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A Tunable, Three-Dimensional *in vitro* Culture Model of Growth Plate Cartilage Using Alginate Hydrogel Scaffolds

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Abstract

Defining the final size and geometry of engineered tissues through precise control of the scalar and vector components of tissue growth is a necessary benchmark for regenerative medicine, but it has proved to be a significant challenge for tissue engineers. The growth plate cartilage that promotes elongation of the long bones is a good model system for studying morphogenetic mechanisms because cartilage is composed of a single cell type, the chondrocyte; chondrocytes are readily maintained in culture; and growth trajectory is predominately in a single vector. In this cartilage, growth is generated via a differentiation program that is spatially and temporally regulated by an interconnected network composed of long- and short-range signaling mechanisms that together result in the formation of functionally distinct cellular zones. To facilitate investigation of the mechanisms underlying anisotropic growth, we developed an *in vitro* model of the growth plate cartilage by using neonatal mouse growth plate chondrocytes encapsulated in alginate hydrogel beads. In bead cultures, encapsulated chondrocytes showed high viability, cartilage matrix deposition, low levels of chondrocyte hypertrophy, and a progressive increase in cell proliferation over 7 days in culture. Exogenous factors were used to test functionality of the parathyroid-related protein–Indian hedgehog (PTHrP–IHH) signaling interaction, which is a crucial feedback loop for regulation of growth. Consistent with *in vivo* observations, exogenous PTHrP stimulated cell proliferation and inhibited hypertrophy, whereas IHH signaling stimulated chondrocyte hypertrophy. Importantly, the treatment of alginate bead cultures with IHH or thyroxine resulted in formation of a discrete domain of hypertrophic cells that mimics tissue architecture of native growth plate cartilage. Together, these studies are the first demonstration of a tunable *in vitro* system to model the signaling network interactions that are required to induce zonal architecture in growth plate chondrocytes, which could also potentially be used to grow cartilage cultures of specific geometries to meet personalized patient needs.

Keywords: growth plate cartilage, IHH/PTHrP signaling, 3D culture model, tissue architecture, alginate hydrogel Introduction

During embryonic and postnatal skeletal development, the shape and length of bones is determined by the activities of the growth plate cartilage. Growth plate cartilage promotes elongation of long bones via regulation of chondrocyte maturation that is reflected in morphologically and functionally unique cellular domains.¹ Toward the distal (epiphyseal) end resides the resting (or reserve) zone that is composed of the least mature chondrocytes.² These resting zone chondrocytes are progressively recruited into the proliferative zone, where cell cycle activation and changes in cell morphology and cell organization result in expansion of isogenic columns of chondrocytes along the axis of growth.³

As columns lengthen, chondrocytes at the proximal (meta-

physeal) end of the column withdraw from the cell cycle and increase in volume (hypertrophy) in two steps that are characteristic of the prehypertrophic and the hypertrophic chondrocytes.^{4,5} Growth is generated from chondrocyte hypertrophy through increased cell mass and deposition of specialized matrix.⁶ Hypertrophic chondrocytes are subsequently replaced by bone through the process of endochondral ossification.⁷ Thus, continuous growth over the two-decade span of human development requires tight coordination between cell production in the resting and proliferative zones, and cartilage loss in the hypertrophic zone.

Chondrocyte maturation in growth plate cartilage is coordinated by a complex paracrine signaling network that is rooted

in reciprocal interactions between two major signal transduction pathways that include the secreted ligands PTHrP (encoded by *Pthlh*), produced by periarticular resting chondrocytes, and Indian hedgehog (IHH), produced by prehypertrophic chondrocytes.^{8–11} In this context, PTHrP acts to maintain resting chondrocytes, and it stimulates the formation of proliferative chondrocytes.^{9–12} In addition, PTHrP signaling represses hypertrophy, in part, through the inhibition of IHH signal transduction.^{12–17} Conversely, IHH induces hypertrophic differentiation in a PTHrP-independent manner while promoting *Pthlh* expression in periarticular resting chondrocytes.^{8,10,18–20} Therefore, crosstalk between PTHrP and IHH paracrine signaling forms the core of a feedback circuit that maintains long-term growth by balancing chondrocyte production with chondrocyte loss through endochondral ossification.^{2,5}

Despite a deep understanding of the regulatory pathways that regulate growth, few options exist for clinical intervention in growth disorders.^{21–24} *In vitro* models of growth plate cartilage that reveal the network structure of regulatory interactions would advance both drug discovery efforts and tissue engineering solutions for growth disorders. One challenge is that chondrocytes readily form cartilage *in vitro*, but they do not spontaneously assemble into functional growth plates.^{25–28} Results using pellet cultures generated from postnatal growth plate chondrocytes suggest that a growth plate-like structure can be induced by the addition of exogenous factors. For example, pellet cultures treated with the hormone thyroxine or the signaling molecule Wnt5a form rudimentary chondrocyte columns and regionally display morphological evidence of chondrocyte hypertrophy.^{29,30}

Although these observations suggest that chondrocytes respond appropriately to growth factor stimulation *in vitro* and that isolated chondrocytes are competent to organize into columns, these studies did not confirm formation of definitive growth plate structure through gene expression or demonstrate that columns formed via known cell biological mechanisms. Therefore, it remains to be determined whether a limited repertoire of signaling molecules is sufficient to establish growth plate structure *in vitro*. Furthermore, pellet cultures significantly limit mechanistic studies by affording minimal control over the spatial distribution of growth factors and matrix structure. A culture system that allows precise spatial and/or temporal manipulation of the cellular microenvironment is needed to finely dissect the molecular mechanisms that regulate vertebrate growth.

To establish an *in vitro* model of growth plate cartilage, we encapsulated primary growth plate chondrocytes in beads composed of alginate hydrogel. Alginate is a linear polysaccharide that forms hydrogels when crosslinked in the presence of divalent cations.³¹ It is widely used in tissue engineering applications because of its inherent nonfouling nature and ease of functionalization of the alginate molecule, and because the pore size of alginate hydrogels allows for the diffusion of waste out and nutrients and growth factors into the tissue-engineered construct.^{31–40} Growth plate chondrocytes that have been encapsulated in “semi-solid” alginate beads maintain a rounded morphology.^{31–33} More recently, groups have utilized alginate to inject growth plate chondrocytes into mice for long-term culture, and they have even observed zonal arrangement and column formation.^{40,41} However, due to the complex nature of the

in vivo culture environment, the crucial factors for growth plate development were not elucidated.

In the current study, we describe a novel three-dimensional (3D) *in vitro* culture model where chondrocytes encapsulated within alginate hydrogel scaffolds display zonal arrangement of gene expression domains consistent with growth plate cartilage architecture. In our culture system, chondrocytes display cell cycle properties and generate extracellular matrix consistent with growth plate chondrocytes. Moreover, we show that chondrocyte hypertrophy can be regulated by exogenous manipulation of PTHrP and IHH signaling. Importantly, treatment of alginate bead cultures with soluble IHH protein or thyroxine induces formation of a spatially distinct zone of hypertrophy. Our results indicate that we have recapitulated the PTHrP/IHH feedback loop in a minimal culture system and, thus, have developed a viable model for studies of the genetic regulatory network that maintains the growth plate cartilage, thereby promoting bone growth.

Materials and Methods

Materials

Unless otherwise indicated, chemicals and reagents were obtained from Sigma Aldrich (St. Louis, MO), EMD (Darmstadt, Germany), or Thermo Fisher Scientific (Waltham, MA); cell culture media from Gibco/Life Technologies (Grand Island, NY); oligonucleotides from IDT (Coralville, IA); and consumables from Thermo Fisher Scientific or VWR (Radnor, PA). Growth factors were obtained from Sigma-Aldrich, EMD Calbiochem (San Diego, CA), Tocris (Ellisville, MO), or R&D Systems, Inc. (Minneapolis, MN).

Chondrocyte isolation

Neonatal Swiss Webster mice (Jackson Laboratories, Bar Harbor, ME) at postnatal day 4 (P4, with P0 being the day of birth) were euthanized, and the growth plate cartilage from both hind limbs was harvested into Hank's balanced salt solution. Cartilage was transferred into complete medium that was composed of Dulbecco's modified Eagle medium containing 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA) and 1% glutamine-penicillin-streptomycin. Collagenase was added to 0.25% (w/v), and the cartilage was digested for 4 h in a passively humidified incubator at 37°C and 8% CO₂ and pipetted several times to break up remaining tissue. Chondrocytes were pelleted by centrifugation at 125 *g* for 5 min by using a Sorvall Legend RT centrifuge (Kendro Laboratories, Newtown CT) and resuspended in Complete Medium. Cell density was quantified by using a hemocytometer. Chondrocytes were maintained in the incubator until time for alginate encapsulation. All procedures performed on animals were consistent with regulatory agency policies and were approved by the Institutional Care and Use Committee at the University of Nebraska Medical Center.

Chondrocyte encapsulation in alginate

Sodium alginate powder (Pronova UP MVG, >60% guluronic acid, 200,000–300,000 g/mol; NovaMatrix, Sandvika, Norway) was dissolved in sterile phosphate-buffered saline (PBS) to a

concentration of 1.5% (w/v), and the solution was filtered sterilized by using 0.20 μm Rapid-Flow PES filters. Growth plate chondrocytes were pelleted by centrifugation at 125 *g* for 5 min and resuspended in an appropriate volume of alginate solution (to 8×10^6 cells/mL) by gentle pipetting and vortexing. Homogenous alginate beads were formed by using the basic “drop” method previously described.³⁴ Briefly, 10 μL of alginate-cell solution was drawn into a pipette tip, and the tip was wiped with a Kimwipe® to ensure the alginate could drop freely from the tip. The plunger was rapidly depressed to release a drop of the cell suspension into 2 mL of crosslinking solution (50 mM CaCl_2 /140 mM NaCl at 37°C) contained in a 24-well tissue culture dish from a height of 2–3 inches. Only one bead was prepared in an individual well. After 2 min of crosslinking, beads were transferred to a well containing 2-mL of Complete Medium for a maximum of 10 min and then washed in minimum essential medium (MEM)-alpha medium without Phenol Red (a-MEM) for a maximum of 10 min. After both washes, the bead was placed into cartilage culture media (described later).

Culture conditions

Cartilage culture media were prepared by using previously described methods⁴: a-MEM supplemented with 50 $\mu\text{L}/\text{mL}$ GPS, β -glycerophosphate (10 mM), and L(+) ascorbic acid (50 $\mu\text{g}/\text{mL}$). In addition, dexamethasone (1 nM), proline (1 mg/mL), 1% antioxidant (Sigma Aldrich), sodium pyruvate (1 mM), 1% nonessential amino acids (Gibco), and 1% Insulin-Transferrin-Selenium +3 media supplement (ITS +3; Sigma Aldrich) were added. In some cultures, parathyroid hormone 1–34 (PTH1–34), IHH, purmorphamine (PMA), bone morphogenetic protein-4 (BMP4), or thyroxine (T4) was added to the media at the concentrations indicated in the text. Beads were cultured in a 48-well plate in a humidified incubator at 37°C and 8% CO_2 for a maximum of 7 days, with medium changes every day.

Evaluation of viability

Viability of encapsulated chondrocytes was determined by using a Live/Dead Cell Viability kit (Invitrogen). Briefly, after culture, beads were incubated in PBS containing 2 μM Calcein AM/4 μM ethidium homodimer-1 for 30 min in the dark, washed in several changes of PBS and the fluorescence signal of live (green) and dead (red) cells was imaged by using a Leica DMI6000B inverted microscope. Live and dead cell counts were quantified from the resulting images by using a basic ImageJ macro (see Supplementary Data in appendix) to calculate percent viability.

Immunofluorescence imaging

For immunofluorescence analysis, all reagent and wash solutions contained 2 mM CaCl_2 to maintain crosslinking of alginate. Beads were fixed with 4% paraformaldehyde (PFA) for 20 min, permeabilized with TBST (0.1% Triton X-100/ 15-mM Tris-HCl/136-mM NaCl, pH 7.6), and blocked for 2 h with 10% heat-inactivated sheep serum (HISS; Sigma Aldrich) in TBST. Subsequently, the beads were incubated with gentle rocking at 4°C in either rabbit-anti-Collagen VI (1:500; Abcam, Cambridge, United Kingdom) or rabbit-anti-Collagen IV (1:500; Abcam) primary antibody in 2%

HISS/ TBST overnight. The beads were then incubated in a secondary anti-mouse AlexaFluor 647 antibody (1:1000; Jackson Laboratories) and phalloidin-labeled AlexaFluor 488 (1:100; Invitrogen) for 2 h in 2% HISS/TBST. After washing for 3 \cdot 5 min in TBST, the beads were mounted with Prolong Gold antifade reagent with DAPI (Invitrogen) on a glass microscope slide with a coverslip. Images were obtained on a Leica DMI6000B inverted microscope or a Zeiss 710 laser scanning confocal microscope. For confocal microscopy, optical sections were collected at 1.8- μm intervals for between 15 and 25 sections (total depth of 30–40- μm), with line averaging = 2, scan speed = 4 or 5, and resolution = 1024 \cdot 1024.

Transcript expression analysis

For each experimental condition, four beads were depolymerized in 50 μL of 50 mM ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) and chondrocytes were recovered by centrifugation. RNA was isolated from cells by using the NucleoSpin® RNA Plus kit (Thermo Fisher) according to the manufacturer’s instructions and quantified by using a Thermo Fisher Scientific NanoDropOne spectrophotometer. cDNA was synthesized by using Superscript IV (Thermo Fisher) according to the manufacturer’s instructions and analyzed by a digital droplet polymerase chain reaction (ddPCR; Bio-Rad Laboratories, Inc., Pleasanton CA). Thermal cycling conditions were 95°C for 5 min, 40 amplification cycles (95°C for 30 s and 60°C for 30 s), and a final signal stabilization step (4°C for 5 min, 90°C for 5 min). The transcript count of each gene of interest was normalized with a geometric average of transcript counts of three normalization control transcripts: actin, RPL13a, and HPRT. Data from this analysis are reported as fold change in the geometric mean \pm standard error of mean (SEM) over the transcript count of the nontreated control, and significance was calculated by using two-way analysis of variance (ANOVA) analysis using Prism software (GraphPad Software, Inc., La Jolla, CA). A full list of primer sequences and gene accession numbers used to generate primers is included in Supplementary Table S1.^{42–48}

Flow cytometry

Fluorescence-activated cell sorting (FACS) was used to analyze cell cycle progression in cultured chondrocytes. Alginate beads were dissociated in 200 μL of 50 mM EDTA for 5 min. Cells were washed with PBS and centrifuged at 125 *g* for 5 min. The cell pellet was resuspended in 200- μL of PBS and fixed by adding 2.8 mL of ice-cold 90% ethanol dropwise while vortexing. Fixed cells were stored at -20°C for a maximum of 7 days. For cell cycle analysis, DNA in fixed cells was stained with 0.05 mg/mL of propidium iodide in Telford’s reagent (33.62 $\mu\text{g}/\text{mL}$ EDTA, 26.8 $\mu\text{g}/\text{mL}$ RNase A at 93 U/mg, and 0.1% Triton X-100) for 1 h at 4°C in the dark. Labeled suspensions of cells were filtered through 5-mL filter-cap polypropylene Falcon tubes (Corning Labs, Tamaulipas, Mexico) and were then immediately analyzed on a BD FACSCaliber 2 flow cytometer (BD, Franklin Lakes, NJ). The resulting cell cycle data were represented as stacked bar graphs of the geometric mean \pm SEM of percentage of cells in each phase of the cell cycle, and significance was calculated by using a two-way ANOVA using GraphPad Prism software.

In situ hybridization

For fluorescence *in situ* hybridization (FISH) analysis of gene expression in cell cultures, whole alginate beads containing chondrocytes were fixed in 4% PFA in TBS containing 5mM CaCl₂ overnight at 4°C. To maintain structural integrity of the sample, beads were infiltrated with 37.5:1 40% acrylamide (JT Baker, Center Valley, PA) overnight at 4°C and the acrylamide was polymerized by using 10% ammonium persulfate (10 μL/mL acrylamide) and TEMED (1 μL/mL acrylamide). Polymerized samples were subsequently incubated in 30% sucrose overnight at 4°C before embedding in Tissue-Tek Optimum Cutting Temperature embedding medium and freezing on dry ice. Sections (60 μm) were collected on glass microscope slides, and FISH was performed by using fluorescein-labeled RNA probes for *collagen2a1* (*ColII*) and *collagen 10a1* (*ColX*), as previously described.⁴⁹ Slides were mounted by using Prolong Gold antifade reagent with DAPI (Life Technologies), and images were obtained on a Leica DMI6000B inverted microscope. The images presented in this article are montages that were assembled by tiling and stitching images from several optical planes as a z-stack (10-μm width between individual slices in the z-stack, 3 total slices per z-stack, total width of the z-stack equaling 20 μm) to visualize the entire section of the bead for downstream quantification. To quantify cell counts, we used a batch processing analysis in Image J using a macro-programmed in-house using FIJI software (<http://fiji.sc/>) to quantify the number of cells expressing the respective gene (for macro, see Supplementary Data). Typically, each image yielded cell counts of ~1000 and the data for each experimental condition were derived from ~10,000 cell counts. The fraction of cells expressing a given gene was determined by dividing the number of cells expressing the gene by the number of

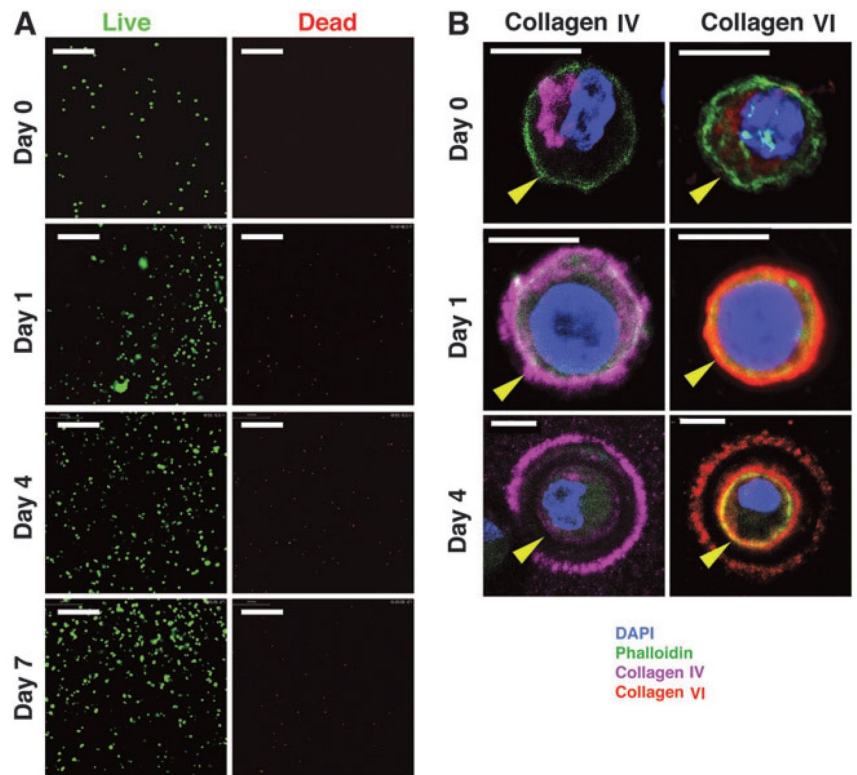
DAPI-positive nuclei observed in the section. The *ColX:ColII* ratio was calculated as the geometric mean of the *ColX:DAPI* ratio for each replicate divided by the geometric mean of the *Col2:DAPI* ratio for each replicate ($n = 3$ or 4 sections for each replicate). The ratio is reported as the geometric mean of the *ColX:ColII* ratio for each replicate \pm SEM. To determine statistical significance, a Student's *t*-test was used. To determine whether a given treatment had significant overall effects on hypertrophy, datasets from different time points were pooled into treated or untreated groups and compared by using a paired Wilcoxon signed-rank test.

Results

Alginate culture maintains the growth plate cartilage phenotype

The objective of this study was to investigate the use of alginate hydrogels as 3D scaffolds for the development of an *in vitro* growth plate model. As a starting point, we analyzed the short-term cellular response of mouse chondrocytes to encapsulation within 1.5% alginate hydrogels for a maximum of 7 days. The culture conditions used resulted in cell viability greater than 90% on days 0, 1, 4, and 7 (Fig. 1A and Supplementary Table S2). Individual chondrocytes progressively elaborated a pericellular matrix, as evidenced by extracellular domains of collagen IV and collagen VI, during the 7-day culture period (Fig. 1B). Since spontaneous dedifferentiation and hypertrophy are common problems in chondrocyte cultures, we next assessed chondrocyte differentiation via quantitative ddPCR analysis of gene expression. Chondrocytes in our alginate bead cultures express high levels of *ColII* (Fig. 2A), low levels of the fibroblast marker *collagen1a1* (*Coll*,

Fig. 1. Culture in alginate hydrogel promotes chondrocyte growth. (A) Invitrogen Live/Dead assay to determine chondrocyte viability for at least a week of alginate culture. Evident cell proliferation is noticed over time, but low amounts of dead cells are seen at all time points ($n = 3$). (B) Immunofluorescence assay demonstrating the formation of pericellular matrix in alginate culture using antibodies against Collagen IV (left) and Collagen VI (right). Freshly isolated chondrocytes lack ECM staining, but they produce *de novo* ECM after 1 day in alginate culture. On day 4 (right), a secondary ECM structure is observed resembling territorial matrices ($n = 4$). Arrowheads in (B) indicate the position of the cell membrane. Scale bar is 150 μm in (A) and 10 μm in (B).



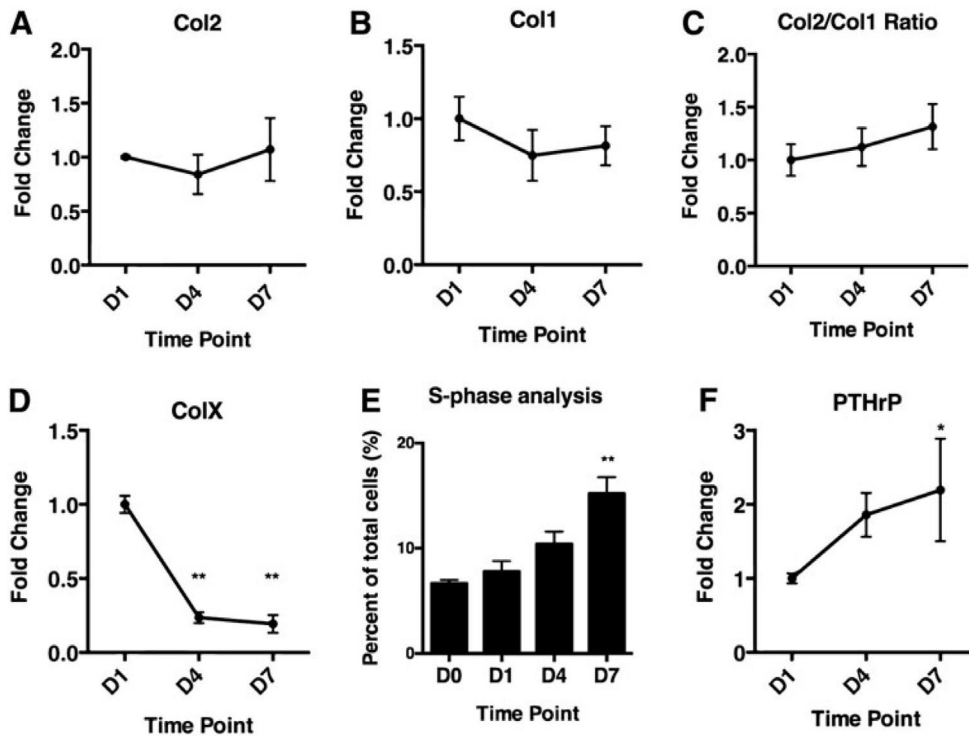


Fig. 2. Culture in alginate hydrogel restrains cartilage differentiation. Gene expression analysis of alginate cultures using ddPCR ($n = 4$) to count mRNA transcripts of Collagen 2 (A), Collagen 1 (B), Collagen X (D), and PTHrP (F). Values are reported as fold change in gene expression relative to day 1. (C) Ratio of mRNA transcript counts of Collagen 2 and Collagen 1. (E) Propidium iodide cell cycle analysis by FACS to quantify percentage of cells in S phase (DNA synthesis for cell proliferation/ growth). Alginate cultures tend to increase the number of proliferating cells over time, compared with D0 and D1 ($n = 3$). * $p < 0.05$, ** $p < 0.01$. ddPCR, digital droplet polymerase chain reaction; FACS, fluorescence activated cell sorting; PTHrP, parathyroid-related protein.

Fig. 2B), and maintain a stable ratio of *Col1:Col2* gene expression (Fig. 2C), a measure commonly used to assess chondrocyte dedifferentiation. Although the *Col1:Col2* ratio did not significantly change over time, *ColX* expression, a marker of chondrocyte hypertrophy, decreased rapidly and significantly (Fig. 2D).

We also noted that cell density within the beads increased over time (Fig. 1A) and many of the initial single cells become doublets in our cultures (data not shown), two observations that are consistent with cell proliferation. Therefore, we performed cell cycle analysis of our cultures by using flow cytometry (FACS). FACS analysis revealed two populations of chondrocytes in our cultures that were distinguished by both forward and side scatter measures of cell size, and, thus, will be referred to as “larger” and “smaller” chondrocyte populations (Supplementary Fig. S1). Smaller chondrocytes constituted less than 10% of the chondrocytes recovered from the beads and were associated with the only observable instances of sub-G1 peaks. In related experiments, we have observed that these small cells remain in the G1 phase of the cell cycle regardless of condition or treatment (data not shown), whereas larger cells are growth factor responsive (see below). Thus, only the larger, growth factor-responsive cells were included in the reported cell cycle analysis.

In this population of larger chondrocytes, FACS analysis revealed that only 5–7% of chondrocytes were in S phase at the initiation of alginate culture (Fig. 2E), whereas 15–20% of cells were in S phase in embryonic growth plate cartilage.⁵⁰ However, during 7 days of culture within the alginate hydrogel beads, we observed a statistically significant increase in the percentage of cells in S phase that approached the level reported *in vivo* (Fig. 2E). Since PTHrP has been shown to both inhibit hypertrophy and promote cell proliferation in cartilage,^{16,17} we next asked whether PTHrP was expressed by chondrocytes in our alginate culture. ddPCR analysis confirmed a significant upregulation of PTHrP expression

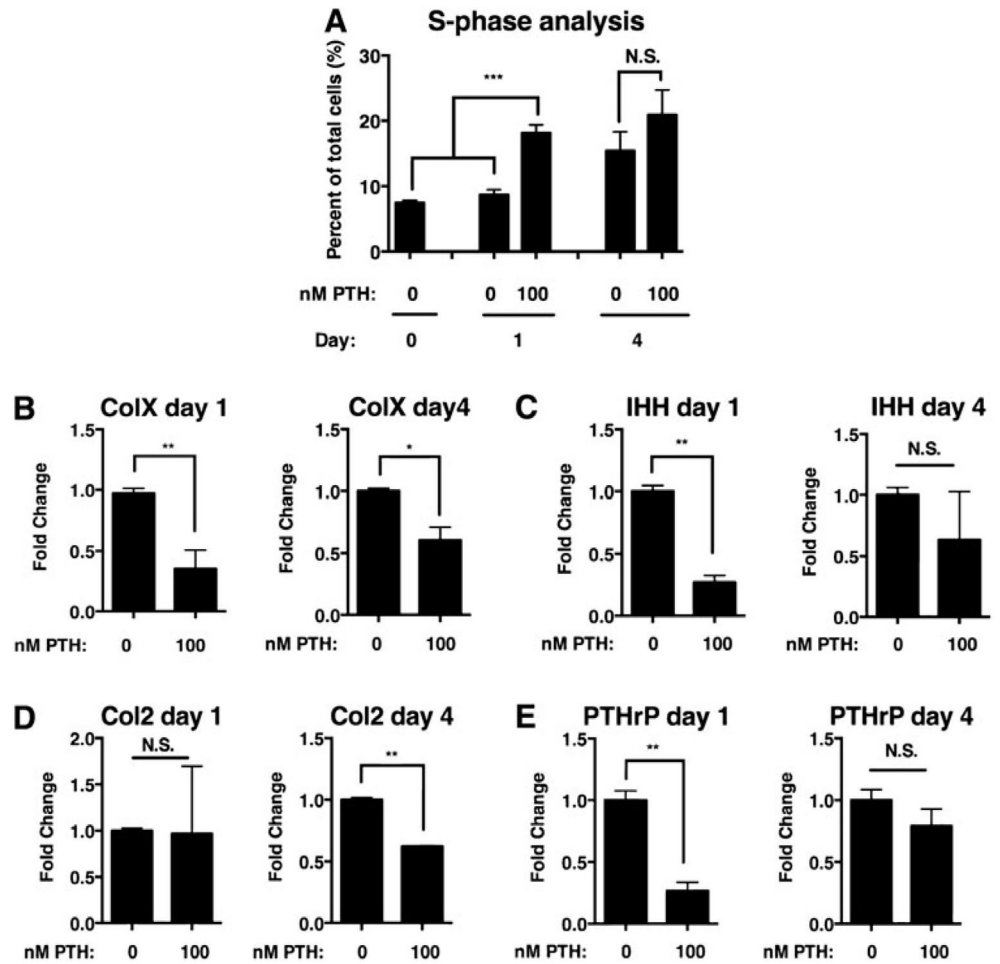
over 7 days of culture (Fig. 2F). Taken together, these data suggest that our minimal culture conditions are sufficient to maintain the growth plate chondrocyte phenotype *in vitro*.

PTH1–34 treatment promotes cell proliferation in alginate bead culture

Given the correlations between PTHrP expression, cell proliferation, and chondrocyte differentiation, we next asked whether the stimulation of PTHrP signaling could further enhance cell proliferation and chondrogenesis. We cultured chondrocytes in the presence of recombinant human PTH1–34, a 34-amino-acid peptide derived from the N-terminus of the parathyroid hormone that has been shown to activate the same receptor and downstream signal transduction pathways as the natural ligand PTHrP in chondrocytes.^{9,17} FACS analysis showed that 1 day treatment with PTH1–34 increased the fraction of cells in S phase as compared with untreated controls, whereas no significant difference was observed after 4 days of treatment even when culture medium and factors were replaced every day (Fig. 3A). It should be noted that the fraction of S-phase cells in PTH1–34 stimulated cultures was identical after 1 and 4 days of treatment (compare 100 nM PTH on day 1 and 4). Therefore, the apparent desensitization to PTH1–34 treatment results from the increase in cell cycle activation over time in untreated cultures (compare 0 nM PTH on day 1 and day 4). Thus, exogenous stimulation of PTHrP signaling enhances chondrocyte cell proliferation in the short term, but it does not appear to have long-term benefits.

In addition to stimulating cell cycle progression, PTH1–34 also significantly reduced the expression of *ColX* (hypertrophic chondrocytes, Fig. 3B) and *Ihh* (prehypertrophic chondrocytes, Fig. 3C), as well as endogenous expression of PTHrP after 1 day of treatment as compared with control cultures (Fig. 3E). One

Fig. 3. Treatment with PTH1–34 accelerates the chondrocyte response to alginate bead culture. (A) Propidium iodide cell cycle analysis by FACS to quantify percentage of cells in S phase (DNA synthesis for cell proliferation/ growth). PTH treatment on day 1 stimulates proliferation similar to levels that are typically seen in day 4 cultures. However, 4 days of PTH treatment has little effect ($n = 4$). (B–E) Gene expression analysis was performed on PTH-treated alginate cultures with ddPCR to count mRNA transcripts. On day 1 (*left*), PTHrP signaling inhibits expression of Collagen X (B), IHH (C), and PTHrP (E), but not Collagen 2 (D). After 4 days of culture (*right*), the effects of PTHrP on gene expression are greatly attenuated ($n = 3$). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. IHH, Indian hedgehog; N.S., not significant.



interesting observation was that PTH1–34 treatment continued to repress *ColX* expression after 4 days of treatment, whereas the expression of other genes (i.e., *Ihh* and *PTHrP*) had already returned to the level of untreated cultures. Therefore, we decided to validate the ddPCR results by analyzing gene expression at single-cell resolution by using FISH. *In situ* hybridization in alginate beads is difficult because the elevated hybridization temperatures, denaturing conditions, and metal ion chelation of hybridization buffers promote depolymerization of the alginate scaffold. To address this issue, we devised a novel tissue processing protocol in which alginate beads are stabilized before cryo-embedding by infiltration with acrylamide and induction of polymerization with ammonium persulfate.

Using this method, we were able to carry out FISH on slides without disrupting the integrity of bead architecture, which allowed the spatial relationships between cells in our culture to be accurately determined. We visualized gene expression in each cell with fluorescence microscopy (Fig. 4A, left), and quantified the total cell number (DAPI), as well as the number of hypertrophic chondrocytes (*ColX*) and immature chondrocytes (*Col11*) by using an object-counting macro in ImageJ (see Supplementary Data). These values were used to calculate the hypertrophic index (ratio of *ColX*-positive cells:*Col11*-positive cells) that describes the state of chondrocyte maturation in regions of the alginate beads. Using this approach, we found that PTH1–34 treatment reduced the hypertrophic index after both 1-day and 4-day

treatments (Fig. 4B). Although the downward trend on each day was not statistically significant, the overall effect of PTH1–34 treatment across days was a significant reduction in hypertrophic index. Thus, together, *in situ* hybridization image analysis and ddPCR transcript quantification demonstrate long-term repression of *ColX* expression by continuous PTH1–34 treatment.

Activation of IHH signaling induces chondrocyte hypertrophy in alginate bead culture

In vivo, IHH signaling both promotes chondrocyte hypertrophy^{8,10,15} and activates *PTHrP* gene expression,^{2,5} which antagonizes chondrocyte maturation, including hypertrophy.^{14–17} Therefore, we next asked which effect would dominate after exogenous activation of IHH signaling in alginate culture. To answer this question, we used three known activators of IHH signaling: PMA, a commonly used agonist of Smoothed, the signaling component of the hedgehog receptor complex⁵¹; thyroxine, a hormone that activates IHH expression in chondrocytes^{52,53}; and BMP4, which is known to upregulate IHH expression.^{12,54} At all time points analyzed, IHH-activating agents increased the hypertrophic index, as determined by FISH analysis (Fig. 5A, B) and increased the fraction of large cells as determined by FACS analysis (Supplementary Fig. S2). After pooling data from different time points, it was determined that overall, 1 and 5 ng/mL BMP4, 0.5 μ g/mL PMA, and 500 ng/mL thyroxine

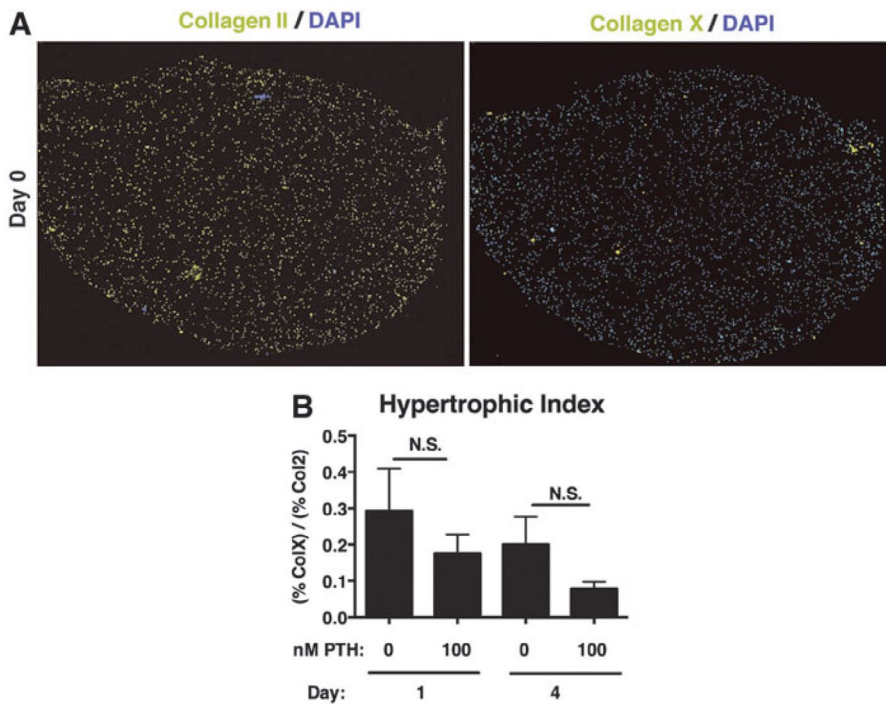


Fig. 4. Visualizing chondrocyte gene expression in alginate bead culture. (A) Fluorescent images generated by using our improved protocol exploiting acrylamide embedding of fixed beads in conjunction with fluorescent *in situ* hybridization (FISH) to visualize cells that highly express Collagen II (*left*) and Collagen X (*right*) as a cell-by-cell measure of hypertrophic differentiation. Images are analyzed via a fully automated macro in ImageJ to quantify the percentage of cells expressing each gene relative to a nuclear DAPI stain. Ratio of these percentages was calculated (ColX/Col2) to yield a hypertrophic index (refer to Methods for more details). (B) Quantification of hypertrophic index in PTH1–34 treated and untreated cultures ($n = 3$). PTH1–34, parathyroid hormone 1–34.

each significantly increased the hypertrophic index of bead cultures. In addition, both PMA and thyroxine maintain the proportion of large (growth factor responsive) cells in alginate culture (Fig. 5C, D). Given that direct activation of IHH signaling by the Smoothed agonist PMA increased the hypertrophic index to a greater extent than the indirect activators thyroxine and BMP4, we next asked whether recombinant IHH protein could similarly induce chondrocyte hypertrophy. As expected, hypertrophy was induced by IHH, as determined by the *ColX:ColIII* ratio

(Fig. 5A, B). However, the induction of hypertrophy (*ColX*-positive cells) was observed exclusively in cells near the bead surface, appearing as a ring illuminating the bead edges when analyzed by epifluorescence microscopy (Fig. 6A). Conversely, *ColIII*-positive cells were enriched within core of the bead (Fig. 6A, quantification in Fig. 6B, C). Interestingly, treatment with thyroxine, but not PMA, also induced the formation of a peripheral *ColX*-expression domain (Fig. 6D). These data demonstrate regionalization of chondrocytes within alginate bead cultures into a layer

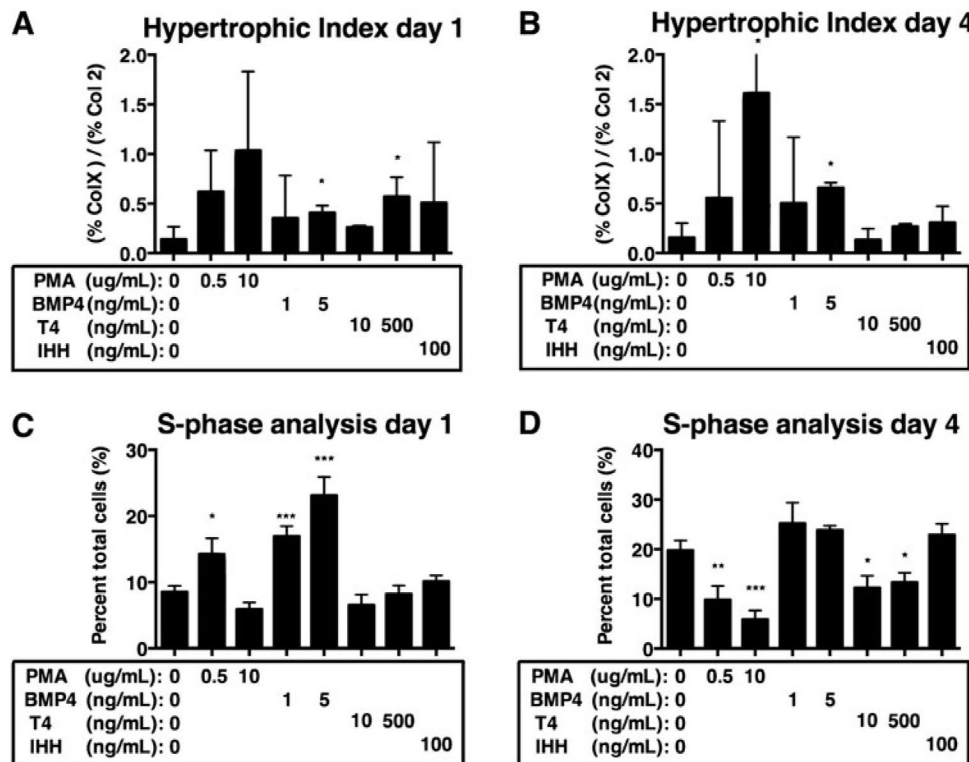
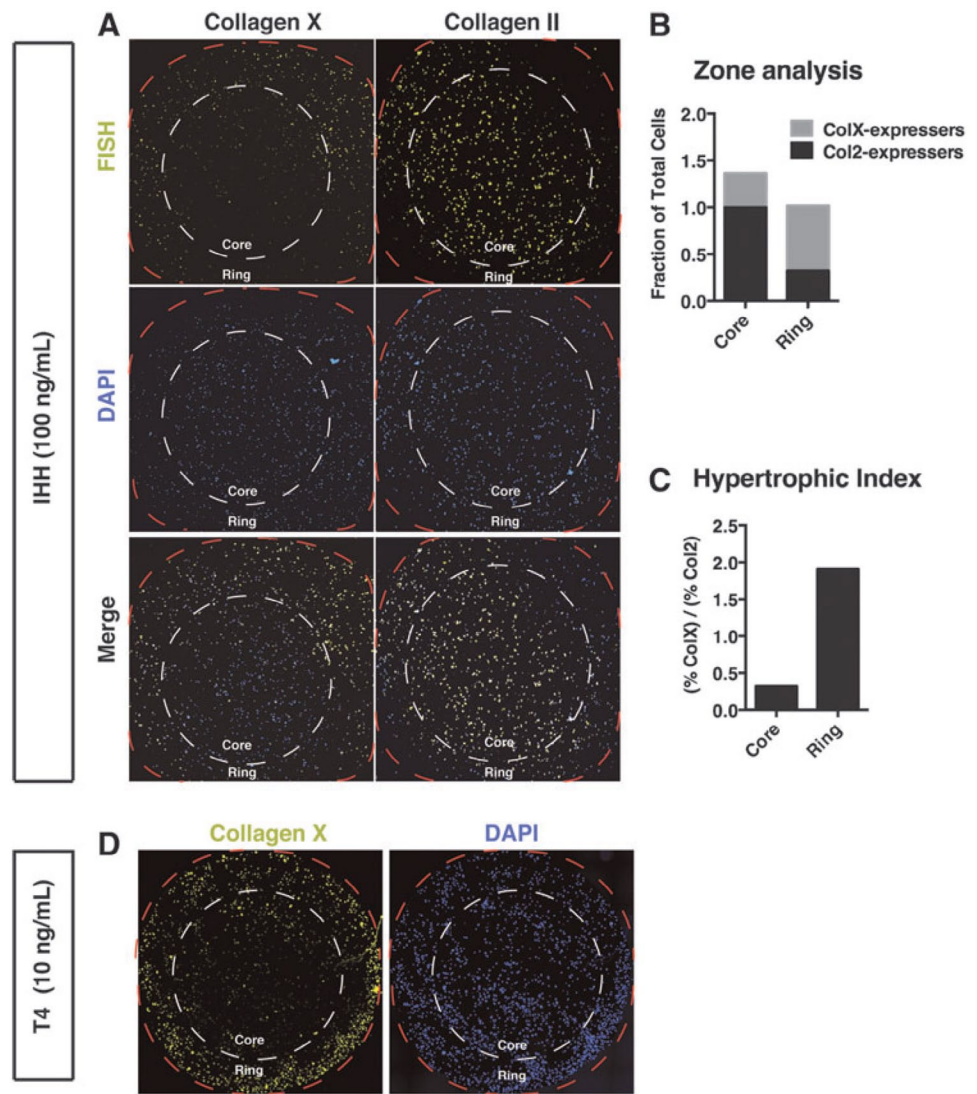


Fig. 5. Induction of hypertrophy after exogenous activation of IHH signaling. (A, B) Analysis of FISH derived images to calculate the extent of hypertrophy in alginate cultures treated with thyroxine (10, 500 ng/mL), BMP4 (1, 5 ng/mL), PMA (0.5, 5 μ g/mL), and IHH (100 ng/mL) for a single day (A) and for 4 days in culture (B) ($n = 3$). In panel B, PMA 20 day 4, the error bar is much larger than the rest of the data points and, therefore, is clipped by the axis. (C, D) Propidium iodide analysis of stimulated bead cultures was performed to quantify the percentage of cells in S phase ($n = 3$) on days 1 (C) and 4 (D). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. BMP4, bone morphogenetic protein- 4; PMA, pumorphamine.

Fig. 6. IHH treatment in alginate bead culture is sufficient to establish chondrocyte zonal arrangement. (A) FISH (top) was used to visualize *ColX*-expressing (left) and *ColII*-expressing (right) cells in 100 ng/mL IHH-treated cultures. White dotted lines delineate the observed zones of predominant expression. Red dotted lines delineate the outer boundary of the bead. Beads form a *ColX*-enriched ring (outer portion) surrounding a *ColII*-enriched core (inner portion). DAPI (middle) is shown on the right to demonstrate that cells are still evenly dispersed in hydrogel culture after 4 days, aside from isogenic groups, ($n = 3$). (B) Counts of *ColII*-expressing and *ColX*-expressing cells (divided by the count of DAPI-positive nuclei in each FISH image), stacked in a bar graph to illustrate the relative predominance of hypertrophic versus nonhypertrophic chondrocytes in different domains of the IHH-treated alginate beads. (C) Hypertrophic indexes of different domains of the IHH treated alginate beads. (D) FISH was used to visualize *ColX*-expressing (left) and total (right) chondrocytes in alginate cultures treated with 10 ng/mL thyroxine.



of hypertrophic-zone-like cells on the bead periphery that surrounds a core of less mature chondrocytes. To our knowledge, this is the first example of zonal chondrocyte organization in a tissue-engineered cartilage construct *in vitro*.

Discussion

In children, growth plate cartilage is crucial to skeletal morphogenesis. Defects in growth plate function due to genetics (incidence of chondrodysplasia—1 in 5000 births), metabolic disease, radiation and chemotherapy, and high impact fractures affect skeletal growth, which can lead to deformities, growth arrest, or structural instability of developing long bones.^{22–24} However, an incomplete understanding of the molecular and cellular processes that produce growth has resulted in few clinical options to treat growth defects and has severely limited advances in tissue engineering and regenerative strategies to replace damaged or diseased tissue.^{22–24} Although genetic models have identified many important molecules that regulate cartilage morphogenesis, a deeper mechanistic understanding of growth regulation in vertebrates has been confounded by the lack of tools for precise and combinatorial genetic manipulation that is needed to

determine mechanism. *In vitro* models offer the potential to simultaneously regulate multiple secreted, matrix, and mechanical factors in space and time.^{55–58} Here, we describe the development of a 3D chondrocyte culture system by using mouse neonatal growth plate chondrocytes encapsulated in an alginate hydrogel to investigate the mechanisms underlying growth plate formation, regulation, and growth disorders.

3D alginate culture favors immature chondrocytes

For many tissues, two-dimensional cultures are sufficient to preserve cell phenotypes and cell-specific responses to growth factor signaling. However, in the case of growth plate cartilage, monolayer cultures promote dedifferentiation of chondrocytes, abnormal gene expression profiles, and inappropriate responses to signaling factors.^{59–62} The 3D culture of chondrocytes in pellets, micromass, or artificial scaffolds has been shown to maintain the chondrogenic phenotype.^{27–31,61,62} Although pellet cultures have been induced to generate a growth plate-like architecture, the difficulty of manipulating these cultures to generate regional differences in signaling factor concentrations or matrix composition inspired us to explore culture models in which

chondrocytes are encapsulated in hydrogel scaffolds that have defined composition. The choice of scaffold is potentially important, since a physical interaction of scaffold materials with cells and growth factors might significantly alter outcomes of these models. Therefore, we chose alginate as a scaffold material because the low cell adhesion, limited protein binding, and minimal cell toxicity of this material render alginate a “blank slate” for the introduction of defined signaling and matrix factors. The many advantages of alginate also include mild gelation conditions, fluid diffusion of nutrients and wastes into and out of the construct, and accessible functional groups for modifications.³⁹ For these reasons, many cell types, including articular chondrocytes,²⁸ have been successfully cultured in alginate gels.

Our basal culture conditions display high viability and extracellular matrix deposition that are consistent with previous reports,²⁷ which suggests that the basal culture conditions are permissive for chondrogenesis. However, unlike previously described growth plate cartilage cultures, we observed a rapid and significant decrease in chondrocyte hypertrophy and a concomitant increase in cell proliferation during 7 days in culture. These findings are important because the induction of hypertrophy and cell cycle exit are common observations in monolayer and micromass culture of chondrocytes.^{25,26} In these contexts, hypertrophy is often accompanied by expression of *IHH* and BMPs, and, thus, it has been assumed that isolated growth plate chondrocytes are unable to maintain expression of factors that are required to repress chondrocyte maturation.⁵⁴ By contrast, our results demonstrate that these alginate culture conditions promote the maintenance of proliferating and growth factor-responsive chondrocytes that exhibit minimal hypertrophy. Although the mechanism remains to be defined, endogenous production of PTHrP might underlie preservation of the chondrocyte phenotype in alginate since *PTHrP* expression continued to increase during the 7 day culture period and the addition of exogenous PTH1–34 inhibited the expression of both *ColX* and *IHH*, transcriptional markers of chondrocyte hypertrophy. These data strongly suggest that resting/proliferative chondrocytes, not hypertrophic chondrocytes, are the default maturation state in our alginate cultures.

***PTHrP* signaling is the primary driver of cell proliferation in alginate culture**

Although endogenous PTHrP production likely plays an important role in the maintenance of chondrogenesis, *PTHrP* expression in our culture system increases at a rate that is probably suboptimal for chondrocyte cultures. For example, during 7 days in basal medium, our cultures display increased cell cycle entry from ~5% of cells in S phase at day 0 to ~15% by day 7. In contrast, ~20% of resting and proliferative chondrocytes are in S phase in embryonic growth plate cartilage.⁵⁰ However, after only 1 day of treatment with PTH1–34, cell proliferation within our cultures was restored to *in vivo* levels in our cultures. PTH1–34 stimulation of cell proliferation occurs concomitant with a loss of *IHH* expression, suggesting that PTHrP signaling alone is the primary driver of cell proliferation in these cultures.

The apparent primacy of PTHrP signaling is surprising given the genetic evidence for roles of *IHH*, *Wnt5a*, and *Wnt5b* in cell cycle control.^{12,20,50} This paradox could be explained if these growth factors regulate *PTHrP* expression or if PTH1–34 induces

the expression of additional endogenous growth factors. The *in vivo* observation of 20% S phase cells likely represents a limit to the rate of proliferation in heterogeneous chondrocyte populations, since this rate was not exceeded by using lower or higher concentrations of PTH1–34 (data not shown). Together, our data suggest that the establishment of PTHrP signaling is a crucial step to restore normal cell proliferation levels in growth plate chondrocyte cultures. However, one potential challenge to using PTH1–34 in long-term cultures is the potential for desensitization of the PTHrP signaling pathway (Fig. 3), which could alter the cell proliferation rate and possibly lead to the induction of cell hypertrophy. Results presented here suggest that chronic treatment is not detrimental to cultures since continuous stimulation with PTH1–34 maintained maximum levels of cell proliferation and minimum levels of *ColX* and *Ihh* expression for at least 7 days in our cultures. Given these results, we suggest that PTHrP signaling plays a crucial, yet underappreciated role in maintaining cultures of growth plate chondrocytes.

***IHH* signaling establishes a hypertrophic zone in alginate culture**

PTH1–34 stimulation of the cell cycle and repression of hypertrophy (Fig. 3A, B) demonstrate that PTHrP pathway functions are maintained in chondrocytes within our alginate cultures.^{3,6} Likewise, our data show that functions of the *IHH* signaling pathway

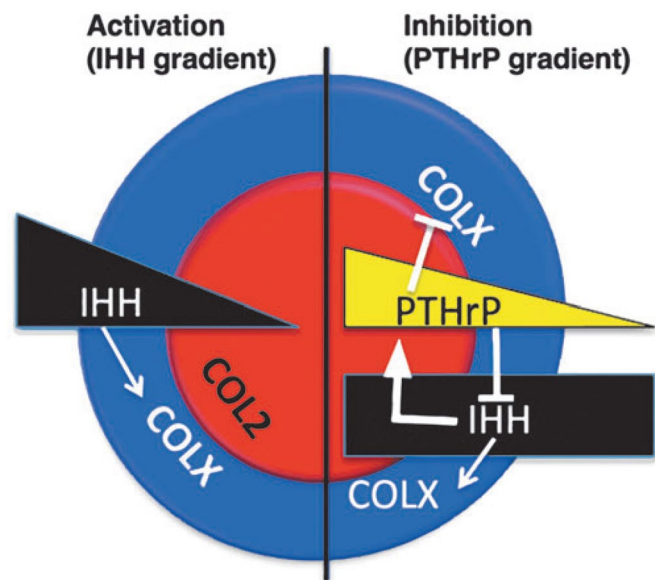


Fig. 7. Model exploring the potential mechanisms of *IHH*-driven zonal demarcation. Possibilities include an outside-in *IHH*-diffusion gradient (left) where cells that are exposed to high levels of *IHH* are induced to hypertrophy, but at the center of the bead, cells are seeing less *IHH* and maintain a static differentiation state. Alternatively, *IHH* diffuses into the bead rapidly, creating a uniform *IHH* concentration in media (right). Under this model, *IHH* induction of hypertrophy is balanced by endogenous PTHrP expression (differentiation block). Since PTHrP rapidly diffuses out of alginate but is constantly produced, this forms a radial gradient of PTHrP from the inside out where cells on the outer ring are exposed to a higher *IHH*/ PTHrP ratio, causing induction of hypertrophy.

are also preserved in these cultures. Specifically, the activation of IHH signaling either directly (IHH or PMA) or indirectly via BMP signaling (thyroxine or BMP4) increased the proportion of *ColX*-expressing chondrocytes in bead cultures (Fig. 5A, B). Induction of chondrocyte hypertrophy is an important PTHrP-independent function of IHH *in vivo*.⁸ Thus, in combination with the data from PTH1–34 treatments, our results demonstrate preservation in alginate culture of the crucial functions that comprise the PTHrP-IHH signaling feedback loop, a key node in the regulatory network of growth plate cartilage.^{2,5}

Although treatment with IHH, PMA, or thyroxine resulted in similar effects on chondrocyte hypertrophy, we observed one crucial difference—both IHH and thyroxine treatments produced regional differences in chondrocyte hypertrophy in alginate beads. Specifically, treatment with IHH or thyroxine was sufficient to stratify alginate bead cultures into an inner core consisting of nonhypertrophic (resting/proliferative) chondrocytes and an outer ring of *ColX*-expressing hypertrophic chondrocytes, which mimics the layered organization of growth plate cartilage *in vivo*.

Regional differences were unexpected from treatment with a single factor, but they could occur if diffusion of the hypertrophy-inducing factors into the bead was rate limiting. Restricted diffusion of IHH might be expected since the pore size in 1.5% alginate gels restricts the diffusion of molecules in the 10–70 kD range to varying degrees³⁷ and mature IHH protein has a molecular mass of 20 kD. However, IHH is sufficiently small that it could be expected to equilibrate in concentration throughout the bead during 4 days in culture,³⁷ and, therefore, a distinction between the core and the periphery should lessen over time. An additional argument against a requirement for sustained exogenous concentration gradients is the observation that the small molecule thyroxine also induced a hypertrophic domain. Thyroxine has a negative charge that should limit interaction with the alginate scaffold and a low molecular weight (0.78 kD) that should allow unimpeded diffusion in alginate gels.⁵² Therefore, the current data do not support a model in which sustained concentration gradients of signaling molecules are required to establish zonal differences in growth plate chondrocytes.

If not by sustained concentration gradients of exogenous signaling molecules, what mechanism might account for regionalization of chondrocytes in alginate beads? An alternative model is that a concentration gradient is only transiently required to initiate the formation of distinct maturation domains. In this context, the current results most likely indicate induction of PTHrP expression in the nonhypertrophic core chondrocytes in response to IHH produced by the peripheral hypertrophic chondrocytes (Fig. 7). In turn, endogenously produced PTHrP from the core chondrocytes might diffuse toward the periphery, inhibiting IHH-dependent hypertrophy. Consequently, an initial anisotropic growth factor stimulus can activate the endogenous IHH/PTHrP signaling feedback interaction that generates distinct zones of chondrocyte maturation as observed *in vivo*.

Conclusion

Our results are the first demonstration of a tunable, 3D culture model of growth plate cartilage. This culture system will be an important tool for elucidating the mechanisms underlying

chondrocyte differentiation, cartilage architecture. In addition, our *in vitro* model could be used to advance drug discovery by facilitating high-throughput screening for molecules that regulate cartilage growth. Finally, our results encourage future studies that aim at using alginate hydrogel scaffolds to engineer cartilage constructs of defined proportions *in vitro*.

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Supplementary Data

Supplementary ImageJ Macro

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SUPPLEMENTARY TABLE S1. SEQUENCE OF PRIMERS USED FOR DIGITAL DROPLET POLYMERASE CHAIN REACTION AMPLIFICATION

<i>Gene</i>	<i>Forward sequence</i>	<i>Reverse sequence</i>
Actin NM_007393.3 ⁴²⁻⁴⁴	AAATCGTGCGTGACATCAAAGA	GCCATCTCCTGCTCGAAGTC
HPRT NM_013556.2 ⁴²⁻⁴⁴	GACCTCTCGAAGTGTGGATAC	TCAACAGGACTCGTATTTG
RPL13-a NM_009438.4 ⁴⁵	ATCCCTCCACCCTATGACAA	GCCCCAGGTAAGCAAACCTT
Col1 NM_007742.4 ⁴⁶	GCAACAGTCGCTTCACCTAC	GTGGGAGGGAACCAGATTG
Col2 NM_031163 ⁴⁷	CGAGTGGGAAGAGCGGAGACTAC	CCAGTTTTTCCGAGGGACAGT
ColX NM_009925 ⁴⁶	TTCTGCTGCTAATGTCTTGACC	GGGATGAAGTATTGTGTCTTGGG
PTHrP NM_008970	GAAACGCAGAGAACAGGAGAA	AATTTCAATGCGTCCCTTAAGCTG
IHH NM_010544 ⁴⁸	CCCCAACTACAATCCCCGACA	TCATGAGGCGGTCCG

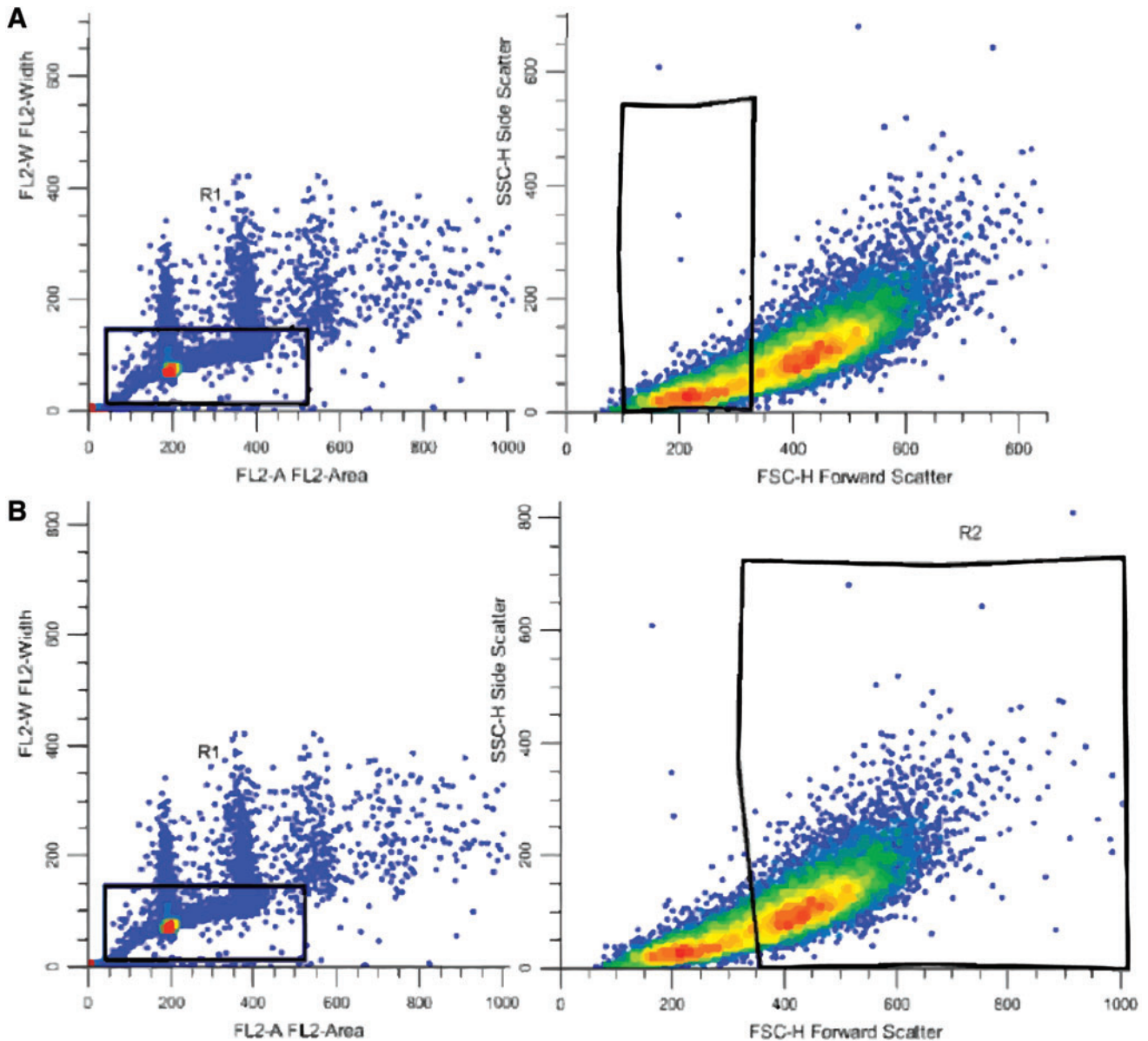
Primers used for ddPCR gene expression analysis. The table lists provided earlier give the name of all genes analyzed by ddPCR in the current study, the gene accession numbers that were used to generate each set of primers, and the publications that guided the selection of primers, where applicable (left column). Full sequences for each primer used in the current study (forward and reverse) are shown to the right.

ddPCR, digital droplet polymerase chain reaction; IHH, Indian hedgehog.

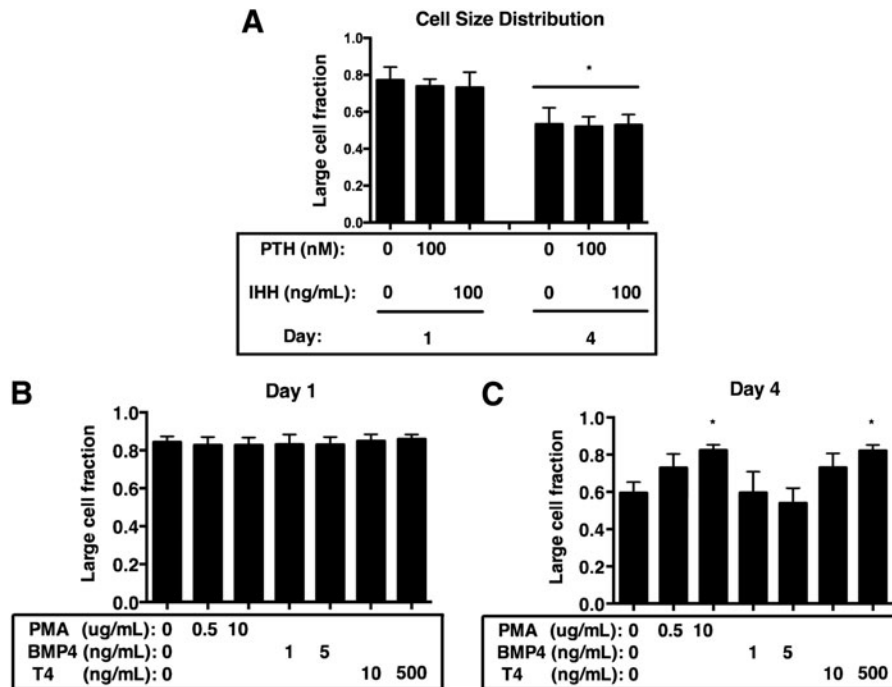
SUPPLEMENTARY TABLE S2. QUANTIFICATION OF THE LIVE-DEAD STAINS FROM FIGURE 1A

Time point	Live count	Dead count	Total cells	Live/total	Percent viability
Day 0	100	9	109	0.92	92
Day 1	143	11	154	0.93	93
Day 4	314	10	324	0.97	97
Day 7	258	13	271	0.95	95

Fluorescence images resulting from our viability assay were input to our automated ImageJ object-counting macro. Results from all time points analyzed are shown in each row. Counts in the green channel (live cells) and red channel (dead cells) were summed to calculate total cells. The ratio of living and total cells was multiplied by 100 to calculate percent viability (rightmost column).



SUPPLEMENTARY FIG. S1. Two distinct populations of chondrocytes isolated from growth plate cartilage and cultured alginate beads. (A, B) Examples of scatterplots generated in ModFit LTE software showing distributions of cell area with respect to cell width (left) and forward scatter with respect to side scatter (right) of propidium iodide-stained chondrocytes isolated from alginate bead culture. Rectangles show the gating strategy used to separate FACS data pertaining to smaller (A) versus larger (B) chondrocytes. * $p < 0.05$.



SUPPLEMENTARY FIG. S2. Cell size distribution modulated by exogenously activating IHH signaling. For all panels, counts of large, small, and total cell populations derived from propidium iodide S-phase analysis and used to calculate the fraction of total cells that were in the larger cell population. (A) Large cell fraction of chondrocytes cultured for 1 (*left*) and 4 (*right*) days in the presence or absence of PTH1-34 or IHH ($n=3$). (B, C) Large cell fraction of chondrocytes cultured in the presence or absence of IHH-stimulating factors for 1 day (B) and 4 days (C) in culture. BMP4, bone morphogenetic protein-4; IHH, Indian hedgehog; PMA, purmorphamine; PTH1-34, parathyroid hormone 1-34. For all graphs, $*p < 0.05$.