenoldopam rescues ciliogenesis and mechanosensing

Cells treated with IFT88 siRNA had decreased cilia length compared to scramble control, while fenoldopam treatment appears to recover cilia length (n ≥ 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 displayed a decreased OPN response to fluid flow, while fenoldopam treatment was able to recover flow stimulated OPN expression (B). Treatment with IFT88 siRNA decreases cilia incidence, but is recovered with fenoldopam treatment; n ≥ 8 fields of view (C). Fenoldopam treatment on untransfected cells has no effect on cilia incidence; n ≥ 8 fields of view (D). Fenoldopam also does not alter IFT88 mRNA expression in untransfected cells (E). Mean ± SEM; n ≥ 4; ***p < 0.001.

2.4.4. Fenoldopam increases cilia length through an AC6-cAMP mechanism

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. We increased primary cilia length by fenoldopam
treatment, and then briefly stimulated the cells with the adenylyl cyclase agonist, forskolin (Fig. 2.6A). Fenoldopam treatment significantly enhanced the forskolin stimulated cAMP response by 130% ± 25% compared to vehicle control. Additionally, fenoldopam treatment stimulated a 20% ± 8% increase in AC6 mRNA expression (Fig. 2.6B). 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% ± 3% (Fig. 2.6C), while AC6 inhibition had no effect on cilia incidence (Fig. 2.6D). 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% ± 3% and 30% ± 3% respectively, which was not recovered with fenoldopam treatment (Fig. 2.6E, F).
2.5. Discussion

Our results demonstrate that primary cilia length plays a significant role in cell mechanosensitivity. We employed two distinct, clinically utilized, small molecules to increase cilia length, and both resulted in enhanced mechanosensitivity. Cells with impaired ciliogenesis have impaired mechanosensing, but this may be recovered with fenoldopam treatment. Finally, we show that fenoldopam modulates osteocyte mechanosensitivity through a mechanism involving AC6, and cells with diminished AC6 have 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 in 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 \(^{113,114}\). Lithium has a much less defined function and is clinically used to treat a wide range of mental disorders, including bipolar disorder \(^{115}\). Furthermore, lithium is an inhibitor of GSK-3\(\beta\) and can have downstream effects on various signaling 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 \(^{116,117}\).

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 sensitizes 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. Our results support this possible molecular pathway by demonstrating an increase in AC6 mRNA expression in response to fenoldopam stimulation. Previously, we have demonstrated that AC6 localizes to the osteocyte primary cilium and is critical for primary cilia-mediated mechanotransduction. Besschetnova et al, reported that stimulating the cAMP signaling pathway results in increased cilia length. Using an siRNA mediated knockdown, they then 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 recovering and elongating primary cilia by stimulating IFT particle transport. It has previously been reported that stimulation of the adenylyl cyclase-cAMP-PKA signaling pathway augments anterograde transport of IFT particles to promote cilia elongation. 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 utilized 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. This does not, however, 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 hypothesized that longer cilia would experience greater membrane strain to increase opening of stretch-activated ion channels on the ciliary membrane. Alternatively, longer cilia may simply allow for the presence of more cilia-specific proteins and signaling molecules within this microdomain. 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 signaling cascade. It is also possible that cilium-lengthening agents actually enhance ciliary protein production and trafficking to promote cillum elongation.

The correlation between cilia length and critical ciliary proteins involved in mechanosensing was examined with AC6 siRNA treatment. This 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 to 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. For example, endothelial cell primary cilia are flow sensors in regions of low shear, specifically, because they are cleaved off as shear stresses increase. Interestingly, electron microscopy has shown that primary cilia structure is not constant along the ciliary axoneme and becomes increasingly disorganized and asymmetric at the distal tip. 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.

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. 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. A computational model by Vaughan et al, simulated osteocytes
exposed to fluid flow within the lacunar-canaliculal network\textsuperscript{92}. The authors modeled a free-standing cilium, 0.5 $\mu$m 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. The authors, however, 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 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\textsuperscript{124,125}. Furthermore, many antidepressants and antipsychotics that block dopamine D2 receptors can cause decreased estrogen and testosterone levels leading to significantly reduced bone mass\textsuperscript{126}. 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 signaling 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 characterized by impaired primary cilia function. Mutations of PC2, polycystin 2, are attributed to polycystic kidney disease and skeletal deformations. Bardet-Biedl syndrome is characterized by malfunctioning BBS proteins at the base of the primary cilium, causing retinopathy, polydactyly, and renal failure \(^{84,127,128}\). Recently, primary cilia have even been implicated in tumor development. Primary cilia help regulate Wnt signaling, changes in which have been correlated with cancer cell progression \(^{129}\). Furthermore, some cancer cell types lose their primary cilia, which potentially contributes to their insensitivity to repressive signals \(^{130}\). Additionally, atherosclerotic plaques form in areas of low 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 low fluid flow in order to promote an adequate cellular response \(^{59,131}\). Within bone, osteocytes utilize 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 \(^{36,82,83}\). 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.

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.
Chapter 3

3. High-throughput drug screening allows identification of ciliogenesis modifying compounds

3.1. Abstract

In Chapter 2 we identify two small molecules, fenoldopam and lithium, as compounds that increase primary cilia length. Furthermore, we find the cells with longer cilia are more mechanosensitive. Primary cilia play a critical role in mechanosensing, chemosensing, and signal transduction in a myriad of cell types. Moreover, numerous different human conditions are being attributed to aberrant ciliogenesis. Disrupted ciliogenesis can result in improper embryogenesis, skeletal patterning disorders, polycystic kidney disease, anosmia, and retinitis pigmentosa, among numerous other conditions. While we have identified fenoldopam as a compound with potential to be repurposed for bone applications, our list of candidate therapeutics to treat such a range of ciliopathies is limited. In this chapter we utilize high-throughput drug screening to recognize compounds that manipulate ciliogenesis. This work allows us to identify individual small molecules, as well as classify compounds based on mechanism of action to help inform the development of novel cilia-targeted therapeutics.
3.2. Introduction

As previously discussed, primary cilia are solitary immotile organelles serving chemosensing and mechanosensing roles in numerous different cell types. Primary cilia are comprised of microtubule doublets that are highly acetylated\textsuperscript{132}. Furthermore, primary cilia form a unique microdomain within the cell to which particular proteins localize, and through which many signaling pathways are transduced. Impaired primary cilia formation is being implicated with an increasing number of different health conditions being termed ciliopathies. The most notable of these being disrupted embryonic patterning and polycystic kidney disease\textsuperscript{31}. Primary cilia also serve chemosensing roles in olfaction as well as in neurons and in the retina controlling vision\textsuperscript{133}.

Due to the acetylated microtubule composition of the ciliary axoneme, it is anticipated that compounds targeting microtubule stability and microtubule acetylation, as well as cell cycle progression, will significantly impact ciliogenesis. Primary cilia formation is tightly regulated with the cell cycle, with ciliogenesis occurring during the G1 phase of the cell cycle\textsuperscript{134}. As cells approach the G2 and S phase the cilium is disassembled before cell division. Taxol, and its derivatives, stabilize microtubules to prevent them from depolymerization, and can also arrest cells in the G1 phase, when ciliogenesis occurs\textsuperscript{135}. Histone deacetylases (HDACs) increase α-tubulin acetylation to also stabilize microtubules\textsuperscript{136}.

Primary cilia formation can be altered, pharmacologically, having a direct impact on cell function. Besschetnova et al. demonstrated that inhibiting intracellular calcium or stimulating the cAMP-PKA signaling pathway can significantly enhance primary cilia length\textsuperscript{112}. Recently it has been demonstrated that increasing primary cilia length with fenoldopam can potentiate
mechanosensing in endothelial cells and osteocytes, resulting in enhanced nitric oxide production to reduce blood pressure, and increased osteogenic signaling, respectively. These works demonstrate that ciliogenesis can be pharmacologically enhanced, having a direct impact on cell function.

Targeting primary cilia is a potent way to manipulate cell activity, such as by sensitizing cells to chemical or mechanical stimuli. This has implications in combating diseases ranging from osteoporosis, atherosclerosis, and polycystic kidney disease, to cancer and primary ciliary dyskinesia. While several compounds are known to manipulate cilia length, the list is minimal. Here, we utilize high-throughput drug screening to expand our library of candidate bioactive small molecules that increase primary cilia length, to both further our understanding of cilia biology and identify potential disease treating compounds.

### 3.3. Materials and Methods

3.3.1. **Summary**

In this chapter we perform high-throughput drug screening to expand our list of candidate compounds that increase primary cilia length. We established a high-throughput screening system in collaboration Dr. Charles Karan and his team at the High-Throughput Screening Center at the Columbia Genome Center. This platform involves an automated process of cell culture on 384-well plates, drug treatment with 6931 compounds, staining, imaging, and image analysis.
3.3.2. **Cell culture**

MLO-Y4 osteocytes were maintained on collagen I-coated dishes in MEM\(\alpha\) (Life Technologies) supplemented with 5% fetal bovine serum (FBS), 5% calf serum (CS), and 1% penicillin/streptomycin (P/S) at 37°C and 5% CO\(_2\). Osteocytes were then seeded onto 384-well collagen-coated plates for 72 hours before fixation. Cells were treated, in triplicate, with 6931 biologically active small molecules from a collection of libraries for 16 hours before fixation. These libraries include Tocris, Sigma LOPAC, Selleck Chemical, Prestwick, and Spectrum commercially available compound libraries. All cell seeding and plate handling was performed with the Perkin Elmer cell::explorer workstation.

3.3.3. **High-throughput imaging**

Cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton-X 100 (Sigma). 10% goat serum (vendor) was used for blocking. Cilia were stained using a monoclonal antibody against acetylated \(\alpha\)-tubulin (1:20) from a C3B9 hybridoma cell line, and visualized with AF488 (1:1000, ThermoFisher). Nuclei were visualized with Hoechst. Cells were imaged using a GE INCell 2000 high-throughput imager at 40X magnification. 4 fields of view were taken per well.

3.3.4. **Automated cilia detection and measurement**

Each pair of nuclei and cilia images was first analyzed individually, then the entire population was examined to evaluate each compound’s effect on cilia length and incidence. A custom MATLAB script was developed to perform all image analysis. This script first utilizes an enhanced implementation of a watershed transform, which is a segmentation tool which enables detection of similarly shaped overlapping particles in noisy images by the procedural whittling
down of pixels found close to the edge of such particles. Our augmentation of the algorithm incorporates thresholding for noise removal based on signal intensity and object size, nuclei dilation to exclude non-cellular artifacts, as well as skeletonization of cilia to quantify length. The results of the analysis are number of nuclei, number of cilia, and average cilia length in each image.

3.3.5. Identification and classification of hits

Compounds were selected based on deviation from the population mean. Each well was normalized to vehicle control images on each plate, allowing the establishment of a normalized population mean. Hits were chosen based on compounds whose triplicate mean was furthest (based on standard deviation away) from the population mean. This was performed to identify the 105 individual compounds with the greatest deviation in terms of increased cilia length, and can also be performed for changes in cilia incidence. Compounds were classified based on mechanism of action.

3.4. Results

3.4.1. High-throughput screening platform

To identify compounds that modulate primary cilia formation we developed a high-throughput platform to automate cell culture, staining, imaging, and analysis, Fig. 3.1. This platform was used to examine the effects of 6931 small molecule treatments on osteocyte primary cilia length and incidence. Osteocytes were chosen as the model cell type, as we have repeatedly demonstrated that they form primary cilia of consistent length and incidence. Cells were cultured on 384-well plates, and treated with small molecules with known biologic
activity, enabling classification of mechanisms of action. Drug treatment was applied for 16 hours at 10 µM concentration, performed in triplicate, before fixation and immunostaining of primary cilia. Cells were then imaged using the GE INCell 2000 High Content Analyzer, capturing 4 fields of view per well at 40X magnification. Additionally, each plate contains 8 wells of DMSO vehicle control.

Figure 3.1: High-throughput screening platform
An automated platform was developed to perform the complete high-throughput drug screening and analysis. First, cells are seeded on 384-well plates, then treated with one of 6931 compounds with known biologic activity from commercially available small molecule libraries. Cells are then fixed, stained, and imaged. Images are then analyzed using custom MATLAB scripts, and compounds that increase cilia length were classified based on mechanism of action.
3.4.2. **Primary cilia detection and analysis**

A MATLAB script was developed to detect and analyze changes in ciliogenesis, Fig. 3.2. Nuclei and cilia images were binarized based on object size and signal intensity. A dilated mask was overlaid on the nuclei to identify only cilia that are associated with a nucleus, removing image artifacts. Cilia were skeletonized to quantify cilia length. Primary cilia incidence was quantified as the number of cilia versus nuclei per field of view.

Cilia length and incidence for each field of view were normalized to vehicle control on that individual plate. This allowed development of a normalized population average and standard deviation. Initially, hits were chosen as compounds with a normalized average 1.5 standard deviation above the population average \(^{139}\). To control for toxic effects only small molecules with at least 40% viability, based on number of nuclei versus control, were selected as hits. Furthermore, compounds repeated between multiple libraries eliciting the same effect on cilia length or incidence were excluded, such that only one of the repeated compounds was selected as a hit. For example, bufexamac was present in 3 libraries and was listed as a hit increasing length from each library, but is only counted once for analysis of mechanism of action. This resulted in 103 individual compounds that increase primary cilia length, independent of changes in cilia incidence, and 92 compounds that increase cilia incidence independent of length. 18 individual compounds were conserved between the two lists.
Figure 3.2: Primary cilia imaging, detection, and analysis
Cells stained for primary cilia (acetylated α-tubulin; visualized with AF488, green) and nuclei (Hoescht, blue), and imaged at 40x magnification. A representative image (A) is cropped to better display features, scale bar represents 30 µm. A custom MATLAB script identifies nuclei and cilia for analysis (B). The script detects nuclei (blue), and applies a dilation mask to reject objects not associated with a cell (white). Objects are further filtered based on size and signal intensity. Rejected objects are labeled in (red) and identified cilia are labeled in (green). Cilia length and incidence distributions are displayed as histograms of compound standard deviations from population mean (C, D).

3.4.3. Compound classification
Compounds were classified based on mechanism of action to assess pathways through which primary ciliogenesis is altered, Fig. 3.3, 3.4. The most common class of compounds was DNA/RNA synthesis inhibitors; including anti-folates, topoisomerase inhibitors, purine/pyrimidine (nucleoside) analogs, and general DNA/RNA synthesis inhibitors not falling into these other classes. It was also noticed that several distinct dopamine D₁-like receptor (DR1)
agonists increase length, as well as an adenylyl cyclase activator. As previously discussed, it has been demonstrated that DR1 agonists enhance adenylyl cyclase production. Additionally, compounds affecting microtubule stability, as well as GSK3 and HDAC inhibitors also increase primary cilia length. Of the 103 hits increasing length, 37 did not share a distinct pathway with another compound. 7 of these compounds have biologic activity, but their mechanisms of action have not been explicitly described in mammalian cells. These compounds comprise phytochemicals and flavonoids. 30 of 92 total incidence hits were also individual compounds. All hits increasing both cilia length and incidence are listed in Table 3.1, along with the standard deviation away from the population average. Compounds that are conserved between these lists are highlighted. These conserved hits are categorized by mechanism of action in Fig. 3.5.
Figure 3.3: Classification of compounds that increase cilia length
Compounds that increase cilia length were classified based on mechanism of action, independent of cilia incidence. Only classes with at least 2 hits are listed here to demonstrate repeated mechanisms of action increasing cilia length. Classes marked with dots (anti-folates, general DNA/RNA synthesis inhibitors, topoisomerase inhibitors, and nucleoside analogues) all represent different compounds that inhibit DNA and RNA synthesis, slowing proliferation. Classes marked with slashes (DR1 agonists, Adenylyl cyclase modulators) represent compounds that increase AC activity and cAMP production. DR1 agonists have been demonstrated to stimulate production of ACs.
Figure 3.4: Classification of compounds that increase cilia incidence

Compounds that increase cilia incidence (independent of changes in cilia length) were classified based on mechanism of action, just as in Figure 3.3.
### Cilia length

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<td>110-Phenanthroline monohydrate</td>
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### Cilia incidence

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Table 3.1: List of hits increasing cilia length and incidence

All compounds determined as hits based on statistical analysis of 1.5 standard deviations away from population mean. Lists comprise hits that increase cilia length independent of incidence, and incidence independent of length. Standard deviations away are listed, with compounds increasing length on the left, and compounds increasing incidence on the right. Highlighted compounds are conserved between both data sets.
Figure 3.5: Conserved classes of compounds
Compounds conserved between lists of hits independently increasing cilia length and incidence are classified based on mechanism of action. Below each class is also listed the number of conserved compounds in each group, 18 in total. Anti-folates (DHFR inhibitors) are the most commonly conserved, with topoisomerase, HDAC, and DNA/RNA synthesis inhibitors all having multiple conserved hits.

3.5. Discussion

Using our high-throughput drug screening platform, we identify unique compounds and classes of compounds that enhance ciliogenesis. This has potential to elucidate molecular pathways and targets for therapeutic development of compounds to treat conditions marked by impaired ciliogenesis. Impaired cilia formation has consequences in cell signaling and homeostasis, as well as chemo and mechanosensing.
The final list of hits is comprised only of individual compounds, yet many compounds were repeated in different libraries with the same mechanism of action. This screen utilizes multiple different commercially available libraries, so there is overlap in compounds between libraries. Because of this, many compounds identified by the 1.5 standard deviation threshold are repeated, with 17 of the 103 compounds increasing cilia length having at least one repetition in another library. For example, both bufexamac and methotrexate are listed as hits from Selleck and Prestwick libraries. 92 compounds were identified to increase cilia incidence (independent of cilia length), with 8 of these containing library repetitions with similar effects on incidence. Compound repetition between libraries demonstrates robustness of the entire drug treatment, imaging, and analysis platform.

The results from the screen focus on compounds that increase primary cilia length and incidence. We also analyzed the screening data for compounds that decrease cilia length and incidence. This, however, elicited almost no hits. Compounds that decreased cilia length or incidence by at least 1.5 standard deviations also caused cell death. Of compounds that decreased cilia incidence, none retained at least 40% cell viability. Many of the ciliopathies that we described earlier in this thesis are characterized by impaired cilia formation and function. Because of this, we focused the analysis of our drug screen results on compounds that increase cilia length and incidence.

Our high-throughput screening platform does not necessarily exclude compounds not listed as hits. Through this platform we only tested compounds at 10 µM concentrations over a 16 hour timeframe. Lithium was not classified as a hit, however we have previously demonstrated that lithium increases cilia length over 16 hours, at 500 µM concentration\textsuperscript{137}. Cilia
length changes have also been reported with 3 hours of drug treatment with calcium channel blockers (gadolinium, 30 µM) or adenylyl cyclase activators (forskolin, 100 µM) \textsuperscript{112}. While a full dose-response on this complete bioactive library of 6931 compounds was not feasible, classifying compounds based on mechanism of action still allows examination of pathways through which ciliogenesis may be modified.

The largest group of hits that increase cilia length are DNA and RNA synthesis inhibitors, including anti-folates, topoisomerase inhibitors, purine/pyrimidine analogues, and other less specific DNA/RNA inhibitors. As previously introduced, ciliogenesis coincides with the cell cycle, and deciliation occurs with the initiation of DNA synthesis \textsuperscript{140}. Additionally, topoisomerase inhibitors have also previously been demonstrated to enhance ciliogenesis \textsuperscript{141}. Purine and pyrimidine analogues additionally prevent the continuation of the cell cycle, allowing enhanced ciliogenesis \textsuperscript{142}. The role of anti-folates and dihydrofolate reductase inhibitors (DHFRs) on primary cilia formation has not been explicitly examined, but because DHFR inhibitors impair DNA/RNA synthesis, this result is not unexpected \textsuperscript{143}. In fact, certain doses of prenatal exposure to the DHFR inhibitor methotrexate can result in methotrexate embryopathy, characterized by altered patterning and embryogenesis, consistent with altered primary cilia function \textsuperscript{144}.

Drug classification also supports a purported dopamine receptor–adenyl cyclase–calcium–related pathway to enhance cilia length. Several distinct compounds that act as agonists of dopamine D\textsubscript{1}-like receptors (DR1), as well as compounds that stimulate adenylyl cyclase activity, are demonstrated as hits in our screen. It has previously been demonstrated that fenoldopam, a DR1 agonist enhances cilia length \textsuperscript{56,137}. Furthermore, it has been demonstrated
that DR1 agonists enhance AC6 production, which results in increased cAMP production. Besschetnova et al. previously reported that blocking calcium channels or stimulating cAMP production and PKA activation all increase primary cilia length, in a process that is impaired with AC6 disruption. AC6 is a calcium inhibited isoform of adenylyl cyclase that localizes to the primary cilium. Through our screen we also identify general calcium channel blockers that increase cilia length. Together with existing literature, the compounds classified through our screen help to demonstrate a pathway through which DR1 agonists stimulate an increase in AC6 production to promote cilia elongation. Previously we have also demonstrated that AC6 plays a critical role in load-induced bone formation and mechanotransduction. Our results demonstrate that DR1 and AC agonists have potential use in promoting bone formation for the treatment of bone diseases such as osteoporosis.

Histone deacetylases have been implicated in cilia formation, and suggest a length-stiffness functional relationship. It has been previously demonstrated that HDACs, including HDAC2 and HDAC6, activity results in cilia disassembly. Primary cilia contain heavily acetylated microtubules, and this acetylation stabilizes microtubules from disassembly. HDAC6 inhibition also stabilizes cilia to prevent mechanically-induced disassembly. We have demonstrated that increased cilia length enhances mechanosensitivity; however, we have also shown that increased microtubule acetylation by HDAC6 inhibition increases primary cilia stiffness to abrogate primary cilia-mediated mechanotransduction. This distinction suggests that a relationship exists whereby both cilia length and stiffness can independently impact its function as a cellular mechanosensor.
Our drug screening platform utilized osteocytes as a model system, but modulating primary ciliogenesis has broad implications in treating numerous human conditions. Increasing cilia length can enhance cell mechanosensitivity. In the context of bone augments osteogenic activity and may have potential as a bone disease therapeutic strategy \(^{137}\). Increasing cilia length also has implications in treating hypertension resulting from disrupted cilia mechanosensing \(^{56}\). Modulating ciliogenesis can also restore chemosensing. A mouse model of diminished IFT88 production leads to impaired cilia formation, resulting in disrupted olfaction \(^{133}\). When ciliogenesis is recovered – using an adenovirus to promote IFT88 expression – olfaction in the mice is restored. Primary ciliogenesis has also been linked to tumor progression, with many cancer types reported to lose primary cilia \(^{70}\). In particular, premalignant and invasive breast cancer cells both display significantly fewer cilia than normal breast tissue \(^{75}\). Primary cilia regulate hedgehog signaling, which is known to be disrupted in tumorigenesis \(^{70}\). Thus, restoring ciliogenesis also has significant potential in slowing cancer progression. Understanding ciliogenesis and identifying modulators of this process are critical to developing therapeutic strategies for a myriad of different human health conditions.
Chapter 4

4. Osteocyte cilia mediate paracrine signaling between cells within bone

4.1. Abstract

Bone maintenance and mechanotransduction is regulated by intercellular communication between the cells within bone. Osteocytes residing deep within bone tissue have been demonstrated to be highly mechanosensitive, and it is believed that these cells coordinate intercellular signaling to direct bone formation and osteogenic differentiation. As demonstrated in Chapter 2 of this thesis, primary cilia are critical osteocyte mechanosensors. In this chapter we mechanically stimulate osteocytes and use the conditioned media to culture bone-forming osteoblasts and mesenchymal stem cells (MSCs), to study mechanically-induced intercellular paracrine signaling. We demonstrate that the osteocyte cilium, and ciliary mechanosensing proteins, play a critical role in initiating the mechanotransduction events that result in pro-osteogenic paracrine signaling to osteoblasts. Furthermore, we demonstrate that enhancing osteocyte primary cilia-mediated mechanotransduction with fenoldopam augments osteoblast activity and MSC osteogenic differentiation. Together, this demonstrates a regulation of intercellular bone signaling through manipulation of the osteocyte cilium.
4.2. Introduction

The function of osteocytes was long unknown, since osteoblasts actively form new bone material and mesenchymal stem cells can differentiate into osteoblasts, while osteocytes resorb bone. Osteocytes reside deep within the lacuno-canalicul network in bone, comprising over 90% of all bone cells. Studies now suggest that this dominant cell type actually serves a mechanosensing role in bone; and moreover, that osteocytes direct signaling to other cells within bone. Notably, Tatsumi et al. utilized an in vivo osteocyte-specific ablation model and found impaired bone mechanotransduction in mice lacking osteocytes.

The prevailing paradigm is that osteocytes sense mechanical forces, and transduce these signals to direct the activity of the other cell types within bone. It is believed that osteocytes signal to osteoblasts, osteoclasts, and MSCs through soluble paracrine signals as well as direct cell communication. However, only recently has this phenomena been explicitly demonstrated. Osteocytes subjected to pulsatile fluid flow were found to secrete soluble factors into the media that increase osteoblast proliferation, potentially through activation of a nitric oxide pathway. More recently, it was found that osteocytes exposed to oscillatory fluid flow display enhanced signaling to MSCs, increasing MSC osteogenic differentiation, migration, and proliferation. Moreover, our group also recently demonstrated that paracrine factors secreted by mechanically stimulated osteocytes can elicit epigenetic changes in MSCs that promote osteogenic differentiation.

While we previously described that primary cilia play a significant role in osteocyte mechanosensing, it is unclear how this initial mechanotransduction event may be transduced into downstream signaling to other cell types. As mentioned earlier in this thesis, impairing primary

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cilia formation disrupts osteocyte mechanosensing, and whole bone mechanotransduction. Similarly, knockdown and knockout of ciliary proteins such as stretch-activated ion channels (TRPV4, PC2) and mechanotransductive proteins (AC6) abrogates osteocyte and whole bone mechanosensing. Furthermore, we previously demonstrated that we can pharmacologically sensitize the osteocyte cilium to mechanical stimulation. It is unclear, however, how these proteins or manipulation of the osteocyte cilium impact the intercellular bone signaling axis.

Figure 4.1: Flow-induced osteogenic paracrine signaling
A prevailing paradigm in bone mechanotransduction is that osteocytes sense mechanical stimuli and transduce these signals to direct osteoblast-mediated bone formation and mesenchymal stem cell osteogenic differentiation. In this Chapter we examine soluble paracrine signaling between mechanically stimulated osteocytes and osteoblasts/MSCs.

4.3. Methods

4.3.1. Summary

To study intercellular paracrine signaling, we collect conditioned media from mechanically stimulated osteocytes and use it to culture osteoblasts and MSCs. This model allows RNA and pharmacologic manipulation of cilia and ciliary proteins specifically in
osteocytes, while quantifying resultant osteogenic changes in osteoblasts and MSCs. To study soluble paracrine signaling from mechanically stimulated cells we utilized a rocking platform to supply oscillatory fluid flow and consistently collect conditioned media. Based on the size of the culture vessel, amount of media, placement on rocker, and rocking angle and frequency, the peak shear stress can accurately be calculated \(^\text{153}\). Using this scheme we can study not only the initial ciliary mechanosensing events, but how the resultant downstream signaling impacts intercellular communication.

4.3.2. **Cell culture**

As performed in Chapter 2, MLO-Y4 osteocytes were cultured on collagen I-coated dishes in MEM\(\alpha\) (Life Technologies) supplemented with 5% FBS, 5% CS, and 1% P/S at 37°C and 5% CO\(_2\). Osteocytes were seeded at 2800 cells/cm\(^2\) for 72 hours before application of fluid flow. MC3T3 osteoblasts were cultured in MEM\(\alpha\) supplemented with 10% FBS and 1% P/S. Osteoblasts were seeded at 2000 cells/cm\(^2\) 72 hours before receiving conditioned media. C3H10T1/2 MSCs were cultured in DMEM low glucose (Life Technologies) supplemented with 10% FBS and 1% P/S, and seeded at 1100 cells/cm\(^2\) 72 hours before culture with conditioned media \(^\text{150}\). Osteocytes were treated with 10 \(\mu\)M fenoldopam mesylate (Sigma) for 16 hours or 5 \(\mu\)M tubastatin (Sigma) for 3 hours, prior to experimentation.

4.3.3. **Mechanical stimulation**

Osteocytes were cultured on rectangular flasks (8.2 x 9.2 cm; 12.5 ml of media) on a rocking platform which oscillated at a frequency of 0.33 Hz with an amplitude of 1.3 cm, supplying 0.08 Pa shear stress for 2, 6, 12, or 24 hours, Fig. 4.2. Shear stress generated by oscillatory fluid flow was calculated as previously performed \(^\text{153}\). 12 hours of fluid flow was used
in all experiments. Cells were also cultured in static conditions as a no flow control. Conditioned media was collected from osteocytes immediately upon completion of rocking, and used to culture osteoblasts or MSCs for 24 or 48 hours, respectively.

Figure 4.2: Model of flow-induced paracrine signaling
To study mechanically-induced osteogenic paracrine signaling between osteocytes and osteoblasts/MSCs, we utilized a rocker platform to perform conditioned media studies. In this platform, MLO-Y4 osteocytes are placed on a rocker to apply oscillatory fluid flow mechanical stimulation. The conditioned media is then collected and used to culture MC3T3 osteoblasts and C3H10T1/2 mesenchymal stem cells. With this set-up we can then manipulate osteocyte primary cilia-mediated mechanotransduction (such as by siRNA-mediated knockdown or pharmacologic challenge) and study the resulting changes in paracrine signaling leading to altered osteoblast and MSC activity. All results are compared relative to static (no flow) osteocytes.

4.3.4. RNA interference in osteocytes
Gene silencing was performed only in osteocytes, 48 hours prior to application of fluid flow, by siRNA-mediated knockdown, as described in Chapter 2. All data was compared to scramble siRNA control. Primary cilia were disrupted by IFT88 siRNA (5’-CCAGAAACAGATGAGGACGACCTTT -3’)\(^{137}\). Adcy6 (5’-CCTGCCACCTACAAC-AGCTCAATTA -3’), Pkd2 (5’-CCTCTTGGCAGTTTCAGCCTGTAAA -3’), and TRPV4 (5’-GATGGACTGCTCTCCTTTCTTTGTTGA -3’) were disrupted as previously performed\(^{35,137}\).
4.3.5. **mRNA expression**

RNA isolation and qRT-PCR was performed as described in Chapter 2. Osteoblasts and MSCs were washed with PBS and total mRNA was isolated using TriReagent (Sigma), immediately following 24 hours (48 hours for MSCs) of culture in conditioned media from mechanically stimulated (or static control) osteocytes. Total mRNA was converted to cDNA by TaqMan reverse transcriptase (Applied Biosystems). Gene expression was analyzed by quantitative real-time PCR using primers and probes (Life Technologies) for analysis of osteopontin, \textit{OPN} (Mm00436767\_m1), osterix, \textit{OSX} (Mm04209856\_m1), and \textit{GAPDH} (4351309). Samples and standards were run in triplicate, and all gene expression was normalized to \textit{GAPDH} endogenous control.

4.3.6. **Analysis**

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

4.4. **Results**

4.4.1. **Increased duration of flow enhances signaling to osteoblasts**

Cultured osteocytes were placed on the rocker platform and subjected to oscillatory fluid flow for 2, 6, 12, or 24 hours, each timepoint with a corresponding static control. Media was collected and used to culture osteoblasts for 24 hours. At each timepoint osteoblasts demonstrated a significant increase in osteopontin (OPN) mRNA expression compared to static controls, Fig. 4.3. 12 hours of rocking was used for all further experiments.
Figure 4.3: Osteoblast response to increasing durations of osteocyte mechanical stimulation
Osteocytes were exposed to oscillatory fluid flow (F), or no flow control (NF), for 2, 6, 12, and 24 hours. Conditioned media was collected from osteocytes and used to culture static osteoblasts for 24 hours. Osteoblasts were then immediately lysed for mRNA expression of osteopontin. At each timepoint osteoblasts expressed significantly more osteopontin mRNA relative to no flow control of the respective timepoint. Mean ± SEM; \( n \geq 4 \) for each group; ***\( p < 0.001 \).

4.4.2. **Intercellular signaling is impaired with cilia/associated protein disruption**

We next sought to examine the role of osteocyte primary cilia-mediated mechanotransduction on osteogenic intercellular paracrine signaling. Primary cilia formation was disrupted using siRNA-mediated knockdown of IFT88, while mechanotransduction proteins were impaired by knockdown of the ciliary stretch-activated ion channels TRPV4 and PC2, or knockdown of AC6. Knockdown of osteocyte cilia or ciliary mechanosensing proteins
significantly impairs mechanically-induced pro-osteogenic paracrine signaling to osteoblasts, Fig. 4.4.

Figure 4.4: Osteocyte primary cilia direct osteogenic paracrine signaling
Osteocyte primary cilia-mediated mechanotransduction was disrupted to examine the effects of downstream paracrine signaling to osteoblasts. Osteocyte primary cilia formation was disrupted by IFT88 siRNA-mediated knockdown (A). Components of osteocyte primary cilia-mediated mechanotransduction were disrupted (B) by siRNA-mediated knockdown of AC6 (signal transduction), as well as PC2 and TRPV4 (cilia-localized mechanosensitive ion channels). In both conditions, impairment of osteocyte primary cilia-mediated mechanosensing abrogates pro-osteogenic paracrine signaling to osteoblasts. Mean ± SEM; n ≥ 4 for each group; *p < 0.05, ***p < 0.001.

4.4.3. Fenoldopam treatment enhances intercellular signaling
Finally, we examined how potentiating osteocyte mechanosensitivity may direct intercellular osteogenic signaling. Osteocytes were treated with fenoldopam to increase cilia length and enhance mechanosensitivity, and subjected to oscillatory fluid flow. The collected media was used to culture osteoblasts or MSCs for 24 or 48 hours, respectively. Media collected from the fenoldopam treated osteocytes significantly enhanced osteoblast osteogenic activity, as measured by osteopontin gene expression, and significantly enhanced MSC osteogenic differentiation, as measured by osterix (OSX) expression, Fig. 4.5. Additionally, we have previously demonstrated that HDAC6 (histone deacetylase 6) inhibition increases ciliary bending
stiffness to decrease mechanosensitivity. Osteoblasts cultured with conditioned media from mechanically stimulated osteocytes treated with the HDAC6 inhibitor, tubastatin, displayed impaired osteopontin expression, Fig. 4.5.

**Figure 4.5: Pharmacologically manipulating osteogenic intercellular communication**

Osteocytes were treated with fenoldopam, tubastatin, or vehicle control, and the resulting response of osteoblasts and MSCs was examined. Fenoldopam treatment sensitizes osteocyte mechanosensitivity to enhance mechanically-stimulated pro-osteogenic paracrine signaling promoting osteoblast activity (A), and MSC osteogenic differentiation (B). Additionally, tubastatin treatment diminishes the flow-mediated osteocyte paracrine signaling to osteoblasts. Mean ± SEM; n ≥ 4 for each group; *p < 0.05, ***p < 0.001.
4.5. Discussion

In this work we have demonstrated that cilia, and cilia-associated proteins not only impact osteocyte mechanosensitivity, but the entire mechanotransduction cascade leading to pro-osteogenic intercellular communication. By manipulating osteocyte mechanosensitivity, we can direct the intercellular signaling axis between cells within bone.

Intercellular communication within bone utilizes several different paracrine factors to mediate osteogenic signaling. Mechanically stimulated osteocytes have been demonstrated to increase production of prostaglandin E2 and cAMP, as well as release of nitric oxide, Ca\(^{2+}\), and ATP \(^{154,155}\). Expression of larger peptides involved in bone formation such as IGF-1, RANKL, and OPG have also been demonstrated to be increased in osteocytes exposed to physical stimulation \(^{82,156}\). One major limitation of our model is that MLO-Y4 osteocytes do not produce sclerostin, a significant regulator of osteoblast activity \(^{24,155}\). As previously described, sclerostin is produced by osteocytes as a negative regulator of osteoblast activity. Our results, however, demonstrate that sclerostin production by osteocytes is not exclusively necessary for the regulation of osteoblast activity by paracrine signaling.

Paracrine signaling is not the sole method of intercellular communication. Osteocyte dendritic processes extend throughout the lacuno-canalicular network, allowing for direct cell contact between osteocytes, and other cells within bone. This allows for the formation of gap junctions between cells through which ions such as Na\(^+\), K\(^+\), Ca\(^{2+}\), and Mg\(^{2+}\) can be transported to neighboring cells \(^{154}\). Connexins make up hemicannels which form gap junctions mediating intercellular signaling. In both osteocytes and osteoblasts connexin 43 (Cx43) has been demonstrated to open in response to fluid flow, enabling signal transduction between
neighboring cells. While our model does not recapitulate the entire complexity of bone, our data do demonstrate that manipulation of cellular mechanosensitivity can have profound impacts on pro-osteogenic intercellular signaling.

By manipulating osteocyte primary cilia and ciliary associated proteins we can direct mechanically-induced intercellular communication. We have previously demonstrated that knockdown of AC6 impairs osteocyte mechanosensing, in a similar manner to impaired ciliogenesis by IFT88 knockdown. It has also been shown that polycystin 2 is involved in NO production in osteocytes exposed to oscillatory fluid flow. In vivo, skeletal deletion of the polycystin ion channel complex impairs whole bone mechanosensitivity. We have also demonstrated that knockdown of TRPV4 impairs osteocyte mechanosensing. TRPV4 has been implicated with a key role in cartilage mechanosensing, and mutations in this channel have been associated with numerous skeletal dysplasias. Interestingly our previous data suggests that TRPV4 and not PC2 is the main ciliary mechanosensitive ion channel over short periods of mechanical stimulation. TRPV4 and PC2, however, may form a heteromeric channel within the cilium suggesting that their function may in fact be linked, warranting further investigation into their long term ciliary mechanosensing function. Our data here also demonstrate that enhancing osteocyte primary cilia-mediated mechanosensing significantly potentiates pro-osteogenic paracrine signaling to osteoblasts and MSCs. While osteoblasts and MSCs are certainly both mechanosensitive, the current paradigm is that osteocytes are the predominant mechanosensors within bone, directing osteogenic activity of other bone cells.
Chapter 5

5. Targeting primary cilia to promote load-induced bone formation \textit{in vivo}

5.1. Abstract

Bone actively responds to mechanical stimuli, yet none of the current treatment options for low bone mass and osteoporosis leverage the inherent mechanosensitivity of bone. Our group has previously identified the primary cilium as a critical mechanosensor within bone, and that pharmacologically targeting the primary cilium with fenoldopam can enhance osteocyte mechanosensitivity. In chapter 4, we demonstrate that potentiating osteocyte mechanosensing with fenoldopam \textit{in vitro} promotes pro-osteogenic paracrine signaling to activate osteoblasts and stimulate osteogenic differentiation of mesenchymal stem cells. Here we utilize an \textit{in vivo} model of load-induced bone formation to demonstrate that fenoldopam treatment sensitizes bone to mechanical stimulation, in a dose-dependent manner. We then examine minimal adverse effects of this treatment as assessed by bone quality, and kidney and liver morphology and histology. Finally, we demonstrate that fenoldopam treatment can augment load-induced bone formation in osteoporotic bones, using a mouse model of postmenopausal osteoporosis. This work is the first to examine the efficacy of targeting primary cilia-mediated mechanosensing to treat osteoporotic bones.
5.2. Introduction

As previously discussed in this thesis, osteoporosis is a devastating condition that contributes to increased risk of fracture and extended hospitalization, with over 50% of the US population over 50 years old having low bone mass leading to osteoporosis\textsuperscript{162}. It has long been known that bone actively responds to mechanical stimulation, with load being a potent anabolic stimulus of bone formation\textsuperscript{163}. Despite this, none of the current osteoporosis therapeutics leverage the inherent mechanosensitivity of bone. Bisphosphonates and RANKL inhibitors are antiresorptive agents that mitigate bone resorption, while the anti-sclerostin antibody prevents inactivation of bone-forming osteoblasts\textsuperscript{15,164}. Furthermore, these compounds are starting to be met with patient concern of increased microdamage with reports of mandibular necrosis and atypical fracture\textsuperscript{17,18}. Thus, the need for a new class of osteoporosis therapeutics is growing.

Bone is known to actively respond to mechanical stimulation, with load being a potent anabolic stimulus of bone formation. Previous work from our group demonstrated that primary cilia play a key role in whole bone mechanosensitivity. When primary cilia are removed from osteocytes and osteoblasts using a DMP1-CRE Kif3A knockout mouse model, the animals display significantly attenuated load-induced bone adaptation\textsuperscript{83}. Furthermore, we have also shown that mice lacking the cilia-localized protein, adenylyl cyclase 6 (AC6) also display significantly diminished load-induced bone formation\textsuperscript{86}. Interestingly, these global AC6 knockout animals display no alteration in normal bone development, suggesting a key role of AC6 in the response specifically to heightened mechanical stimuli.

The work in this thesis suggests that primary cilia can be pharmacologically targeted to manipulate cellular mechanotransduction. We believe that increasing primary cilia length
increases the amount of ciliary membrane strain in response to mechanical stimulation, to further stimulate stretch-activated ion channels \(^{137}\). However, it is also likely that increasing primary cilia length promotes production of ciliary proteins to potentiate mechanotransduction events. Dopamine-like 1 receptors localize to the primary cilium, and treatment with the agonist, fenoldopam, increases primary cilia length in endothelial cells, kidney epithelial cells, and osteocytes \(^{56,102,137}\). Intravenous injection of fenoldopam lowers blood pressure in hypertensive patients and mice, by increasing flow-induced nitric oxide production \(^{56}\). Furthermore, we previously identified in Chapter 2 that increasing primary cilia length with fenoldopam significantly enhances osteocyte mechanosensitivity and osteogenic signaling \(^{137}\). Besschetnova et al. found that stimulating the cAMP/PKA signaling pathway increases primary cilia length, in an adenylyl cyclase-mediated manner \(^{112}\). It has also been described that fenoldopam treatment increases adenylyl cyclase 6 production in human renal proximal tubule cells \(^{118}\).

Ovariectomized mice (OVX) are a commonly used estrogen depletion model simulating post-menopausal osteoporosis \(^{165,166}\). OVX animals display significant changes in bone architecture such as decreased cortical thickness, decreased bone mineral density (BMD), and increased marrow area in cortical bone; as well as decreased bone volume fraction, trabecular number and thickness, and increased trabecular spacing in trabecular bone \(^{166}\). Four weeks post-ovariectomy in skeletally mature mice has been demonstrated to elicit an osteoporotic phenotype characterized by decreased bone volume fraction, trabecular spacing, trabecular number, and bone mineral density in trabecular bone; and decreased cortical thickness, bone mineral density, and increased marrow area in cortical bone \(^{167}\). It has also been suggested that ovariectomy may lead to reduced mechanosensitivity of osteocytes \(^{165}\).
Fenoldopam is a clinically approved treatment for patients with extreme hypertension but, until our recent \textit{in vitro} work, has never been examined in the context of bone\textsuperscript{137}. The goal of the work in this chapter is to examine how fenoldopam affects whole bone mechanosensitivity to alter load-induced bone formation. Furthermore, we explore the potential of this cilia-targeted therapy to promote bone formation in osteoporotic animals.

5.3. Materials and Methods

5.3.1. Summary

To study the potential of fenoldopam for use in promoting bone formation, we utilized a mouse model of compressive ulnar load to stimulate load-induced bone adaptation. Mice were subcutaneously injected with fenoldopam and subjected to mechanical stimulation. Periosteal bone formation was quantified in response to indicate drug-induced changes in whole bone mechanosensitivity. Potential adverse effects of drug treatment were also examined.

5.3.2. Animals and injections

16 week old, skeletally mature, C57Bl/6 mice were injected subcutaneously with 20 or 50 mg/kg fenoldopam (US Pharmacopeial Convention), or vehicle control, for 7 consecutive days. Mice were housed in the Columbia University Barrier Facility and fed ad libidum. All procedures performed were in accordance with Columbia University Institutional Animal Care and Use Committee guidelines.
5.3.3. **Mechanical stimulation, in vivo**

On the final three days of drug or control injection, animals were also subjected to compressive ulnar load at a peak load of 3 N using a 2 Hz sine wave for 120 cycles, as previously described\(^{83,86}\). Contralateral limbs served as a non-loaded control. 2 days following load, mice were treated with 10 mg/kg calcein (Sigma), and 4 days later 70 mg/kg alizarin (Sigma), with mice being sacrificed 6 days later.

5.3.4. **Dynamic histomorphometry**

Dynamic histomorphometry was performed as previously described, measuring the amount and separation of calcein and alizarin labels to quantify mineralizing surface (rMS/BS), mineral apposition rate (rMAR), and bone formation rate (rBFR/BS) relative to non-loaded controls\(^{83,86}\). Left and right ulnae were dissected and preserved in ethanol. Samples were then infiltrated with methyl methacrylate, and embedded in methyl methacrylate and benzoyl peroxide. Transverse sections of the ulnar midshaft were imaged on a laser scanning confocal microscope (Olympus Fluoview FV1000). Measurements of outer bone perimeter (OP), single label perimeter (SL), double label perimeter (DL), and double label area (DA) were made in ImageJ. The mineralizing surface relative to outer bone surface (MS/BS), mineral apposition rate (MAR), and bone formation rate (BFR/BS) were calculated as:

\[
\frac{MS}{BS} = \frac{0.5 \times SL + DL}{OP} \times 100; \%
\]

\[
MAR = \frac{DA}{DL} \times \# \text{ of days between labels}; \mu m \text{ per day}
\]

\[
BFR/BS = \frac{MAR \times MS/BS}{3.65}; \mu m^3/\mu m^2 \text{ per year}
\]
Relative measurements – rMS/BS, rMAR, rBFR/BS – were determined by subtracting non-loaded from loaded ulnae to display differences due to mechanical load.

5.3.5. mRNA expression

For gene expression analysis, mice underwent the first 5 days of fenoldopam or vehicle injection, and 1 day of ulnar load. 24 hrs later mice were sacrificed, and ulnae were dissected, flash-frozen, and pulverized as previously described. Total mRNA was converted to cDNA by TaqMan reverse transcriptase (Applied Biosystems). Gene expression was analyzed by quantitative real-time PCR using primers and probes (Life Technologies) for analysis of adenylyl cyclase 6, Adcy6 (Mm00475772_m1), and GAPDH (4351309). Samples and standards were run in triplicate, and all gene expression was normalized to GAPDH endogenous control, as previously performed.

5.3.6. Osteoporotic animals

Ovariectomized (OVX) and Sham animals were purchased directly from Jackson Laboratories, with surgery performed at 12 weeks old. Mice followed the same injection (20 mg/kg fenoldopam or vehicle control) and loading timepoints described above beginning at 16 weeks old, a timeline previously demonstrated to exhibit an osteoporotic phenotype in mouse ulnae. Dynamic histomorphometry was only performed on OVX animals, while Sham animals were used to confirm osteoporotic phenotype based on µCT analysis.

5.3.7. µCT analysis

Non-loaded ulnae were used for µCT analysis. Samples were imaged with a Scanco ViaCT 80 at 10.5 µm isotropic resolution. Images were processed using a Gaussian filter and global threshold to segment bone volumes. Ulnar midshafts were imaged and used to assess
cortical bone, while the proximal epiphysis was used to examine ulnar trabecular bone. Cortical bone analysis was performed to determine total area, cortical area, marrow area, bone volume fraction (BV/TV), cortical thickness, moment of inertia ($I_{\text{max}}$ and $I_{\text{min}}$), and bone mineral density (BMD). Trabecular analyses included BV/TV, connectivity density, trabecular number, trabecular thickness, trabecular spacing, and BMD.

5.3.8. Kidney/Liver analysis

Kidneys and liver were dissected from animals, weighed, fixed in 10% formalin, and stored in 70% ethanol before being paraffin embedded. 5 µm thick longitudinal sections were made, and samples were stained with hematoxylin and eosin to visualize tissue structure. Images were captured at 4X magnification. Kidney function was broadly examined by urine creatinine concentration. Mice were subcutaneously injected with 20 mg/kg fenoldopam 3 days per week (Monday, Wednesday, Friday) for 5 weeks, with urine collected once per week. Hydrophobic sand (Lab Sand) was used for urine sample collection, and analysis was performed using a total urine creatinine kit (Cayman Chemical).

5.3.9. Analysis

All data were analyzed with one-way ANOVA followed by Bonferroni post-hoc correction. Dynamic histomorphometry data were initially analyzed with 2-way ANOVA to determine no sex-based differences in drug-enhanced bone adaptation. Values are reported as mean ± SEM, with $p < 0.05$ considered statistically significant. Sample size, n, represents biological replicates.
Figure 5.1: Injection and loading timeline
16 week old mice were subcutaneously injected with fenoldopam (20 or 50 mg/kg) or vehicle control on 7 consecutive days. On the final 3 days mice were also subjected to compressive ulnar loading. Schematic of mouse ulna placed between loading platens was adapted from Warden and Turner, Bone, 2004. 2 days following the completion of applied load, mice were injected with calcein, and then alizarin 4 days later. These fluorochrome labels allow quantification of regions of newly deposited bone mineral for analysis of load-induced bone formation rates.

5.4. Results

5.4.1. Fenoldopam enhances load-induced bone formation

First, we sought to translate our in vitro findings into an in vivo model of load-induced bone formation. Skeletally mature, 16 week old, mice were subcutaneously injected with fenoldopam (20 mg/kg or 50 mg/kg) or vehicle control for 7 consecutive days, Fig. 5.1. On the final 3 days, mice were also subjected to compressive ulnar load, while contralateral limbs served as non-loaded controls, as previously performed. Animals were then treated with calcein and alizarin 4 days apart to allow quantification of periosteal bone formation by standard dynamic histomorphometry, Fig. 5.2. The relative amount of mineralizing surface (rMS/BS) was quantified as the amount of labeled surface normalize to the bone surface. The mineral apposition rate (rMAR) is quantified by the separation of calcein and alizarin labels, while the
bone formation rate (rBFR/BS) is the product of rMS/BS and rMAR. While neither fenoldopam concentration elicited a significant increase in rMS/BS, 20 mg/kg fenoldopam results in a significant increase in rMAR and rBFR/BS compared to vehicle control, Fig. 5.3. Interestingly, the higher dose of fenoldopam resulted in no statistically significant difference in any of these parameters.

Figure 5.2: Fluorochrome labels enable analysis of new bone formation
Calcein (green) and alizarin (red) labels injected 4 days apart. Greater amounts of labeling around the periosteal surface of bone indicate increase mineralizing surface, while separation of labels indicates mineral apposition. Qualitatively, load greatly enhances bone formation, and fenoldopam treatment increases label separation. Scale bar represents 100 µm.
Figure 5.3: Fenoldopam enhances load-induced bone adaptation
Mice were treated with fenoldopam (20 mg/kg, or “Fen – High” at 50 mg/kg) for 7 consecutive days and also subjected to compressive ulnar load on the final 3 days. Fenoldopam treatment displayed no effect in enhancing the amount of mineralizing surface. However, the lower dose of fenoldopam (20 mg/kg) elicited significant increases in load-induced mineral apposition rate and bone formation rate. The higher dose of fenoldopam (50 mg/kg) had no effect on load-induced bone adaptation. Mean ± SEM; n = 26, 12, 14 for each group, respectively; ***p < 0.001. Male and female animals were grouped as 2-way ANOVA revealed no sex-based differences. All data are reported relative to non-loaded contralateral limbs.

5.4.2. AC6 mRNA expression in ulnae

Our previous work identified adenylyl cyclase 6 as a critical signal transduction protein in load-induced bone adaptation. Mice injected with 20 mg/kg fenoldopam for 5 days were sacrificed, and their ulnae were dissected for analysis of AC6 mRNA expression. In fact, the
effective dose of fenoldopam that enhances load-induced bone formation also elicited a significant increase in AC6 mRNA expression, Fig. 5.4.

**Figure 5.4: Fenoldopam enhances AC6 expression in ulnae**

Mice were treated with fenoldopam (20 mg/kg) for 5 days. 24 hours following the final injection mice were sacrificed and ulnae were dissected, flash frozen, and pulverized for mRNA analysis. Fenoldopam treatment increases AC6 mRNA expression in the ulnar cortical bone relative to vehicle control. Mean ± SEM; n = 8 for each group; **p < 0.01.

5.4.3. **Minimal adverse effects of fenoldopam treatment**

In addition to load-induced bone formation, we also examined potential indicators of adverse effects of drug treatment. Fenoldopam treatment elicited no qualitative change in bone ultrastructure, suggesting no difference in woven versus lamellar bone formed in response to load, Fig. 5.5. Additionally, μCT analysis revealed no difference in normal bone microarchitecture due to drug treatment, Fig. 5.5, Table 5.1. μCT was performed on ulna
midshaft to examine cortical bone at the region where dynamic histomorphometric measurements were taken. No changes in bone architecture were examined in 20 mg/kg fenoldopam treated animals, compared to vehicle control, Table 5.1. Kidney and liver weight was also assessed as an indicator of tissue inflammation, with no change examined relative to vehicle control, Fig. 5.6. Furthermore, no changes kidney and liver structure were observed, as assessed by H&E, Fig. 5.7. Urine creatinine was also examined over long-term drug treatment – 3 days per week with 20 mg/kg fenoldopam for 5 weeks – and suggests no change in kidney function, Fig. 5.8.

Figure 5.5: Examination of bone ultrastructure and microarchitecture
Loaded ulnae from fenoldopam treated mice were examined for bone ultrastructure and microarchitecture. Representative images are shown. Left, Qualitative examination of the mid diaphysis of loaded ulnae suggest no changes in the quality of bone formed. Fenoldopam treatment enhanced load-induced bone formation, without promoting woven bone formation. Right, μCT analysis was performed on non-loaded ulnae to assess potential fenoldopam-induced changes in bone architecture. These results are summarized in table 5.1.
Table 5.1: Short-term fenoldopam treatment does not affect bone architecture
The ulnar midshaft of non-loaded animals treated with 20 mg/kg fenoldopam was examined by µCT. Standard cortical bone parameters were examined. Fenoldopam treatment did not result in any change in bone architecture at the region where dynamic histomorphometry was performed.

<table>
<thead>
<tr>
<th>Bone and parameter</th>
<th>Female</th>
<th></th>
<th>Male</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>Fenoldopam</td>
<td>Vehicle</td>
<td>Fenoldopam</td>
</tr>
<tr>
<td>n</td>
<td>9</td>
<td>5</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Total area (mm²)</td>
<td>0.352 ± 0.006</td>
<td>0.357 ± 0.005</td>
<td>0.380 ± 0.006</td>
<td>0.370 ± 0.010</td>
</tr>
<tr>
<td>Cortical area (mm²)</td>
<td>0.306 ± 0.006</td>
<td>0.310 ± 0.003</td>
<td>0.330 ± 0.006</td>
<td>0.320 ± 0.009</td>
</tr>
<tr>
<td>Marrow area (mm²)</td>
<td>0.046 ± 0.003</td>
<td>0.310 ± 0.003</td>
<td>0.051 ± 0.001</td>
<td>0.049 ± 0.001</td>
</tr>
<tr>
<td>Bone vol. / Total vol.</td>
<td>0.869 ± 0.002</td>
<td>0.310 ± 0.003</td>
<td>0.867 ± 0.001</td>
<td>0.866 ± 0.001</td>
</tr>
<tr>
<td>Cortical thickness (mm)</td>
<td>0.173 ± 0.003</td>
<td>0.173 ± 0.006</td>
<td>0.172 ± 0.002</td>
<td>0.169 ± 0.004</td>
</tr>
<tr>
<td>Iₚ₀ (mm²)</td>
<td>0.028 ± 0.001</td>
<td>0.029 ± 0.001</td>
<td>0.037 ± 0.002</td>
<td>0.033 ± 0.003</td>
</tr>
<tr>
<td>Iₚ₅₀ (mm²)</td>
<td>0.004 ± 0.001</td>
<td>0.005 ± 0.000</td>
<td>0.005 ± 0.001</td>
<td>0.004 ± 0.001</td>
</tr>
<tr>
<td>Bone Mineral Density (mg/mm²)</td>
<td>1236.4 ± 5.0</td>
<td>1224.143 ± 11.750</td>
<td>1234.7 ± 9.1</td>
<td>1218.5 ± 7.7</td>
</tr>
</tbody>
</table>

Figure 5.6: Kidney and liver weights with fenoldopam
The kidneys and liver were dissected from fenoldopam (50 mg/kg) treated animals and weighed relative to total body weight. Even at the higher dose of fenoldopam we see no change in kidney or liver weight. Mean ± SEM; n = 10, 14 for each vehicle and control, respectively.
Figure 5.7: Kidney and liver histology
Kidneys and livers dissected from fenoldopam (50 mg/kg) treated animals were fixed, paraffin embedded, sectioned, and stained with H&E. The higher dose of fenoldopam does not cause any changes in tissue morphology compared to vehicle control.
Figure 5.8: Kidney function is not altered with fenoldopam
Urine creatinine was assessed in animals treated with fenoldopam (20 mg/kg) 3 days per week for 5 weeks. Urine was collected at the same time once per week. After 5 weeks of drug treatment, there is no significant difference in urine creatinine concentration relative to pre-drug treatment values. Mean ± SEM; n = 7 for each group.

5.4.4. Fenoldopam is efficacious in osteoporotic animals
We next used ovariectomized (OVX) mice as an estrogen-deficiency model of postmenopausal osteoporosis. Mice underwent OVX surgery at 12 weeks old. 4 weeks post surgery mice were injected with 20mg/kg fenoldopam for 7 days and subjected to compressive ulnar load. Fenoldopam treated animals displayed significantly augmented load-induced bone adaptation as measured by increased rMS/BS, rMAR, and rBFR/BS compared to vehicle control, Fig. 5.9. µCT was performed on ulnar cortical (mid diaphysis) and trabecular (proximal epiphysis) bone to confirm effects of OVX on ulnae microarchitecture, Table 5.2.
Figure 5.9: Fenoldopam enhances load-induced bone formation in osteoporotic animals
Ovariectomy (OVX) was used as a model of post-menopausal osteoporosis. Mice were subjected to OVX surgery at 12 weeks old, and fenoldopam (20 mg/kg) injections began at 16 weeks old. In osteoporotic animals, fenoldopam significantly enhances load-induced bone adaptation as quantified by dynamic histomorphometry. Mean ± SEM; n = 10 for each group; *p < 0.05, **p < 0.01.
Table 5.2: μCT analysis of osteoporotic and sham fenoldopam treated animals

Non-loaded ulnae from OVX and Sham control animals were examined by μCT to assess bone microarchitecture. Statistical analyses were performed between OVX-Sham for either vehicle or fenoldopam treatment. (*) denotes values where OVX is significantly smaller compared to Sham counterpart. (+) denotes values where OVX is significantly higher compared to Sham counterpart. Data demonstrate that at this timepoint OVX animals display an osteoporotic phenotype with significantly lower bone volume fraction (bone vol. / total vol.) and bone mineral density, with increased marrow area in cortical bone. In trabecular bone OVX animals display significantly decreased bone volume fraction and bone mineral density, with decreased trabecular number and thickness, and increased trabecular spacing and structure model index (SMI).

5.5. Discussion

Our results demonstrate that targeting primary cilia-mediated mechanosensing with fenoldopam treatment significantly enhances load-induced bone formation. While fenoldopam is clinically used for hypertension, this is the first work studying the effects of this treatment on bone in vivo.

Our loading data suggests that short-term fenoldopam treatment does not affect normal bone architecture. μCT analysis of non-loaded cortical bone demonstrates no change in bone
physical properties after 7 days of fenoldopam injection. This treatment does, however, significantly enhance load-induced bone adaptation. Together this suggests that short-term fenoldopam treatment sensitizes cells to mechanical stimulation rather than eliciting long-term systemic changes in bone quality. This type of treatment could be used to sensitize bone to lower amounts of load, thus preventing progression of limited use-induced bone loss. Potential clinical application of fenoldopam to enhance bone formation would likely require an associated exercise regimen.

It is not yet completely defined how fenoldopam treatment enhances primary cilia-mediated mechanotransduction in bone, and it is likely a combination of both structural and biochemical changes. While in vitro we identify significant increases in cilia length, the limited lacunar space in which osteocytes reside may impede substantial cilia elongation\textsuperscript{137}. Computational models have suggested that specific orientations of primary cilia may allow for elongation leading to enhanced mechanosensitivity\textsuperscript{92}. This model also posits that osteocyte primary cilia may not be free standing organelles in vitro and may anchor to the lacunar wall, in a manner similar to chondrocyte cilia attaching to the extracellular matrix\textsuperscript{63}. Regardless of the means of physical changes to primary cilia structure, our data also demonstrates that fenoldopam treatment significantly enhances AC6 production. As previously described our recent work indicates that deletion of AC6 impairs load-induced bone formation, suggesting that the opposite may also be true, and that increasing AC6 production will augment load-induced bone formation\textsuperscript{86}. Together the increase in cilia length, enabling further stimulation of stretch-activated ion channels, and enhanced AC6 protein levels potentiate bone mechanosensitivity.
There is a therapeutic window in which fenoldopam treatment is effective in promoting load-induced bone formation. While 20 mg/kg fenoldopam significantly enhanced bone adaptation, 50 mg/kg elicited no bone formation response. Previous work identified that fenoldopam treatment increases primary cilia length in a dose-dependent manner. Moreover, when the concentration was increased to 2.5x the peak effective dose, cilia length reduces closer to baseline level. Currently it is not completely evident why this dose-dependence occurs, but it is likely due to toxicity. During injections with the higher dose of fenoldopam, we noticed a small amount of tissue necrosis near the injection site in several animals not experienced in with the effective, 20 mg/kg, dose. While this high dose may be associated with some level of toxicity, we do not examine any change in kidney or liver weight and morphology. Further toxicology would need to be performed to fully characterize adverse events.

Fenoldopam does not specifically target osteocytes, thus allowing potential for adverse effects. While fenoldopam is clinically used to treat extreme hypertension, it resembles other antihypertensive compounds and has been shown to have limited or no effect hypotensive effect in normotensive patients. Furthermore, low enough doses of fenoldopam can still increase endothelial cilia length, and mechanically induced nitric oxide production, without affecting blood pressure. Fenoldopam treatment has, however, also been implicated with an increase in renal blood flow and an increase in natriuresis, diuresis, and glomerular filtration rate, potentially all leading to hypertensive renal damage and kidney disease. High enough doses of fenoldopam, 100 mg/kg, have also been found to be vasotoxic in a rat model. Our effective, 20 mg/kg, treatment dose did not elicit any change in urine creatinine concentration, and even at our higher dose, 50 mg/kg, there was no change in kidney and liver weight or morphology.
Fenoldopam also does not cross the blood brain barrier, so there is minimal risk of dopaminergic central nervous system effects resulting from treatment\textsuperscript{173}. While primary cilia are not unique to osteocytes and fenoldopam does not specifically target bone, our data suggests minimal adverse systemic affects while still enhancing bone mechanosensitivity. A cell-type specific therapy would mitigate any concerns of adverse effects.

Together, these data demonstrate that targeting the primary cilium is a potent strategy for directing cell mechanotransduction, and intercellular communication to dictate whole tissue function. This is the first demonstration of a ciliotherapy enhancing bone adaptation \textit{in vivo} and, moreover, enhancing load-induced bone formation in osteoporotic subjects. Fenoldopam is clinically used, but until now has never been examined in the context of whole bone adaptation. Our results suggest that there is significant potential for repurposing fenoldopam for use in bone indications, and for developing cilia-targeted therapeutics to direct cell and tissue mechanotransduction to combat human diseases.
Chapter 6

6. Conclusion

6.1. Summary

The overall goal of this thesis is to demonstrate the capability of targeting primary cilia to sensitise cells to mechanical stimulation and promote bone formation, as a potential therapeutic strategy for treating bone diseases. In this thesis we examined primary cilia-mediated mechanotransduction in osteocytes, expanded our list of candidate drugs through high-throughput screening, studied implications of ciliary manipulations on intercellular pro-osteogenic signalling, and translated this work into an in vivo model of bone formation in osteoporotic animals. This work elucidates primary cilia and mechanotransduction as viable targets for bone disease therapeutics.

In Chapter 2, we demonstrated that two distinct compounds, fenoldopam and lithium, significantly increase primary cilia length. We then demonstrated that cells with longer cilia are more mechanosensitive. Furthermore, fenoldopam treatment was able to recover ciliogenesis in cells with disrupted cilia formation, and that this compound functions through a DR1-AC6-cAMP-mediated pathway. The increase in cilia length is anticipated to enhance bending under fluid flow to further stimulate stretch-activated ion channels and also stimulate production of ciliary proteins such as AC6, together, potentiating mechanotransduction.

In Chapter 3, we developed a high-throughput drug screening platform to further our list of candidate compounds that modulate ciliogenesis. 6931 compounds with known biologic
activity were tested for their capability to increase primary cilia length. We set up a fully automated platform to culture, drug treat, fix, stain, image, and analyze these compounds. Our analysis revealed 103 compounds that increase cilia length and 92 that increase cilia incidence, 18 of which are conserved between both data sets. Because these compounds have known biologic activity, we classified these drugs based on mechanism of action to help further inform cilia biology research.

In Chapter 4, we began to examine how manipulation of osteocyte primary cilia-mediated mechanotransduction may influence downstream osteogenic signaling to other cells within bone. We utilized a co-culture model where osteocytes underwent siRNA-mediated knockdown or pharmacologic challenge and were mechanically stimulated. The conditioned media was then used to culture osteoblasts or MSCs to study mechanically-induced paracrine signaling. We demonstrated that fenoldopam treatment not only enhances osteocyte mechanosensitivity, but also downstream signaling to promote osteoblast activity and MSC osteogenic differentiation.

Finally, in Chapter 5, we translate our in vitro findings into an in vivo model of load-induced bone formation. Mice treated with fenoldopam displayed significantly greater load-induced bone formation, potentially occurring through an increase in AC6 production. Furthermore, we also utilized µCT to suggest that the increase in bone formation was predominantly due to enhanced mechanotransduction, and not changes in normal bone architecture due to drug treatment. We also began to examine potential adverse consequences of drug treatment, but did not see any alteration in quality of bone formed or serious signs of kidney or liver damage. Most importantly, we demonstrated that this treatment strategy also sensitizes
ovariectomized animal ulnae to mechanical stimulation, increasing load-induced bone formation in osteoporotic animals.

6.2. Future studies

The work in this thesis examines the therapeutic potential of targeting primary cilia-mediated mechanosensing in promoting bone formation; yet, future work is required before this treatment strategy can be fully translated to human applications.

To discern whether the increase in primary cilia-mediated mechanosensing is due to an increase in cilia bending or protein production, further work would need to be performed. Cells endogenously expressing SSTR3-GFP would enable visualization of cilia deflections in real time. Using our existing fluid flow devices and imaging capabilities we can compare ciliary bending between vehicle and drug treated cells\textsuperscript{132,174}. We can also couple this work with ciliary-localized calcium and cAMP biosensors that our group has developed to elucidate changes in intraciliary signaling\textsuperscript{35}.

Next, we plan to expand our drug screening efforts to further identify compounds that modulate ciliogenesis, and further elucidate signaling pathways involved in ciliogenesis. The drug screening platform can be expanded to include dose responses studies, as well as temporal responses. We only tested 10 \( \mu \text{M} \) concentrations for 16 hours. Previous work has demonstrated that higher concentrations of certain compounds can increase cilia length within 3 hours\textsuperscript{112}. Furthermore, our screen only included osteocytes. IMCD kidney epithelial cells are also commonly used to examine primary ciliogenesis. Growing work is also exploring the role of ciliogenesis in chondrocytes\textsuperscript{175}. Mimicking the same screening platform in kidney cells and
chondrocytes serves multiple purposes. Repeated classes of hits between cell types will demonstrate conserved pathways involved in ciliogenesis. Conversely, distinct individual and classes of compound will suggest cell type-specific effects, providing potential for minimal side effects with in vivo treatment. In the immediate future, we hope to perform confirmation studies to validate hits that increase cilia length, as well as incidence.

Our co-culture experiments only examine paracrine signaling from osteocytes to osteoblasts and MSCs. Paracrine signaling is not the only means of intercellular communication, as direct-cell communication also plays a critical role in directing bone adaptation. While it is anticipated the enhanced osteocyte mechanosensing will also potentiate mechanotransduction events through direct-cell contact, further co-culture experiments could more accurately describe this pathway. In addition, the consequence of manipulating osteocyte mechanosensing on osteoclast activity would be a significant advance in this work as the majority of first-in-line osteoporosis treatments manipulate osteoclast activity. It is anticipated that enhanced osteocyte mechanosensitivity would limit, but not abolish, osteoclast activity.

Finally, we wish to build upon our in vivo model of fenoldopam-sensitized bone formation. Numerous avenues exist to further test the therapeutic potential of this treatment strategy before it is ready for human trials. We only utilized subcutaneous injections, but oral delivery may provide a more patient-friendly alternative drug delivery mechanism. Long-term treatment and characterization of microdamage accumulation would allow for comparison between this treatment strategy and existing options. We have begun these long-term studies, treating ovariectomized and sham mice with fenoldopam for 5 weeks, and will examine trabecular and cortical bone architecture changes by μCT and bone strength with three-point
bending in response to prolonged drug treatment. We also plan to move to larger animal models, starting with rat, to establish efficacy in a more human physiologically relevant model. Currently, fenoldopam is only used in short doses, < 48 hours, in patients with extreme hypertension or renal failure. As such, no current patient data exists to infer potential implications on bone quality. Finally, expanding our drug screening efforts to include more compounds and multiple cell types will elucidate alternative compounds which may prove more efficacious or further reduce any potential adverse effects on other cell types.

6.3. Significance

Osteoporosis is a devastating condition affecting over 50 million Americans. The most significant consequence of osteoporosis is the severely heightened risk of fracture, with hip fractures being the most devastating at 20% patient mortality within the first year following fracture \(^3,4\). Surviving patients are still left with dramatically impaired quality of life, with 60% requiring additional assistance, and 33% entirely reliant on caretakers, leaving less than 7% reaching full recovery \(^5,6\). While current treatment options exist for conditions of low bone mass, there is increasing concern with these as risks of atypical fracture and necrosis emerge. As such, it is estimated that after one year only 40% of patients will continue with their prescribed treatment, and after two years only 20% will continue treatment \(^20,21\). New therapeutic strategies are required.

The work in this thesis is the first use of a cilia-targeted therapy to promote load-induced bone formation in osteoporotic bones. As mentioned, current osteoporosis therapeutics are targeted towards inhibiting normal bone pathways. Bisphosphonates and RANKL inhibitors
disrupt osteoclast-mediated resorption, while the anti-sclerostin antibody prevents osteoblast inactivation. Our results demonstrate that manipulation of bone mechanotransduction is a potent and viable strategy to promote bone formation. We believe that the primary cilium is not only a potential, but a viable and robust therapeutic target for manipulating cell mechanosensitivity and whole bone adaptation. This work opens the door to an entirely new class of bone disease therapeutics that leverage the inherent mechanosensitivity of bone to stimulate bone formation. Our proposed treatment strategy has the potential to enable patients to reap the benefits of mild ambulation to restore bone quality to slow and even treat the progression of osteoporosis.

While the work in this thesis focuses on the bone disease applications of targeting primary cilia, we believe that this work has significant implications in numerous human diseases. Mechanotransduction is a process critical to a wide range of cell and tissue types, and primary cilia are present on nearly every human cell type. Polycystic kidney disease, atherosclerosis, skeletal patterning, and osteoarthritis are all impacted by mechanotransduction. Furthering our understanding of how various cells sense and respond to mechanical cues, and elucidating ways to manipulate this process are critical in developing therapeutics for such a myriad of conditions. The impact of primary cilia and mechanotransduction are also increasingly being studied with regards to tumorigenesis. Breast and prostate cancer cells are known to lose primary cilia during tumorigenesis, and both cancer types engage in mechanotransduction, responding to changes in substrate stiffness through altered proliferation and migration \(^{70,75,79}\). Recent work has even examined the potential role of mechanotransduction in bone metastasis \(^{176–178}\). Manipulating primary ciliogenesis and mechanosensing may prove to be a therapeutic strategy for slowing tumorigenesis and metastasis. We believe that targeting primary cilia-mediated
mechanotransduction is a potent therapeutic strategy that has implications in the treatment of a wide range of significant human conditions.
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