Published in final edited form as:

Differentiation. 2017 ; 95: 54–62. doi:10.1016/j.diff.2017.04.002.

Phosphate Regulates Chondrogenesis in a Biphasic and Maturation-Dependent Manner

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Abstract

Inorganic phosphate (Pi) has been recognized as an important signaling molecule that modulates chondrocyte maturation and cartilage mineralization. However, conclusive experimental evidence for its involvement in early chondrogenesis is still lacking. Here, using high-density monolayer (2D) and pellet (3D) ATDC5 chondrogenic models treated with ITS+, β -glycerophosphate (β GP), or ITS+/ β GP, we demonstrate that the cell response to Pi does not correlate with the Pi concentration in the culture medium but is better predicted by the availability of Pi on a per cell basis (Pi abundance). Three levels of abundance were found: basal (Pi/DNA < 10 ng/ μ g), moderate (Pi/DNA=25.3 – 32.3 ng/ μ g), and high abundance (Pi/DNA > 60 ng/ μ g). In chondrogenic medium alone, the abundance levels were at the basal level in 2D culture and moderate in 3D cultures. The addition of 10mM β GP resulted in moderate abundance in 2D and high in 3D cultures. Moderate Pi abundance enhanced early chondrogenesis and production of aggrecan and type II collagen whereas high Pi abundance inhibited chondrogenic differentiation and induced rapid mineralization. Inhibition of sodium phosphate transporters also reduced expression of chondrogenic markers. When 3D ITS+/ β GP cultures were treated with levamisole to reduce ALP activity, Pi abundance was decreased to moderate levels, which resulted in significant upregulation of chondrogenic markers, similar to the response in 2D cultures. Delay of phosphate delivery until after early chondrogenesis occurs (7 days) no longer enhanced chondrogenesis, but instead accelerated hypertrophy and mineralization. Together, our data highlights the dependence of chondrogenitor cell response to Pi on its availability to individual cells and the chondrogenic maturation stage of these cells and suggest that appropriate temporal delivery of phosphate to ATDC5 cells in 3D cultures represents a rapid model for mechanistic studies into the effects of exogenous cues on chondrogenic differentiation, chondrocyte maturation, and matrix mineralization.

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Keywords

Phosphate; Chondrogenesis; Chondrocyte Mineralization; 2D vs. 3D; Matrix Production; Endochondral Ossification; Culture-Dependent Differentiation; Biphasic

INTRODUCTION

Understanding the factors regulating chondrogenesis and endochondral ossification is important to treating developmental disorders and the cartilaginous diseases, as well as improving stem cell-based cartilage tissue regeneration. Dysfunction in chondrocyte proliferation can lead to developmental issues, such as achondroplasia¹ and hypochondroplasia². Hypertrophic differentiation of chondrocytes, a process that occurs normally during endochondral ossification, is associated with pathogenic changes in osteoarthritic articular cartilage^{3–5}. Mesenchymal stem cells (MSCs) also express hypertrophic markers after chondrogenic induction *in vitro*^{6,7} and *in vivo*⁸, hindering their clinical application for cartilage tissue regeneration.

One potent factor that mediates cell fate in chondrocytic cells is inorganic phosphate (Pi). Studies in ATDC5^{9–11}, primary growth plate¹², and limb bud cells¹³ have established that Pi in the range of 2–4mM upregulates collagen type X expression and apoptosis in mature chondrocytes, thus, inducing terminal differentiation. The role of Pi as a signaling molecule has also been demonstrated in several other cell types^{14–18}, including osteochondral progenitor cells in which it induces osteogenic differentiation^{14,15}. Furthermore, many of these cell-mediated activities are regulated by other exogenous cues, such as growth factors^{19–21} and matrix composition¹³.

The response to Pi is orchestrated by the activity of phosphatases, such as alkaline phosphatase (ALP)^{22–24}, the availability of pyrophosphate (PPi)^{25,26}, and activity of sodium-dependent phosphate transporters in skeletal^{27,19,28} and non-skeletal systems^{29,30}. ALP cleaves PPi to release Pi³¹, and ALP expression in the growth plate progressively increases from being hardly detectable in the proliferative zone to higher in the maturing zone, and highest in the hypertrophic zones³². Deficiency of ALP activity causes hypophosphatasia, which is a skeletal disease associated with diminished or absent hypertrophic zones in the growth plate³³. The expression profile of sodium-dependent phosphate cotransporter 1 (PiT-1) has been found to be variable during endochondral ossification. *In vitro*, expression levels were found to be highest during the early stages of chondrogenesis in the CFK2 chondroprogenitor cell line³⁴ and during the proliferative and the early phase of hypertrophy in ATDC5 cells^{28,35}. *In vivo*, the expression profile of PiT-1 has been variable depending on species³⁶. The effects of Pi during terminal differentiation require the activity of PiT-1^{27,19,28,37}, as inhibition of its activity negates Pi induced apoptosis during terminal differentiation³⁷.

Although ALP activity is highly upregulated in hypertrophic and terminally differentiated chondrocytes during endochondral ossification³², Pi is present in the resting and proliferative zones of the growth plate³⁸. However, few studies have investigated whether chondrogenic differentiation or early events in the endochondral ossification pathway are

regulated by Pi. Kimata *et al.* demonstrated that Pi treatment upregulated cell proliferation through ERK1/2 mediated cyclin D1 expression in the ATDC5 cell line and primary chondrocytes²⁸. Wang *et al.* also showed that transient Pi treatment upregulated collagen type II gene expression³⁴. Taken together with the previously reported expression profile of PiT-1 and activity of ALP, these studies suggest that Pi plays a direct regulatory role in early chondrogenic differentiation and proliferation during endochondral ossification.

To elucidate the role of phosphate in early chondrogenic events, we used ATDC5 cells in 2D and 3D cultures. This cell line, established by Atsumi *et al.* from the mouse teratocarcinoma cells AT805, recapitulate the phases of endochondral ossification, from chondrogenic commitment to hypertrophic differentiation, with the addition of insulin^{39–41}. In these cells, chondrogenic maturation and matrix mineralization occur on an accelerated timescale if given an exogenous source of phosphate^{10,11}. Since ALP activity has also been shown to change as chondrocytes differentiate and mature^{32,42}, we used β -glycerophosphate (β GP) as our phosphate source to ensure cells control the release of phosphate. Although 10mM β GP is considered a supraphysiologic concentration of organic phosphate for *in vitro* studies⁴³, it was found in this study that the maximum Pi concentration in the media using this level of β GP is 4mM, the concentration employed in many previous mechanistic studies^{28,9,34}. So that the $\text{Ca} \times \text{Pi}$ never reaches levels that lead to dystrophic precipitation of CaPO_4 , calcium concentration was maintained at 1.3mM^{30,44}. Using this media formulation, we found that the response of chondroprogenitor cells was regulated by Pi availability on a per cell basis (Pi abundance). Specifically, moderate Pi abundance upregulates markers of chondrogenesis whereas high abundance levels inhibit chondrogenesis and stimulate rapid matrix mineralization. We also show that this biphasic response to Pi concentration is mediated by ALP activity and cellular uptake of Pi and is dependent on the maturation stage of chondrocytes. Finally, delaying the addition of exogenous phosphate to ATDC5 cells in 3D cultures upregulates the expression of hypertrophic markers and accelerates terminal differentiation without dystrophic mineral formation, demonstrating that this culture system may serve as a rapid and physiologic model of endochondral ossification.

METHODS

Cell culture

ATDC5 cells (Sigma) were maintained in DMEM/F12 (Life Technologies) supplemented with 5% FBS (Life Technologies) and 1% Antibiotic-Antimycotic (Life Technologies). Chondrogenesis of ATDC5 cells was induced with chondrogenic differentiation medium (ITS+) consisting of the growth medium supplemented with 1% ITS+ Premix (Corning) and 50 $\mu\text{g}/\text{ml}$ ascorbate acid-2-phosphate (Sigma). Co-treatment medium (ITS+/ β GP) consists of ITS+ medium supplemented with 10 mM β -glycerophosphate (Sigma). Non-chondrogenic mineralizing medium (β GP) consists of growth medium supplemented with 10mM β GP. Calcium concentration was maintained at 1.3mM.

Monolayer (2D) culture—Four days prior to chondrogenic induction, cells were seeded at the density of 6000 cells/ cm^2 in multiple well plates. When cells reached 100% confluence

(D0), differentiation was initiated by replacing the growth medium with differentiation medium. Cultures were fed with 2ml medium every other day, and maintained for 21 days.

Pellet (3D) culture—Four days prior to chondrogenic induction, cells were seeded at the density of 6000 cells/cm². When cells reached 100% confluence (D0), they were trypsinized and centrifuged into pellets containing 2.5×10^5 cells in round-bottomed polypropylene 96-well-plate. Cultures were fed with 200μl medium every other day. Pellet cultures were maintained for 21 days with differentiation medium. In delayed treatment, pellets were switched to ITS+βGP medium after 7 or 14 days in ITS+ medium.

Biochemical analysis

Cartilage-specific matrix production was measured by the 1,9-dimethylmethylene blue (DMMB) assay as previously described⁴⁵. Both monolayer cell tissues and pellets were digested with 1 mg/ml proteinase K in 0.1M ammonium acetate at 50°C for 16 hours. Digested samples were mixed with DMMB dye (pH 1.5) at a ratio of 1:20, and the GAG content of the samples were determined by comparing the ratio of 525 nm to 595 nm readings to the standard curve derived from shark chondroitin sulfate. Measured GAG content of each sample was normalized by its DNA content using Hoechst 33258 dye (Sigma) as previously described⁴⁶. To measure mineral content of each sample, insoluble residues from the proteinase K digestion were collected and hydrolyzed by with 10% (w/w) acetic acid for 48 hours at 40°C. Hydrolyzed solution was added to Arsenazo III dye (Pointe Scientific) at ratio of 1:15, and the absorbance was read at 650 and 500 nm. The mineral content was calculated by comparing the ratio of these two absorbance readings to a standard curve created from calcium chloride.

Gene expression

Total RNA of each sample was extracted using TRI Reagent® RT (Molecular Research Center). For pellet culture, five pellets were combined and homogenized in the same reagent using the Micro Tube Homogenizer. Equal amounts of extracted RNA were reverse-transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kit (Life Technologies). Synthesized cDNA was amplified using SYBR® Green PCR Master Mix (Life Technologies) on Applied Biosystems® 7500 Fast platform. The mean cycle threshold of the housekeeping genes ($\overline{Ct}_{hk}$) *Hprt* and *Ppia*⁴⁷ were used to calculate the fold change in transcript levels compared to day 0 samples using the Ct method. Relative expression levels were calculated as $x = 2^{-Ct}$, in which $Ct = E - C$, $\Delta E = Ct_{exp} - \overline{Ct}_{hk}$ at the time-point of interest, and $\Delta C = Ct_{exp} - \overline{Ct}_{hk}$ at day 0. The forward and reverse primer sequences are listed in Table 1.

Histological analysis

Monolayer cultures were washed with PBS, and fixed in 70% ethanol at 4°C for 16 hours. Differentiated pellets were washed with PBS, and fixed in 5% buffered formalin at 4°C for 16 hours. Fixed pellets were washed with 70% ethanol, embedded in paraffin wax, and then 7 μm sections were taken. Proteoglycan accumulation in monolayer tissues and pellets was

evaluated by staining the sectioned tissue with 3% Alcian blue dye (Poly Scientific), and calcium deposition was visualized by staining with 2% Alizarin red S dye (pH 4.3).

Alkaline Phosphatase Activity Analysis

Alkaline phosphatase activity was measured by the QuantiChrom™ Alkaline Phosphatase Assay Kit (BioAssay Systems). Both monolayer cell tissues and pellets were washed with PBS, then lysed in 0.2% Triton-100 solution for 30 minutes at room temperature. Upon addition of lysis buffer, pellet samples were ground with a pestle to break apart the tissue. Alkaline phosphatase activity was determined by taking the ratio of the difference between 405 nm readings taken immediately after adding the assay buffer (t=0) and after 4 minutes on a plate reader to the difference between the 405 nm readings of the calibrator (Tartrazine) and deionized water using the equation from the manufacturer's protocol.

Phosphate Concentration Analysis

Concentration of phosphate in the media was determined using the QuantiChrom™ Phosphate Assay Kit (BioAssay Systems). Media from each test condition was harvested and diluted 1:20 with water prior to the addition of assay reagent. Phosphate concentration was determined by reading optical density at 620 nm, followed by calculation using equations given in the manufacturer's protocol.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. All comparisons over multiple time points were analyzed by two-way ANOVA with Tukey's multiple comparison method. Comparisons in experiment at a single time point were analyzed by one-way ANOVA followed by Sidak correction. Significant difference is indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. All error bars indicate the standard error of the mean (s.e.m.).

RESULTS

The influence of β GP supplementation on early chondrogenesis in 2D and 3D cultures

To better understand the individual and synergistic effects of β GP on early chondrogenesis, we monitored the gene expression of early chondrogenic markers, *Acan* and *Col2a1* gene expression as well as the amount of cartilaginous matrix produced by ATDC5 cells in both high density monolayer (2D) and pellet (3D) cultures. Both culture types were treated with ITS+, β GP, or ITS+/ β GP for 14 days. Notably, the expression of genes and matrix components were 2–10 times higher in 3D cultures compared to 2D. Nonetheless, both 2D and 3D cultures exhibited a similar trend of chondrogenic gene expression and matrix accumulation when they were treated with ITS+ or β GP individually. Chondrogenesis and proliferation (not shown) of chondrogenic cells occurs within the first 7 days of culture in ITS+ medium in 2D and 3D. Upregulation of *Acan* and *Col2a1* gene expression (Fig 1a) and production of sGAG-rich matrix (Fig 1b & 1d) in both monolayer and pellet cultures treated with ITS+ confirmed early chondrogenesis. In the absence of ITS+, cells cultured in medium supplemented with 10mM β GP did not express chondrogenic markers in either culture type (Fig 1a, b & d) and induced rapid mineralization of the matrix (Fig 1c & e). The response to

combined ITS+/ β GP treatment, however, was dissimilar in the two systems. In monolayer culture, cells differentiated with ITS+/ β GP showed significantly higher mRNA levels of *Acan* and *Col2a1* (Fig 1a) as well as a 1.6-fold increase in accumulation of an sGAG-rich matrix (Fig 1b & 1d) compared the ITS+ alone group. Mineral also formed in these cultures, but did not appear to be associated with nodules of chondrogenic cells. Conversely, the addition of β GP to ITS+ treated pellet cultures suppressed expression of *Acan* and *Col2a1* genes and matrix accumulation and stimulated mineralization.

The culture-dependent effect of ITS+/ β GP on early chondrogenesis is regulated by ALP activity and Pi abundance

To determine if the effects of β GP supplementation on ATDC5 cells is controlled by cell-mediated regulation of Pi availability, we measured the ALP activity of the cells and subsequent Pi concentration in the media of both systems during early chondrogenesis (days 1, 3, 5, and 7). The ALP activity of cells in monolayer cultures (Fig 2a) induced with ITS+ alone or ITS+/ β GP increased steadily over the first 7 days. In comparison, cells treated with β GP only had a higher level of ALP activity, which peaked at day 3 and was maintained until day 5. By day 7, ALP activity was similar in all 2D culture conditions. In contrast to 2D cultures, ALP activity was highest in cells on day 1 in 3D-ITS+ cultures and then rapidly decreased as differentiation proceeded during the first week. When pellets were treated with β GP or co-treated with ITS+/ β GP, ALP activity was maintained at this high level through day 3 and then decreased to levels similar to the 3D-ITS+ control group (Fig 2d).

The basal concentration of Pi in the medium of 2D and 3D-ITS+-treated cultures was 1mM (the concentration in DMEM/F12). With β GP treatment, Pi concentration in the medium increased to 4mM on average in 2D cultures from days 3–7 (Fig 2b) regardless of ALP activity. Slightly lower Pi levels (~3.6mM on average) were found in 3D cultures treated with β GP or ITS+/ β GP (Fig 2e).

We further estimated the Pi availability on a per cell basis by normalizing the total Pi content in the medium by total DNA content (Fig 2c & f), which we are defining as Pi abundance. Three levels of Pi abundance were observed and were found to be dependent on culture condition (2D vs. 3D) and ITS+ and/or β GP supplementation: low abundance (Pi/DNA < 10 ng/ μ g) was found in 2D-ITS+ cultures; moderate abundance (Pi/DNA=25.3 – 32.3 ng/ μ g) was found in 2D-ITS+/ β GP and 3D-ITS+ cultures; and high abundance (Pi/DNA > 60 ng/ μ g) was detected in culture conditions with the highest-level ALP activity—2D- β GP, 3D-ITS+/ β GP, and 3D- β GP. As was shown in Fig. 1, groups exposed to moderate Pi abundance levels had enhanced expression of *Acan* and *Col2a1* as well as increased sGAG accumulation (Fig 1b & 1d) whereas high Pi abundance suppressed these markers in 3D cultures. Thus, high Pi abundance correlates with inhibition of early chondrogenesis while moderate Pi abundance correlates with enhanced chondrogenic differentiation.

To explore the possible pathways that could be involved in mediating the response to available levels of Pi to the cells, we examined the gene expression of mediators of chondrogenesis (*Sox9*), chondrocyte hypertrophy (*Runx2*), and a known inhibitor of chondrogenesis, β -catenin (*Ctnnb1*) as well as their gene targets (*Col2a1*, *Col10a1*, and

Axin2, respectively) before, during, and after the change in Pi abundance at day 1, day 3, and day 7. Although Pi abundance increased in response to additional β GP supplementation starting at day 3, there were not significant discrepancies in gene expression between groups with different Pi levels until day 7. The increase in Pi abundance from low to moderate (comparing 2D ITS+ to 2D ITS+/ β GP) correlated with higher mRNA levels of *Sox9* and its gene target *Col2a1*, as well as *Col10a1*. However, no significant difference in the expression of *Runx2*, *Ctnnb1*, or *Axin2* was observed (Fig 3a). The shift from moderate to high Pi abundance (comparison of 3D-ITS+ to 3D-ITS+/ β GP) resulted in elevated *Col10a1* gene expression with no associated change in the expression levels of *Runx2*. High Pi abundance also reduced the level of *Col2a1* gene expression and increased the gene expression of *Ctnnb1* and its target *Axin2* (Fig 3b).

Inhibition of ALP Activity restores early chondrogenesis in 3D

The next step was to examine if the activity of ALP and Pi uptake by ATDC5 cells played a role in the β GP-induced inhibition of early chondrogenesis in 3D cultures. ALP activity and Pi uptake were individually inhibited using levamisole and PFA, respectively. In 3D-ITS+/ β GP cultures, levamisole treatment reduced the Pi abundance to Pi/DNA=48.4 ng/ μ g after day 3 (Fig 4a). This is a level slightly higher than the moderate range, but the difference was not significant. ALP inhibition in the ITS+/ β GP group enhanced markers of early chondrogenesis compared to 3D-ITS+ cultures (moderate Pi abundance). There was a slight increase in *Col2a1* gene expression over ITS+ controls (Fig. 1b; not significant) and significantly higher sGAG expression (Fig 4c & d). Furthermore, levamisole treatment reduced expression of the phosphoprotein osteopontin (OPN), a marker sensitive to phosphate treatment⁴⁸. Without the addition of β GP, levamisole treatment had no effect on chondrogenesis (data not shown). Pi abundance was reduced with treatment of PFA, but remained in the high range (Pi/DNA>64.3 ng/ μ g; Fig 4a). Accordingly, PFA treated pellets were noticeably smaller than other conditions. In these pellets, *Col2a1* expression and matrix deposition were inhibited (Fig 4b & c). Taken together with the response of the cells exposed to moderate Pi abundance in 2D cultures, the response of ATDC5 cells to both inhibitors in 3D indicates that phosphate uptake is required for early chondrogenesis and that a moderate level of phosphate will enhance matrix production of chondrocytes.

Delayed β GP supplementation expedites late chondrogenesis in 3D cultures

To determine if the biphasic response to β GP continues after early chondrogenesis, β GP treatment was delayed for 7 or 14 days in pellet cultures. While Pi abundance was significantly increased in both delayed groups once β GP was added, the level remains in the moderate range (Pi/DNA<42 ng/ μ g; Fig 5a). The expression of *Acan* or *Col2a1* genes on days 14 and 21 of culture was comparable to or slightly higher than the ITS+ group (not significant; Fig 5c). Accordingly, the β GP-induced inhibition of sGAG production was not observed as demonstrated by histological staining (Fig 5b) and quantification of sGAG production (Fig 5d). Instead, sGAG accumulation was increased in the 7-day delayed group 1 week after the addition of β GP. By day 21 of culture, sGAG levels in both delayed groups were equivalent to the ITS+ group.

Col10a1 gene expression was upregulated by day 21 in the 7-day delayed group over cultures treated with ITS+ alone or ITS+/βGP from day 0 (Fig 5c). Calcium accumulation was the same 7 days after βGP addition in both delayed groups, which was 13.5% of the amount accumulated when βGP was added from day 0 (Fig 5e). Notably, calcium deposition in the delayed group did not occur linearly as observed in cultures with βGP from day 0. Thus, moderate Pi abundance induces hypertrophy and mineralization in ATDC5 cells without altering their chondrogenic response.

DISCUSSION

Here we demonstrate that the availability of Pi on a per cell basis and the maturation point of chondroprogenitor cells both determine their response to phosphate during endochondral ossification. Using the measure of Pi abundance, we show that there is a biphasic response to phosphate during early chondrogenesis – moderate levels enhance chondrogenesis and matrix production whereas, if the differentiating chondrocytes are exposed to high levels during early endochondral ossification, chondrogenesis is inhibited and rapid calcification ensues. The observed discrepancy in Pi abundance between 2D and 3D systems also highlights the culture-dependent ALP activity in response to βGP. It was possible to reduce Pi abundance to moderate levels by partially inhibiting ALP activity, which reversed βGP-induced suppression of chondrogenesis. Similar to 2D cultures, moderate Pi abundance increased expression of chondrogenic markers and deposition of sGAG-rich matrix in 3D cultures. After early chondrogenesis (7–10 days), the response to βGP delivery by these cells is no longer biphasic and hypertrophy makers are upregulated. Thus, the cell response to Pi is maturation-dependent.

We show that it is important to evaluate the effect of Pi on chondrogenesis by examining its availability on a per cell level. Emerging evidence has shown that Pi can function as a signaling molecule to regulate cellular functions and differentiation of MSCs¹⁴, osteoblasts¹⁵, and chondrocytes^{49,9}. In both osteoblasts and chondrocytes, the signaling function of Pi was shown to be mediated by phosphate influx through sodium-dependent phosphate transporters^{14,34}. In culture, the volume of medium and number of cells present, in addition to the concentration of Pi, would affect the Pi influx among individual cells. Taking these factors into consideration, the availability of Pi on a per cell level is significantly different in two scenarios reported here. First, 2D and 3D culture have different ratios of medium volume to the number of cells in individual wells, resulting in culture-dependent discrepancy in Pi abundance. Second, significant proliferation occurs during the chondrogenic differentiation in both systems, changing the cell number in the culture. Thus, Pi abundance decreases as differentiation proceeds, which, as we have shown, alters cellular response. Thus, these factors should be accounted for in studies of the effects of exogenous phosphate on cell function.

The literature indicates that Pi both negatively and positively regulates early chondrogenesis^{28,34,50–56}. Our results strongly suggest a biphasic model that may explain these discrepancies. For instance, recent studies that have shown that the Wnt/β-catenin pathway prevents MSCs chondrogenesis^{50,51}, possibly promoted by A2b adenosine receptor activity that is mediated by cellular phosphate uptake through PiT-1^{5253–55}. In accordance

with these studies, we observed increased gene expression of *Ctnnb1* (β -catenin) and its target gene, *Axin2*, as well as decreased Sox9 activity, extrapolated from decreased expression of *Col2a1*, in the high Pi culture, which suggests that high Pi abundance inhibits early chondrogenesis through upregulation of β -catenin activity. The measured increase in Sox9 expression and activity in response to moderate Pi conditions in our studies agrees with the results of Wang et al., which reported upregulation of PiT-1 and Col2a1 gene and protein expression in response to short-term Pi treatment (24hr) in differentiating CFK2 cells³⁴. Additionally, Kimata et al. demonstrated that 4mM Pi enhanced proliferation in early stages of chondrogenic differentiation of ATDC5 cells and primary chondroprogenitors through phosphorylation of ERK1/2²⁸, a pathway also involved in the upregulation of aggrecan expression⁵⁶. To our knowledge, most literature on the signaling function of phosphate reports Pi level in the form of medium concentration. Therefore, it is difficult to correlate previous results with the biphasic effect observed here without calculating the numbers of cells used in those studies. Future studies that monitor Pi abundance may resolve the biphasic response observed here and in the literature.

Delaying β GP supplementation in pellet culture until after the progression of early chondrogenesis prevented exposing the cells to high Pi abundance during this stage, as cells rapidly reduced their ALP activity after differentiation was initiated. This model may better reflect *in vivo* conditions, as ALP activity is detected in bone marrow chondroprogenitor cells but not in resting or proliferative chondrocytes in growth plate³². The moderate increase in the Pi abundance in the delayed model also led to a mineral deposition profile that resembles the physiological mineralization of cartilage matrix^{57,58}, unlike the linear mineral formation observed in non-delayed group. This may be due to the presence of matrix deposited during early chondrogenesis. For example, aggrecan, a high molecular weight proteoglycan, has been reported to inhibit the growth mineral crystals^{59–61}. Degradation and reorganization of collagen are also important for correct mineral formation in bone^{62,63}. Therefore, this model may be used to further evaluate the role of matrix accrual and modification on the response of chondroprogenitor cells to phosphate and cartilage mineralization during endochondral ossification as well as diseases, such as osteoarthritis.

Some limitations of this study need to be considered. Our analyses are based on extracellular Pi concentration in the medium, which may not directly reflect Pi uptake by the cells. It would be interesting to measure the phosphate influx into the cells to further clarify the role of Pi in regulating early chondrogenesis. Moreover, the ATDC5 cell line used in this study is derived from embryonic teratocarcinoma. While the response of this cell line has been verified in primary cells in many studies^{28,64,65}, additional experiments designed to investigate the role of Pi in primary chondrocytes and stem cells will help validate our findings. Furthermore, we have neglected the contribution of other regulatory proteins, such as glycoprotein-1 (PC-1) and ankylosis protein (ANK) and phosphatases such as phosphatase orphan 1 (PHOSPHO1), that have been shown to mediate the balance of PPI/Pi in mineralizing cultures. Future studies will determine the role of these factors in mediating the biphasic response of ATDC5 cells to Pi.

In conclusion, our results demonstrate that chondroprogenitor cell response to Pi is biphasic and is regulated by the availability of Pi on a per cell basis. Our delayed model demonstrates

that such a biphasic response to Pi depends on the maturation stage of chondroprogenitor cells. Delayed delivery of β GP in pellet culture of ATDC5 cells can be used as a rapid model to conduct mechanistic studies of chondrogenesis, chondrocyte maturation, and matrix mineralization during endochondral ossification. By ensuring that Pi abundance is moderate for the first 7 days of differentiation and then high thereafter, chondrogenesis and accelerated hypertrophy can be achieved in a short timeframe. Finally, the findings that moderate Pi abundance enhances the expression of chondrogenic markers during early chondrogenesis and extended exposure increases deposition of a cartilaginous matrix suggests that phosphate delivery may also be a cost-effective method to improve the outcome of cartilage tissue engineering strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Sanjana Murali for assistance in histological staining. The work was supported by University of Michigan's TMJD and Orofacial Pain Interdisciplinary Consortium UM-TOPICs Institutional Career Development Training Grant (K12DE023574).

Abbreviations

βGP	β -glycerophosphate
Pi	inorganic phosphate
2D	two-dimensional
3D	three-dimensional
qPCR	quantitative polymerase chain reaction
sGAG	sulfated glycosaminoglycan
ALP	alkaline phosphatase
PiT1	sodium-dependent phosphate cotransporter 1; pyrophosphate-PPi

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Figure 1. Differential effects of ITS+/βGP co-treatment on early chondrogenesis of ATDC5 cells in 2D and 3D cultures

(a) Quantification of mRNA expression (relative to day 0 and the housekeeping genes *Hprt* and *Ppia*) of early chondrogenic markers (*Acan* and *Col2a1*) in monolayer (2D) and pellet (3D) cultures over 14 days of differentiation. Fold change (relative to day 0) in sGAG accumulation (b) and calcium deposition (c) normalized to DNA content over 14 days of differentiation in 2D and 3D cultures treated with ITS+ or ITS+/βGP. (d) Alcian blue staining of 2D and 3D cultures at day 14 to show accumulation of sGAG-rich matrix. 3D cultures stained also with nuclear fast red counterstain to show cell structure. (e) Alizarin Red staining of 2D and 3D cultures at day 14 to show mineral content in each sample. Data represented as mean ± s.e.m. Significant difference from the ITS+ group at the same time point (n = 8) is indicated by * P<0.05, ** P<0.01, ***P<0.001, ****P<0.0001 by two-way ANOVA with Tukey's multiple comparison method. NS, not significant.

Figure 2. The culture-dependent effect of ITS+/βGP on early chondrogenesis is regulated by ALP activity and Pi abundance

(a & d) Normalized ALP activity, (b & e) Pi concentration (mM) present in the medium, and (c & f) Pi availability on a per cell basis during the first seven days of differentiation in 2D and 3D cultures treated with ITS+, ITS+/βGP, or βGP (n=3). Data represented as mean ± s.e.m. Significant difference from the ITS+ group at the same time point is indicated by * P<0.05, ** P<0.01, ***P<0.001, ****P<0.0001 by two-way ANOVA corrected with Tukey's multiple comparison method. NS, not significant. ¥ indicates significance difference between ITS+/βGP and βGP conditions at the same time point (P<0.0001).

Figure 3. Gene expression reveals possible pathways involved in the biphasic regulation of Pi
 Quantification of mRNA expression (relative to day 0 and Hprt and Ppia) of genes and downstream effectors associated with early chondrogenesis and hypertrophy in 2D (a) and 3D (b) cultures treated with ITS+ and ITS+/βGP during the first seven days of differentiation. Data represented as mean $\pm$ s.e.m. Significant difference from the ITS+ group at the same time point is indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ by two-way ANOVA with Tukey's multiple comparisons. NS, not significant.

Figure 4. Inhibition of ALP Activity or Pi uptake restored early chondrogenesis in 3D
 (a) Pi availability on a per cell basis in 3D culture treated with ITS+, ITS+/βGP, ITS+/βGP/levamisole, or ITS+/βGP/PFA during the first seven days of differentiation. (b) Quantification of mRNA expression (relative to day 0 and *Hprt* and *Ppia*) of the chondrogenic marker *Col2a1* and phosphate uptake marker *OPN* at day 7 of differentiation. (c) Alcian blue and alizarin red staining of 3D cultures treated with ITS+, ITS+/βGP, ITS+/βGP/levamisole, or ITS+/βGP/PFA at day 14 of differentiation. Pellets stained with Alcian blue were also stained with nuclear fast red counterstain to show cell structure. (d) Fold change in sGAG accumulation normalized to DNA content (relative to day 0) over 21 days of differentiation in 3D cultures treated with ITS+ or ITS+/βGP/levamisole. Data represented as mean ± s.e.m. Significant difference from the ITS+ group at the same time point is indicated by * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001 by one-way or two-way ANOVA with Tukey's multiple comparisons. NS, not significant. n = 3.

Figure 5. Delayed βGP supplement expedites late chondrogenesis

(a) Pi availability on a per cell basis in 3D culture treated with ITS+, and ITS+βGP supplemented from day 0, day 7, day 14 during 21 days of differentiation. (b) Alcian blue and alizarin red staining of delayed 3D cultures at day 21. Fold changes in mRNA expression (relative to day 0 and the housekeeping genes *Hprt* and *Ppia*) of early and late chondrogenic markers (*Acan*, *Col2a1*, and *Col10a1*) (c), normalized sGAG accumulation (d), and normalized calcium deposition (e) in delayed 3D cultures over 21 days of differentiation. (f) Comparison of fold changes in normalized calcium deposition between 7-day delayed and no delay 2D and 3D cultures after βGP supplementation. Data represented as mean ± s.e.m. Significant difference between ITS+ and other conditions at the same time point is indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ by two-way ANOVA with Tukey's multiple comparison method. NS, not significant. n = 4.

Table 1

The sequences of primers used

Gene		Primer Sequences (5'→3')	
<i>Acan</i>	Forward	CGCCACTTTCATGACCGAGA	Reverse CAAATTGCAGAGAGTGTCCTG
<i>Col2a1</i>	Forward	ACGAGGCAGACAGTACCTTG	Reverse AGTAGTCTCCGCTCTTCCACT
<i>Col10a1</i>	Forward	CCAAACGCCCACAGGCATAA	Reverse TGCCTTGTTCTCCTCTTACTGG
<i>Hprt</i>	Forward	CTGGTGAAAAGGACCTCTCGAA	Reverse CTGAAGTACTCATTATAGTCAAGGGCAT
<i>Ppia</i>	Forward	CGCGTCTCCTTCGAGCTGTTTG	Reverse TGTAAGTCACCACCCTGGCACAT
<i>Sox9</i>	Forward	CGGAACAGACTCACATCTCTCC	Reverse GCTTGACGTCGGTTTTGG
<i>Runx2</i>	Forward	CCACGGCCCTCCCTGAACTCT	Reverse ACTGGCGGGGTGTAGGTAAAGGTG
<i>Ctnnb1</i>	Forward	CACAGCTCCTTCCTGAGTG	Reverse CTGCCCCGTCATATCAGCTACT
<i>Axin2</i>	Forward	AAGCCCCATAGTGCCCAAAG	Reverse GGGTCCTGGGTAAATGGGTG
<i>Slc20a1</i>	Forward	GGAACGGCTTGATAGATGTGG	Reverse GCAGAACCAAACATAGCACTGAC
<i>Slc20a2</i>	Forward	CCGTCCAGTGGCTTCACTAT	Reverse AGTCACGAACCAGGCAACAA
<i>Osteopontin</i>	Forward	AGCAAGAACTCTTCCAAGCAA	Reverse GTGAGATTCGTCAGATTCATCCG
<i>Osteocalcin</i>	Forward	GCCCTGAGTCTGACAAAGGTA	Reverse GGTGATGGCCAAGACTAAGG