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Primary Cilia Modulate IHH Signal Transduction in Response to Hydrostatic Loading of Growth Plate Chondrocytes

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Abstract

Indian Hedgehog (Ihh) is a key component of the regulatory apparatus governing chondrocyte proliferation and differentiation in the growth plate. Recent studies have demonstrated that the primary cilium is the site of Ihh signaling within the cell, and that primary cilia are essential for bone and cartilage formation. Primary cilia are also postulated to act as mechanosensory organelles that transduce mechanical forces acting on the cell into biological signals.

In this study, we used a hydrostatic compression system to examine Ihh signal transduction under the influence of mechanical load. Our results demonstrate that hydrostatic compression increased both Ihh gene expression and Ihh-responsive Gli-luciferase activity. These increases were aborted by disrupting the primary cilia structure with chloral hydrate.

These results suggest that growth plate chondrocytes respond to hydrostatic loading by increasing Ihh signaling, and that the primary cilium is required for this mechano-biological signal transduction to occur.

Keywords

Growth plate chondrocyte; hydrostatic compression; primary cilium; Indian hedgehog

Introduction

Many studies of mechanical loading of cartilage and bone tissue have demonstrated clear effects at the level of gene expression, protein translation, extracellular matrix production, and in membrane transport processes [1–5]. These studies also suggest that there are multiple cellular pathways capable of responding to the physical stimulation resulting from mechanical forces.

It has been well-established that the hedgehog (Hh) family plays an important role not only during chondrogenesis and limb formation, but also during longitudinal growth at the growth plate [6–7]. Loss of Indian hedgehog in skeletal tissues results in severe dwarfism

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due to reduced proliferation and abnormal maturation of growth plate chondrocytes. When Ihh is over-expressed in mice, chondrocyte proliferation is enhanced [8–9]. Smoothened (Smo) is one of the two hedgehog receptor proteins involved in the Ihh signal transduction pathway. Binding of Ihh to its receptor Patched (Ptc) allows Smo to initiate the signaling cascade that leads to activation of the Cubitus interrupts (Ci) transcription factor family members Gli1, Gli2, and Gli3 [10–11].

Primary cilia have a unique hair-like structure, and act as a cellular sensory organelle. The intraflagellar transport (IFT) complex is required for ciliary function, which occurs through a complex of multi-subunit proteins resulting in transfer of precursors back and forth from the flagellar tip to the cell body. Recent studies have shown that a key step in Ihh activation occurs when Smo moves to the tip of the primary cilium [12–14], a translocation process that can be disrupted by the Smo antagonist cyclopamine [13–14]. Studies in mice have shown that mutations that cause IFT dysfunction result in the loss of the primary cilia, abnormal Ihh signaling, defects in limb growth and bone formation [15–19].

Primary cilia were originally linked to mechano-transduction in kidney epithelial cells, in which the cilia detect urine flow and transduce this fluid-flow signal into a transient intracellular calcium signal, resulting in increased cell proliferation. Other studies have shown that chloral hydrate treatment can abolish this cilium-dependent flow-induced Ca^{++} signaling event in different cell types [20–21]. Furthermore, studies of a core component of the IFT, the Tg737 gene, reveal that mice with a mutated Tg737 gene have shorter primary cilia, are unable to mount a fluid flow response, and develop unregulated cell proliferation and cyst formation (polycystic kidney disease) [22–23].

It has long been known that cartilage is sensitive to mechanical forces; however, no specific cellular mechano-transduction signaling pathway has been discovered in chondrocytes. In this study, we used our previously described [24] chondrocyte cell pellet model subjected to a hydrostatic compression loading system to determine if primary cilia transduce mechanical forces into biological signals in growth plate chondrocytes.

Materials and Methods

Epiphyseal chondrocyte culture

Primary epiphyseal chondrocytes were isolated from two day-old Sprague Dawley rats (Harlan, Indianpolis, IN) as previously described [24]. Animal use was according to a protocol approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic. The primary cells were cultured either as high-density monolayers (6.6×10^5 cells/cm^2) or as three-dimensional cell pellets (3×10^5 cells/pellet) in DMEM/F12 medium (Invitrogen, Carlsbad, CA) supplemented with 50 μg/ml L-ascorbic acid phosphate, 100 μg/ml sodium pyruvate, 1% penicillin-streptomycin, and ITS+ premix (Sigma Chemical, St. Louis, MO) to a final concentration of 0.625 μg/ml bovine insulin, 6.25 μg/ml transferrin, 6.25 ng/ml selenous acid, 1.25 mg/ml BSA, and 5.35 μg/ml linoleic acid. Primary chondrocyte pellets were cultured for five days before being subjected to hydrostatic compression, with or without treatment with recombinant sonic hedgehog (Shh 5 nM, R & D System, Minneapolis, MN) or chloral hydrate (4 mM, Sigma) as described below. The control samples were cultured unloaded and without any pharmacological treatment.

Hydrostatic compression

A custom-designed mechanical loading system (Figure 1) was used to apply an intermittent 1 MPa hydrostatic compression force (one hour on, one hour off). The hydrostatic loading system consists of two separate compartments, a sterile bioreactor chamber which houses the chondrocyte pellets (up to 9 pellets at a time) and a non-sterile hydraulic section of the

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system. The two sections were separated by a gas-permeable flexible fluoro-ethylene-propylene membrane (McMaster-Carr, Cleveland OH). Computer-controlled tandem high-speed micro-gear pumps (Micropump, IDEX Health & Science, Oak Harbor, WA) force water (maintained at 37 °C and equilibrated with 7.5% CO_2 in air) through the hydraulic section thereby generating hydrostatic pressures inside the bioreactor. Heat- and gas-exchange occurs through the membrane. The pressure in the chamber is monitored by a compensated transducer, and controlled in real time by modulating pump speed and a water flow outlet restrictor. Automatic control was achieved by using custom-written C software running on an IBM PC. Half the culture medium was replaced every 12 hours during the unloaded interval. At the end of the compression period, the pellets (loaded and unloaded control) were collected and terminal assays performed.

Cell proliferation

The cell proliferation assay was carried out immediately after the end of the compression period. Cell viability was evaluated using the CellTiter 96 Aqueous One assay kit following the manufacturer's protocol (Promega, Madison, WI). The reagent contains both a tetrazolium compound (MTS) and phenazine ethosulfate (PES), an electron coupling reagent. In metabolically active cells, reduced MTS is a soluble and colored formazan product, which is quantified by measuring the absorbance at 490 nm, and is directly proportional to the number of living cells.

Gene expression

To investigate the effect of hydrostatic loading on chondrocyte gene expression, total RNA was isolated from the cells at designed time points using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Total RNA was reverse transcribed using a SuperScript III Kit (Invitrogen, Carlsbad, CA), and quantitative real-time PCR was performed to measure Ihh and Smo gene expression. The expression of 18s RNA was used for normalization. An ABI prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) program was used for all measurements of minimum threshold cycle (Ct), and the differences in gene expression between control and treated sample were calculated using the manufacturer's protocol. The PCR primers were designed using the Primer Express Software (Applied Biosystems):

Gene	Primer pair
Indian Hedgehog	5'-TGCCGACCGCCTCATG 5'-CATGACAGAGATGGCCAGTGA
Smoothened	5'-TTCTCTAAGCGGCGTGAACTG 5'-AAACCGGCAACAGGTCCAT

Gli-luciferase reporter assay

Ihh signaling was examined by measuring the activity of Gli, a downstream transcription factor in the Ihh pathway. Approximately 2×10^6 cells per 60 mm plate were transiently co-transfected overnight with an Ihh-responsive Gli-luciferase reporter plasmid and a Renilla luciferase control reporter plasmid (Promega) using Fugene 6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol. The Gli-luciferase reporter was kindly provided by Dr. Jeremy Reiter, University of California [19] and contains an enhancer with 8 repeating sequences which include a Shh response element and a Gli binding site. The day after transfection, the cells were collected, counted, and then cultured as a three-dimensional cell pellet (3×10^5 cells/pellet) for four days. The chondrocyte cell

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pellets were then subjected to hydrostatic compression loading as described above for two days prior to measurement of luciferase activity (Dual luciferase assay, Promega). Luciferase reporter activity (n = 9, repeated twice) was normalized for transfection efficiency using the Renilla luciferase activity. Gli-reporter activity was also measured following hydrostatic compression, but in the presence of either the hedgehog antagonist cyclopamine [13–14] (10 μM, Logan Natural Products, Plano, TX), or the primary cilia destabilizing chloral hydrate [20–21] (4 mM) during the compression period.

Immunostaining

Primary chondrocytes were seeded on cover slips at 1.5×10^5 cells/200 μl/cover slip in 200 μl of DMEM/F12, and cultured as monolayer for 4 days prior to a treatment with or without chloral hydrate (4 mM) for another two days. The cells were then fixed in 4% paraformaldehyde at room temperature for 10 minutes. After washing with PBS, the cells were incubated overnight at 4 °C with an antibody against acetylated-α-tubulin (Invitrogen 32–2700, Carlsbad, CA) diluted 1:20 in PBS with 1% normal donkey serum and 0.1% saponin (Sigma Chemical Co, St. Louis, MO). On the following day the cells were washed, and incubated with a tetramethyl rhodamine-coupled goat anti-mouse secondary antibody (715-026-151, Jackson ImmunoResearch, West Gove, PA), diluted 1:100 in the same dilution buffer as the primary antibody for 1 hour at room temperature. The sections were then mounted in Vectashield® mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Images were captured using a fluorescence microscope with a 63 × oil lens (Spectral Laser Scanning confocal microscope, Leica Microsystems, GmbH, Wetzlar, Germany). The length of cilia in control and chloral hydrate treated cells was measured using the Image J software package [25].

Immunoblotting

Monolayer cells were cultured for five days and with or without chloral hydrate treatment; the protein lysates were collected in RIPA buffer with Mini complete protease inhibitor (Roche) and the protein concentration determined using the Dc protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of protein (60 µg) were separated on 9% SDS-PAGE gels, and transferred onto nitrocellulose membranes (n = 4, repeated twice). The membranes were probed with antibodies to Ihh (1:50 dilution, Santa Cruz Biotechnology sc-1196, Santa Cruz, CA) or Smo (1:100 dilution, MBL International Corporation, Woburn, MA, LS-A2666) in 5% non-fat dry milk in TBS-T overnight, and subsequently incubated with horseradish peroxidase-conjugated secondary antibody (1:500 dilution, Santa Cruz) for one hour prior to detection using a chemiluminescent detection system (Santa Cruz). The protein loading was normalized to the immunoreactivity level of actin (1:400, sc-8432, Santa Cruz). The Kodak 1D image analysis software (Eastman Kodak Company, Rochester, NY) was used for densitometric quantitation.

Statistical analysis

All data are presented as mean ± SD. Statistical analyses were performed using either the Student's *t*-test, or analysis of variance, as appropriate. Results were considered statistically different at $p < 0.05$.

Results

Effects of hydrostatic compression on cell proliferation and Ihh gene signaling

Growth plate chondrocyte pellets subjected to hydrostatic compression forces showed a transient increase in cell proliferation after 24 hours of compression before returning to

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control levels by 48 hours (Figure 2), but there was no change in Caspase activity on both day (data not shown).

Quantitative RT-PCR analysis demonstrated that the expression of Ihh was increased more than 7-fold over the unloaded control after one day of compression, and then further increased to 20-fold over control after two days of compression.

To further investigate the time-course of this marked increase in Ihh gene expression in response to hydrostatic compression, the experiments were repeated at shorter intervals of 0.5, 1, 2, and 4 hours after the initiation of loading. Ihh expression was found to increase significantly as early as 30 minutes after the onset of hydrostatic compression (Figure 3B).

To study the effect of hydrostatic loading on downstream activity of the Ihh signaling pathway, we measured Gli-luciferase activity after two days of hydrostatic compression. This resulted in a more than 3-fold increase Gli reporter activity (Figure 4A). This activity was blocked by the addition of either 10 nM cyclopamine or 4 mM chloral hydrate during the hydrostatic loading.

A similar pattern was observed when cell pellets were treated with 5 nM sonic hedgehog (Shh). In this case, the Gli-luciferase activity increased 19-fold (Figure 4B), and this induced activity was also completely inhibited by the addition of 10 nM cyclopamine. This suggests that the same pathway was involved in both two experiments.

Disrupting primary cilia structure inhibits compression-induced Ihh signaling

To confirm the disruptive effects of chloral hydrate on cilia structure in epiphyseal chondrocytes, immunohistochemical staining against acetylated-α-tubulin was preformed. Untreated primary chondrocytes grown in monolayer had long, smoothly curved ciliary structures (Figure 5A), while the cilia in chloral hydrate-treated cells appeared shorter, curled, and deformed (Figure 5B). The length of cilia from normal cells was 5.05 ± 2.08 μm, and after 2 days of treated with chloral hydrate cilia treatment, cilia were 2.28 ± 0.30 μm in monolayer culture system.

In addition to examining the effects of this disturbed ciliary structure on Ihh and Smo expression in the absence of loading, quantitative RT-PCR showed that chloral hydrate treatment reduced Ihh expression to 57% of control and decreased Smo expression to 44% of control (Figure 6A). A similar effect was shown at the protein level by immunoblotting. Immunoreactive Ihh protein decreased to 61% of control levels in the chloral hydrate-treated cells, while Smo levels were reduced to 73% of control (Figure 6B).

Discussion

The Hueter-Volkmann principle of physeal growth holds that compressive forces across the growth plate inhibit physeal growth, while tensile forces (or release of compressive forces) accelerate physeal growth. Although this principle was first articulated more than 150 years ago [26], the cellular and molecular mechanisms underlying this phenomenon have not yet been elucidated. Mechanical compression can reduce longitudinal growth; experimental data have shown that in extreme cases, for example if the growth plate was stapled, it became irreversible disrupted [27–28]. It should be noted that the Hueter-Volkmann principle was formulated based on a continuous compressive or tensile loading environment, as opposed to the intermittent loading found in nature or even the hydrostatic loading system used in these experiment. Thus, the relationship between loading and growing is likely much more complex. A theoretical cartilage growth force response curve was proposed by Frost [29], it suggests that with no mechanical stimulus, growth stay at basal rate. Mild tension and

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compression increase growth, while large compression forces can stop the growth quickly. In this study, proliferation was increased transiently but statistically significantly by compression on the first day and returned to normal on the second day (Figure 2); however the nature of this change still remains unclear. This differs from other studies of compression [30]. It should however be noted that the MTS assay we used to measure this response is sensitive to cellular metabolic activity, thus the increase in the AQ96 assay could be related to a transient increase in metabolic activity.

Many studies have investigated the relationship between mechanical loading and biological signal-transduction in growth plate chondrocytes. Animal models in which growth was restricted using a wire loop [31–32], physeal stapling [33], or overloading the growth plate using weighted backpacks [34] all demonstrate that the growth plate is biologically responsive to changes in physical forces. *In vitro* loading experiments using chondrocytes seeded in scaffolds, such as agarose, alginate, poly (L-lactide-co-ε-caprolactone (PLCL), and gelfoam all indicate that compression-induced changes in the chondrocyte's environment modulates the synthesis of extracellular matrix components at the mRNA and protein levels [3–4, 35–38].

In this *in vitro* study, primary growth plate chondrocytes were cultured as an aggregated cell pellet with maximum cell density in all three dimensions, which is how growth plate chondrocytes are organized *in vivo* [24]. We have previously shown that this pellet culture system allows cells to terminally differentiate into hypertrophic chondrocytes that express high levels of alkaline phosphatase and type X collagen [39]. We used our custom-designed loading system to apply an intermittent hydrostatic compression force on these cell pellets, which resulted a markedly increased Ihh expression. As a consequence of this stimulation, Gli-luciferase reporter activity downstream in the Ihh signaling pathway was also increased. This effect was abolished by cyclopamine treatment, indicating that hydrostatic compression stimulates the Ihh pathway directly. Cyclopamine inhibits Ihh signaling by direct binding to Smoothened at its heptahelical domain [14]. Therefore it is not surprising that we observed that 2 days cyclopamine treatment completely inhibited Gli-luciferase activity, but only partially reduced Ihh expression (data no shown). We also observed that despite in presence of Shh, chloral hydrate treatment reduced Ihh expression; this partial loss of Ihh expression implies that variables other than Smo, cyclopamine, chloral hydrate treatment and mechanical loading can alter Ihh expression.

Ihh serves an important role in cartilage development. In experimental studies of Ihh as a mechnotransduction mediator in the mandibular condylar cartilage, Tang *et al*. showed that Ihh expression, and the number of proliferating cells were significantly increased on day 3 and day 7 of mandibular advancement [40]. Wu and his colleagues have reported that mechanical stress stimulated chondrocyte proliferation and Ihh expression [41] in a three-dimensional collagen sponge scaffold system. However, the nature of this loading model makes it difficult to resolve the type of mechanical load experienced by cell within the sponge.

There is increasing evidence that the primary cilium is involved and acts as a signaling mediator in the hedgehog family pathway [42–44]. Hh pathway activation is mediated by the receptors Patched (Ptc) and Smoothened (Smo). Singla and Reiter have proposed a dynamic model for Hh signal transduction [45], that suggests that in the presence of Hh, Smo was released by Ptc, then moves to the ciliary tip where Gli activator forms and moves down the cilium to turn on Hh dependent genes. Experimental data have shown that Ptc [10], Smo [19], and Gli [46] expression in cilia were affected by Shh treatment. Furthermore failure to form a functional primary cilium inhibits chondrocyte differentiation, and results in delayed chondrocyte hypertrophy in the growth plate. Thus, Koyama *et al.* [47] reported

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that mice deficient in the kif3a gene, which encodes an essential component of the primary cilium, had rare detectable cilia and a disorganized growth-plate pattern. In their study, the expression of genes encoding Ihh, collagen type X, VEGFA, MMP-13 and osterix-7 were barely detectable. Orpk mice containing mutations in the Tg737 gene, which encodes a protein required for cilium assembly, also have skeletal patterning and growth defects associated with significantly shorter primary cilia in chondrocytes [17]. More recent work has shown that Ellis-van Creveld (Evc and Evc2) gene products co-localized at the basal body of primary cilia and on the ciliary membrane, and are essential for Ihh signaling in the growth plate cartilage [48–49]. Mice lacking Evc had short limbs, ribs, and dental abnormalities. Although, the *in situ* data in this study showed normal expression of Ihh, the mRNA levels of downstream targets patch1, Gli1, and Pthrp were diminished. As the ciliary structure did not reveal any abnormalities, the authors suggested that Evc is a specific modifier of Hh signal transduction.

In chondrocytes, two major bending patterns of the primary cilia have been described by Jensen *et al.* in a shear stress associated model [50]. Normal primary cilia structure appeared either straight or smoothly curved, while deformed cilia were short or showed acute bends. The author suggested that, in chondrocytes, the primary cilia acted as a potential mechanosensor in skeletal patterning and growth. Our immunofluorescent staining for acetylated α-tubulin showed that chloral hydrate disturbed chondrocyte ciliary structure. The cilia were short and sharply bent or curled in the chloral hydrate-treated cells. Following withdrawal of chloral hydrate for 2 days, the appearance of the cilia recovered, approximating that of the untreated control cells. Our data indicate that primary cilia structures are functionally linked to the Ihh pathway in growth plate chondrocytes and that primary cilium function can be disrupted by chloral hydrate in these cells. Although chloral hydrate exposure affected the structure of the cilia, it otherwise did not have a notable effect on cellular morphology, which has been observed in other studies [21, 51]. We have also noticed that cilia length in cell differ between monolayer *vs* pellet cells, but those cells had similar response to chloral hydrate treatment (data not shown), and further, more detailed studies of cilia structure are needed. In a chondrocyte/agarose compression model, McGlashan *et al* [52] have observed that mechanical loading reduced cilia incidence and length, and believed that primary cilium length was an adaptive mechanism in response to mechanical loads, since cilia length and incidence are varied with location in cartilage [53]. In addition, a 48 hour structure study by Kennedy [54] showed that chloral hydrate treatment caused ciliary filament breakdown at junction of the cilium with the basal body. The electron microscope revealed 5mM chloral hydrate caused the filament breakdown while cells and all the basic component of the kinetosome appeared to be normal in structure; but after 44–48 hours cell death began to increase. Perhaps, in our study chloral hydrate disrupted Ihh signaling pathway was due to loss of Smo,Gli in those broken cilary filaments.

Conclusions

Previous studies have shown that the primary cilium is a mechanosensory organelle which transduces mechanical forces into biological signals, and that it plays an important role in the Ihh-PThrP feedback loop. Our present study demonstrates that growth plate chondrocytes respond to hydrostatic loading by increasing Ihh signaling, and that the primary cilium is required for this mechano-biological signal transduction to occur.

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Highlights

>High density primary chondrocyte pellets were examined under a hydrostatic compression> Hedgehog's signal pathway was affected by this compression>it was through primary cilia.

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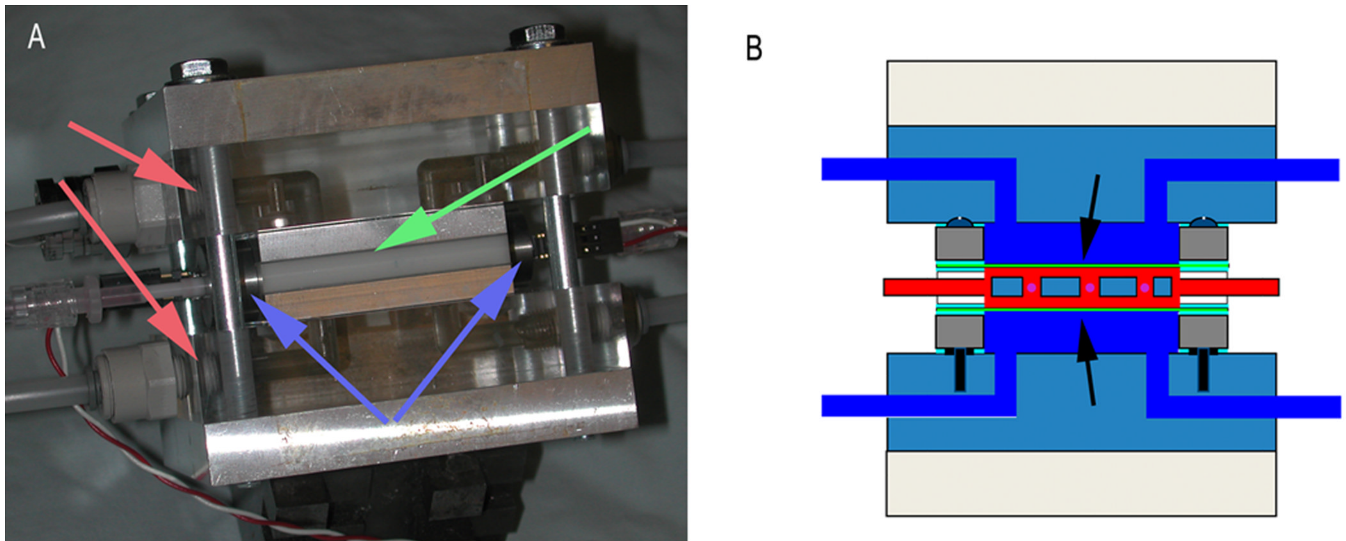

Figure 1. Custom-designed hydrostatic loading system

The module consists of a PMMA/aluminum (**A**, red arrows) frame surrounding the bioreactor chamber (**A**, green arrow) with the intervening space filled with water (**B**, dark blue). When pressuring the bioreactor, the media ports are closed using valves (**A**, blue arrows), and pressure is applied to the sample across the flexible FEP membrane (**B**, arrows). A perforated insert holds up to nine aggregates. Automatic control was achieved by using custom-written C software running on an IBM-PC. This module allows the application of arbitrary hydrostatic pressure waveforms on the construct in the bioreactor.

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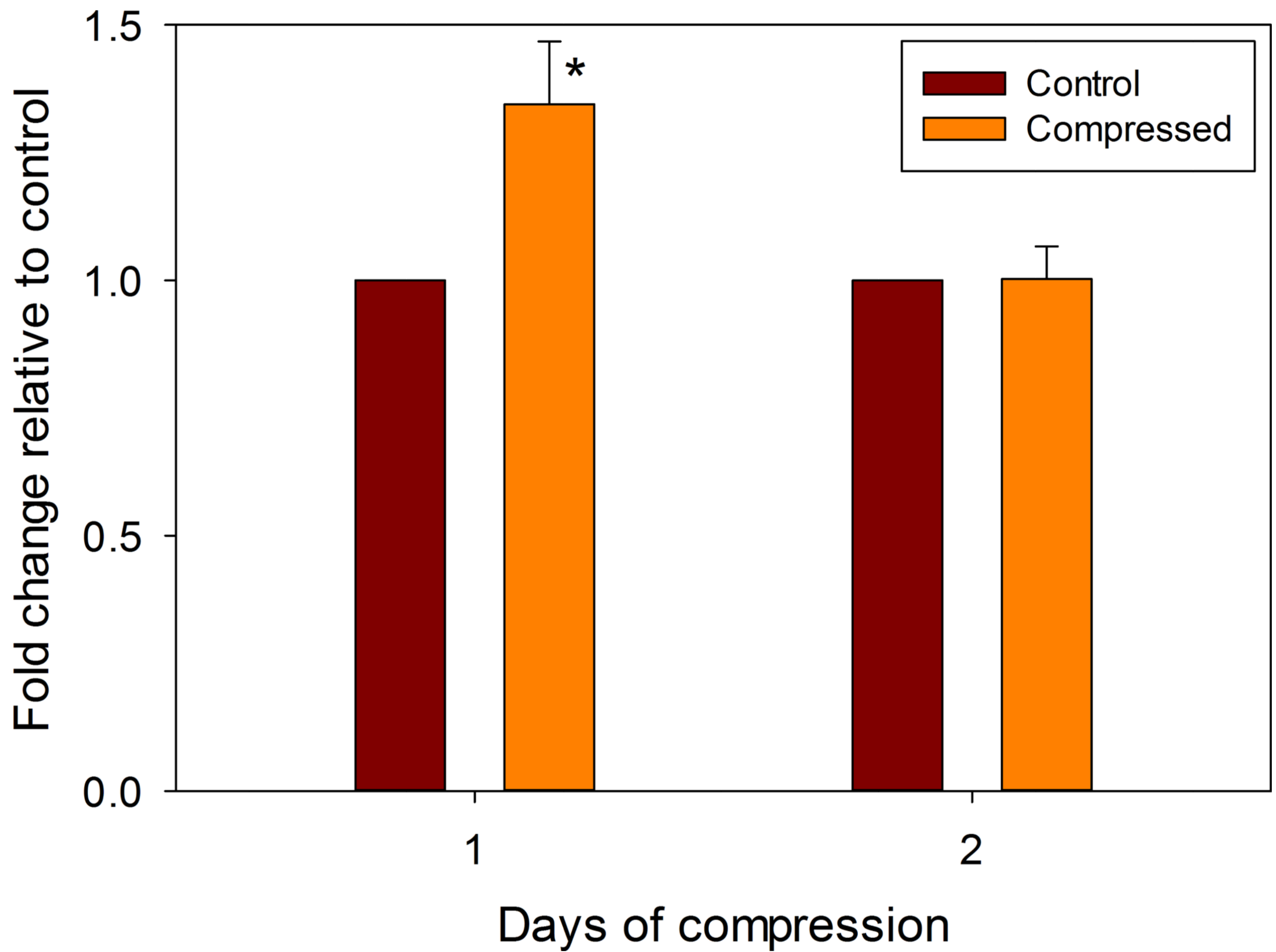

Figure 2. Proliferation Assay

Aggregates either were left unloaded (Control) or were hydrostatically loaded for 24 or 48 hours as described (Compressed). Immediately after compression, the cells were incubated at 37 °C in 200 μl of medium with 40 μl of the MTS/PMS solution (from the Titer AQ kit) for 1 hour. Absorbance was read at 490 nm in a 96-well plate reader. Data are shown normalized to the control group (* indicates $p = 0.0005$).

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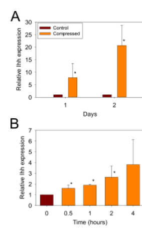

Figure 3. Effects of hydrostatic compression loading on Ihh gene expression in growth plate chondrocytes

Unloaded (Control), and compressed samples were collected after either **A:** 1 (*$p = 0.014$), or 2 days (*$p = 0.0002$) or **B:** After 0, 0.5, 1, 2, and 4 hours of intermittent hydrostatic compression (p =0.017 by ANOVA, * $p = 0.017$, 0.03, 0.02 respectively).

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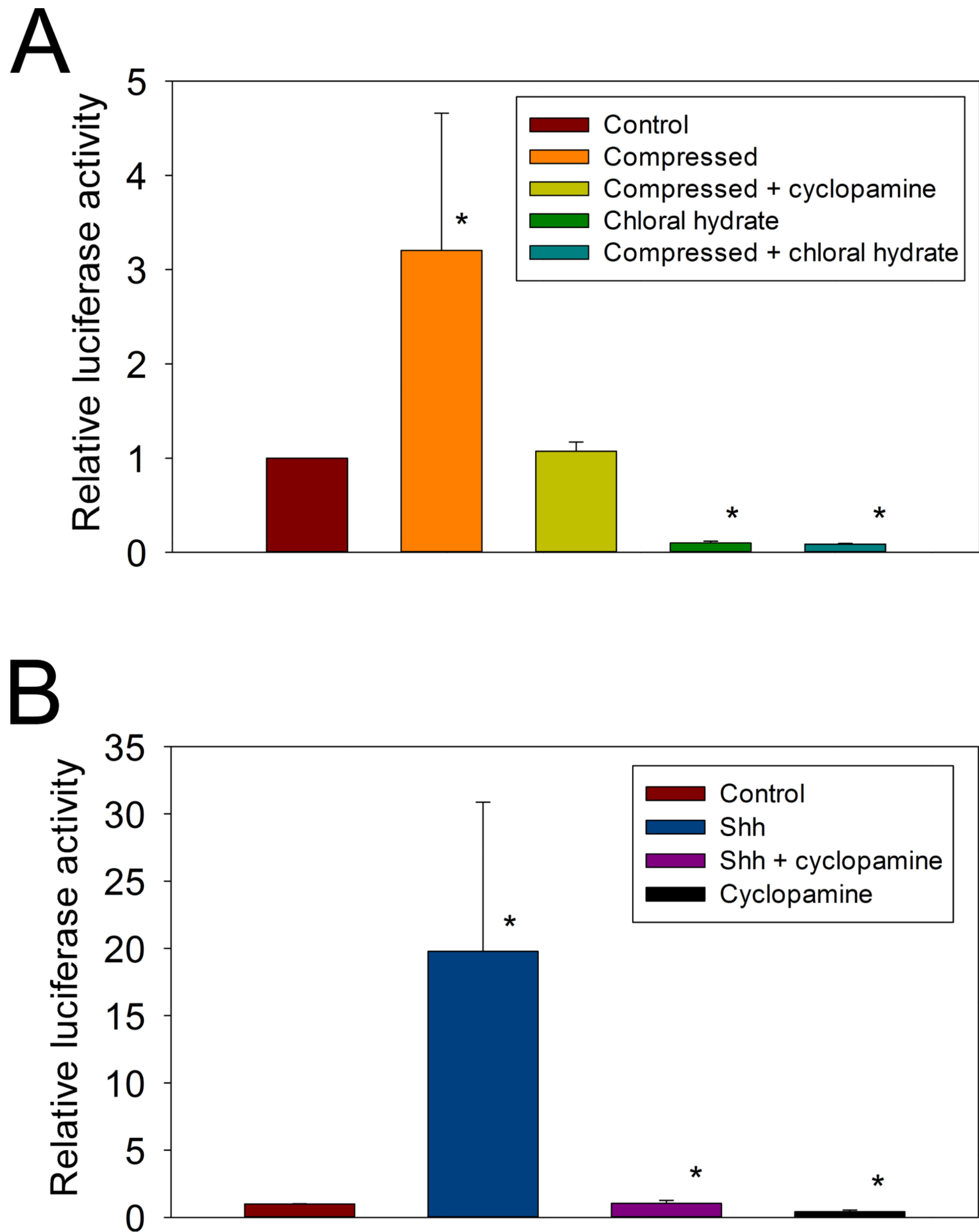

Figure 4. Gli-dependent luciferase activity

Gli-luciferase reporter construct was transiently transfected into primary growth plate chondroctyes, which were then cultured as cell pellets. **A:** Luciferase activity was measured after 48 hours of treatment without (Control) or with compression (Compression, * $p = 0.04$). The compression induced increase in activity was abolished by the addition of 10 nM cyclopamine (Compressed + cyclopamine). Four mM chloral hydrate essentially eliminated Gli-luciferase activity either without (Chloral hydrate, * $p = 0.00002$); or with hydrostatic compression (Compressed + chloral hydrate, * $p = 0.00002$); **B: Cyclopamine abolishes the Shh-induced increase in Gli expression.** Gli-Luciferase activity was measured after 48

hours of treatment with or without 5nM sonic hedgehog (Shh, * $p = 0.006$ compared to control), Shh + Cyclopamine, or Cyclopamine alone (* $p < 0.05$, compare to Shh).

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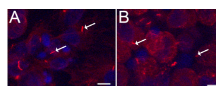

Figure 5. Cilia structure in growth plate chondrocytes with or without chloral hydrate treatment
Confocal image of primary cilia (arrows) immunostained with an anti-acetylated α-tubulin antibody. **A:** Control; **B:** After 2 days of chloral hydrate (4 mM) treatment. Scale bar = 10μm.

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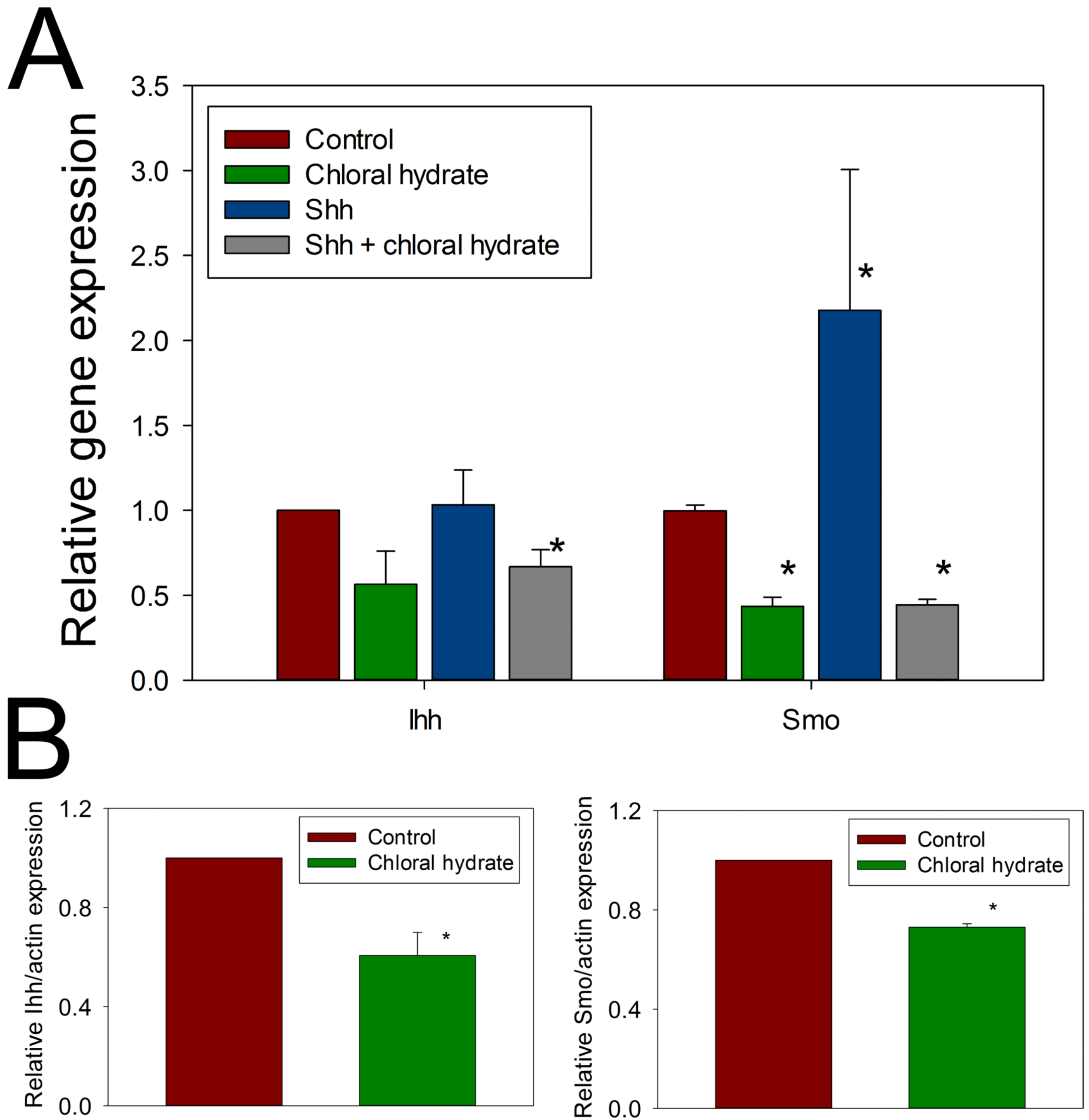

Figure 6. Ihh and Smo expressions were affected by chloral hydrate

A: Chloral hydrate treatment significantly reduced mRNA level (measured by real time PCR) of Ihh (* $p = 0.012$) and Smo (* $p = 4 \times 10^{-6}$), Shh treatment significantly (* $p = 10^{-2}$) increased Smo, but not Ihh expression, and the increase was abolished by chloral hydrate treatment (* $p = 10^{-6}$). **B:** Protein levels of Ihh and Smo were also significantly reduced (* $p = 0.027$ and 0.001, respectively) as measured by Western blotting.