INTRODUCTION: Primary cilia are ubiquitous to the surface of nearly all vertebrate cells as a microtubule-based appendage with a 9+0 axonemal symmetry. While primary cilia have been identified on chondrocytes for over 40 years, little is understood of the role of these non-motile cell surface extensions. Past studies identified the function of primary cilia as flow sensors in renal tubule epithelial cells. A recent study on the primary cilia of bone cells has provided evidence these cell surface extensions, though not actively motile, may deflect during dynamic fluid flow in a manner of mechanosensation independent of calcium intake. Based on these studies, we hypothesize that primary cilia are involved in mechano-responsiveness of hypertrophic chondrocytes. It has been shown that increased local strain from cyclic loading in a three dimensional chondrocyte culture results in increased expression of type X collagen (Col X), a marker for hypertrophic chondrocytes. Treatment of cells with chloral hydrate is one of the few chemical methods to remove primary cilia from cell surface. In this report, we look to examine the role of primary cilia in hypertrophic chondrocytes by mechanically testing the cells whose cilia are removed by chloral hydrate treatment.

METHODS: Cell Culture and Isolation: Primary chick embryonic chondrocytes were isolated from the cephalic part of 17d embryonic chick sternal cartilage and grown in monolayer on culture slides. 

Abrogation of Primary Cilia: To remove the primary cilia from the chondrocytes, the cells were treated for 72 hours with 4mM chloral hydrate (Spectrum Laboratory Products) in F12 medium and then placed in fresh medium for another 24 hours before fixation, both steps at 37°C. Controls were incubated in F12 medium without added chloral hydrate.

Immunocytochemistry: The cells were fixed in paraformaldehyde and incubated with a primary antibody of anti-acetylated α-tubulin (1:500, Sigma) overnight at 4°C. The secondary antibody used was tetramethyl rhodamine isothiocyanate (TRITC)-conjugated donkey anti-mouse IgG (1:200, Jackson ImmunoResearch) incubated at room temperature for 2 hours, and the cells were then stained and mounted with VectorShield containing DAPI (Vector Laboratories). Negative controls were incubated in PBS without the primary antibody.

3D Culture with the Application of Cyclic Mechanical Stimulation: The cells were seeded to Gelfoam collagen sponges, and experimental groups were treated with 4mM chloral hydrate for 72 hours. The medium was then changed and the cells were stretched for 24 hours in fresh F12 medium with 5% cyclic load applied by the computer-controlled BioStretch system (iCT Tech, Canada). Five percent strain was chosen because it is thought to be comparable to the amount of matrix deformation of growth plate cartilage in vivo. 

Real Time RT-PCR: Sponges were removed at 24 hours, and RT-PCR was performed to determine relative mRNA levels of Col X. Total RNA was isolated using the RNAqueous-4PCR kit (Ambion, Austin, TX). Reverse transcription and PCR amplification was performed using iScript cDNA Synthesis kit (Bio-Rad Laboratories). Quantitative real time PCR was performed on a DNA Engine Opticon Real-Time Detection System, using the QuantiTect SYBR green PCR kit (Qiagen). Samples were loaded in triplicate and signal levels were normalized to 18S rRNA.

RESULTS: After treatment of hypertrophic chondrocytes with chloral hydrate, immunocytochemistry was performed to determine the drug’s effect on primary cilia. We used a monoclonal antibody against acetylated α-tubulin, a major component of primary cilia. Often the chondrocytes showed a region of increased signal intensity at one edge of the cell which was then scored as a primary cilium since the cilium itself as well as its anchoring centrosome both contain acetylated α-tubulin (Fig 1A). When counting cells using this method, 44.6% of control chondrocytes displayed primary cilium, while 0% of chloral hydrate treated cells displayed any primary cilium (Fig 1C). Next we quantified cells with a distinct projection of immuno-fluorescent signal intensity extending into extracellular space (Fig. 1B). 2.2% of control chondrocytes contained cilia extending into extracellular space, while 0% of chloral hydrate treated chondrocytes contained such extended cilia (Fig. 1D). Thus, by both criteria, chloral hydrate treatment completely removed primary cilium from hypertrophic chondrocytes. To test whether such chondrocytes devoid of any primary cilia still respond to mechanical signals, we cultured these chondrocytes in a three dimensional collagen sponge. After applying cyclic mechanical loading, we performed real-time RT-PCR to determine the mRNA level of Col X. Under non-loading conditions, chloral hydrate treatment did not affect Col X mRNA level significantly (Fig 2). Thus, chloral hydrate by itself did not affect Col X mRNA level. Under loading conditions, the Col X mRNA level in control chondrocytes increased 3.2 fold while that in chloral hydrate treated cells only increased 2 fold (Fig. 2). Thus, chemical removal of primary cilia reduced but did not eliminate mechanical stimulation of Col X mRNA.

DISCUSSION: The use of chloral hydrate was an effective method to remove the primary cilia from hypertrophic chondrocytes, as visualized by the complete removal of cilia using two different characterizing criteria during immunocytochemistry. The drug caused an increase in cell size as well as disruption of the cytoskeleton, leading to increased cytoplasmic staining and removal of cilia. The drug has a known inhibitive effect on mitosis, namely by affecting the spindle apparatus, which may explain a decreased number of chondrocytes in the drug-treated group. However, it has no side-effect on Col X gene expression as shown by real-time RT-PCR data. Surprisingly, the complete removal of primary cilia from hypertrophic chondrocytes did not eliminate their mechano-responsiveness completely, as the mechanical stimulation of Col X mRNA level was reduced but not abolished in cilium-free chondrocytes. From the data, we conclude that the primary cilium of chondrocytes have a role in sensing changes in the mechanical environment of the cell which leads to increased expression of mechanosensitive genes, and that other mechanosensing pathways may exist in hypertrophic chondrocytes in addition to primary cilia.