Published in final edited form as:

Med Eng Phys. 2013 March ; 35(3): 392–402. doi:10.1016/j.medengphy.2012.06.005.

Primary cilia act as mechanosensors during bone healing around an implant

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Abstract

The primary cilium is an organelle that senses cues in a cell's local environment. Some of these cues constitute molecular signals; here, we investigate the extent to which primary cilia can also sense mechanical stimuli. We used a conditional approach to delete Kif3a in pre-osteoblasts and then employed a motion device that generated a spatial distribution of strain around an intra-osseous implant positioned in the mouse tibia. We correlated interfacial strain fields with cell behaviors ranging from proliferation through all stages of osteogenic differentiation. We found that peri-implant cells in the *Col1Cre;Kif3a^{fl/fl}* mice were unable to proliferate in response to a mechanical stimulus, failed to deposit and then orient collagen fibers to the strain fields caused by implant displacement, and failed to differentiate into bone-forming osteoblasts. Collectively, these data demonstrate that the lack of a functioning primary cilium blunts the normal response of a cell to a defined mechanical stimulus. The ability to manipulate the genetic background of peri-implant cells within the context of a whole, living tissue provides a rare opportunity to explore mechanotransduction from a multi-scale perspective.

Introduction

The primary cilium is a non-motile microtubule-based organelle that projects from the cell surface and has been described as both a chemosensor and a mechanosensor [1, 2]. Proteins are shuttled into and within the primary cilium via intraflagellar transport (IFT) and

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Disclosure

None.

Ethical approval: not required

Conflict of interest statement

None of the authors have any conflict of interest.

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kinesin motors drive this anterograde translocation and ciliary assembly (reviewed in [3]). Kif3a is one of the subunits of this anterograde motor and disruption in Kif3a leads to inappropriately assembled and truncated primary cilia [4].

Kif3a is essential for tissue formation [5] and, in the skeleton, Kif3a function is essential for proper skeletal morphogenesis. For example, removal of Kif3a from collagen type II-expressing cells produces a variety of skeletal patterning defects including the formation of ectopic cartilage condensations (also known as exostoses) and the premature fusion of cranial bone sutures, a class of malformations referred to as synchondroses [6]. These skeletal defects are primarily observed in the growth plates of the appendicular skeleton, where the embryonic cartilage anlagen is replaced by bone through endochondral ossification.

The skeletal defects observed in Kif3a^{fl/fl}Col2Cre embryos have been attributed to disruptions in Hedgehog signaling [6, 7]. Hedgehog (Hh) signals are transduced via the Patched and Smoothened receptors, both of which must be localized to the primary cilium in order to be activated (reviewed in [8, 9]). Binding of a Hedgehog ligand to Patched leads to activation of the Gli transcription factors, which in turn coordinately repress and activate transcriptional targets. The Gli proteins have been shown to localize to the tip of the primary cilium, where the balance of activator and repressor forms of Gli control Hedgehog pathway activation [10].

The primary cilium has also been implicated as a mechanosensor [11]. Primary cilia may act as flow and shear stress sensors in various organs [12–14] and tissues [15]. In the skeleton, primary cilia may transduce fluid flow shear stress that occurs within the narrow canaliculi that interconnect osteocytes [16]. Rather than being embedded in a bony matrix, however, pre-osteoblasts reside on bone surfaces or in the bone marrow cavity. Consequently, it is not clear whether a pre-osteoblast utilizes a primary cilium as a mechanosensory organelle.

Here we asked whether primary cilia are essential for mechanotransduction in these osteogenic precursors. To do so, we conditionally deleted an essential component of the primary cilium, Kif3a, in *Collagen type I*-expressing pre-osteoblasts. Then using a biomechanically defined method for generating strain in vivo, we studied how truncating the primary cilium affected how pre-osteoblasts sense a mechanical stimulus. Our results provide evidence that Kif3a and by association, the primary cilium of pre-osteoblasts, functions as a mechanosensor for load-induced bone formation. These findings have direct relevance to the issue of bone formation in response to loading, especially with regards to orthopedic and dental implant osseointegration.

Materials and methods

Conditional inactivation of Kif3a in collagen type Ia1 expressing cells

All experiments were performed in accordance with Stanford University Animal Care and Use Committee guidelines. Animals were housed in a light- and temperature-controlled environment and given food and water ad libitum. The Cre-loxP system was used to generate mice in which Kif3a is conditionally inactivated in *collagen type I*-expressing cells, under control of the collagen type Ia promoter [17].

Mono-cortical defect model

Adult mice (males, between 3 and 5 months old) were anaesthetized with an intraperitoneal injection of ketamine-xylazine. A 5.0mm incision was made over the right anterior-proximal tibia, and the tibial surface was exposed while carefully preserving the periosteum. A 0.8–1.0mm hole was drilled through the anterior cortex with a high-speed dental drill. Wounds

were closed with size 6–0 Vicryl sutures. After surgery, mice received subcutaneous injections of carprofen for analgesia and were allowed to ambulate freely. Five mice were sacrificed at days 3, 7, 14 and 28 after surgery.

Implant device and loading paradigm

The mouse model involved two unique features: 1) placement of a 0.5mm- diameter, surface-characterized polymer pin-shaped implant (Poly(L-lactide- co-D,L-lactide), i.e., 70% L-lactide and 30% D,L-lactide, material grade LR706, Midwest Plastics, MN and Medical Micro Machining, Inc., Simi Valley, CA) in a 0.8mm diameter drill hole in the mouse tibia in order to create a pure gap interface; and 2) a micromotion device enabling either stabilization or controlled motion of the implant in the wound site. Implant micromotion was generated by a separate hand-activated system that was connected to the cap attached to the center column of the bone plate in order to deliver short sessions of micromotion (e.g., lasting 1 min). This system consisted of: a) a linear variable differential transducer (LVDT, TransTek Inc., Ellington, Connecticut Model #0240-00000); b) a load cell (Honeywell Sensotec, Columbus, Ohio Model #11, load range of 0 to 2.27 kg); and c) a core for the LVDT, one end of which was connected to the load cell, and the other end consisting of a small (1 mm diameter) tip that could pass through a 1.1-mm-diameter hole in the cap on the center column of the bone plate. Data were collected at 200 Hz sampling rate via a DaqBook system (iO Tech Inc., Cleveland, Ohio). With the series connection of LVDT and load cell, it was possible to simultaneously measure axial motion of the implant and the force required to produce this motion. A part of the force measured by the load cell was the force needed to compress a rubber o-ring (of known stiffness) beneath the head of the implant, while the remainder of the force was due to the resistance of the interfacial tissue, which we observed to vary over the post-surgical healing time.

Finite element modeling

We formulated a 3-D linear finite element model of the in-vivo experiment using COMSOL Multiphysics® (e.g., Fig. 4). The tibia was modeled as a bi-material cylinder of cortical bone and marrow space. The implant had a main diameter of 0.8mm; a tip of 0.5mm-diameter; and two circumferential ridges of 0.05mm radius to act as strain concentrators, as described later. The implant's length was 5.33mm, and the tip's length was 1.33mm. The uppermost circumferential ridge of the implant was positioned in the upper region of a trans-cortical hole (0.8mm diameter) in the tibia. The cortical thickness of the tibia was 0.16mm and the bone marrow diameter was 1.18mm. The length of the tibial segment was 11.0mm.

The Young's modulus and Poisson's ratio for the cortical bone were set at 15 GPa and 0.33, respectively; 10 MPa and 0.33 for the bone marrow; and 432 MPa and 0.35 for the Poly(L-lactide-co-D,L-lactide) implant. The Young's modulus of the gap tissue around the portion of the implant within the bone was allowed to vary with post-surgical time according to in vivo data collected on the stiffness of the tissue in this gap region. Specifically the values of E were: 3.103, 4.766, 6.567, 9.331, 11.036, 10.854, and 17.559 MPa over days 0 to 7, respectively. (note that on day 6 E was maintained at 11.054 MPa since input parameters in Comsol's parameter sweep have to be monotonic.) Poisson's ratio of the gap tissue was set to 0.33 for all days.

An axial inward displacement of 150µm was prescribed at the top of implant. The outer cortical surface over the bottom half of the tibia was fixed. Interfacial strain fields were determined due to implant displacement 60 times per day (in one 60-second session). Calculated output included plots of the principal compressive and tensile strains (and related combined strain parameters) in the interfacial region. To validate our finite element (FE) modeling, the first step was to place pin and screw implants in actual-sized "mock"

interfaces comprised of silastic rubber (ReproRubber) and tantalum powder (particle size 17 μm) – the former having known mechanical properties and the latter serving as fiducial markers in micro-CT images of interfaces before and after implant motion. We then compared strains measured using image analysis via DISMAP [18–20] – versus strains predicted by axisymmetric as well as 3D FE models of the mock situation. Good agreement between measured and predicted strains in this step gave confidence in the ability of the FE modeling to simulate the interfacial situation, at least in a situation of known geometry and mechanical properties. However, the mechanical properties of the silastic/tantalum rubbery interface were not meant to match the actual properties of interfacial tissues that existed *vivo*. Therefore, our second validation step involved determination of the mechanical properties of interfacial tissues around the implants *vivo*, so that these properties could be input into 3D FE models for predicting strain. We determined the *in vivo* mechanical properties of the interfacial tissue indirectly, by measuring the force required to displace the implant by 150 μm during the motion trials that occurred each day. By knowing the properties of the rubber O-ring in our implant motion system, we could back-calculate the *in vivo* stiffness of the interface at each time point in the experiment [21]. Then, to determine the interfacial mechanical properties associated with these measured stiffness values at each time point, we selected a Young's elastic modulus (E) and Poisson's ratio (ν) in the FE model such that the model's stiffness matched the measured *in vivo* stiffness at each time point. We then went on to use such validated FE models to assess the interfacial strain fields at each day of our *in vivo* trials.

Tissue processing, histology, immunohistochemistry, and *in situ* hybridization

The right limbs were dissected, skinned and then fixed in 4% paraformaldehyde overnight. Decalcification was achieved by introducing the samples into 19% ethylenediaminetetraacetic acid (EDTA) for 10 days at 4°C. After demineralization, the implant was gently pulled out of the bone. Specimens were dehydrated through an ascending ethanol series prior to paraffin embedding. Eight-micron-thick longitudinal sections were cut and collected on Superfrost-plus slides (Fisher Scientific, Pittsburg, PA) for histology using a modification of Movat's Pentachrome staining. Adjacent sections were stained with Aniline blue and Safranin-O/Fast green; slides were mounted with. Proliferating cells were detected by immunohistochemistry for Proliferating Cell Nuclear Antigen (PCNA, Zymed) by incubating tissue sections with biotinylated mouse anti-PCNA antibody at room temperature for 45 min. Streptavidin-peroxidase was used as a signal generator, diaminobenzidine (DAB; Zymed) as a chromogen to stain PCNA-positive nuclei dark brown. For whole-mount bone and cartilage staining, osteoid tissues were stained with Alizarin red and chondrogenic tissues were stained with Alcian blue following previously published protocols [22]. For *in situ* hybridization, the relevant digoxigenin-labeled mRNA antisense probes were prepared from complementary DNA templates for *Collagen type I*. Sections were prepared as described [23] and incubated in hybridization buffer containing the relevant RNA probe. Probe was added at an approximate concentration of 0.25mg/ml and stringency washes of saline sodium citrate solution were conducted at 52°C and further washed in maleic acid buffer with 1% Tween20. Slides were treated with an antibody to digoxigenin (Roche). For color detection, slides were incubated in nitro blue tetrazolium chloride (Roche) and 5-bromo-4-chloro-3-indolyl phosphate (Roche). After developing, the slides were cover-slipped with aqueous mounting medium.

Histomorphometric and statistical analyses

To quantify new bone, we represented the 1.0mm circular monocortical defect across typically forty 8-mm-thick tissue sections. Of those 40 sections, we used a minimum of 8 sections to quantify the amount of aniline blue-stained new osteoid matrix. Tissue sections were photographed with a Leica digital imaging system (5× objective). The resulting digital

images were analyzed with Adobe Photoshop CS2 software. We chose a fixed, rectangular ROI that in all images corresponded to 10^6 pixels. The injury site was always represented inside this ROI by manually placing the box in the correct position on each image. Aniline blue-positive pixels were automatically selected with the magic wand tool set to a color tolerance of 60. This tolerance setting resulted in highlighted pixels with a range of blue that corresponded precisely with the histological appearance of new osteoid tissue in the aniline blue-stained sections. Cortical surfaces, or bone fragments resulting from the drill injury, were manually de-selected. The total number of aniline blue-positive pixels for each section was then recorded. The pixel counts from individual sections were averaged for each tibia sample ($n=5$), and the differences within and among treatment groups were calculated on the basis of these averages.

Results

Kif3a function in pre-osteoblasts is expendable for skeletal patterning

In addition to their function as chemosensors, primary cilia are purported to function as mechanosensors and here we set out to explore the role of Kif3a and the primary cilium in mechanically-induced bone formation. We began by asking whether Kif3a function in *collagen type I*-expressing pre-osteoblasts was important in skeletal development. Using a *Collagen type I* α 1-specific Cre driver [17, 24], we genetically inactivated Kif3a and examined the resulting *Col1Cre;Kif3a^{fl/fl}* embryos, pups, and adult mice at multiple time points. In contrast to the phenotypes reported in *Kif3a^{fl/fl}Col2a1Cre* mice, we failed to detect any aberrations in skeletal patterning [25]. Even within the cranial skeleton, which forms via both intramembranous ossification and endochondral ossification, we failed to detect any changes in skeletal patterning (Fig. 1A,B) or in the onset and rate of chondrogenic and osteogenic mineralization (Fig. 1C–H). For example, loss of Kif3a function in *Collagen type I*-expressing cells does not disrupt the formation of the persistent cartilage that underlies the parietal bones, nor does it disrupt the intramembranous ossification of the overlying parietal bone (Fig. 1C,D). Conditional loss of Kif3a in *Collagen type I*-expressing cells did not alter any aspect of chondrogenic (Fig. 1E,F) or osteogenic differentiation (Fig. 1G,H) in the head skeleton.

Indian Hedgehog loss-of-function does not phenocopy Kif3a loss-of-function

In *Kif3a^{fl/fl}Col2a1Cre* embryos the observed skeletal defects were attributed to perturbations in Hedgehog signal transduction [6]. Hedgehog signaling, including localization of the Patched/Smoothed receptor complex and processing of the Gli transcription factors, largely takes place within the primary cilium (reviewed in [26]). We were therefore particularly interested in determining if Hedgehog signaling was perturbed in *Col1Cre;Kif3a^{fl/fl}* animals. We compared e17.5 *Col1Cre;Kif3a^{fl/fl}* embryos with e17.5 *Ihh^{-/-}* embryos and focused on sites of endochondral ossification, such as the supraoccipital bone (Fig. 2A–C) and sites of intramembranous ossification, such as the frontal and parietal bones (Fig. 2D). For example, at e17.5, wild-type parietal bones have expanded to the point where the ossification fronts of the frontal and parietal bones are nearly juxtaposed, separated only by a thin zone of undifferentiated mesenchyme that constitutes the coronal suture (Fig. 2E). *Col1Cre;Kif3a^{fl/fl}* embryos look like their wild-type counterparts (Fig. 2F) but *Ihh^{-/-}* embryos have much smaller parietal bones and the intervening undifferentiated mesenchyme was considerably wider (Fig. 2G). In multiple sites of intramembranous ossification and endochondral ossification, *Col1Cre;Kif3a^{fl/fl}* embryos did not exhibit skeletal abnormalities that were obvious in *Ihh^{-/-}* embryos (Fig. 2 and see [27]). Thus, we conclude from these analyses that loss of Kif3a does not phenocopy the loss of the *Ihh* ligand, and that in pre-osteoblasts, the primary cilium is serving some role other than

Hedgehog signal transduction. Our next experiments focused on identifying this additional function for primary cilia.

Reparative osteogenesis is unperturbed in *Col1Cre;Kif3a^{fl/fl}* mice

The act of bone injury causes a mechanical deformation of skeletal tissues, and this physical stimulus serves as one of the earliest signals to activate skeletal stem cells in response to damage [28]. Given the proposed function of the primary cilium as a mechanosensor [29], we were keen to evaluate how mechanically sensitive bone repair was impacted by deletion of *Kif3a*.

We first carried out a series of control experiments using a modified injury model [30], in which biomechanical inputs to the bone repair process are minimized. The injury involves drilling a small hole through one cortical bone surface that impinges on the bone marrow cavity but which leaves the far cortical bone surface intact. As a consequence, there is minimal contribution of biomechanical stimuli to the healing process [31].

Using this model, we characterized the process of implant osseointegration. We found that within 3 days, the injury site was filled with cells derived from the bone marrow cavity (Fig. 3A). By post-injury day 7, *Collagen type I*-expressing pre-osteoblasts initiated their differentiation into osteoblasts (Fig. 3B). Over the next two weeks, the extracellular matrix mineralized (Fig. 3C). By day 28, the cortical bone was restored to its pre-injury morphology (Fig. 3D).

The bone repair response occurred exclusively via intramembranous ossification. Consequently, it was an ideal model in which to evaluate the functional importance of *Kif3a* in the process of bone formation. Using histomorphometric analyses to quantify the osteogenic repair response in wild-type and *Col1Cre;Kif3a^{fl/fl}* mice, we found that both the control and mutants generated equivalent amounts of new bone in the injury site (Fig. 3E,F; quantified in G). Consistent with our analyses of *Col1Cre;Kif3a^{fl/fl}* embryos, we concluded that *Kif3a* function in pre-osteoblasts is not essential for the program of bone formation.

Strain fields are generated by implant displacement

Within bounds, mechanical forces and related quantities such as strain can have a stimulatory effect on osteoblasts, inducing them in some cases to proliferate (reviewed in [32]) or to differentiate [33] and thus enhance bone formation. Since loss of *Kif3a* did not impede bone formation per se, we were in a position to specifically ask whether mechanically induced osteogenesis was impacted when the primary cilium was rendered non-functional. We employed the identical mono-cortical defect model with the addition of an implant (Fig. 4A) supported by a bone plate screwed onto the tibial surface. The plate acted as a platform for stabilizing the implant, and for allowing application of micromotion to the implant itself (Fig. 4B and see [31]). Using this set-up we axially displaced the implant 150µm (60 cycles in 1 minute per day) within the mono-cortical defect, which in turn induced interfacial strain distributions around the implant (Fig. 4C–G). *Collagen type I* transcription was up-regulated in areas of moderate strain (Fig. 4H), and its expression persisted in response to the mechanical loading until at least day 7 (Fig. 4I). Because implant motion induced an early and persistent expression of *Collagen type I* in cells adjacent to the implant, we were able to utilize the same osteoblast-specific *Col1a1Cre* mouse crossed with mice carrying a floxed *Kif3a* allele to generate progeny in which *Kif3a* was inactivated in peri-implant cells.

Using the *Col1Cre;Kif3a^{fl/fl}* mice, and wild-type, age- and sex-matched controls, we next evaluated how the loss of a functioning primary cilia impacted how cells responded to the principal compressive and tensile strain in the implant interface (Figs. 5A,C,E,G and

5I,K,M,O). We first used Safranin O/Fast Green histology to identify the presence of cartilage proteoglycans (Fig. 5B). Principal compressive strains were found to reach their largest magnitude near the uppermost circumferential ridge of the implant (Fig. 5C). Occasionally these strains were in excess of -40% (where a negative sign indicates compressive strain) but in contrast with the compressive strains associated with chondrogenesis in fracture sites [34], these regions of high strain corresponded to sites of fibrous tissue and bone formation, without any evidence of a cartilage intermediate (Fig. 5D). Moderately large compressive strains, between -15 to -20% , occurred adjacent to the bottom ridge of the implant (Fig. 5E). In wild-type mice, this region also corresponded to sites of fibrous tissue formation, again with no evidence of cartilage (Fig. 5F). Compressive strains on the order of -15 to -20% , were also predicted adjacent to the bottom surface of the implant (Fig. 5G), and this site similarly corresponded to regions of fibrous tissue formation without evidence of chondrogenesis (Fig. 5H). In the remainder of the gap-type interface, at a distance from the implant surface, the compressive strain magnitudes were smaller, e.g., 0 to -10% (e.g., see Fig. 5A,C,E,G) and in wild-type mice, these regions corresponded to sites of bone formation (Fig. 5J,L,N,P).

The spatial extent of principal tensile strains generally co-localized with that of the principal compressive strains, i.e., tensile and compressive principal strains reached peak magnitudes adjacent to the surface of the implant at the strain-concentrating circumferential ridges (Fig. 5C,E,K,M), and at the corners of the implant's base (Fig. 5G,O). Our model predicted that the peak tensile strains were between 25 to 40% immediately adjacent to the implant surface at the circumferential ridge, but fell off rapidly to more moderate magnitudes (e.g., 15 to 25%) with distance from the implant surface (Fig. 5K,M,O). In wild-type controls these moderate-strain regions in the gap interface were the sites of abundant Aniline blue-positive osteoid matrix (Fig. 5J,L,N,P) while the higher-magnitude tensile strain regions were filled with fibrous tissue.

Collectively, these data indicate that excessively large principal compressive and tensile strains interfered with bone formation at specific regions that were immediately adjacent to the implant surface, whereas moderate-level strains farther out in the interface act as an osteogenic stimulus to peri-implant cells.

Primary cilia as mechanotransducers for in vivo bone formation

The previous experiments set the stage for evaluating whether primary cilia act as mechanical sensors in vivo. Using the same method for generating interfacial strain, we placed implants into *Col1Cre;Kif3a^{fl/fl}* mice and wild-type controls, and subjected them to the implant-displacement paradigm (Fig. 4). We then calculated the interfacial strain created by implant loading, and simultaneously evaluated how the cellular response to this strain was altered as a result of the *Kif3a* deletion.

In wild-type mice both the principal strains (purple and orange lines) and combined strain parameters (blue and green lines) were highest within the first 2 days of loading (Fig. 6A) and this window of high strain corresponded to the onset of cell proliferation (Fig. 6B). In addition to this temporal correspondence we found a tight correlation in the location of PCNA-positive proliferating cells and the highest principal strains, where both were localized next to the implant surface, in the vicinity of the ridge (Fig. 6C).

We next consider the *Col1Cre;Kif3a^{fl/fl}* mice. Although the implant displacement was the same, the cellular response to this mechanical stimulus was markedly different: unlike wild-type cells, peri-implant cells in the *Col1Cre;Kif3a^{fl/fl}* mice showed almost no evidence of cell proliferation (Fig. 6D). There are two possible explanations for this finding. First, the mutant cells were simply unable to proliferate; second, the *Col1Cre;Kif3a^{fl/fl}* cells didn't

proliferate because they were insensitive to the mechanical stimulus associated with implant displacement. We ruled out the first possibility when we evaluated other sites in the *Col1Cre;Kif3a^{fl/fl}* skeleton and found evidence of robust cell proliferation in the adjacent periosteum (Fig. 6E). Therefore, *Col1Cre;Kif3a^{fl/fl}* mutant cells are capable of mounting a proliferative response; they simply do not do so around the implant.

We further addressed this possibility by examining in more detail the responses of wild-type cells to implant displacement. For example, implant displacement stimulates the formation of an oriented collagenous matrix within the peri-implant space [35]. Using Picrosirius red staining and polarized light to visualize collagen fiber orientation we demonstrated that, between day 3 (Fig. 6F) and day 7 (Fig. 6G) there was an increase in both the amount and organization of collagen around the implant. We also measured the interfacial stiffness and showed that the material properties of wild-type interfacial tissue increased with time (Fig. 6H). Furthermore, the calculated interfacial strains decreased with time (from 3–6 days; Fig. 6A). For example, between days 3–6 the interfacial strains decreased by approximately 30% (For both principal and deviatoric strains), consistent with the more mature collagen arrangement at day 7 (e.g., Fig. 6G). Principal strains less than 30% favor the differentiation of cells into osteoblasts [36], which is consistent with our finding that on day 7, when the region having moderate strains in the wild-type peri-implant space was filled with new bone (Fig. 6I,J).

Are cells lacking the primary cilium insensitive to the mechanical stimulus related to implant displacement?

Picrosirius staining of the peri-implant tissues on day 7 in *Col1Cre;Kif3a^{fl/fl}* mice revealed less collagen (Fig. 6K). This deficit, however, was not attributable to a lack of cells in the peri-implant space (Fig. 6L). Rather, the decreased amount of collagen and its disorganized arrangement culminated in a lack of peri-implant bone formation (Fig. 6M,N). Histomorphometric analyses verified that the loss of Kif3a in peri-implant cells resulted in a significantly reduced osteogenic response (Fig. 6O). Collectively, the inability of *Col1Cre;Kif3a^{fl/fl}* cells to proliferate in response to a mechanical stimulus; their failure to deposit and then orient collagen fibers to the strain fields caused by implant displacement; and finally their resistance to differentiate into bone-forming osteoblasts demonstrates that the lack of a functioning primary cilium blunts the normal response of a cell to a defined mechanical stimulus.

Discussion

Skeletal cells are mechanosensitive

Skeletal cells are uniquely sensitive to physical forces (and related quantities such as strain) and employ a number of methods to detect and decipher these mechanical stimuli (reviewed in [37, 38]. For example, integrins and the focal adhesions with which they are associated span the cell membrane to connect the cell's internal cytoskeleton to its external environment and in doing so, they fulfill a basic requirement for a cellular mechanosensory apparatus [39]. In previous work we showed that, by deleting an essential component of the pathway, integrin signaling is also essential for skeletal mechanotransduction *in vivo* [17]. Other candidate mechanosensors include ion channels, whose flux is influenced by mechanical stimuli [40]. Even deformation of a cell's membrane may be sufficient to elicit a mechanically induced response [41].

Here, we tested the hypothesis that a cell's primary cilium could also act as a mechanosensor. We used a motion device that generated a spatial distribution of strain around an intra-osseous implant that was positioned in the marrow cavity of a mouse. This

system allowed us to control the mechanical stimulus delivered to peri-implant cells, to model the resulting strain fields, and then to correlate how these strain fields affected cell behaviors ranging from proliferation through all stages of osteogenic differentiation. The ability to manipulate the genetic background of the peri-implant cells within the context of a whole, living tissue provided us the rare opportunity to evaluate mechanotransduction at the molecular level.

Strain states play a role in directing cellular responses during healing

We characterized in detail how the material properties of the peri-implant tissues change over time, and how this impacts the strain states produced by implant displacement. When the implant is initially placed the peri-implant space is filled with a pliable, low-stiffness fibrin-rich blood clot [42]. Consequently, displacement of the implant by 150 μm occurs in a relatively low-modulus matrix, which lead to principal compressive and tensile strain magnitudes that reached as large as 80 to 90% adjacent to the implant, and 30 to 40% in the middle of the interface (Fig. 5). Within a 24h period the peri-implant space is replaced by densely packed fibroblastic cells that form strong cell-cell contacts [30]; consequently, implant displacement at this time point leads to a decrease in the magnitudes of the principal strain, from 50 to 60% nearest the upper ridge of the implant to about 10 to 20% farther away in the interface (Fig. 5). Soon thereafter, peri-implant cells begin producing collagenous proteins that are secreted into the extracellular space and these proteins mineralize to create bone (Fig. 6F,G). Our measurements (Fig. 6H) and others [43] indicate that the stiffness of the matrix on day 7 corresponds to an interfacial Young's modulus of approximately 17 MPa. At this stage of healing, implant displacement produces maximal principal tensile and compressive strain values of about 40% right near the ridges and corners of the implant, and about 10% or less in the regions of the interface farther away from the implant's surface.

Taken together, these data argue that cellular responses in the peri-implant space are regulated by mechanical input. We propose the following model, whereby the act of injury causes an immediate, dramatic disruption in tissue integrity. This mechanical stimulus likely serves as one of the earliest inputs to activate a wound healing response. In an undisturbed, mechanically neutral wound site, cells exist in an environment with low-magnitude ambient strains. In a mechanically active site, such as the peri-implant space, cells exist in an environment that can have both high-magnitude as well as lower magnitude strains. High magnitude strains induce cell proliferation. As this division ensues, new cells intercalate themselves into the pre-existing cellular "meshwork" surrounding the implant. This larger cellular mass helps to distribute the strain over a larger volume, which in turn effectively reduces the strain level. Our data demonstrate that reduced strain favors the differentiation of pre-osteoblasts into osteoblasts. These mature osteoblasts deposit a collagenous matrix that eventually mineralizes. Implant displacement in this inherently stiff matrix effectively reduces strain states further, and eventually returns the tissue to its mechanically neutral state. However, if some of the tissues continue to experience dangerously high strains (as they do in certain strain-concentrating regions of our implants), proper mineralization is forestalled; instead, cells continue to proliferate if they retain this capacity, and may go on to form non-osseous matrix. This proposed scenario is consistent with theories of strain-regulated fracture healing [44, 45].

The role of primary cilia as molecular sensors

Skeletal anomalies reported in *Kif3a^{fl/fl}Col2Cre* embryos have been attributed to disruptions in Hedgehog signaling [6]. To determine if our *Col1Cre;Kif3a^{fl/fl}* embryos also had a defect in Hedgehog signaling, we undertook a comparative analysis of the skeletal phenotypes of *Ihh^{-/-}* and *Col1Cre;Kif3a^{fl/fl}* embryos and focused our analyses on sites of bone formation

where pre-osteoblasts play a critical role in establishing and maintaining bone mass (Fig. 2). In these locations we observe an arrest in the onset and the rate of osteoid mineralization in *Ihh*^{-/-} embryos (Fig. 2G and see [27]). *Col1Cre;Kif3a^{fl/fl}* embryos, however, exhibited none of these defects (Figs. 1,2 and see [25, 27]). Thus, we conclude that in pre-osteoblasts the principal function of the primary cilium is not Hedgehog signal transduction. This leads to the question, what other roles might this organelle play?

The role of primary cilia in mechanotransduction

The deletion of *Kif3a* results in a truncated, dysfunctional primary cilium [46]. Our data demonstrate that *Kif3a*^{-/-} pre-osteoblasts are incapable of elaborating a normal primary cilium (Fig. 3E–G and see reference [47]), and these mutant cells are defective in their response to defined physical stimuli (Fig. 6). But precisely what aspect of a physical stimulus is sensed by a primary cilium is unknown. Primary cilia can detect fluid flow [4, 11, 14, 48, 49] and fluid flow in the bone marrow cavity and the peri-implant space might accompany implant displacement. There are other possibilities, however: Primary cilia may be sensitive to deformations in the cell's membrane; or they could respond to gradients of strain in the cell's environment. Strain can alter the orientation of a cell [50, 51]; we observe cells and the matrix become organized parallel to principal strains in the peri-implant space (Fig. 6F,G). This orientation does not occur in *Col1Cre;Kif3a^{fl/fl}* cells (Fig. 6K), indicating that the primary cilia is required for cells to be responsive to strain. In vitro analyses will likely shed more light on the mechanosensitivity of primary cilia.

Mechanical influences on bone healing: implications of regenerative medicine

All cells are mechanosensitive. Physical forces associated with growth and morphogenesis shape the response of embryonic stem cells [52], and the mechanical effects of microgravity clearly influence adult cells [53]. The mechanical environment also provides cells with essential clues about the appropriate differentiation program [54, 55]. For example, a stiff substrate supports the differentiation of stem cells into osteoblasts whereas a soft substrate supports their differentiation into neurons [56]. Mechanosensitivity is also an elemental part of a stem cell's capacity to repair and regenerate damaged tissues [57], and an age-related loss of mechanosensitivity may be a primary risk factor for delayed healing in the elderly [58].

Deciphering which aspects of a mechanical stimulus are most relevant to cells has proven to be challenging. At a tissue level, the mechanical environment can involve a nearly unlimited combination of fluid flow-induced strain, tensile and compressive normal strains, or shear deformations that all act upon cells embedded in a heterogeneous extracellular matrix. This situation is further complicated by injury, where the healing process itself alters the mechanical properties of the extracellular matrix, as well as the constituency of cells occupying the injury site [59, 60].

Despite this complexity, some key concepts have emerged. For example, a number of cellular mechanosensors have been identified, and their basic architecture as well as their mechanisms of action are partially characterized [28, 37]. In some cases, the phenotypes resulting from mechanosensor loss-of-function have been identified, and these studies have been particularly illustrative of how cells sense and respond to some kinds of physical stimuli [16, 17, 35].

Here, we demonstrate that the primary cilium is essential for the ability of pre-osteoblasts to sense strain-related mechanical stimuli at a healing bone-implant interface. Deleting an essential IFT protein, and thereby truncating the primary cilium renders pre-osteoblasts incapable of an appropriate response to the physical stimulus. But it must be emphasized

that the primary cilium is also a molecular sensor ([26] reviewed in [61]), suggesting a role as an integrative signaling nexus. Within one cellular organelle we have the potential to dissect how mechanical stimuli and molecular signals are integrated [11]. Ultimately, these studies will help us understand how to reverse the untoward effects resulting from a loss of mechanosensitivity.

Acknowledgments

Funding: This work was funded by R01-EB000504 to J.B. and J.A.H, AR45989 & AR62177 and New York State Stem Cell grant (N089-210) to C.R.J..

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Figure 1. During development, *Kif3a* function in pre-osteoblasts is dispensable for chondrogenic and osteogenic differentiation

Pentachrome staining of coronal sections of e16.5 calvaria demonstrate equivalent skeletal morphology in (A) wild-type and (B) *Col1Cre;Kif3a^{fl/fl}* embryos. (C) In wild-type embryos intramembranous ossification of the parietal bone occurs normally and is indistinguishable from (D) the parietal bone in *Col1Cre;Kif3a^{fl/fl}* embryos. White dotted line separates persistent cartilage from the overlying bone of the calvaria. (E) The chondrocytes in Meckel's cartilage undergo hypertrophy and the adjacent bone of the developing mandible (dotted black line) forms lateral to the cartilage template. (F) In the *Col1Cre;Kif3a^{fl/fl}* mutants, chondrocytes undergo hypertrophy and their position and size, as well as the developing mandible, is indistinguishable from the wild-type littermates. (G) In wild-type embryos, Aniline blue staining demarcates osteoid matrix, and the location, amount and mineralization pattern are indistinguishable from (H) the same osteogenic condensations in *Col1Cre;Kif3a^{fl/fl}* embryos. (Abbreviations: b: bone; br: brain; ca: cartilage e: eye; Mc: Meckel's cartilage; nc: nasal cavity; t: tongue. A,B scale bar = 200 μ m; C–H scale bar = 50 μ m).

Figure 2. Loss of Kif3a does not phenocopy loss of Indian Hedgehog

Safranin O/Fast Green staining of e16.5 embryos demonstrates (A) sites of both intramembranous and endochondral ossification of the head skeleton. (B, C) Endochondral ossification is shown in the supraoccipital bone. (D) Aniline blue staining shows new osteoid matrix of the interparietal bones, formed by intramembranous ossification. Alcian Blue and alizarin red staining of whole embryo show at e17.5, (E) wild-type parietal bones are separated by a thin coronal suture and (F) *Col1Cre;Kif3a^{fl/fl}* embryos are indistinguishable from their wild-type counterparts. (G) *Ihh^{-/-}* embryos exhibit smaller parietal bones and a wider coronal suture.

Figure 3. During skeletal repair, Kif3a function in pre-osteoblasts is dispensable for osteogenic differentiation

Pentachrome staining of wild-type mice defects at (A) post-injury day 3 shows the injury site contains cells from the bone marrow cavity. (B) Post-injury day 7 shows cells are beginning to differentiate into osteoblasts. (C) By post-injury day 14 cells have begun to deposit a matrix. (D) By post-injury day 28, the cortical bone is restored to its pre-injury morphology. (E) Acetylated tubulin staining of *Col1Cre;Kif3a^{fl/fl}* cells show a lack of primary cilia. (F) Wild-type cells stain positive for acetylated tubulin. (G) Aniline blue staining of a tibial injury site indicates new osteoid matrix deposition in the bone marrow cavity of skeletally mature (H) wild-type and (I) *Col1Cre;Kif3a^{fl/fl}* mice. (J) Histomorphometric analyses demonstrate no statistical difference in the amount of new osteoid matrix that has formed on post-injury day 7. (Abbreviations: cb: cortical bone; bmc: bone marrow cavity. H,I scale bar = 100µm).

Figure 4. An in vivo model of mechanical stimulation

(A) Three-dimensional mesh of the finite element model of the implant in the tibia. (B) Implant motion system installed on a mouse tibia. The implant (1) slides in a channel within the bone plate (2). The head of the implant rests against an O-ring (3). The bone plate is secured onto the mouse tibia by two Reto-pins (4). (C) Via a finite element model, an XY slice through the middle longitudinal plane of the tibia shows the interfacial region of interest around the tip of the implant in the 0.8mm diameter hole in the tibia. (D) Principal tensile strains around an implant for near-zero displacement of 5 μ m. (E) Principal tensile strains in the interfacial region around the implant's tip for axial downward motion of 150 μ m. (F) Principal compressive strains around an implant for near-zero displacement of 5 μ m. (G) Principal compressive strains in the interfacial region around the implant's tip for axial downward motion of 150 μ m. In situ hybridization of *collagen type-I* at (H) post-injury day 3 and (I) post-injury day 7 in wild-type mice. (Abbreviations: cb: cortical bone; bmc: bone marrow cavity; im: implant. H,I scale bar = 50 μ m).

Figure 5. Kif3a acts as a mechanosensor of compressive and tensile strain

(A) The distribution of principal compressive strain at day 7 around a mechanically-stimulated pin implant corresponds to (B) fibrous and bone tissue rather than sites of chondrogenic differentiation, as shown by the absence proteoglycan-rich red matrix in Safranin O/Fast Green stained tissues in wild-type mice. Cortical bone (white dotted line) and the outline of the pin implant (black dotted line) are indicated; boxes correspond to higher magnification images. (C) The distribution of largest (e.g., 30–40%) strain appear in the region between the implant ridge and the intact cortical bone. (D) Compressive strains did not induce chondrogenesis in this model (note absence of proteoglycan-rich red staining). (E) In regions where principal compressive strains are moderate (e.g., 10%), (F) bone formed in wild-type mice. (G) Principal compressive strains at the base of the implant exhibit large strains. At such high strains, (H) peri-implant cells in wild-type mice fail to form bone. (I) The distribution of principal tensile strains around a mechanically-stimulated pin implant correspond to (J) the distribution of new osteoid matrix, as shown by Aniline blue staining in wild-type mice. Cortical bone (white dotted line) and the outline of the pin implant (black dotted line) are indicated; boxes correspond to higher magnification images. (K) The distribution of highest (e.g., 30–40%) strain is the region between the implant ridge and the intact cortical bone. (L) In wild-type mice, the region of high strain corresponds to sites of new osteoid matrix. (M) In regions where principal tensile strains are low (e.g., 10%) mechanically-induced bone formation is robust in (N) wild-type mice. (O) Principal tensile strains at the base of the implant illustrate a region where interfacial strains are about 15%, which (P) in wild-type mice, correspond to sites of minimal osteoid deposition. (Abbreviations: bmc: bone marrow cavity; cb: cortical bone; im: implant. B,J scale bar = 100µm; all other panels, scale bar = 50µm).

Figure 6. Kif3a function is required for the maturation of pre-osteoblasts in the peri-implant space

(A) Predicted principal strains and combined strain parameters on days 1–6 are shown for the region of the uppermost ridge of the implant in wild-type tissue. (B) On post-injury day 3, PCNA staining shows abundant proliferation in wild-type peri-implant cells localized to (C) regions of highest compressive strain (left) and tensile strain (right). For compressive strain plot, green-blue to dark blue indicates the range between –40–60%. For the tensile strain plot, yellow to orange indicates the range between 40–60%. (D) PCNA staining of peri-implant cells from *Col1Cre;Kif3a^{fl/fl}* mice demonstrates a lack of proliferation. (E) PCNA staining of the periosteum demonstrates that cells are capable of proliferating. Picrosirius red staining at (F) day 3 shows minimal collagenous extracellular matrix, but by (G) day 7 there is an increase in collagenous extracellular matrix. (H) Graphs illustrate how the measured interfacial stiffness (left) changes with time and how the derived interfacial Young's modulus (right) changes with time. (I,J) Aniline blue staining of bone formation demonstrates that on post-injury day 7 peri-implant tissues are already mineralized in wild-type mice. (K) Polarized light shows minimal collagenous extracellular matrix and (L) Picrosirius red staining show abundant cells in the peri-implant space of *Col1Cre;Kif3a^{fl/fl}* mice at day 7. (M,N) Aniline blue staining of new bone formation demonstrates that there is

minimal bone formation in *Col1Cre;Kif3a^{fl/fl}* mice at day 7. (**R**) Histomorphometric analyses indicate that in response to implant displacement wild-type mice form significantly more peri-implant bone than *Col1Cre;Kif3a^{fl/fl}* mice. (n=5 for each genotype) (Abbreviations: bmc: bone marrow cavity; cb: cortical bone; im: implant. J,N scale bar = 100µm; all other panels, scale bar = 50µm).