Primary cilia act as gravity transducers in bone cells

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October, 2014

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Abstract

Environmental mechanical signals play important roles in the cell fate determination of metazoans, including bone tissues, which utilize mechanical stress to balance the amount of bone formation versus bone resorption. In this case, the force due to gravity is essentially a constant mechanical signal on Earth and has been a key factor in the evolution of skeletal systems in vertebrates. Accordingly, changes in this force, such as is experienced under hyper- or microgravity conditions, leads to various cellular responses including altered proliferation, migration, and differentiation. Although the modes of response in vertebrate cells to changes in gravitational acceleration have been widely studied, little is known about how cells initially perceive and transduce these mechanical cues into cellular responses. Previous studies suggest an involvement of primary cilia, non-motile sensory antennae in mammalian cells, which may act as gravity transducers due to their mechanosensory characteristics. However, direct experimental evidence supporting this idea has not yet been established. In my study, I show that primary cilia in bone cells play a role in the mechanotransduction of hypergravity acceleration that influences the regulation of cell proliferation. I also demonstrate that this transduction through primary cilia involves a downstream regulation of global β-catenin levels, particularly at cell-cell contact regions, as well as a loss in the contact inhibition of cell growth.

Keywords: Mechanotransduction; hypergravity; microgravity; β-catenin; contact inhibition of cell growth.

Abbreviations: CH, chloral hydrate.

Significance Statement

Gravity is a crucial force governing homeostasis of the skeletal system. While on the surface of Earth, gravity is essentially constant; however, exposure to hyper- or microgravity (during space flights) leads to various cellular responses that may cause health issues, such as reduced bone formation and/or increased resorption. It remains unclear how bone cells perceive and transduce these gravitational cues into biochemical responses, but with this work I present new evidence that primary cilia in bone cells influence mechanotransduction of hyperacceleration into the up-regulation of proliferation. I also propose a transduction mechanism in which β-catenin functions as an intermediary molecule between the transduction of hyperacceleration stimulus at primary cilia and the ultimate proliferative responses in a cell-cell contact dependent manner.

I dedicate this honors thesis to my mother, my father, and my sister as a reflection of my utmost love and affection to them for not only mentally and financially supporting me under any circumstances, but also standing by any life decision that I make.

Introduction

Extracellular mechanical signals are crucial cues for cell fate determination involving proliferation and differentiation (1-4). The most impactful among these mechanical signals, which affect cellular regulatory processes, include fluid flow, shear stress, stiffness of extracellular matrix, as well as the level of gravity experienced by the cell (1). Although physiological and biochemical responses to these mechanical signals have been widely studied (5), little is reported about how cells actually perceive these mechanical cues, transduce them into biochemical signals, and ultimately trigger cellular responses such as proliferation and differentiation (2).

Gravity is one of the most fundamental forces that all organisms on Earth have to cope with, including the organisms that are living in water. Without gravity, water would behave completely different, and water pressure would not exist. For instance, evolution of skeletal systems in mammals not only facilitates mobility, but is also a natural method of counteracting a possible inner sedimentation of organs due to gravity force (6). While gravity is roughly constant within Earth's biosphere, it is possible to experience significant changes of gravitational force over various timeframes such as a few seconds or minutes during aircraft flights (may cause short term hypo- or hypergravity), or over several weeks during space travel (microgravity). In particular, prolonged exposure to microgravity has been shown to cause serious health concerns, including bone loss (6-9). Various studies have shown that microgravity causes a decrease in bone formation and/or an increase in bone resorption, which reduces bone density (7, 8, 10). Conversely, bone-forming osteoblasts have been shown to react to hypergravity as

well, and their common response to increasing acceleration is to increase cell proliferation (11-13). Hence, micro- versus hypergravity conditions cause cellular responses in opposite directions, but the underlying biological mechanisms giving rise to these cellular responses (e.g., bone formation or resorption) appear similar (14, 15). However, it is still unclear how bone cells physically perceive a change in acceleration and transduce this information into the ensuing biochemical responses.

Primary cilia are non-motile, microtubule-containing cellular structures extending from the surface of many mammalian cells (16). Their structure is composed of nine outer microtubule doublets, similar to that of a motile cilium; however, primary cilium lacks the two central microtubules (9+0 axoneme) that are crucial for motile cilia (9+2 axoneme) (16, 17). Primary cilia function as sensory antennae for the transduction of various mechanical signals into biological responses (2, 16-19). In kidney cells primary cilia have been shown to sense fluid flow by a Ca^{2+}-dependent mechanism as well as by perceiving shear stress (20, 21). Furthermore, in bone cells primary cilia act as sensory antennae in the mechanotransduction of fluid flow by a Ca^{2+} independent mechanism, and mediate osteogenic responses at the same time (2). Primary cilia are also involved in the coordination of various signaling pathways that regulate mammalian cell proliferation and differentiation, including Platelet-Derived Growth Factor (PDGF), Wnt, and Ca^{2+} signaling pathways (16, 19, 22). Moreover, being able to transduce mechanical cues into intracellular biochemical signals (i.e., mechanotransduction) constitutes a pivotal skill that allows primary cilia to initiate and regulate the aforementioned signaling pathways (4, 16, 23).

Primary cilia's sensory characteristic in the transduction of mechanical signals and their involvement in mediating various signaling pathways that control cell fate render primary cilium a prime target to be investigated as a transduction device that can perceive long term changes in gravitational acceleration. Despite previous suggestions that primary cilia might act as gravity

transducers (24, 25), direct empirical evidence in support of this idea has not yet been established. Here I present novel evidence for a direct involvement of bone cell primary cilia in the mechanotransduction of hyperacceleration stimulus (10 x *g*) into up-regulation of proliferation. I also demonstrate that the transduction of hyperacceleration through primary cilia involves down-regulating the contact inhibition of cell growth as well as controlling the β-catenin levels in nucleus and cytoplasm, including at cell-cell contact regions.

Results

Observing primary cilia in bone cell cultures.

Malone et al. (2) established that primary cilia extend from the surface of MC3T3-E1 osteoblasts and can be scored by immunofluorescence staining with acetylated α-tubulin, which is strongly enriched in ciliary microtubules. I therefore used fluorescently labeled, acetylated α-tubulin to mark the primary cilia in MC3T3-E1 cells, as well as using fluorescently labeled β-tubulin to display cytosolic microtubules and DAPI for DNA [Fig. S1 *A* and *B*; Fig. 1 *B* – *D* (white arrowheads exemplifying primary cilia in *B* and *C*)] (See SI Methods for details).

Increasing the number of and removing the primary cilia.

4 mM chloral hydrate treatment for 72 hours has previously been shown to remove the majority of primary cilia from MC3T3-E1 cells (2). Similarly, Praetorius and Spring (20) have demonstrated that treating MDCK cells with 4 mM aqueous chloral hydrate for 68 hours significantly revokes the formation of primary cilia. Actively proliferating mammalian cells possess primary cilia particularly in growth 1 (G1) phase of the cell cycle as well as at early synthesis (S) phase (22, 26). Applying mimosine to MC3T3-E1 osteoblasts has been shown to arrest the cells in late G1 phase (27), where primary cilia should be fully established (22, 26). Therefore, before removing primary cilia to test whether they act as transduction elements for changes in gravitational acceleration, I sought to maximize the fraction of cells possessing primary cilia. I quantified the fraction of MC3T3-E1 cells having primary cilia in the absence of drugs and compared it with the cultures treated with mimosine (Fig. S1; see SI Methods for details). In my MC3T3-E1 cultures mimosine treatment caused ~20% increase of cells with primary cilium (Fig. S1*C*; $P < 0.05$). Also, mimosine treatment did not seem to interfere with the overall cellular morphology and cytoskeletal structure of the MC3T3-E1 osteoblasts used here

(Fig. S1 *A* and *B*).

After arresting cells in G-phase by mimosine treatment, I next treated MC3T3-E1 osteoblasts with 4 mM chloral hydrate for 72 hours. This protocol led to a nearly complete removal of primary cilia as determined by acetylated α-tubulin staining (Fig. 1*A*). After the chloral hydrate treatment, it took the cells 48 hours for a full recovery, monitored by the reappearance of cytosolic microtubules, which was evaluated through β-tubulin staining (Fig. 1 *C* and *E*). During this recovery period primary cilia were not rebuilt. Even 48 hours after the chloral hydrate treatment, the percentage of MC3T3-E1 cells that did not possess primary cilia was ~90% (Fig. 1 *A*, *B*, and *D*; $P < 0.001$).

Hyperacceleration causes a significant increase in bone cell proliferation, and primary cilia are required for the mechanotransduction of hyperacceleration into proliferative responses.

Mechanotransduction is the machinery of transducing the mechanical stimuli acting on a cell into biological responses (1). In my study, I applied 10 x *g* hyperacceleration as the mechanical stimulus and sought to determine whether MC3T3-E1 cells respond to the hyperacceleration stimulus by up-regulating their proliferation. When MC3T3-E1 cells were centrifuged at 10 x *g* for 1 hour, a substantial increase in cell growth over the next 48 hours was observed in comparison with the proliferation of uncentrifuged control culture at 1 x *g* (Fig. S2 *A1-A3 and C1-C3*). The proliferation of cells that were centrifuged was about 2 times higher than in control cells at 1 x *g* ($P < 0.001$). Unlike the centrifuged cells, the control culture reached its cellular growth plateau after 36 hours (Fig. 2*A*; blue line for the uncentrifuged control group at 1 x *g*). I then asked whether the growth difference between the control and centrifuged cultures was merely because the centrifuged cells continued proliferating long after the control group reached its stationary growth phase (as seen in Fig. 2*A*; red line for the centrifuged culture); or also due to an additional reduction in the doubling time of centrifuged cells. A shorter doubling time would

indicate a more rapid cell cycle overall. To clarify this issue, I calculated the doubling time of both, the uncentrifuged control and centrifuged cultures for every 12 hours until 36 hours after the centrifugation (Fig. 2*B*). Within the first 12 hours and 12 to 24 hours after the centrifugation, the doubling time of centrifuged cells was 1.3 times shorter than that of the control cells (b/t 0-12 h: $P < 0.01$; b/t 12-24 h: $P < 0.05$) (Fig. 2*B*). The doubling time difference between the control and centrifuged group was even more pronounced between 24 and 36 hours after the hyperacceleration stimulus: the centrifuged group's doubling time was 3 times shorter than that of the control group at 1 x *g* (b/t 24-36 h: $P < 0.001$) (Fig. 2 *A* and *B*).

Chloral hydrate treatment has previously been shown to effectively remove the cilia from certain mammalian cells, such as bone and kidney cells (2, 20). However, chloral hydrate treatment on PtK cells caused a sudden increase in Ca^{2+} levels, which might affect mitosis through microtubule breakdowns (28). I therefore investigated whether chloral hydrate treatment unfavorably affects the proliferation of bone cells. I treated the bone cells with 4 mM chloral hydrate for 72 hours and subsequently incubated them for 48 hours in regular medium with no drug in order for the full recovery of cytosolic microtubules while the majority of primary cilia did not reappear (Fig 1 *B – D*; see *Removal of Primary Cilia* in *Methods*). After the 48 h incubation period in regular medium with no drug, the growth and doubling times of MC3T3-E1 cells that were treated with chloral hydrate did not seem to remarkably deviate from that of the untreated control group (Fig. S2 *A* and *B*; Fig. 2 *A* and *B*).

After establishing the effects of chloral hydrate treatment on MC3T3-E1 cells and ensuring a lack of potentially serious side effects, I proceeded to investigate the main question of my research: Do the primary cilia in MC3T3-E1 cells play a crucial role in the mechanotransduction of hyperacceleration into proliferative responses? My experimental strategy was to remove the primary cilia from MC3T3-E1 cells and let the cells recover from their chloral hydrate treatment for 48 hours until they reestablished their regular cytoskeletal morphology, but still lacking

primary cilia. I subsequently centrifuged cells at 10 x *g* hyperacceleration for 1 hour and finally analyzed the proliferative responses in comparison to a control group kept at 1 x *g*. Within the first 24 hours after the hour-long 10 x *g* stimulus, chloral hydrate treated MC3T3-E1 cells shared similar growth and doubling times with the uncentrifuged, untreated control group (Fig. 2 *A* and *B*: Blue vs. gray.). The doubling times of both chloral hydrate treated groups were quite similar throughout the whole growth curve. Compared to uncentrifuged, chloral hydrate treated cells (Fig. 2*B*: Green vs. gray.) the 10 x *g* hyperacceleration stimulus did not change the overall proliferative behavior of chloral hydrate treated cells. Also, the overall shape of the growth curve of both chloral hydrate groups (Fig. 2*A*: Green vs. gray.) were very similar despite the hyperacceleration stimulus on one of the groups (Fig. 2*A*: Gray.). Furthermore, within the first 12 hours after the stimulus the centrifuged, chloral hydrate treated cells had a 1.6-fold greater doubling time than that of the centrifuged, but chloral hydrate untreated cells (b/t 0-12 h: $P < 0.001$) (Fig. 2*B*: Gray vs. red.).

The uncentrifuged control group with no drug treatment reached its stationary growth phase at 36 hours, while all other groups continued to grow even beyond the 48 hour cutoff (Fig. 2: Blue vs. red, gray, and green). At 36 hours, the doubling time of both chloral hydrate treated groups, either centrifuged (Fig. 2: Gray.) or uncentrifuged (Fig. 2: Green.), was quite similar (Fig. 2*B*). However, the centrifuged untreated group (Fig. 2: Red.) had a ~1.7-fold shorter doubling time in comparison to both the chloral hydrate treated groups which were uncentrifuged (b/t 24-36 h: $P < 0.01$) or centrifuged (b/t 24-36 h: $P < 0.05$) (Fig. 2*B*). On the other hand, between 24 and 36 hours after the centrifugation, the doubling times of chloral hydrate groups [both centrifuged (Fig. 2: Gray.) and uncentrifuged (Fig. 2: Green)] were ~1.7-fold shorter than that of the uncentrifuged control group with no drug treatment (Fig. 2: Blue.) ($P < 0.001$) (Fig. 2*B*).

The essence of my study revealed that in untreated bone cells applying a 10 x *g* stimulus triggered a substantial up-regulation of proliferation (Fig. S2 *C1 – C3*; Fig. 2A: Red.). This

significant response to the 10 x *g* stimulus, however, was almost completely absent on cells where the primary cilia were removed by chloral hydrate treatment (Fig. S2 *D1* – *D*3; Fig. 3A: Gray.).

Mechanotransduction of hyperacceleration through primary cilia involves regulating the contact inhibition of cell growth and the levels of β-catenin at cell-cell contact regions. Apart from a substantial up-regulation of proliferation due to the hyperacceleration stimulus, at the same time I observed a remarkable reduction in cell-cell contact inhibition of cell growth in the centrifuged cultures (Fig. S2). This reduction was recognizable 24 hours after the stimulus [compare Fig. S2 *A3* with *C3* (black arrowheads); Fig. 2*A*: Red vs. blue.]. However, contact inhibition remained almost at control levels in MC3T3-E1 cells that were treated with chloral hydrate to remove primary cilia and exposed to 10 x *g* hyperacceleration (Fig. S2 *A3* and *D3*; Fig. 2*A*: Gray).

In vertebrate cells cadherin-catenin complexes play important roles in maintaining cell-cell contact and the inhibition of proliferation upon reaching a critical density in a 2-D culture (29, 30). Growth inhibition through cell-cell contacts constitutes a crucial regulation that is often perturbed in cancer cells leading to tumor formation. β-catenin is one pivotal element in the formation and functionality of cadherin complexes (30-32; Figs. 3 and 5*C*), but it is also an essential downstream component of both mechanical and chemical signal transduction that enables primary cilia to regulate cell proliferation (4, 22). Hence, I asked whether β-catenin levels are regulated as a biochemical response to the hyperacceleration stimulus, and whether the mechanotransduction pathway of primary cilia initiates this regulation as an early downstream response to the hyperacceleration stimulus. In MC3T3-E1 cells with intact primary cilia, the hyperacceleration stimulus led to a 2-fold decrease in the β-catenin levels of the nucleus ($P < 0.001$) as well as a 1.5-fold decrease in the β-catenin levels of cytoplasm ($P < 0.05$) (Fig. 4). Expression levels were monitored by fluorescence intensity measurements (See

Immunofluorescence and *Confocal Imaging and Quantifying the Fluorescence Intensity of β-catenin* in SI Methods; Fig. 4*B*). The purpose of measuring nuclear β-catenin levels (besides the cytoplasmic levels) was to see whether the decrease in cytoplasmic levels is partially due to a translocation of β-catenin into nucleus as it would be the case during canonical Wnt signaling (3). Cells treated with chloral hydrate to remove their primary cilia and furthermore incubated for another 48 hours in regular medium (without chloral hydrate) displayed no change in β-catenin levels, neither in their nucleoplasm nor cytoplasm ($P < 0.05$) (Fig. 4). Once cells were treated with chloral hydrate, the hyperacceleration stimulus did not change nuclear or cytoplasmic levels of β-catenin, and these cells displayed similar levels of β-catenin as the unstimulated, untreated control group ($P < 0.05$) (Fig. 4*B*).

Hyperacceleration not only reduced β-catenin levels in cytoplasm and nucleus, but I also found a striking reduction of β-catenin levels at cell-cell interaction sites [compare Fig. 3*C* (white arrows pointing at cell-cell interaction sites) with *A*]. In all experimental groups, but the centrifuged group with no chloral hydrate treatment, the β-catenin levels were similar to the levels of the uncentrifuged, untreated control group (compare Fig. 3 *B* and *D* with *A*). I thus proceeded to examine in more quantitative terms whether β-catenin levels are indeed down-regulated in cell-cell contact regions, the most important location where β-catenin participates in the formation of cadherin-catenin complexes and activates their inhibitory function on cell growth (Fig. 5*C*). In centrifuged cells that were not treated with chloral hydrate, the levels of β-catenin measured directly at cell-cell contact regions was 50% less than in control cells (compare red bar to the others in Fig. 5*B*). The β-catenin levels were quantified by the analysis procedure described in Adams et al. (33) that utilizes β-catenin's mean fluorescence intensity at cell-cell contact sites and the mean intensity at non-contacting membranes to calculate the fluorescence intensity ratio ($P < 0.001$; Fig. 5 *A* and *B*; see SI Methods). The uncentrifuged, chloral hydrate treated group (1 x g CH in Figs. 3*B* and 5*B*) showed similar β-catenin levels at contact sites as the uncentrifuged, untreated control group (1 x g in Figs. 3*A* and 5*B*). Finally,

once the primary cilia were removed by chloral hydrate, the 2-fold reduction effect on β-catenin levels by the hyperacceleration stimulus was no longer observed (Fig. 5*B*: Grey bar.).

Discussion

Gravity is an ever-present force on Earth's surface with a nearly constant value that influences the morphology and physiology of land and water organisms in various ways. Changing the magnitude of gravity, e.g. to microgravity during space flights, or hyper-gravity on a centrifuge, affects the physiology of cells and organisms. These effects are particularly obvious in vertebrate tissues such as bone, cartilage, and tendons which provide stability, shape, and structural integrity to the overall skeletal system (6, 7, 9, 34). However, the mechanisms by which mammalian cells transduce these mechanical stimuli into cellular responses are not yet fully established. In this study I demonstrated that hyperacceleration (hyper-gravity) causes cultured bone cells to increase their proliferation rates, and I showed that primary cilia are required for the perception and mechanotransduction of hyperacceleration underlying these proliferative responses. Furthermore, I demonstrated that a hyperacceleration stimulus leads to a substantial loss in contact inhibition of cell growth, as well as a decrease in both nuclear and cytoplasmic levels of β-catenin. At cell-cell contact sites, β-catenin binds to cadherins building a functional complex that inhibits cell proliferation over the formation of cell-cell interactions (30, 31, 35, 36). The post-stimulus results of my study indicated a significant reduction in the levels of β-catenin at cell-cell contact sites. These observations suggest an explanation for why the stimulated cells achieved much higher cell densities than unstimulated controls as well as stimulated cells that lacked primary cilia.

In mammalian cells, primary cilia develop during early G1 phase after mitosis (22, 26). They disassemble again in late S phase, shortly before G2 phase and the formation of the mitotic spindle (22, 26, 37). Thus, I assumed that arresting bone cells within the late G1 phase would synchronize cells in culture and thereby increase the fraction of cells possessing primary cilia. In my study, I first demonstrated that synchronizing MC3T3 cells in late G1 phase via mimosine

treatment (27) substantially increases the number of cells that display functional primary cilia. Optimizing the number of cells possessing primary cilia was crucial for all the cilium-based mechanotransduction tests I performed with MC3T3-E1 osteoblasts. As a control, I treated MC3T3-E1 osteoblasts with chloral hydrate that resulted in cells shedding off their primary cilia (2, 20). Lacking primary cilia, this control group should not experience any mechanotransduction effects from the primary cilia. This procedure provided convincing evidence that the primary cilia are involved in the process of transducing hyperacceleration into an up-regulation of cell proliferation.

Nevertheless, while chloral hydrate treatment is one of the few – though efficient – chemical ways of removing primary cilia (2), one must keep in mind that immediately after its application chloral hydrate might disrupt mitotic events through microtubule breakdowns (28). Thus, after the chloral hydrate treatment, I let cells recover for 48 hours prior to the hypergravity stimulation by centrifugation. During that recovery period, osteoblasts reverted to their regular cytoskeletal morphology while still lacking primary cilia in at least 90% of all cases. Successful recovery of cells from chloral hydrate treatment was indicated by a normal looking microtubule cytoskeleton and cell cycle resembling that of the control cells. Moreover, the slight difference between the growth curves of unstimulated cultures with and without primary cilia indicates the importance of primary cilia for cell growth regulation.

Hyperacceleration has previously been shown to affect the cell proliferation of several organisms in various ways. For instance, Kumei et al. (38) stated that conditions of 18-, 35-, and 70 x *g* caused an increase in cell proliferation of HeLa cells by shortening the G1 phase during centrifugation. Among the accelerations tested, 35 x *g* was the most effective one in terms of promoting cell proliferation (38). Additionally, *Xenopus laevis* A6 cells also increased their cell proliferation by ~40% upon being exposed to 5 x *g* hyperacceleration stimulus (39). In the context of bone cell responses, osteoblast-like ROS17/2.8 cells that experienced gravitational

accelerations from 1.5 to 2 x *g* increased their rate of proliferation by 30-35% (40). Moreover, Miwa et al. (12) presented that 30 minutes of 5 x *g* hyperacceleration stimulus triggered MC3T3-E1 osteoblast cells, the same cell type studied here, to increase their DNA synthesis by ~50% in comparison to that of uncentrifuged control groups. With my experimental setup, I also demonstrated that hyperacceleration stimulus increases proliferation in bone cells, thus complementing the results from previous studies (12, 13, 41).

My study showed for the first time that the primary cilium is a key element for the mechanotransduction of hyperacceleration into the regulation of proliferation. Considering that primary cilia have been previously found to act as transducers for fluid flow and shear stress in vertebrate cells including MC3T3-E1 osteoblasts (2, 20, 21), my finding on their role in transducing hyperacceleration was not unexpected. Based on the observation that MC3T3-E1 osteoblasts display reduced cell growth under microgravity conditions (12, 41) and release prostaglandin E_2 (PGE_2) as an osteogenic response to hypergravity (12), my evidence on primary cilia's role in hyperacceleration transduction suggests that they might also be responsible for transducing hypoacceleration (microgravity) into reduced osteogenic activity during space flights, which has been shown to result in decreased bone formation and/or increased bone resorption (8, 10).

In my next set of experiments I demonstrated that hyperacceleration enhances the cell growth of osteoblasts by decreasing the doubling times, possibly by a down-regulation of the effect of contact inhibition on cell proliferation. In vertebrate cells N- and E-cadherin-catenin complexes play an essential role in maintaining cell-cell contact and the inhibition of proliferation once cells grow to a critical density (30, 31, 35, 36). These cadherin-catenin complexes are also present in MC3T3-E1 osteoblasts (42; also see Figs. 3 and 5), and like elsewhere, their formation at cell-cell adhesion zones inhibits further cell growth after these contacts are established (30, 31, 35, 36). One essential factor for the formation and functionality of cadherin-catenin complexes is β-

catenin (30-32; Fig. 5*C*). Depletion of β-catenin eliminates contact inhibition of cell proliferation by cadherin complexes, and the down-regulation in the amount of these complexes reduces the recruitment of cytoplasmic β-catenin to the plasma membrane (30, 32). β-catenin is thus important in the formation of cadherin-catenin complexes that inhibits cell growth, but it is also an essential downstream element for mechanical and chemical signal transduction that enables primary cilia to regulate cell proliferation (4, 22). Armstrong et al. (2007) has shown that osteoblast-like ROS 17/2.8 and primary osteoblast cells regulate β-catenin levels as an early response to a short-term mechanical strain (3). Similarly, I found that a hyperacceleration stimulus decreases both the nuclear and cytoplasmic levels of β-catenin as well as at cell-cell contact regions, and also showed that primary cilia are required for these early biochemical responses to the stimulus. Based on the data of this study, I propose the following mechanism: It has been shown that depletion of cytoplasmic β-catenin abolishes the inhibition of cell growth through cadherin complexes (30, 31), and this strongly suggests that β-catenin constitutes a mediator that connects the mechanotransduction at primary cilia to hyperacceleration-induced proliferative responses by regulating the contact inhibition of cell growth.

My study presents a reduction in contact inhibition of cell growth as a response to hyperacceleration; however, increase in cell-cell contact and elevated cadherin expression in vertebrate cells have been shown as responses to microgravity (43-45). Hence, the data from my study indicate that leaving the normal 1 x *g* environment of Earth's gravitational field apparently triggers similar modes of action, with opposite effects in either direction – i.e., hypergravity decreases cell-cell contact inhibition, while hypogravity increases it (43, 44). This suggests that primary cilia might also play a role in the mechanotransduction of the response to microgravity that suppresses growth, potentially via an up-regulation of cell-cell contact inhibition of proliferation, in a similar but opposite manner with the mechanism that I propose for hypergravity.

Most cells are sensitive to extracellular mechanical signals (1) and respond to them by regulating their metabolism. With bone cells, for example, changes in gravitational acceleration affect the balance between bone formation and resorption (46). Here I investigated potential pathways of the machinery with which cells perceive these mechanical signals and translate them into cellular responses. My study was able to produce evidence that primary cilia in bone cells mediate the mechanotransduction of hyperacceleration into proliferative responses. I then proposed a mechanism in which β-catenin acts as an intermediary molecule connecting the upstream mechanotransduction by primary cilia to the downstream regulation of proliferation by a cell-cell contact dependent manner. Highlighting the role of osteoblast primary cilia in gravity transduction, I expect my study to lay the foundation for more sophisticated research investigating how cells and organisms perceive and respond to changes in gravitational acceleration.

Methods

Cell culture.

MC3T3-E1 cells (Subclone 14, ATCC® CRL-2594™) were grown on 35 mm uncoated glass bottom gridded dishes (P35G-2-14-CGRD, Mattek Corporation, Ashland, MA) and cultured in Minimum Essential Medium α (MEM α; Life Technologies / Gibco®) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S) as well as being incubated at 37°C and 5% CO_2 (ref. 47). (See SI Methods for *Cell Cycle Synchronization*.)

Fluorescence Imaging of Primary Cilia.

Primary cilia MC3T3-E1 cells were stained for acetylated α-tubulin as well as staining with anti-β-tubulin to display cytosolic microtubules, and DAPI to image DNA (See SI Methods for immunofluorescence staining details). Fluorescence imaging was done via 20x [0.4 N.A.; Nikon] and 100x-oil [1.3 N.A; Nikon] objective lenses on a Nikon Eclipse 80i microscope with Photometrics CoolSNAP HQ^2 CCD camera.

Removal of Primary Cilia.

In order to abrogate primary cilia from MC3T3-E1 osteoblasts, cells were treated with MEM α supplemented with 10% FBS, 1% P/S, and 4 mM aqueous chloral hydrate (courtesy of Prof. J.R. McIntosh, University of Colorado, Boulder, CO) for 72 hours at 37°C and 5% CO_2 (2, 20). Immediately after the treatment, cells were washed three times with PBS, and fresh medium was added. 24 hours after the chloral hydrate treatment, confluent cell cultures were diluted back to 50,000 cells/ml, which was followed by hyperacceleration experiments in 24 hours.

Stimulation of Hyperacceleration.

MC3T3-E1 cells, both untreated and chloral hydrate treated, were grown on 35 mm uncoated glass bottom gridded dishes. The dishes were embedded in 6-well plates and tightly sealed with parafilm. The plates with embedded dishes were then placed on 6-well plate adapters in a swing-out rotor equipped, table top centrifuge (Eppendorf® 5804; courtesy of Prof. N.R. Pace, University of Colorado, Boulder, CO), which was kept in a controlled 37°C temperature room. Cells were centrifuged for 1 hour at 240 RPM (revolutions/min) for a constant 10 x g hyperacceleration stimulus. Following centrifugation, no pelleting was observed on the bottom of the centrifuged dishes. While these cells were being centrifuged, the matched static control groups (also tightly sealed with parafilm) were placed in the same 37°C room for the same time period in order to maintain identical incubation conditions with the experimental groups except for the hyperacceleration stimulus, which was the intended independent variable (See SI Methods for post-stimulus quantification of cell growth and fluorescence intensity of β-catenin).

Statistical Analysis

In order to understand whether the mimosine treatment increased the number of cells possessing primary cilia, the mimosine-treated group was compared to the control group with no drug by unpaired *t*-testing with Welch's correction in which sample variance was not assumed to be equal (48). A one-way ANOVA with Bonferroni's selected-pairs multiple comparison test was utilized for any observational comparison of more than two groups. All the statistical analyses were carried out using GraphPad Prism 5.01 software.

Acknowledgements

Firstly, I would love to express my wholehearted thanks to my supervisor, Prof. Andreas Hoenger, and my secondary advisor, Prof. David M. Klaus. The greatness in their patience, tolerance, and knowledge was what, I must confess, made my research project and thesis feasible. Since the day I knocked on their doors to ask for a research opportunity, they have taken great care of me in the world of science. Hence, I cannot thank them enough for providing me with this wonderful research opportunity, which will undoubtedly influence the course of my scientific future.

Secondly, I would like to deliver my deepest appreciations and gratitude to whom I consider as an exceptional mentor, Prof. J. Richard McIntosh for his insightful scientific discussions and sincere friendship over the period of my honors research. Also, I must acknowledge the immense help from and the genuine company of the following members of Hoenger Lab and Boulder Electron Microscopy Services, whom I felt as if they were the members of my family in the United States: Dr. Eileen O'Toole, Mary Morphew, Cynthia Page, Dr. John Heumann, Prof. David Mastronarde, Dr. Joanna Brown (now at University of Edinburgh, Edinburgh, U.K.), and Minghua Liu.

Next, I would like to give my genuine thanks to my friend-for-life, Joel Wetzsteon, for his tremendous philosophical and theoretical contributions to the basis and design of this work. Also, I express my warmest thanks to my other everlasting friend, John Chisolm, for his help with the English grammar and vocabulary throughout my grant proposal writings, as well as discussing the social and interdisciplinary aspects of this project with me while bearing my mad company. Furthermore, I am sincerely grateful to Ryan Hogrefe for being one of my enduring best friends and mentally supporting me throughout my honors research.

Lastly, I wish to thank Dr. Christy Fillman for being a committee member for my thesis defense and for helping me complete the official work which was required for the departmental honors application process. I acknowledge the support from Profs. James Orth and Susan Reynolds for antibodies, Prof. Norman Pace and Dr. Benjamin Barthel for chemicals as well as instruments, and finally Prof. Christopher Jacobs (University of Columbia, New York, NY) and his student An Nguyen (University of Columbia, New York, NY) for experimental protocols. I also thank Prof. Jordan Raff and his laboratory members (University of Oxford, Oxford, U.K.) for being a courteous host during the time of my thesis writing. My work was supported by National Institutes of Health / National Center for Research Resources Grant P41-GM103431 to my supervisor Andreas Hoenger, University of Colorado Undergraduate Research Opportunities Program 2013 Summer Team Grant (in part with Joel G. Wetzsteon), Howard Hughes Medical Institute Biosciences Undergraduate Research Grant 2013-2014 (The Biological Sciences Initiative, University of Colorado, Boulder, CO), and British Society for Cell Biology 2014 Summer Studentship (taken place at the University of Oxford).

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Figure Legends

Fig. 1. Chloral hydrate treatment removes primary cilia from MC3T3-E1 osteoblasts. (*A*) Mimosine treated cells were stained with DAPI (blue) displaying DNA and acetylated α-tubulin (green) scoring primary cilia (*B* – *E*), then the percentage of cells having primary cilia was calculated at pre-treatment (control) condition as well as at 1 h, 12 h, 24 h and 48 h after the chloral hydrate treatment (n ≥ 720 cells per condition). (*B* and *C*) Cells with no drug treatment possess primary cilia (exemplified by white arrowheads) displaying similar numbers with the control group from the graph (*A*) and have an ordinary cytoskeletal morphology determined by anti-β-tubulin antibody staining (red) (Scale bars: 40 and 10 μm respectively). (*D* and *E*) On the other hand, at 48 hours after the chloral hydrate treatment, cells display their regular cytoskeletal morphology (*E*), while most cells did not rebuild their primary cilia (*D*) (Scale bar in *D*: 40 μm; *E* shares the same scale with *C*). (Error bars: SD from at least 3 independent experiments; *** for $P < 0.001$)

Fig. 2. Primary cilia in bone cells are required for the mechanotransduction of hyperacceleration into proliferative responses. (*A*) Growth curve of MC3T3-E1 osteoblasts at static conditions (1 x *g*) and after the hyperacceleration stimulus (10 x *g*) with or without (CH) primary cilia's presence. (*B*) Reference doubling times of MC3T3-E1 cells were calculated within different phases of growth including between 0-12, 12-24, and 24-36 hours after the stimulus. [Error bars: SEM (Per condition: n ≥ 40 labeled gridded dish zones from 3 independent experiments); * for $P < 0.05$, ** for $P < 0.01$, *** for $P < 0.001$.]

Fig. 3. Immunofluorescence images illustrates that hyperacceleration down-regulates the levels of β-catenin at cell-cell contact regions, depending on the presence of primary cilia. (*A* – *D*) Cells in represented conditions were stained with an antibody against β-catenin (magenta),

rhodamine-conjugated phalloidin scoring actin (green), and DAPI visualizing DNA (blue). Dashed yellow rectangles with asterisks represent zoomed random cell-cell contact regions (white arrows in *C* pointing towards cell-cell contact sites). (Scale bars: 20 μm)

Fig. 4. Hyperacceleration reduces the nuclear and cytoplasmic levels of β-catenin, and primary cilia are required for this stimulus-induced biochemical response. (A) Cells in represented conditions were co-stained with an antibody against β-catenin as well as rhodamine-conjugated phalloidin scoring actin. (B) Nuclear and cytoplasmic β-catenin levels determined for the following experimental conditions: untreated (no CH) and uncentrifuged (1 x *g*; blue), CH-treated and uncentrifuged (1 x *g* CH; green), untreated and centrifuged (10 x *g*; red) as well as CH-treated and centrifuged (10 x *g* CH; grey). β-catenin intensities were quantified using ImageJ software (see SI Methods). [Scale bars: 20 μm; error bars: SEM (n ≥ 35 cells per condition); * for $P < 0.05$ and *** for $P < 0.001$.]

Fig. 5. Hyperacceleration stimulus leads to a decrease in β-catenin levels at cell-cell contact regions through a mechanical transduction path for which primary cilia are essential. (*A*) Maximum projection of a confocal z stack demonstrates a cell-cell contact region displaying β-catenin (magenta), which accumulates at cell-cell contact sites. Actin (green) is scored by rhodamine-conjugated phalloidin. DNA is stained with DAPI (blue). Labeled regions in *A* exemplify the regions that were utilized to calculate fluorescence intensity ratio (FIR) of β-catenin at cell-cell contact sites (see *Confocal Imaging and Quantifying the Fluorescence Intensity of β-catenin* in *Methods*): *(1)* contact region; *(2)* non-contacting membrane; *(3)* background. (Scale bar: 20 μm). *(B)* FIR of β-catenin at cell-cell contact regions in centrifuged (10 x *g*) and uncentrifuged (1 x *g*) cells that were untreated or chloral hydrate treated (CH). (*C*) The cartoon depicts a cadherin-catenin complex maintaining cell-cell contact. (Error bars: SEM for n ≥ 20 cell-cell contact regions per condition; *** for $P < 0.001$.)

Figure 1:

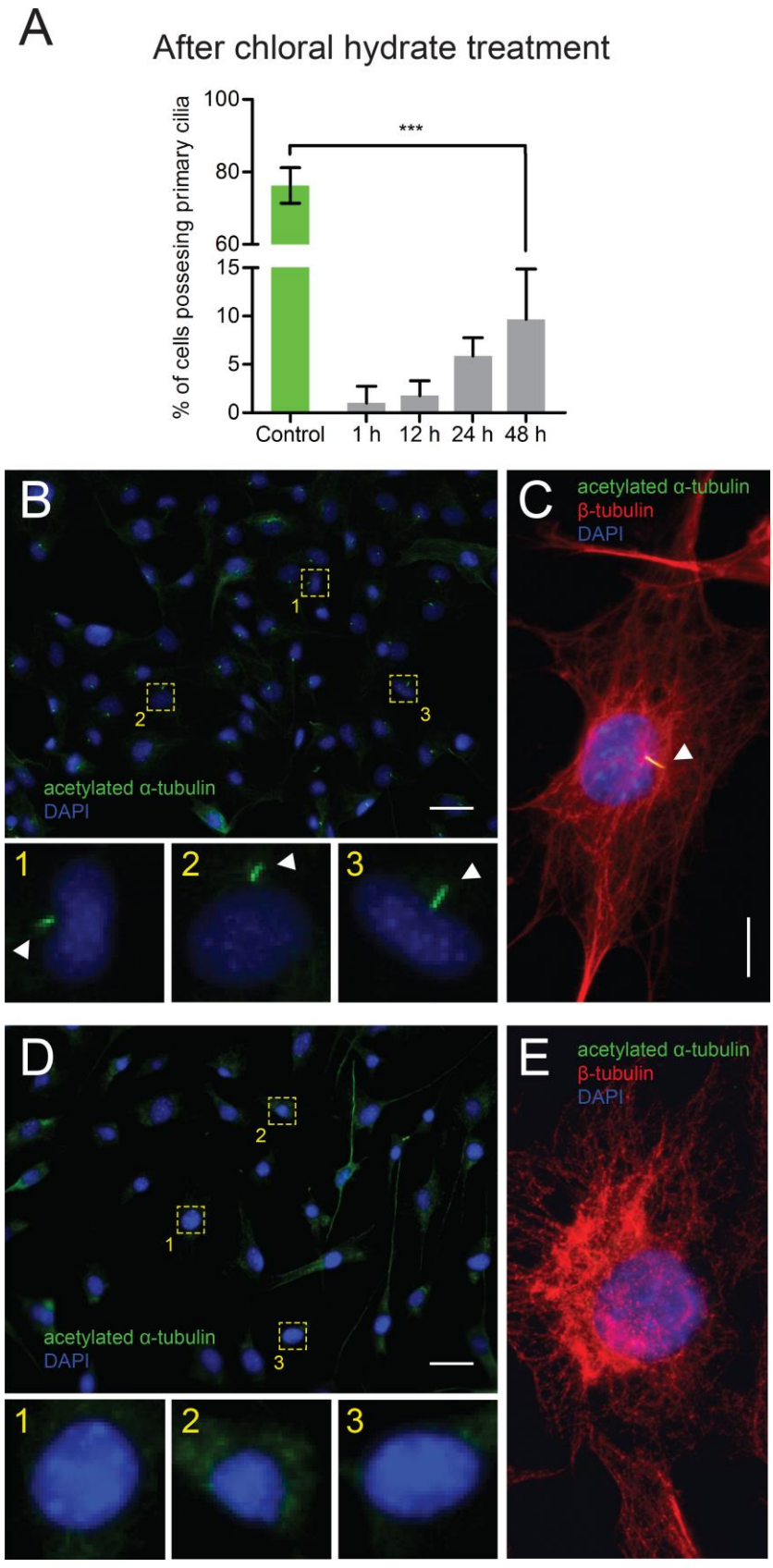

A
After chloral hydrate treatment
% of cells possesing primary cilia
100
80
60
15
10
5
0
***
Control
1 h
12 h
24 h
48 h
B
acetylated α-tubulin
DAPI
1
2
3
C
acetylated α-tubulin
β-tubulin
DAPI
D
acetylated α-tubulin
DAPI
E
acetylated α-tubulin
β-tubulin
DAPI

Figure 2:

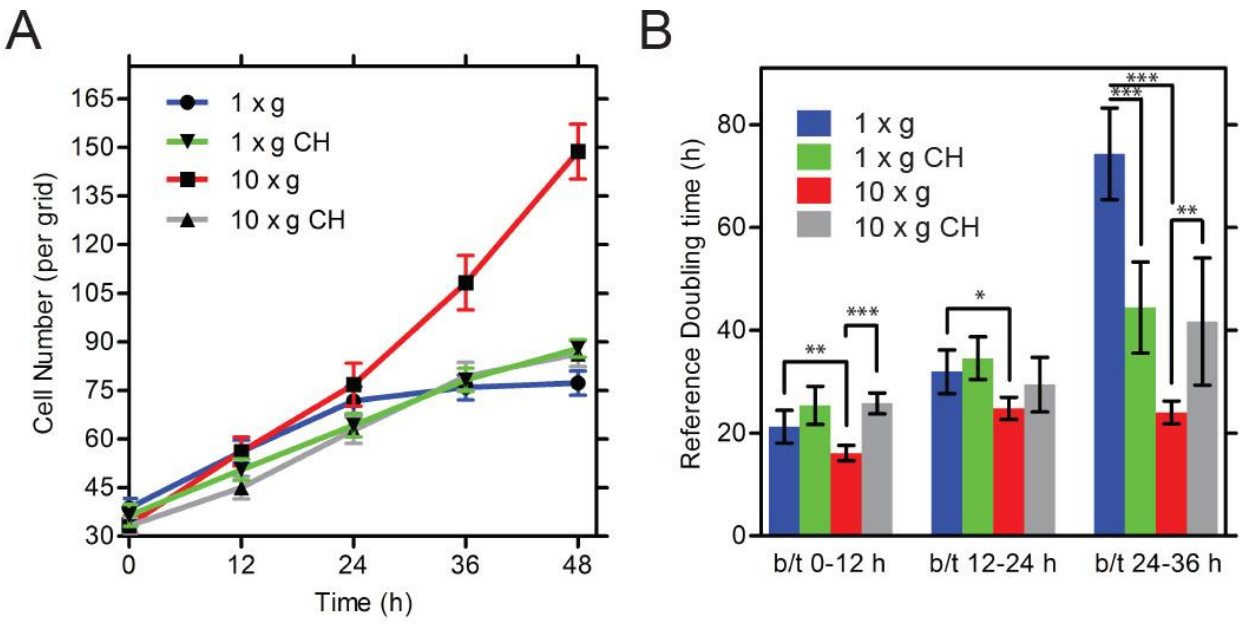

A
Cell Number (per grid)
Time (h)
1 x g
1 x g CH
10 x g
10 x g CH
B
Reference Doubling time (h)
b/t 0-12 h
b/t 12-24 h
b/t 24-36 h

Figure 3:

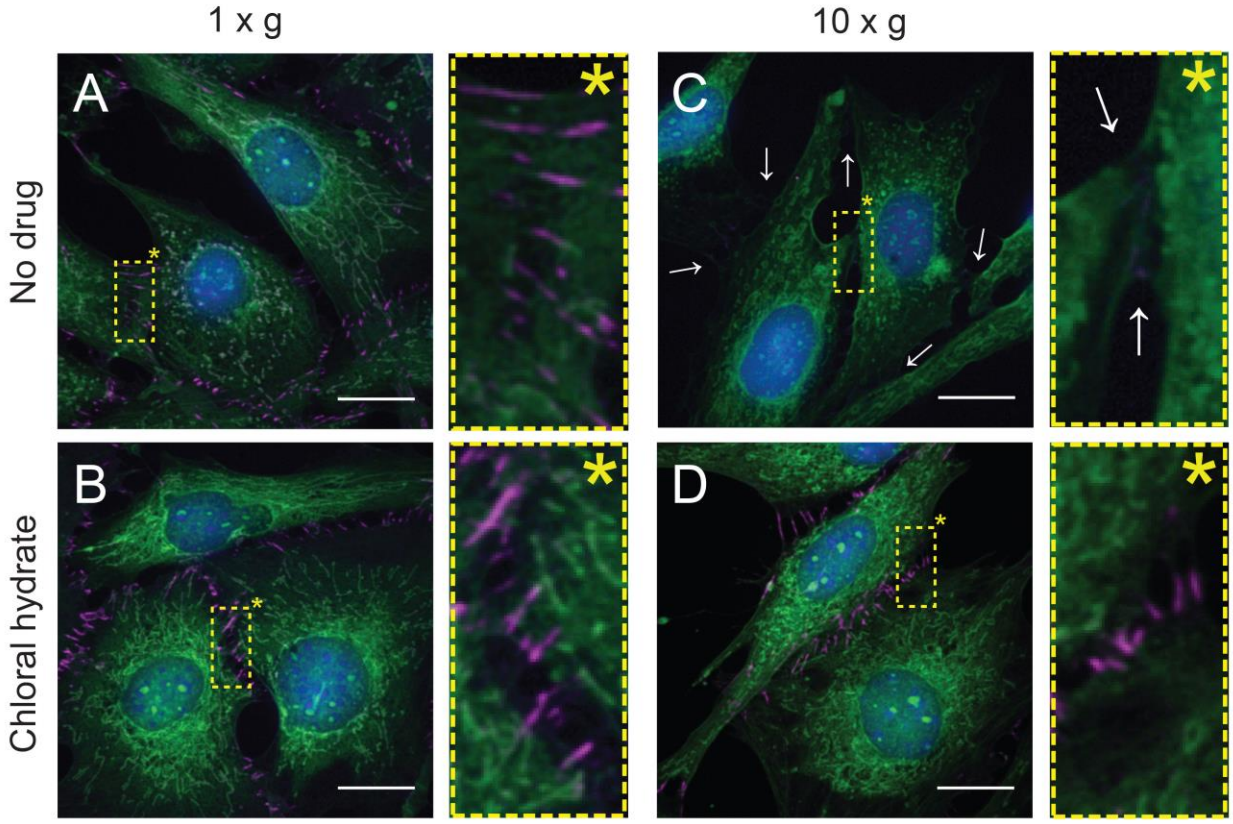


Figure 4:

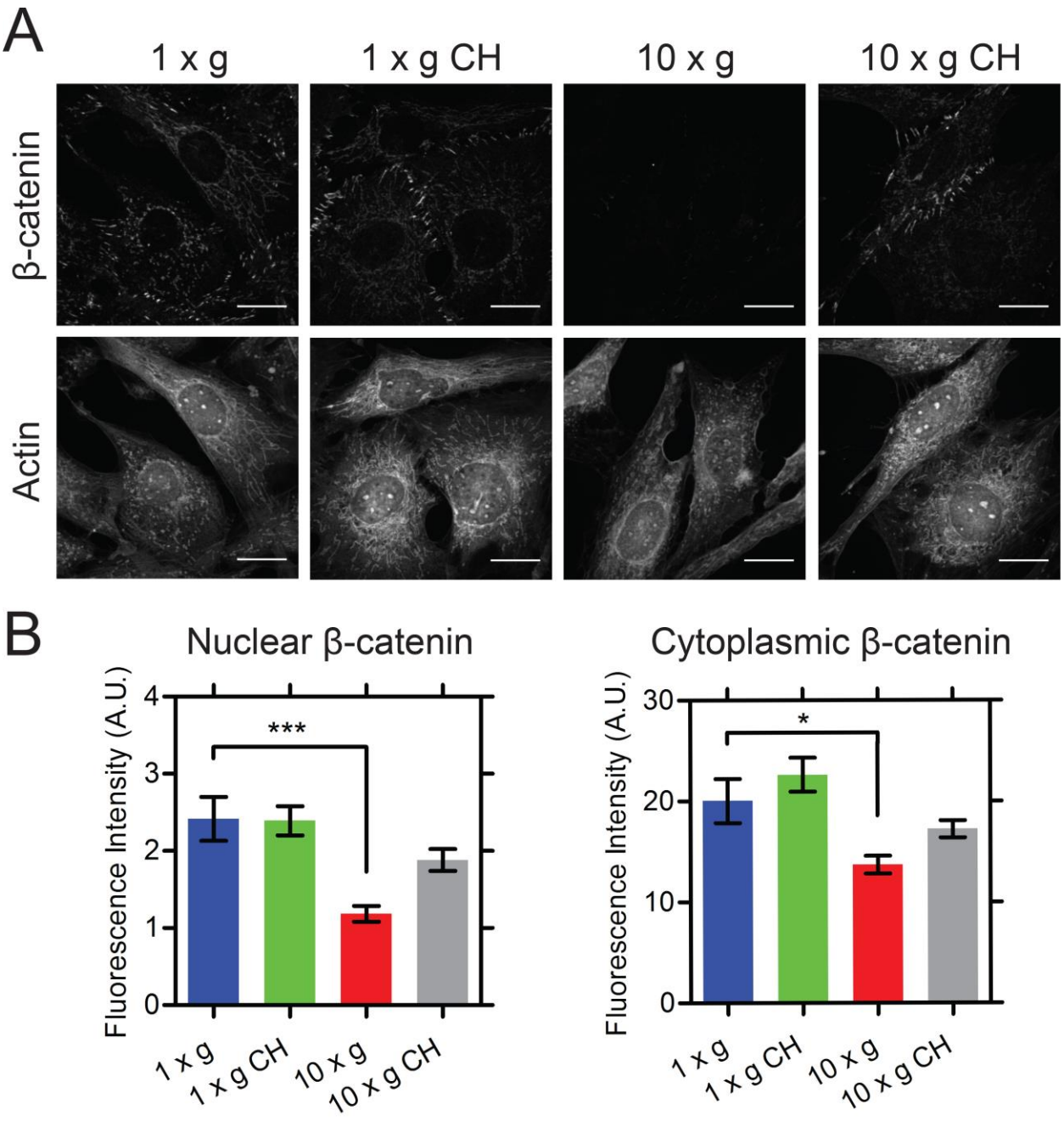
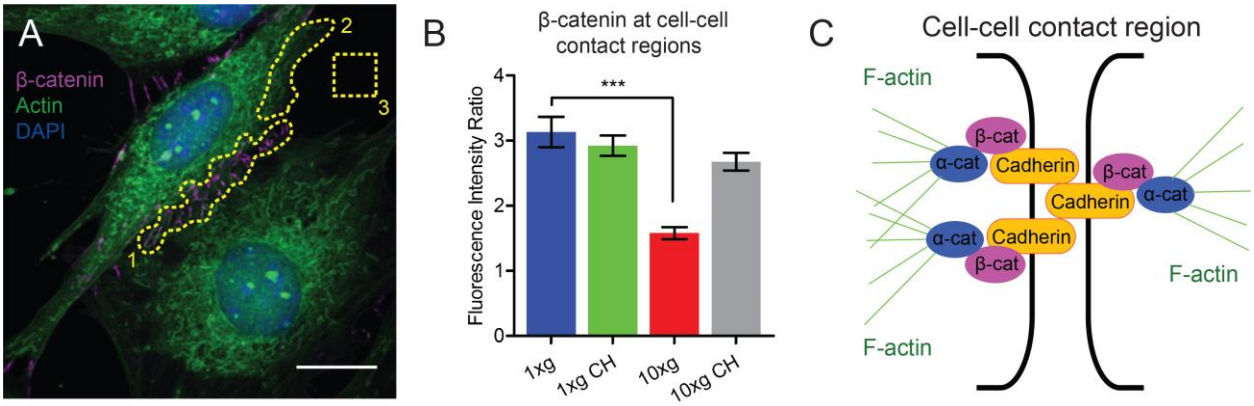

A
1 x g
1 x g CH
10 x g
10 x g CH
β-catenin
Actin
B
Nuclear β-catenin
Fluorescence Intensity (A.U.)
0
1
2
3
4
***
1 x g
1 x g CH
10 x g
10 x g CH
Cytoplasmic β-catenin
Fluorescence Intensity (A.U.)
0
10
20
30
*
1 x g
1 x g CH
10 x g
10 x g CH

Figure 5:


A
β-catenin
Actin
DAPI
1
2
3
B
β-catenin at cell-cell contact regions
Fluorescence Intensity Ratio
***
1xg
1xg CH
10xg
10xg CH
C
Cell-cell contact region
F-actin
β-cat
α-cat
Cadherin
F-actin

Supplemental Info (SI)

Supplemental Methods:

Cell cycle synchronization.

Before the cell cycle synchronization, cells were grown until 60% confluent. In order to synchronize MC3T3-E1 cells in late G1 phase of their cell cycle, they were transferred into Minimum Essential Medium α (MEM α; Life Technologies / Gibco®) supplemented with 10% FBS, 1% P/S, and 400 μM mimosine (courtesy of Dr. B. Barthel, University of Colorado, Boulder, CO), and incubated for 24 hours at 37°C and 5% CO_2 (ref. 27). After the mimosine treatment, cells were washed three times with phosphate buffered saline (PBS) and stimulated to progress in their cell cycle by the re-addition of MEM α supplemented with 10% FBS and 1% P/S as described in ref. 27.

Immunofluorescence.

MC3T3-E1 cells were fixed in -20°C methanol for 10 minutes. Then, coverslips underneath the 35 mm uncoated glass bottom dishes were removed by a coverslip removal fluid (P-DCF-OS-30, Mattek Corporation, Ashland, MA). After being fixed with methanol, cells were washed with PBS twice and incubated in PBS containing bovine serum albumin (BSA) (50 mg/ml) for 1 hour at room temperature (RT). Subsequently, the cells were incubated for 2 hours at 4°C in a humidified chamber with the following primary antibodies: 6-11B-1 mouse anti-acetylated α-tubulin (1:2000, Sigma-Aldrich), rabbit anti-β-tubulin (1:200; courtesy of Prof. J. Orth, University of Colorado, Boulder, CO), rabbit anti-β-catenin (1:500; courtesy of Dr. S.D. Reynolds, National Jewish Health, Denver, CO) (Primary anti-bodies were used depending upon the context of the experiment carried out; i.e., rabbit anti-active-β-catenin and rabbit anti-β-tubulin were not utilized together within any immunofluorescence staining). After the cells were washed thrice with PBS

containing BSA (50 mg/ml), they were incubated for 1 hour at RT in a dark chamber with the following secondary antibodies: goat anti-mouse Alexa 488 and goat anti-rabbit Alexa 594 (1:200; courtesy of Prof. J.R. McIntosh, University of Colorado, Boulder, CO) as well as donkey anti-rabbit Alexa 647 alternatively (1:500; courtesy of Prof. J. Orth, University of Colorado, Boulder, CO). After three times wash with PBS containing BSA (50 mg/ml), DNA was stained with DAPI (1:100,000 of 5 mg/ml stock; Sigma-Aldrich), and F-actin was stained with 20 µg/ml rhodamine-conjugated phalloidin in PM buffer (35 mM KH_2PO_4, 0.5 mM $MgSO_4$, pH 6.8).

Bright-field Imaging and Quantitation of Cell Growth.

35 mm uncoated glass bottom gridded dishes (P35G-2-14-CGRD, Mattek Corporation, Ashland, MA) allowed tracking of adhesive cells overtime through numerically “labeled zones” (For a clear view, see Fig. S2). After centrifugation, 40 or more randomly picked labeled zones for each experimental and control group were tracked and imaged in the context of 3 independent experiments. Imaging was done every 12 hours for the post-stimulus period on a Leica DM IL LED inverted phase contrast microscope with a Leica DFC340 FX digital camera by using 10x lens [0.22 numerical aperture (N.A.); Leica HI PLAN)]. For the quantification of cell growth over time, cells in each collected image were manually counted, and doubling times (D.T.) were calculated using $D.T. = Incubation\ time \times ln2 / ln(N_e/N_b)$, in which N_b and N_e represents the number of cells at the beginning and the end of incubation time respectively.

Confocal Imaging and Quantifying the Fluorescence Intensity of β-catenin.

Immediately after centrifugation, both stimulated and unstimulated cells were fixed in -20°C methanol. Cells were then stained with active-β-catenin antibody. Utilizing DAPI and rhodamine-conjugated phalloidin (staining actin which was pseudo-colored with green in Fig. 3 *A* – *D* and Fig. 5*A*) allowed determining the nucleus and cell body of MC3T3-E1 cells respectively. Images were collected through a 63x oil objective lens (1.4 N.A.; HCX PLAN APO, Leica) with an iris

collar on a Leica DMRXA spinning disk confocal microscope with a Hamamatsu ImagEM EM-CCD camera (C9100-13).

After confocal imaging, nuclear and cytoplasmic fluorescence intensities of β-catenin were quantified in ImageJ software (National Institutes of Health, Bethesda, MD), using a technique that is similar to the analysis method described in Zhurinsky et al. (S1). For the nuclear fluorescence intensity, the region of interest (ROI) was defined using where DAPI stained in MC3T3-E1 cells. Once the ROI for nucleus was defined, corrected fluorescence intensity for nuclear β-catenin was calculated through multiplying the area of nucleus by the fluorescent intensity of image background, and subtracting this value from the total fluorescence intensity of nuclear region (Fig. 4*B*). For the total cell intensity of β-catenin, ROIs were defined utilizing the boundaries of where rhodamine-conjugated phalloidin (targeting F-actin) stained in cells. Corrected total cell intensity of β-catenin was then calculated in the same way explained for nuclear β-catenin intensity. With this, cytoplasmic fluorescence intensity was quantified by subtracting the corrected nuclear intensity from the corrected total cell intensity (Fig. 4*B*).

The levels of β-catenin at cell-cell interaction sites were determined by calculating the fluorescence intensity ratio (FIR) of β-catenin at cell-cell contact regions, as described in the analysis method in ref. 33; ROIs were drawn on the fluorescently brightest areas of the contact sites (Fig. 5*A*; region 1), the edge of a non-contacting membrane (Fig. 5*A*; region 2), and a cell-free zone in the image for background correction (Fig. 5*A*; region 3). FIR was equal to (mean β-catenin fluorescence intensity at contact site – background intensity) / (mean β-catenin fluorescence intensity at non-contacting membrane – background intensity) (ref. 33).

Supplemental References:

1. Zhurinsky J, Shtutman M, Ben-Ze'ev A (2000) Differential Mechanisms of LEF/TCF Family-Dependent Transcriptional Activation by β-Catenin and Plakoglobin. *Mol Cell Biol* 20(12):4238–4252.

Supplemental Figure Legends:

Fig. S1. Mimosine treatment increases the fraction of MC3T3-E1 cells possessing primary cilia in comparison to that in untreated control cells. (*A* and *B*) In control cells (no drug) (*A*) and in the cells after 24 hours of mimosine treatment (*B*), primary cilia were stained with antibodies against acetylated α-tubulin (green), while cytoplasmic microtubules were displayed using β-tubulin antibody (red). DNA was stained with DAPI (blue) (Scale bars: 40 μm). (*C*) Graph shows the percentage of cells having primary cilia in control cultures with no drug and in cultures treated with mimosine (n ≥ 720 cells per condition; error bars: SD from 3 independent experiments; * for $P < 0.05$).

Fig. S2. Hyperacceleration stimulus causes a substantial increase in MC3T3-E1 cell growth. 24 hours after the chloral hydrate treatment cells were diluted to 50,000 cells/ml and seeded on glass bottom gridded dishes in order to track exact locations of cell populations over time (see *Bright-field Imaging and Quantitation of Cell Growth* in *Methods*). At 24 hours after the dilution, the centrifugation experiments were carried out. (*A1 – A3*) Growth of MC3T3-E1 cells at unperturbed conditions (1 x *g*) with no drug treatment. (*B1 – B3*) Growth of cells, which were treated with chloral hydrate, but remained at static conditions (1 x *g*). (*C1 – C3*) Growth of stimulated cells (10 x *g*) with no drug exposure. These cells kept their primary cilia and display a loss in contact inhibition of cell proliferation at 48 h after the stimulus (*C3*; arrowheads pointing the examples of where cells continue growing on top of each other). (*D1 – D3*) Growth of

stimulated cells (10xg), which were treated with chloral hydrate in advance. (Scale bar in A1 representing the scales in all other images: 70 µm)

Figure S1:

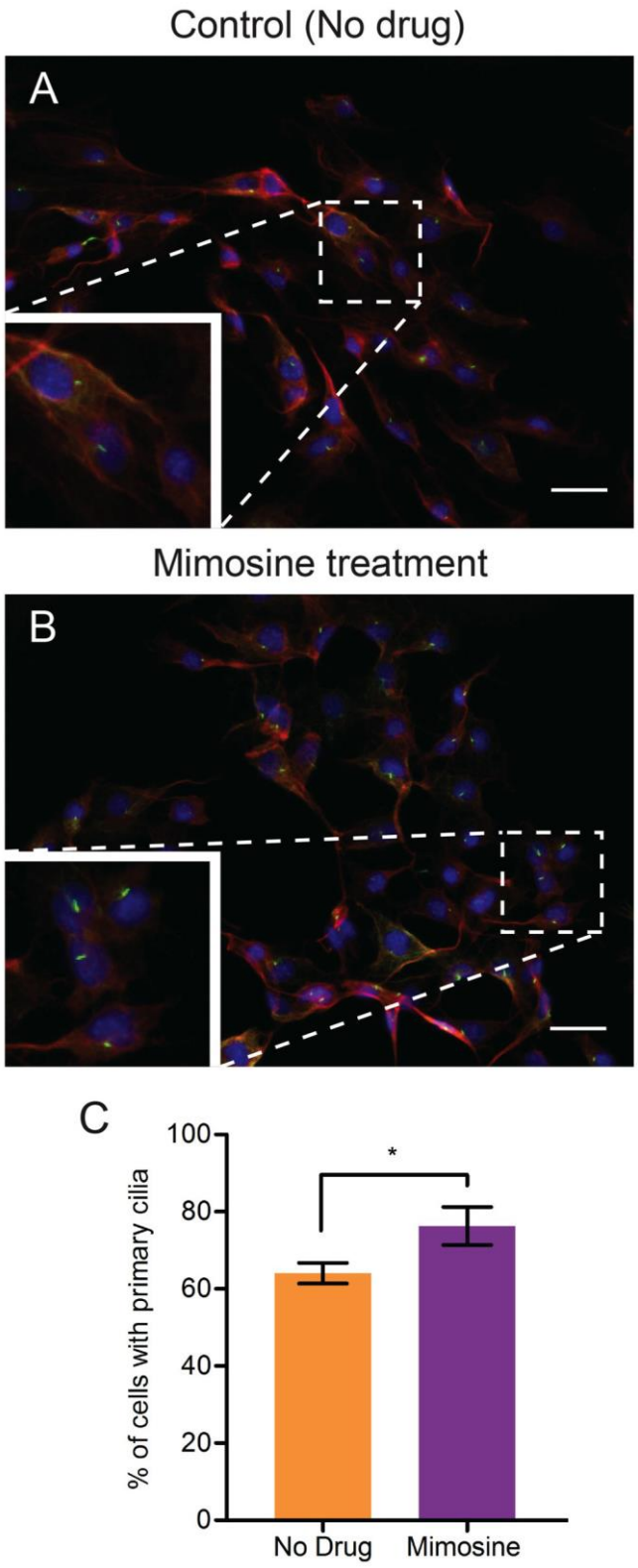
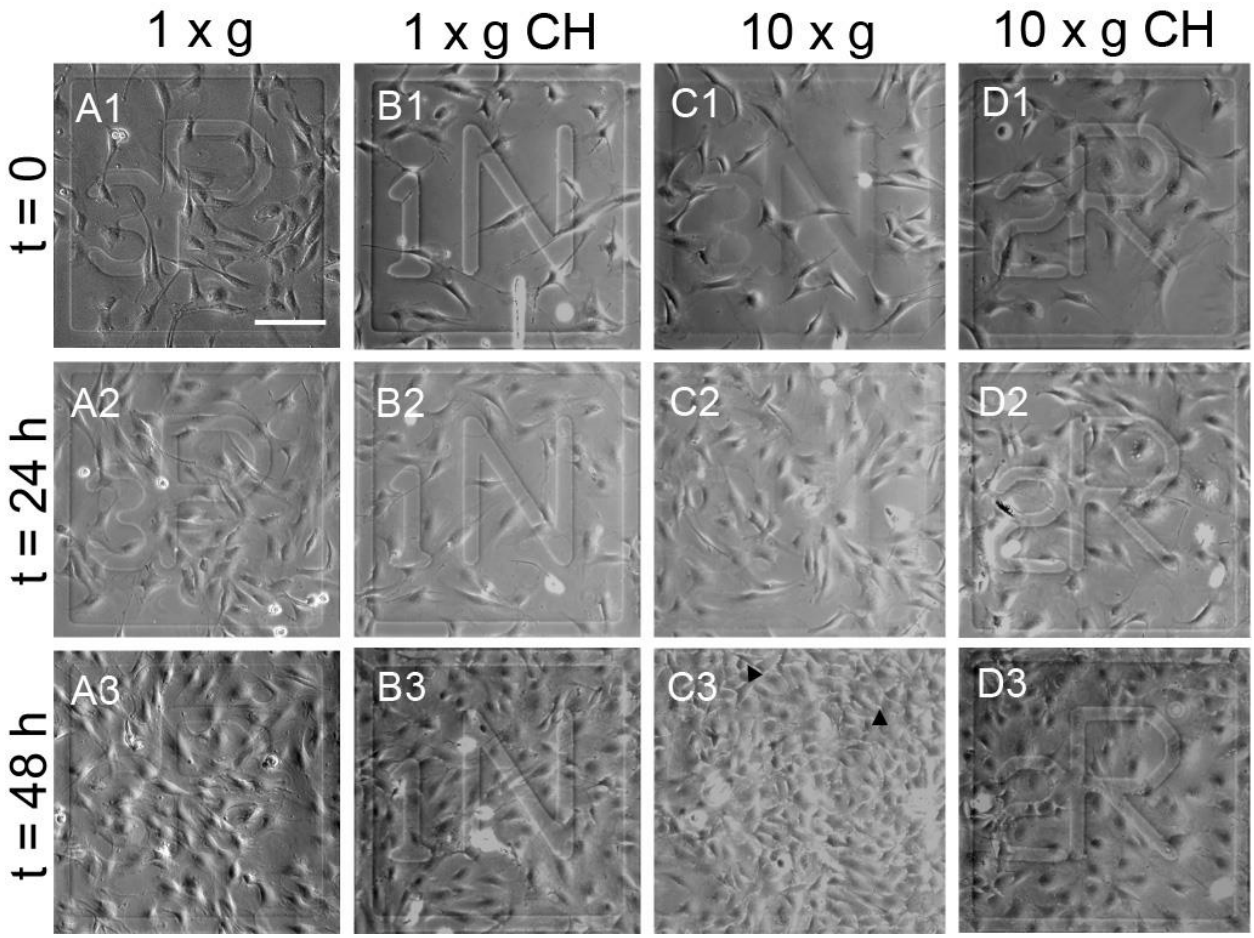

Control (No drug)
A
Mimosine treatment
B
C
% of cells with primary cilia
100
80
60
40
20
0
*
No Drug
Mimosine

Figure S2:


1 x g
1 x g CH
10 x g
10 x g CH
t = 0
t = 24 h
t = 48 h
A1
B1
C1
D1
A2
B2
C2
D2
A3
B3
C3
D3