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Chondrocyte primary cilia shorten in response to osmotic challenge and are sites for endocytosis

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SUMMARY

Objective: The purpose of this study was to examine the influence of cartilage site and osmolarity on primary cilia incidence, length and orientation in live chondrocytes in undisturbed cartilage. Additionally, we imaged endocytotic markers to test our hypothesis that the ciliary pocket is a site for endocytosis.

Materials and methods: We measured primary cilia incidence, length and orientation in the coronal plane using *ex vivo* live cell confocal imaging of intact murine femoral chondrocytes. Measurements were taken from five regions of the medial and lateral condyles of the left and right femur and also after one minute of osmotic challenge. Transmission electron microscopy and immunocytochemistry were used to characterize the orientation and position of chondrocyte primary cilia in the saggital plane and to determine the colocalization of clathrin coated vesicles, endosomal and lysosomal proteins and CD44 with the ciliary pocket.

Results: Chondrocyte primary cilia length decreased significantly after a one minute hypo- or hyper-osmotic challenge and varied between condyles and across the surface of each condyle. The majority of the length of the chondrocyte primary cilia was positioned within a membranous invagination rather than projecting out from the cell membrane and clathrin coated vesicles, endosomal proteins and CD44 colocalised with the ciliary pocket.

Conclusions: We demonstrate that live *ex vivo* chondrocyte primary cilia are capable of shortening within minutes in response to osmotic challenge and provide subcellular and cellular evidence that chondrocyte primary cilia are deeply invaginated in a ciliary pocket which contains sites for endocytosis.

Introduction

Primary cilia have been identified in almost all vertebrate cells[1,2]. They are immotile consisting of a 9 + 0 arrangement of microtubules, lacking the inner doublet and corresponding molecular motors necessary for motility. Microtubules themselves are polymers of α and β tubulin with both a (+) and a (−) end[3,4]. Microtubules elongate and resorb from the (+) end and therefore primary cilia shortening occurs from the distal tip in a complex process involving intraflagellar transport[3,4].

Electron microscopy studies have shown that primary cilia often reside in invaginations of the plasma membrane called the ciliary pocket[5–9]. In early work, clathrin coated pits and vesicles were frequently reported along the membrane of the ciliary pocket and at the ciliary base suggesting a potential involvement in receptor mediated endocytosis[5,6]. Most recently it has been shown that an actin-cilium interface is able to modulate the local environment of the ciliary pocket[7,8]. The ciliary pocket-primary cilia complex is a site of endocytosis as well as primary cilia based signal transduction pathways in synoviocytes[8,10].

In addition to their role in endocytosis, primary cilia may play a critical role in signal transduction. Numerous cell signalling and cell adhesion proteins have been found on chondrocyte primary cilia including the integrin subunits α2, α3, and β1[11], the mechano-sensitive ATP release channel, Connexin 43[12] and the osmotically sensitive cation channel transient receptor potential vanilloid 4 (TRPV4)[13]. Together, these findings indicate that the chondrocyte is able to respond to changes in the extracellular matrix by utilizing proteins and signalling molecules associated with the primary cilium.

Most recently it has been shown that compression produces a reversible reduction of both primary cilia length and incidence in chondrocytes[14]. The presence of TRPV4 along the length of the primary cilia[13] together with the potentially heightened sensitivity

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of the cilium to fluctuations in osmotic pressure due to its 4,000 fold smaller volume relative to the cell body[5] may suggest that primary cilia are involved in the sensing and transduction of osmotic as well as mechanical challenges to chondrocytes. Chondrocytes experience fluctuations in their osmotic environment during cartilage compression and relaxation as water is extruded and then imbibed into the negatively charged proteoglycan rich extracellular matrix[15,16]. Additionally, during osteoarthritis the cartilage matrix loses proteoglycan molecules and the surface integrity is compromised leading to tissue swelling and a chronic decrease in interstitial osmolarity[17]. It is currently unknown if the incidence and length of chondrocyte primary cilia are modulated by osmotic challenge.

Chondrocyte primary cilia incidence and length have been reported extensively in the literature and have been shown to vary greatly with species, cartilage surface, and tissue preparation (explants, agarose constructs or culture). Early studies on equine and murine chondrocyte primary cilia of the femoral condyle found that virtually all chondrocytes have at least one cilium[18]. Later studies on bovine patella explants found a primary cilia incidence of 46%[19] and reported primary cilia lengths ranging from 1.1 μm in the superficial layer of cartilage to 1.5 μm in the deep layer[19]. Compared to chondrocytes in explants, chondrocytes in culture tend to have longer primary cilia (1.3–2.2 μm) but a lower primary cilia incidence (26–28%)[14,20].

The disparity between *in situ* and *in vitro* measurements of chondrocyte primary cilia incidence and length underlines the importance of the extracellular matrix in influencing these attributes. The explant studies in the literature have machined the subchondral bone[18,19] perhaps disrupting the anchoring of the collagen fibrillar network into the bone[21] and thus altering the pre-stressed conditions experienced by the chondrocytes in the proteoglycan rich matrix[22]. These properties of the extracellular matrix and the interconnectedness of the primary cilia with it *via* integrin molecules[11], necessitate the utilization of whole cartilage-bone constructs in these studies. To date chondrocyte primary cilia incidence and length have not been measured in fully intact and undisturbed cartilage.

In addition to sample preparation, the animal species and/or the specific joint surface from which the cartilage was harvested may influence chondrocyte primary cilia. It is well established that cartilage and chondrocyte properties vary significantly between different joints of the same body and even across different surfaces of the same joint. For example, even under identical load magnitudes the cartilages of the patellofermoal joint differ in histological, material and compositional properties and their chondrocytes in anabolic and catabolic metabolism[23]. In addition, cartilage thickness and collagen fibre split line orientation have been shown to vary significantly across the articulating surfaces of the femur[24]. To date, the influence of site across the same cartilage surface on primary cilia incidence, length and orientation has not been measured.

The purpose of this study was to characterize primary cilia in femoral chondrocytes *in situ* using transmission electron microscopy and immunocytochemistry and *ex vivo* using live cell confocal imaging. For the first time, we examine the influence of cartilage site and osmolarity on primary cilia incidence, length and orientation in live chondrocytes on the condyles of intact murine femora. We hypothesised that primary cilia incidence and length would be greater on the more central, constantly loaded regions of the cartilage compared to peripheral intermittently loaded regions and that changes in osmolarity would also influence primary cilia incidence and length. Additionally, we imaged endocytotic markers at the cellular and subcellular level to test our hypothesis that the ciliary pocket is a site for endocytosis.

Materials and methods

Animals

All animal procedures were approved by the University of Calgary Animal Care Committee. Skeletally mature (age = 4.0 ± 0.5 months, mass = 26 ± 12 g (mean ± sd)) female Balb/C mice were euthanized and the femora were isolated and placed in fixative for electron microscopy and immunocytochemistry or iso-osmotic (300mOsm) Dulbecco's Modified Eagle Medium (DMEM) without phenol red (Invitrogen, Burlington, ON) for live cell confocal imaging.

Transmission electron microscopy

Femora were fixed in 3% glutaraldehyde in Millonigs buffer (300mOsm) at room temperature overnight prior to post fixation in 2% osmium tetroxide for 2 hours. Samples were dehydrated in ethanol and then infiltrated with Polybed 812 resin (Polysciences, Warrington, PA). Polymerization was performed at 37°C for 24 hours. Silver grey sections were cut with a diamond knife on an ultra microtome (Leica, Concorde, ON). Sections were stained with uranyl acetate and lead citrate and examined in a Hitachi H7650 electron microscope.

Immunocytochemistry

Femora were fixed in 4% paraformaldehyde, decalcified (CalEX, Fisher Scientific, Ottawa, ON) and then submerged in 25% sucrose overnight. Samples were embedded in optimal cutting temperature compound and flash frozen. Sagittal sections (12 μm) were cut, washed (phosphate buffered saline (PBS)) and blocked (normal goat serum and Triton X (Sigma, Oakville, ON)) before going through sequential wash (PBS and Tween 20 (Sigma, Oakville, ON)) and antibody application steps. Primary antibodies included acetylated α-tubulin (clone 6-11B-1, 1:500, Sigma, Oakville, ON), early endosome antigen-1 (EEA-1) (1:200, Abcam, Cambridge, MA), Lysosomal-associated membrane protein 1 (LAMP-1-FITC conjugated) ([1D4B], 1:10, Abcam, Cambridge, MA) and CD44 (1:10, Abcam, Cambridge, MA). Secondary antibodies included Alexa Fluor® 488 and Alexa Fluor® 647 (Invitrogen, Burlington, ON). Finally, the nucleic acid stain Hoescht 33342 (0.1 mM, Invitrogen, Burlington, ON) was applied to all sections. After staining, sections were mounted in ProLong® Gold (Invitrogen, Burlington, ON) and coverslipped.

Slides were imaged using an oil immersion lens (40×, 1.4 N.A.) on an LSM 7 DUO (Carl Zeiss Canada Ltd., Toronto, ON) confocal microscope in either differential interference contrast (DIC), channel or Lambda scanning confocal modes. In the channel scanning confocal mode dyes were sequentially scanned; Hoescht (excitation 405 nm, emission 429–684 nm) and Alexa Fluor® 647 (excitation 633 nm, emission 638–755 nm). At least 30 cells located in each of the superficial zone, middle zone, calcified cartilage region or within a chondron were identified. In Lambda scanning confocal mode, sections stained with either Hoescht, Alexa Fluor® 488 or Alexa Fluor® 647 were excited and the resulting emission spectra recorded across 8.8 nm bands from 411–692 nm. An additional autofluorescence curve was recorded from an unstained section simultaneously excited at 405 nm, 488 nm and 633 nm. On a triple stained section simultaneously excited at all three wavelengths a cell of interest was scanned and the resulting emission spectra linearly unmixed using the prerecorded emission curves.

Live cell confocal imaging

Chondrocytes of the intact femora were incubated with 125 nM Tubulin Tracker (Invitrogen, Burlington, ON) to stain polymerized