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Primary Cilia Exist in a Small Fraction of Cells in Trabecular Bone and Marrow

Thomas R. Coughlin\textsuperscript{1+}, Muriel Voisin\textsuperscript{2+}, Glen L. Niebur\textsuperscript{1}, Laoise M. McNamara\textsuperscript{2}

\textsuperscript{+} Authors contributed equally to this work

\textsuperscript{1}Tissue Mechanics Laboratory, Bioengineering Graduate Program
University of Notre Dame, IN, 46556 USA

\textsuperscript{2}Dept of Mechanical and Biomedical Engineering
National University of Ireland, Galway, Ireland

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Address Correspondence to:
Laoise M. McNamara
Biomedical Engineering,
National University of Ireland, Galway
Galway,
Ireland

Phone: +353-91-492251
Fax: +353-91- 563991
e-mail: Laoise.McNamara@nuigalway.ie
Abstract

Primary cilia are potent mechanical and chemical sensory organelles in cells of the bone lineage in tissue culture. Cell culture experiments suggest that primary cilia sense fluid flow and this stimulus is translated through biochemical signaling into an osteogenic response in bone cells. Moreover, *in vivo* primary cilia knockout in bone cells attenuates bone formation in response to loading. However, understanding the role of the primary cilium in bone mechanotransduction requires knowledge of their incidence and location *in situ*. We used immunohistochemistry to quantify the number of cells with primary cilia within the trabecular bone tissue and the enclosed marrow of ovine cervical vertebrae. Primary cilia were identified in osteocytes, bone lining cells, and in cells within the marrow, but were present in only a small fraction of cells. Approximately 4% of osteocytes and 4.6% of bone lining cells expressed primary cilia. Within the marrow space, only approximately 1% of cells presented primary cilia. The low incidence of primary cilia may indicate that cilia either function as mechanosensors in a select number of cells, function in concert with other mechanosensing mechanisms, or that the role of primary cilia in mechanosensing is secondary to its role in chemosensing or cellular attachment.

Keywords

Mechanobiology
Mechanotransduction
Primary cilia
Marrow
Osteocyte
MSCs
Trabecular bone
**Introduction**

Bone adapts to mechanical loading through the coordinated activities of osteocytes, osteoblasts, and osteoclasts. Osteoblasts and osteocytes are derived from marrow stromal cells (MSCs), while osteoclasts are derived from hematopoietic stem cells (HSCs) [1]. MSCs, HSCs, and their progeny reside in the trabecular bone marrow forming a complex multicellular niche [2, 3], while osteocytes are entombed within the mineralized bone. All of these cells are subjected to mechanical cues due to skeletal loading [4].

MSCs, osteoblasts, osteocytes, and osteoclasts are all mechanosensitive and both intra- and inter-cellular signaling are driven by a range of mechanical loads [5]. However, the precise mechanisms of mechanosensation are unknown. Osteocytes are exposed to fluid flow *in vivo* [6], and respond to fluid flow in culture by producing biochemicals and proteins associated with osteogenic differentiation [7-9]. MSCs and osteoblasts also respond to fluid flow, intercellular forces, and matrix strain *in vitro* [10, 11], and may be subjected to these stimuli *in vivo* [12]. A number of mechanosensing cellular structures that could sense these signals have been proposed [13], including ion channels, gap junctions [14], integrins [15], and the actin cytoskeleton [16]. Recently, primary cilia have been cited as a potential important mechanosensor in bone cells and MSCs [16, 17].

Primary cilia are cell appendage organelles formed during the quiescent/G1 phase of the cell cycle, comprised of nine microtubule doublets that attach to the basal body and are encased in the plasma membrane [18-21]. Primary cilia sense and respond to mechanical cues in many cell types, most notably acting as essential flow sensors in the epithelial cells of the kidney [22]. When the cilium bends in response to flow, an influx of Ca^{2+} occurs and spreads to neighboring cells [23].
Recent studies have implicated primary cilia in bone cell mechanotransduction. *In vitro*, primary cilia inhibition in MSCs blocks osteogenic gene transcription, but increases MSC proliferation [17]. Primary cilia abrogation [16] or inhibition [24] in MC3T3-E1 osteoblasts also attenuates normal osteogenic responses to fluid flow. Primary cilia abrogation in MLO-Y4s abolishes upregulation of osteogenic factors normally experienced in response to fluid flow *in vitro* [16, 24]. *In vivo*, mice with an osteoblast- and osteocyte-specific knockout of Kif3a, a gene essential to primary cilia formation and function, exhibited bone formation in response to loading, but it was diminished compared to control mice [25]. Conditional knockout of Kif3a also decreases the number and length of primary cilia in osteoblasts, which results in osteopenia [26]. In addition to its role as a mechanosensor, primary cilia are known to facilitate various physiological functions including chemical sensory processes [27] and matrix attachment [28]. Moreover, the length of cilia decreases in response to mechanical loading in both tenocytes [29] and chondrocytes [30], suggesting that cilia may respond to local matrix deformation, as the fluid flow velocities are small in these models.

The role of primary cilia as mechanosensors in bone cells *in vivo* depends on the types and numbers of cells with cilia, and their local mechanical environment. In particular, primary cilia in osteocytes may sense fluid flow through the lacunar-canalicular network [6]. However, there are conflicting reports regarding *in vivo* expression of cilia in osteocytes, in cortical bone. Tonna *et al.* found primary cilia in only 4% of osteocytes in aging mice using transmission electron microscopy (TEM) [31], while 94% of osteocytes in rat tibia exhibited immunohistochemical (IHC) staining for the primary cilia marker, acetylated α-tubulin [32]. In the trabecular bone compartment, the incidence of ciliated osteocytes in bone tissue and ciliated cells in the bone marrow space are not known. In bone marrow, cells with elongated cilia may be
preferentially located in areas of the marrow where they are likely to be subjected to mechanical or chemical stimuli. For example, near the trabecular bone-marrow interface, cells are subject to high shear stresses during physiological loading [4], and biochemical signals between osteocytes, bone lining cells, and osteoblastic precursors in the marrow are prevalent. As such, quantifying the presence of primary cilia in the trabecular bone and marrow may lend understanding to their role as mechano- or chemosensors or indicate other putative functions. While osteocytes are believed to reside in a mechanically stimulated environment [6], where mechanotransduction via cilia is possible, cells in the centralized marrow may not experience significant mechanical stimulation and as such presentation of an elongated cilia may not be necessary, but this has never been investigated. The goal of this study was to determine the fraction of cells expressing primary cilia in osteocytes, in marrow cells near the bone surface, and in cells in the central marrow cavity. In order to achieve this, we validated an immunostaining protocol for sheep trabecular bone and marrow; and applied it to quantify the number and length of cilia within the marrow, on the bone surface, and within the bone matrix.

**Materials and Methods**

*Animal Model*

Primary cilia expression was studied in trabecular bone from the cervical vertebrae of young sheep, six to eight months of age. The sheep is a grazing animal and bone from this site would be subjected to regular cyclic loading from the neck musculature. Ovine kidney was studied as a positive control for the antibodies. Bone and kidney were obtained from an abattoir (Brady’s, Athenry, Ireland) within four hours of the time of slaughter, and maintained on ice throughout processing.
Antibody Validation

To verify the specificity of the antibodies in ovine tissue, we conducted preliminary staining on kidney and bone. Acetylated α-tubulin and intraflagellar transport protein 88 (IFT88), both of which are vital for ciliogenesis [21], were used to target primary cilia.

Antibody specificity was verified in the kidney, a tissue where primary cilia are known to be present [33]. The tissue was fixed in 4% w/v paraformaldehyde (PFA) for 72 hours and cut into small sections that were processed (Leica ASP300) and paraaffin embedded (Leica EG1150H). Longitudinal sections were cut at 20 µm using a microtome (Leica RM2235), collected on SuperFrost® slides (Menzel Glaser), and left to dry overnight at 60°C. Slides were stored at room temperature, until dual immunostaining as described below. Slides were imaged using a Nikon confocal microscope.

Paraffin embedded samples were rehydrated as per routine protocols (xylene and descending grades of ethanol) and were stained after proteinase K antigen retrieval (20 μg/mL in TE buffer, 20 minutes at 37°C). Slides were rinsed twice for 2 minutes with 0.5% v/v Phosphate Buffered Saline (PBS)- Tween and blocked for 1 hour with 3% Normal Goat Serum (NGS)/ 1% Bovine Serum Albumin (BSA) in PBS.

For acetylated α-tubulin staining, blocked sections were incubated with anti acetylated α-tubulin (Abcam), 1/20 dilution in NGS 3%/BSA 1% in PBS, overnight in a humidified chamber at 4°C, followed by three 10 minute rinses in BSA 1% w/v in PBS. Goat Dylight 488 anti mouse 1/200 (Jackson ImmunoResearch) secondary antibody was then applied for 1 hour at room temperature in a dark humidified chamber. Three 10 minute rinses with BSA 1% w/v in PBS were performed in the dark and slides were mounted with a fluoroshield mounting media.
(Sigma) containing propidium iodide (PI) as a nuclear counterstain with coverslips (#1 thickness, Menzel Glaser). For dual immunostaining, anti acetylated $\alpha$-tubulin, at 1/20 dilution, was targeted with goat anti mouse Dylight 594 (Jackson ImmunoResearch), at 1/200 dilution, and then anti IFT88, at 1/100 dilution, as a second primary antibody targeted with Dylight 488, at 1/200 dilution. Dual immunostained slides were counterstained with 4' (DAPI) nuclear stain with coverslips. The antibody specificity validation was repeated in trabecular bone. One of the three ovine trabecular bones harvested from the cervical vertebrae was selected for dual immunostaining. The samples were harvested, processed, and immunostained using acetylated $\alpha$-tubulin and IFT88, as described in detail above.

_Cilia in Isolate Marrow Stromal Cells_

Marrow cells were extracted from the trabecular bone marrow of three ovine cervical vertebrae using centrifugation and expanded to passage three on collagen coated (Sigma) slides. On four consecutive days of expansion, subpopulations of MSCs were fixed using 4% w/v PFA for 20 minutes, permeabilized for 5 minutes at 4°C (2mM sodium chloride (NaCl), 1.5 mM magnesium chloride (MgCl$_2$), 16 mM sucrose and 0.5% Triton X in PBS), and immunostained with anti-acetylated $\alpha$-tubulin similar to the method described above using anti acetylated $\alpha$-tubulin at 1/50 dilution.

_Cilia in Trabecular Bone and Marrow_

Three cylindrical cores of trabecular bone and marrow were obtained from the C2 vertebrae of three sheep. The end plates of the vertebrae were removed and cylindrical cores were prepared using an 8.25 mm hollow diamond-tipped drill bit (Starlite) under constant
irrigation. The cores were fixed in 4% w/v PFA for 48 hours and demineralized for 21 days using 10% w/v ethylenediaminetetraacetic acid (EDTA) buffered to a pH of 7.5 at 4° C. After demineralization, samples were rinsed in dripping tap water overnight to drain excess EDTA. Processed and paraffin embedded samples were sectioned at 20 µm, collected on SuperFrost® slides, and left to dry overnight at 60°C. Slides were stored at room temperature. The sections were immunostained for acetylated α-tubulin as described above.

Confocal Microscopy

Slides were imaged on an inverted confocal laser-scanning microscope (LSM 510; Zeiss, Germany). Because the slides had multiple labels, multitracking was used to reduce bleaching and bleed through. The image acquisition software used was AIM 4.2 (Zeiss, Germany). Low magnification (10X) and high magnification (63X, oil immersion objectives) micrographs were obtained, using a low scan speed (7 s), averaging 4 images, using a 1024x1024 pixel definition. An argon laser (488 nm) was used to excite Dylight 488 and a Neon laser (550 nm) was used to excite PI.

For dual immunostained slides, a fluorescent Revolution confocal microscope (Andor), with spinning disk (Yokagawa CSU22) was used. The software used was Andor iq 2.3 acquisition software (Andor). Low and high magnification (10X and 63X oil immersion objectives, respectively) micrographs were obtained using a low scan speed, averaging 4 images, using a 1004x1002 pixel definition. An argon laser (488 nm) was used to excite Dylight 488, a Neon laser (561 nm) was used to excite Dylight 561 nm, and 350 nm to excite DAPI.

False color imaging was used to generate micrographs. Image stacks were acquired at 63x magnification, using a 0.5 µm step, and an average of four images per optical slice were
taken. For imaging the marrow, two randomly selected marrow pores were imaged per section generating three to five image stacks per pore. When imaging the bone matrix, five randomly selected areas were acquired per section.

**Primary Cilia Assessment**

Primary cilia were quantified under confocal microscopy in three locations in each bone: in the marrow space away from the endosteal surface (marrow >50 μm from the bone wall), marrow near the endosteal surface (marrow <50 μm from the bone wall), and in osteocytes (Fig. 1 A-D). Cells near the bone surface were further divided to identify primary cilia in bone lining cells. Image stacks were analyzed using ImageJ software (NIH). Primary cilia were identified based on the presence of stained acetylated α-tubulin proximal to a nucleus. Because we were interested in cilia that were extended, only stained cilia at least 1 μm in length were counted. Positive acetylated α-tubulin was excluded if there were two adjacent stained structures pointing toward one another, indicating two cells at a late stage of mitosis. Primary cilia length was measured in three dimensions by combining the measured lengths in three orthogonal directions by the Pythagorean theorem.

To determine the percentage of cells with primary cilia, the number of cells in the different locations was determined. The number of cells in a marrow region was found using Automatic Nuclei Counter on ImageJ software (NIH). The number of bone lining cells on the trabecular bone surface and osteocytes in trabecular bone were counted directly.

**Results**

*Antibody Validation*
In ovine kidney, anti IFT88 was colocalized with anti acetylated α-tubulin on cilia like structures (Fig. 2A), and the stains were not observed separately. In the absence of either primary antibody, only small regions of diffuse staining by the secondary antibodies were observed (Fig. 2C). Similarly, IFT88 and anti acetylated α-tubulin were colocalized and specific in sections of trabecular bone (Fig. 2B). As such, the anti acetylated α-tubulin antibody was specific to cilia in ovine tissue.

In cultured MSCs, 25% of 299 cells observed exhibited positive staining for acetylated α-tubulin (Fig. 2D and 2E). Cilia were observed on the apical side of cells, extending into the media.

*Primary Cilia Occurrence in Marrow and Trabecular Bone*

Primary cilia greater than 1 μm in length were observed in all locations that were analyzed within the trabecular bone samples (Fig. 3 A-C). In the marrow, cilia protruded into the intercellular space (Fig. 3A and 3B). In the bone matrix, cilia appeared in the lacunae adjacent to osteocyte nuclei (Fig. 3C). A total of 13,752 marrow cells at a distance further than 50 μm from the bone wall, 4,983 marrow cells within 50 μm of the bone wall, 758 cells on the bone surface, and 642 osteocytes were analyzed. The percentage of cells with primary cilia (mean±S.D.) in the marrow space away from the endosteal surface was 0.91±0.20%. Near the endosteal surface, the percentage was 1.48±0.74%, and increased to 4.60±1.80% when only considering bone lining cells. Similarly, 4.04±1.04% of osteocytes presented primary cilia. A greater percentage of osteocytes and bone lining cells presented primary cilia than did marrow cells in the interior of the pores (p<0.05; Fig. 4).
On average, the measured cilia were 1.62 µm long, with cilia within the marrow reaching up to 7 µm. The mean length of cilia only differed between osteocytes and marrow cells near the bone surface, while the lengths did not differ between the other locations (Fig. 5).

**Discussion**

In this study, we found that cells with primary cilia were rare in trabecular bone and the encompassed marrow space. As such, if cilia are important mechanosensory or chemosensory organelles, then those cells expressing them must be uniquely located to sense and act on these signals. The greater proportion of bone lining cells and osteocytes that expressed cilia compared to cells in the central marrow cavity is consistent with this idea, as osteocytes and marrow cells near the bone are known to affect bone mechanobiological adaptation and reside in mechanically stimulated areas [4, 6, 12]. The relatively small number of primary cilia in osteocytes and bone marrow cells may indicate that they do not function as mechanosensors in osteocytes or MSCs or that only a small number of MSCs and osteocytes are responsible for sensing loading-induced fluid flow using cilia. Taken together, the primary cilium may be only one of several mechanosensory structures with only a subset of cells relying on them at a given time.

This study provides the first measure of primary cilia incidence in trabecular bone and bone marrow, and also in the bone of large animals. Since cilia may play different roles in cortical vs. trabecular bone, in normal vs. elevated loading conditions, or across species, it is essential to quantify their presence to fully understand their function. The methods employed to detect and measure cilia in this study were carefully validated, and should be applicable across a range of species and anatomic sites. Indeed, the validation of the antibodies in the ovine model provides further evidence of the conservation of cilia specific proteins across species.
Some minor limitations of this study must be considered. Foremost, the phenotype of ciliated cells in the marrow and bone tissue was not characterized. While osteocytes embedded in bone tissue can be identified based on their morphology and physical location, the marrow contains cells of both the hematopoietic and mesenchymal lineage. Although we did not identify specific cell types that expressed cilia in the marrow, we did analyze the marrow near the trabecular bone surface separately. This region likely contains osteoprogenitor cells [34], committed osteoblasts, ostal macrophages [35], and HSCs, which are believed to reside adjacent to the bone lining cells [36]. In addition, it is known that proliferation and recruitment of osteoprogenitors occurs close to the trabecular bone surface in the marrow [37]. Although these are only general guides to the cell phenotypes in each region, the large number of cells analyzed made the use of rigorous identification, as with in situ hybridization, unfeasible.

The relatively low incidence of cilia in bone marrow stromal cells harvested from these bone samples is comparable to recent reports. From 30 to 60% of cells were reported to have cilia in human adipose derived stem cells [27]. In contrast, MSCs, MC3T3-E1s and MLO-Y4s demonstrated a high incidence of primary cilia in cell culture [16, 38] in other studies. These latter reports may be reflective of murine cells, or differences in media used to culture the various cell lines [27].

Previous in situ measurements of primary cilia expression in osteocytes have produced conflicting results. Primary cilia were detected in only 4% of osteocytes in aging mice using TEM [31], which is similar to the results presented here. In contrast, 94% of osteocytes in rat cortical bone expressed primary cilia based on acetylated α-tubulin immunostaining [32]. However, the TEM analysis conducted as part of that same study [32] revealed that structures representative of the basal portion of the cilia (mother and daughter centriole) were only detected
in approximately 50% of osteocytes. Moreover, many cilia were less than 0.2 μm in length, which suggests that the distal portion of the ciliary axoneme for these cells was very short or absent. In contrast, we only measured cilia that were at least 1 μm, as we presumed that such a length would be necessary to facilitate mechanosensation. Finally, these studies quantified primary cilia in osteocytes of cortical bone in rodents, while the focus of our study was trabecular bone. The role of the cilia as a mechanosensor may differ in these two tissues or between species, as the tissue strains and the distance of the osteocytes from the bone surface differ.

The length of cilia measured in this study are comparable to previous reports for cultured cells. Cilia ranged from 1-4 μm in cultured chondrocytes [39], 4-9 μm in MC3T3-E1 [16], and 2-4 μm in MLO-Y4 cells [40]. We found that the cillum length varied slightly across locations in the trabecular bone compartment that we analyzed. Although, cillum length was up to 7 μm in the marrow near the bone surface and less than 3.5 μm in the rest of the marrow, the average cillum length did not differ between the locations in the marrow. Short cilia in osteocytes may be explained by the spatial constraints of the pericellular space [41]. Similarly, in the marrow short cilia may be due to spatial restrictions in the intercellular space. Cells can alter the length of their primary cilia [42], which may also explain the small number of cilia greater than 1 μm in length. For example, in fresh tendon, the average length of cilia in tenocytes was 1.1 μm, but increased to 3.0 μm after 24 hours in media [29]. Interestingly, subsequent dynamic loading resulted in reduced cilia length similar to fresh tendon [29]. Thus, the short cilia observed in our study may reflect that the cells reside in a mechanically stimulated environment. Similarly, primary cilia increase their length in response to low intracellular calcium levels and vice versa, indicating that calcium levels may be high in the trabecular bone compartment [42].
Overall, this study reveals that primary cilia occur in a small fraction of cells in the trabecular bone and marrow. Our in situ data provides a link between in vitro data on primary cilia expression and how they are expressed in the native in vivo cellular environment. The low incidence of primary cilia may indicate that (1) cilia function as mechanosensors on a select number of cells or (2) cilia function in concert with other mechanosensing mechanisms. This study is the first to examine primary cilia incidence in the bone marrow microenvironment. Studying mechanosensors in their multicellular niche to explore mechanosensing is crucial in developing an understanding of mechanotransduction.
Fig. 1 (A) Ovine trabecular bone pores averaged approximately 0.5 mm across (scale = 200 μm).

Primary cilia stained by an acetylated α-tubulin antibody (green) were measured and quantified in four distinct locations of the trabecular bone compartment: (B) marrow space more than 50 μm from the bone surface, (C) marrow within 50 μm of the bone surface, in the bone lining cells, and (D) in osteocytes (scale = 20 μm).
Fig. 2 Primary and secondary antibodies were validated for sensitivity and specificity in ovine cells and tissues. (A) Ovine kidney, which is known to express primary cilia ubiquitously, was stained by both anti acetylated α-tubulin and by anti IFT88, two markers expressed in the primary cilium (scale = 20 μm). Nearly all cells exhibited cilia stained by both antibodies. The colocalization of staining validates the sensitivity of the antibody for cilia. (B) Primary cilia in trabecular bone were similarly stained by both anti acetylated α-tubulin and by IFT88 (scale = 20 μm). (C) Ovine kidney was immunostained with only secondary antibodies to ensure specificity.
for the primary antibodies (scale = 20 μm). (D) In cell culture, ovine bone marrow stromal cells cultured to passage three displayed primary cilia that were positive for acetylated α-tubulin (scale = 20 μm, N=3). (E) The number of primary cilia in the expanding cells varied across four days of culture (N=3).
Fig. 3 Positive acetylated α-tubulin stained cilia were counted in (A) the marrow, (B) bone lining cells, and (C) osteocytes (scale = 10 μm).
Fig. 4 Osteocytes and bone lining cells had a higher incidence of cilia expression (defined as cilia greater than 1 μm in length) than cells in the central marrow cavity more than 50 μm from the bone surface. Bars marked by the same letter are not statistically different (N=3; mean±STDEV; p<0.05, ANOVA with Tukey’s HSD).
Fig. 5 The average length of primary cilia was 1.49 μm in marrow cells away from the endosteal surface, 1.46 μm in marrow cells near the bone surface, 1.86 μm for the bone lining cells, and 1.65 μm for the osteocytes. Cilia were longer in osteocytes than in cells in the marrow near the bone surface (*p<0.05, Kruskal-Wallis). The box represents the upper and lower quartiles, the middle bar is the median, and whiskers span the 95th percentile. Circles represent outliers.
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