Primary Cilia are Expressed on a Small Fraction of Cells in Trabecular Bone and Marrow

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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) and osteoclasts derive from hematopoietic stem cells, both of which reside in trabecular bone marrow [1]. The marrow cell population is subjected to mechanical cues due to skeletal loading [2], and MSCs and osteoblasts exhibit altered gene expression in response to such stimuli [3]. Many mechanosensing pathways have been proposed as sensors of this mechanotransduction, including primary cilia [4, 5]. The primary cilia is a cell appendage organelle formed during the quiescent/G1 phase of the cell cycle. Primary cilia are expressed in vitro on human MSCs [6], MC3T3-E1 osteoblast-like cells and MLO-Y4 osteocyte-like cells [5]. Primary cilia inhibition in MSCs blocks osteogenic gene transcription [4], whereas abrogation in MC3T3-E1 osteoblasts or MLO-Y4 osteocyte-like cells attenuates normal osteogenic responses to in vitro fluid flow [4, 5]. in vivo, mice with an osteoblast- and osteocyte-specific knockout of Kif3a, a gene essential to primary cilia formation and function, exhibited decreased bone formation in response to loading compared to control mice [7]. As such, primary cilia appear to be a potent mechanosensor in bone cells and their progenitors.

While the mechanosensory capacity of primary cilia in vitro is well established, their role in vivo depends on their presence on cells in the bone or marrow that can affect the differentiation of bone cells. As such, their incidence in bone cells must be quantified in order to understand their function in vivo. However, there are conflicting reports on the incidence of primary cilia in bone [8, 9], and their incidence in the marrow has not been addressed. As such, the goal of this study was to quantify the frequency and distribution of primary cilia in the bone and marrow of the trabecular bone compartment. Specifically, we quantified the fraction of bone marrow cells, bone lining cells, and osteocytes expressing primary cilia in trabecular bone from the cervical vertebrae of young sheep.

Methods: Immunohistochemistry (IHC) was used to identify and count primary cilia in trabecular bone and marrow. Trabecular bone cores were obtained from the second cervical vertebra of three immature sheep, 6 to 8 months of age. The cores were fixed, demineralized, processed and paraffin embedded. Longitudinal sections with a thickness of 20 µm were cut using a microtome. Cilia were labeled by IHC using a primary anti-acetylated α-tubulin (Abcam) at 1/20 dilution, with a secondary Goat dylight 488 anti-mouse (Jackson ImmunoResearch) at 1/200 dilution. Nuclei were counterstained with propidium iodide (PI). The use of primary cilia antibodies was verified using positive controls: ovine kidney sections were immunostained using two established primary cilia antibodies, acetylated α-tubulin and intraflagellar transport protein 88 (IFT88), and bone marrow stromal were extracted from the trabecular bone marrow of ovine cervical vertebra and stained using acetylated α-tubulin. Slides were imaged on an inverted confocal laser scanning microscope (LSM 510; Zeiss, Germany) at low magnification (10X) and high magnification (63X) using a low scan speed (7 s), averaging 4 images. An Argon laser (488 nm) was used to excite Dylight 488 and a Neon laser (550 nm) was used to excite PI. Image stacks were acquired at 63x magnification, with 0.5 µm between images and a pixel size of 0.14 µm.

Primary cilia were quantified in four locations: in the marrow space (i) > 50 µm or (ii) within 50 µm of the trabecular bone surface, (iii) bone lining cells on the trabecular bone surface, and (iv) osteocytes in trabecular bone tissue (Fig. 1 A-D). Primary cilia were identified based on two criteria: (1) proximity to a cell nucleus (PI staining) and (2) staining of at least 1 µm in length. The number of cells in the measured marrow volume was determined using Automatic Nuclei Counter on ImageJ software (NIH), while the bone lining cells and cells in the trabecular bone tissue were counted directly.

Results: Primary cilia expression in trabecular bone and marrow were not common, but were observed in all locations that were analyzed (Fig. 1 E-G). In bone marrow, only 1% of cells expressed cilia, compared to about 4% in bone lining cells and osteocytes (p < 0.05; Fig. 2). In the marrow, cilia protruded into the intercellular space (Fig. 1E and 1F), and in the trabecular bone cilia appeared in the lacunae adjacent to osteocyte nuclei (Fig. 1G). The average lengths of primary cilia in the different locations were between 1 and 2 µm.

Discussion: Primary cilia have been implicated as important mechanosensors and chemosensors [10] in bone, but their presence and distribution in situ has not been fully quantified. This is the first report of primary cilia incidence in trabecular bone and the encompassed marrow space. The key contribution of this study was the determination of the number of primary cilia in the marrow space and bone tissue using a stringent technique, which ensured consistent and rigorous identification of primary cilia. Within the bone marrow compartment, primary cilia incidence was highest proximal to the trabecular bone edge surfaces
compared to the rest of the marrow space. The % of primary cilia was higher at a distance of 50 μm from the trabecular bone edge. In bone cells of the trabecular tissue it was found that primary cilia were present on approximately 3% of the cells.

Our finding that only a small fraction of osteocytes express primary cilia agrees with an earlier TEM study [9], but a more recent study found 94% of osteocytes in rat cortical bone expressed primary cilia [8]. Differences between these studies might be explained by the bone type, the use of a different species, or to the criteria applied to identify primary cilia. Within the marrow, MSCs are known to express primary cilia, but the percentage of cells expressing cilia was greater than the reported incidence of MSCs in marrow [11]. As such, marrow resident cells of other lineages are likely to express primary cilia as well, but we did not attempt to identify the individual cell types.

These findings provide insight that should help refine hypotheses related to the role of primary cilia in the trabecular bone compartment. The small fraction of osteocytes expressing cilia indicates that either osteocytes do not require cilia as a mechanosensor or chemosensor, or that a small number of them act as mechanosensors via primary cilia, possibly dependent on the local mechanical environment. Indeed, a conditional knockout mouse model demonstrates that cilia contribute to bone adaptation, but are not necessary [7]. This subset of osteocytes might, therefore, sense specific stimuli in certain regions, e.g. regions of high fluid flow, and transmit the resultant signals through neighboring progenitor cells and the osteocyte syncytium.

Taken together, these findings provide a novel insight into the function of primary cilia in the trabecular bone compartment.

**Significance:** The primary cilium is a potent mechanical and chemical sensor in cells of the bone lineage in tissue culture. Hence, knowing the location and incidence of cells that express cilia in the skeleton is crucial to understand their role in adaptive bone remodeling.

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Figure 1: (A) Cells in the trabecular compartment were imaged by confocal microscopy (scale=200 μm). Primary cilia were identified by anti-acetylated α-tubulin conjugated to green dylite in four regions: (B) the marrow > 50 μm from the bone, (C) cells < 50 μm from the bone wall, including bone lining cells, and (D) osteocytes in the bone tissue (scale = 20 μm). Positive acetylated α-tubulin staining was observed in the marrow (E), bone lining cells (F), and in osteocytes (G) (scale = 10 μm).
Figure 2. Percentage of cells with primary cilia. Different letters indicate groups that are significantly different (N=3 animals; mean±STDEV; p<0.05, ANOVA with Tukey's HSD).

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