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# Extracellular Phosphate as a Signaling Molecule

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## Abstract

Phosphorus is involved in various biological processes including membrane integrity, maintenance and inheritance of genetic materials, energy metabolism, intracellular signaling and skeletal mineralization. In addition, accumulating evidences have indicated that alteration in the levels of extracellular inorganic phosphate (Pi) itself triggers signaling to regulate gene expression and cellular functions in some cell types. In bone cells such as osteoblasts and chondrocytes, extracellular Pi modulates cell proliferation, differentiation, mineralization and apoptosis. In extraskeletal tissues, extracellular Pi also exerts various effects. For example, increased extracellular Pi results in the calcification associated with the upregulation of osteoblast marker genes in vascular smooth muscle cells. As to the mechanistic aspects, it is suggested that an increase in extracellular Pi triggers signal transduction via the PiT1 type III sodium/phosphate (Na<sup>+</sup>/Pi) cotransporter and ERK1/2 pathway. Unicellular organisms such as bacteria and yeast sense the environmental Pi with a protein complex located in the plasma membrane, which regulates the expression of multiple genes involved in Pi uptake and metabolism to adapt to its availability. In mammals that are multicellular organisms, Pi availability should be sensed both at a cellular level to regulate the function of each cell and as a whole body to maintain the Pi homeostasis of the extracellular fluid. Although the responsiveness to the increased extracellular Pi suggests the existence of Pi-sensing mechanism in mammalian cells as well, it is unknown whether the sensing of Pi availability at a cellular level and that at a whole-body level share the same pathway or not. This chapter will review the findings regarding the regulation of various cellular functions by extracellular Pi, and also discuss the current concept on the mechanism for Pi-sensing.

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Phosphorus is critical in various biological processes including membrane integrity, synthesis of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), energy metabolism and intracellular signaling. In vertebrates, phosphorus is also

essential in skeletal mineralization. In human adults, approximately 85% of the total phosphorus exists in bone as complex with calcium in hydroxyapatite crystals deposited onto the collagen matrix. Fifteen percent of phosphorus is present in soft tissues with only 0.1% in the extracellular fluids [1]. Phosphorus in serum is present mainly as inorganic phosphate (Pi) in the form of free ions  $\text{HPO}_4^{2-}$  and  $\text{H}_2\text{PO}_4^-$ , and the former is dominant in physiological pH. The majority of the intracellular phosphate ion exists as bound form or inorganic phosphate esters, phospholipids, or phosphorylated intermediate molecules involved in various biochemical processes. The concentration of Pi in the extracellular fluid and cytosol is about  $10^{-4}$  and  $2 \times 10^{-4}$  M, respectively. The intracellular concentration of Pi is maintained by membrane transporters, which function dependently on sodium ( $\text{Na}^+$ ) gradient across the plasma membrane [1].

In mammals,  $\text{Na}^+$ -coupled Pi cotransporters have been classified into three families, based on the amino acid sequence and structural similarity [2]. All cotransporters are multispinning membrane proteins. The type I  $\text{Na}^+$ /Pi cotransporter belongs to the solute carrier family 17 (SLC17), and is known as NPT1 in humans. In addition to the Pi transport activity, type I cotransporters transport organic anions, and their physiological roles are not fully established. Type II  $\text{Na}^+$ /Pi cotransporters are assigned to SLC34, and include NPT2a, NPT2b and NPT2c. Npt2a and Npt2c were shown to be predominantly expressed at the brush border membrane of proximal tubular epithelial cells, and responsible for the renal reabsorption of Pi. On the other hand, Npt2b is expressed in various tissues including small intestine, and has been revealed to be responsible for intestinal apical  $\text{Na}^+$ /Pi cotransport. Type III  $\text{Na}^+$ /Pi cotransporters are classified as SLC20 family, which includes PiT1 (SLC20A1) and PiT2 (SLC20A2). These proteins are widely expressed, and were originally identified as retroviral receptors Glvr-1 and Ram-1, respectively [2].

In addition to the profound roles of phosphorus in various biological processes, it has been implicated that extracellular Pi itself functions as a signaling molecule that regulates gene expression and modulates cell behavior. This chapter will review the recent findings on the regulation of cellular function by extracellular Pi. In addition, current concept on the sensing mechanism of environmental Pi will be discussed.

## Roles of Extracellular Pi in Skeleton

In mammals, renal reabsorption and intestinal absorption of Pi are central to control Pi balance [1]. Prolonged deficiency of Pi due to the abnormal absorption or reabsorption results in the poorly mineralized bone characteristic of rick-

ets or osteomalacia, indicating the critical role of phosphorus in skeletal mineralization. Recently, it has been revealed that extracellular Pi regulates the various cellular functions in skeleton other than mineralization.

Alkaline phosphatase is the enzyme that hydrolyses pyrophosphates and the protein-bound form of phosphate to produce Pi and increase its concentration in the extracellular microenvironment. During the early phase of osteoblast differentiation, the expression of the gene for alkaline phosphatase gradually increases. Using a murine osteoblastic cell line MC3T3-E1 cells, Beck et al. [3] found that a decrease in extracellular Pi production due to the inhibition of alkaline phosphatase activity caused a decreased expression of osteopontin, which is involved in the regulation of mineralization. Then, they further demonstrated that the increased Pi levels in the culture medium strongly induced the expression of osteopontin, and firstly indicated that extracellular Pi itself functions as a specific signal that regulates the gene expression in osteoblastic cells [4]. The authors also performed a combined proteome and microarray investigation in MC3T3-E1 cells to identify the molecules comprehensively whose expression levels were altered in response to treatment with the higher concentration of extracellular Pi [5]. Among the molecules whose amount was increased in response to Pi, some were found to be involved in the regulation of cell cycle, proliferation and DNA synthesis [5]. Thus, the extracellular Pi in microenvironment seems to regulate both proliferation and differentiation of osteoblasts.

Extracellular Pi plays a role in regulating chondrogenesis as well. Chondrogenesis is a multistep process involving the mesenchymal condensation of undifferentiated cells, the proliferation of chondrocytes, and differentiation into hypertrophic chondrocytes, followed by mineralization. As chondrocytes mature, the concentration of Pi increases in the extracellular milieu. The progressive accumulation of Pi ion starts from the proliferative zone and peaks in the hypertrophic zone [6].

Terminally differentiated chondrocytes in growth plate undergo mineralization associated with apoptosis. Several studies have indicated that high level of Pi in the microenvironment of mature chondrocytes induces apoptosis [7–9]. Sabbagh et al. [7] demonstrated that the decreased level of extracellular Pi impaired the apoptosis of hypertrophic chondrocytes and lead to rickets in the hypophosphatemic *Hyp* mouse, a murine model for human X-linked hypophosphatemia. Their data implicate that extracellular Pi plays a critical role for the regulated apoptosis of hypertrophic chondrocytes via the activation of the caspase-9-mediated mitochondrial pathway. Mansfield et al. [8] linked the apoptosis of mature chondrocytes directly to the elevation in environmental Pi concentration and the concomitant rise in intracellular Pi levels, and showed that the effect of Pi on chondrocyte apoptosis was calcium dependent. More recently, Sugita et al. [9] reported that Pi-regulated apoptosis of hypertrophic

chondrocytes involved the intracellular adenosine 5'-triphosphate synthesis mediated by type III Na<sup>+</sup>/Pi cotransporter Pit1.

Wang et al. [10] examined the expression of Na<sup>+</sup>/Pi cotransporters in a chondrocytic cell line CFK2 cells, and detected the expression of type III cotransporters Pit1 and Pit2 [7]. Transient increase in the expression of these genes was observed at 1 week of differentiation, which preceded maximum expression of markers of chondrocyte differentiation such as type II collagen [10]. Their data suggest that Pi may have some role in early chondrocytes as well as mature chondrocytes. We investigated the role of Pi in early chondrocytes at the proliferating stage and found that the treatment with higher concentration of extracellular Pi induced the upregulation of cyclin D1 within 24 h [11]. Cyclin D1 is a positive regulator of progression through the G1 phase of the cell cycle, and is induced to express by many mitogenic stimuli. Consistent with the upregulation of cyclin D1 expression, the extracellular Pi facilitated the proliferation in early chondrocytes [11].

The effects of extracellular Pi on the maturation of chondrocytes were also demonstrated. ATDC5 is a murine mesenchymal cell line and used as a model for chondrogenesis. In differentiated ATDC5 cells, Pi accelerated the expression of type X collagen, a marker for hypertrophic chondrocytes [12]. The expression of matrix Gla protein, which is related to mineralization, was also increased in response to high extracellular Pi [13]. Thus, the signals of extracellular Pi are involved in various processes in chondrogenesis including cell proliferation, maturation and apoptosis, and the effects might differ among the stages of the chondrocytes.

## Effects of Extracellular Pi in Extraskeletal Tissues

Altered levels in extracellular Pi influence the gene expression and cell function in extraskeletal tissues as well. Kido et al. [14] reported that feeding mice with a low-phosphorus diet resulted in the increased level of *Npt2a* Na<sup>+</sup>/Pi cotransporter in kidney, and identified a DNA sequence responsible for Pi in the *Npt2a* gene. The Pi-response element of the *Npt2a* gene was found to consist of the motif related to the E-box, 5'-CACGTG-3'. They cloned a transcription factor that bound to the Pi-response element by yeast one-hybrid system, and identified the mouse transcription factor muE3 (TFE3).

In vascular smooth muscle cells, a higher concentration of extracellular Pi induced the expression of osteoblast-specific genes such as *Runx2* and *osteocalcin*, and caused the phenotypical change that predisposed to calcification [15, 16]. This altered gene expression by increased extracellular Pi was suggested to be mediated by Pit1 [16]. Shuto et al. [17] investigated the effects of high dietary

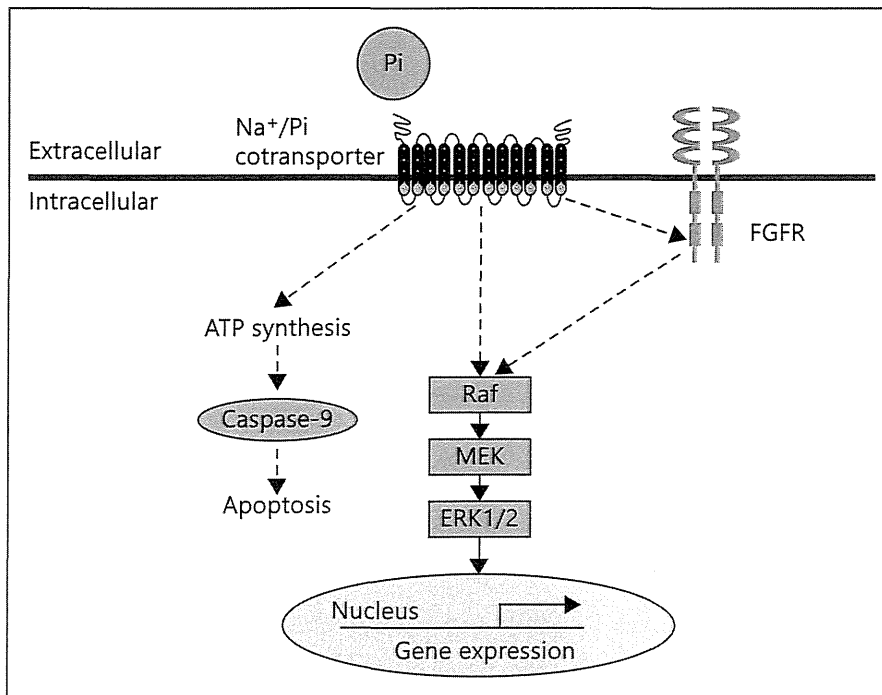
phosphorus in healthy human adults, and found that high dietary phosphorus load resulted in the increased the serum Pi levels at 2 h after the meal and acutely impaired the endothelial function. These results suggest the involvement of high extracellular Pi in the pathogenesis of cardiovascular events associated with chronic kidney disease.

Extracellular Pi directly exerts its effects on the parathyroid gland as well. It was reported that the elevated level of Pi in media increased the parathyroid hormone (PTH) secretion from fresh rat parathyroid gland in vitro [18]. Thus, a great variety of cells are able to respond to the environmental Pi.

### How Is the Pi-Induced Signal Transduced?

As described above, extracellular Pi itself modulates the functions in various cells. Then, how is the extracellular Pi-triggered signal transduced into the cells? It was demonstrated that the increased extracellular Pi induced the activation of ERK1/2 pathway, but not that of the other pathways such as p38MAPK pathway or JNK pathway, in several cells including MC3T3-E1 cells and ATDC5 cells [11, 13, 19]. In these cells, inhibition of the ERK1/2 pathway abolished the regulation of the genes by extracellular Pi, indicating its importance in the transduction of the signals triggered by extracellular Pi.

Another important question is whether Pi has to enter the cell to exert its biological effects or not. It was reported that treatment with phosphonoformic acid (PFA), a well-documented competitive inhibitor of SLC34 family of Na<sup>+</sup>/Pi cotransporters, prevented the calcification of vascular smooth muscle cells expressing Pit1 and Pit2 by inhibiting calcium-phosphate deposition [20]. Since PFA hardly inhibits the Pi transport mediated by SLC20 (PiT) family cotransporters [2], its effect on vascular smooth muscle calcification might be independent of Pi uptake. On the other hand, Li et al. [16] showed that knockdown of the expression of *Pit1* impeded the Pi-induced calcification of vascular smooth muscle cells. In our study, knockdown of *Pit1* in ATDC5 cells abrogated the Pi-induced activation of the Raf/MEK/ERK pathway, suggesting involvement of Pit1 in the responsiveness to extracellular Pi [11]. These results indicate the involvement of Pit1 in transduction of the signals triggered by altered levels of extracellular Pi in some cell types. However, it still remains unclear whether the entry into the cell is required or not for extracellular Pi to trigger signal transduction. As described above, transporters of Pit family were initially identified as receptors for retroviruses, and recent studies have demonstrated some roles of Pit1 that are independent of its role in Pi transport [21]. Further studies are needed to uncover the precise mechanism by which Pit1 mediates the effects of altered extracellular Pi.



**Fig. 1.** Transduction of the signal triggered by extracellular Pi. In several cell types such as osteoblastic MC3T3-E1 cells and chondrocytic ATDC5 cells, increased extracellular Pi induces the activation of Raf/MEK/ERK1/2 pathway and regulates gene expression, a process that is mediated by the Na<sup>+</sup>/Pi cotransporter. In human embryonic kidney cell line HEK293 cells, FGFR is involved in the Pi-induced activation of the ERK1/2 pathway. In hypertrophic chondrocytes, increased extracellular Pi induces apoptosis via intracellular ATP synthesis and caspase-9-mediated mitochondrial pathway.

In a human embryonic kidney cell line HEK293, we found that an increase in extracellular Pi induced the phosphorylation fibroblast growth factor receptor substrate 2 $\alpha$  (FRS2 $\alpha$ ) as well as its downstream molecule ERK1/2. Knockdown of *fibroblast growth factor receptor 1* (FGFR1) expression as well as that of *PiT1* diminished the phosphorylation of both FRS2 $\alpha$  and ERK1/2 induced by extracellular Pi. Moreover, overexpression of FGFR1 rescued the decrease in Pi-induced phosphorylation of ERK1/2 in the cells where the expression of *PiT1* was knocked down [22]. These results suggest that increased extracellular Pi triggers signal transduction via PiT1 and FGFR in HEK293 cells, although the mechanism that mediates the signaling from PiT1 to FGFR1 remains to be elucidated (fig. 1).

Data on the transcription factors that might be involved in the gene regulation by extracellular Pi in mammals are limited. As described above, TFE3 was identified to bind to the Pi-response element in mouse *Npt2a* gene [14]. Early growth response-1, a downstream transcription factor of ERK1/2, was upregulated in response to the increased extracellular Pi in HEK293 cells [22]. In MC3T3-E1 cells, Beck et al. [23] found the transcription factor Nrf2 to be regulated by extracellular

Pi. Moreover, by a combined proteome and transcriptome analyses in MC3T3-E1, they identified that Fra-1, a member of the AP-1 family of transcription factors, was not regulated at the mRNA level, but its protein level was increased in response to extracellular Pi [5]. It was reported that the regulation of matrix Gla protein expression by extracellular Pi in differentiated ATDC5 cells involved the facilitation of the DNA binding of Fra-1 to specific sequences in the matrix Gla protein promoter [13]. It is unknown whether a transcription factor exists or not that functions specifically to mediate the signals of extracellular Pi in mammals.

## Sensing of Environmental Pi in Bacteria and Yeast

Responsiveness to the alteration in the levels of extracellular Pi suggests the existence of the mechanism by which the cells sense it. In unicellular cells such as bacteria and yeast, the systems for sensing the environmental Pi have been extensively studied.

In bacteria, environmental Pi is sensed by ATP-binding cassette (ABC)-type Pi specific transporter (Pst) and a protein called PhoU. The Pst transporter is composed of four subunits, PstS, PstA, PstB and PstC. Pi binds to Pst, and activates a two-component system (TCS) composed of sensory histidine kinase (HK) PhoR, which is an integral membrane protein, and its partner DNA-binding response regulator (RR) PhoU, which is a transcription factor. Under conditions of Pi limitation, phosphorylation of the histidine residues in PhoR is increased, and then the phosphorylated PhoR in turn activates PhoB by phosphorylation on its aspartic residues. The phosphorylated PhoB increases the transcription of multiple genes in the Pho regulon by binding to the specific DNA sequences called PHO boxes, which allows the bacteria to adapt the reduced Pi concentration. If the Pi concentration in the environment is adequate, PhoR is dephosphorylated, and PhoB remains unphosphorylated and is incapable of inducing the transcription of the genes in the Pho regulon. Estimates for the number of Pi-regulated genes vary widely, but proteomic data revealed that amounts of nearly 400 proteins altered in response to the environmental Pi level [24, 25].

Similar to the case of bacteria, environmental Pi controls gene expression through a specialized transcription factor, named Pho4, in yeast. The activity and subcellular localization of Pho4 is regulated by the cyclin-dependent kinase complex Pho80-Pho85. Reduced level of environmental Pi results in the inactivation of the Pho80-Pho85 by a cyclin-dependent kinase inhibitor Pho81. Consequently, Pho4, which remains unphosphorylated and active, translocates into the nucleus to induce the transcription of the target genes belonging to the yeast Pho regulon. One of them encodes Pho84 protein, which functions as a high-affinity

Pi transporter. Under the Pi-starved condition, the expression of Pho84 is strongly induced. Pho84 belongs to the major facilitator superfamily and catalyzes a bidirectional proton (H<sup>+</sup>)-coupled Pi uptake, where the direction of transport is determined by the directionality of the driving force rather than by the orientation of the protein. When adequate level of Pi exists in the environment, Pho84 is rapidly internalized from the plasma membrane and degraded, a process that involves the activation of protein kinase A pathway. The degradation of Pho84 leads to phosphorylation of Pho4. The phosphorylated Pho4 is exported from the nucleus, and the expression of PHO genes is turned off [24, 26].

### **Pi-Sensing to Maintain Homeostasis in Mammals**

Although various mammalian cells respond to the altered levels of extracellular Pi in vitro, little is known about how mammals sense the level of Pi. In multicellular organisms, Pi levels should be sensed at a whole-body level to maintain the homeostasis of extracellular Pi, as well as at a cellular level to regulate the effects of Pi on cell function. Pi homeostasis in mammals is controlled by a variety of hormones that regulate the renal reabsorption and intestinal absorption, including FGF23, PTH, and 1,25-dihydroxyvitamin D [27]. The availability of Pi at a whole-body level should be sensed to control the production of these hormones.

FGF23 is produced by osteocytes and osteoblasts in bone, and plays the central role in Pi homeostasis by reducing the Pi reabsorption and suppressing the synthesis of 1,25-dihydroxyvitamin D in kidney. Reflecting the endocrine function of FGF23, its circulating levels are controlled by various systemic factors, of which 1,25-dihydroxyvitamin D has the best defined regulatory role [28]. Although the extracellular Pi itself might regulate the production of FGF23, an increase in extracellular Pi did not directly stimulate FGF23 expression in osteoblasts [28]. Because of the lack of suitable in vitro cell culture models, it remains unclear whether Pi directly regulates the expression and/or secretion of FGF23. In addition to FGF23, osteocytes express dentin matrix protein 1 (DMP1) and phosphate regulating gene with homologies to endopeptidases on the X-chromosome (PHEX). These three molecules are responsible for inherited hypophosphatemic rickets/osteomalacia, and the loss of function in DMP1 or PHEX results in the increased level of FGF23, suggesting the intimate functional relationship among them [27]. Further studies on the regulatory mechanisms of these molecules might clarify the mechanism for sensing the Pi levels.

Martin et al. [29] investigated the response of serum PTH to changes dietary Pi using rats, and found that duodenal infusion of Pi increased serum PTH with-



in minutes. Intravenous administration of Pi also rapidly increased serum PTH, suggesting the existence of Pi-sensing mechanism in parathyroid gland [29]. On the other hand, Berndt et al. [30] suggested the sensing of Pi in the intestine. They administered Pi into the duodenum of intact or parathyroidectomized rats, and examined the renal Pi excretion. The duodenal Pi loading resulted in the increased renal Pi excretion without alteration in the levels of PTH and FGF23, and the parathyroidectomy did not influence the response to Pi loading [30]. Their results suggest that the intestine can sense the increase in the luminal Pi concentration, although how the signal leads to increase in renal Pi excretion remains to be elucidated.

## Conclusion

Phosphorus is an essential element for all living organisms. Unicellular organisms such as bacteria and yeast have been revealed to adapt the concentration of environmental Pi by sensing it through a system involving a protein complex in plasma membrane and a specialized transcription factor. Various mammalian cells also respond to the alteration of the extracellular Pi levels, and the signal triggered by Pi was found to be transduced through Na<sup>+</sup>/Pi cotransporter and ERK1/2 pathway. Although the responsiveness to extracellular Pi suggests the existence of mammalian Pi-sensing system, Pi availability should be sensed both at a cellular level and at a whole-body level in mammals. Further studies will be required to clarify the underlying mechanisms of phosphate metabolism [31].

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# Regulatory mechanisms for the development of growth plate cartilage

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**Abstract** In vertebrates, most of the skeleton is formed through endochondral ossification. Endochondral bone formation is a complex process involving the mesenchymal condensation of undifferentiated cells, the proliferation of chondrocytes and their differentiation into hypertrophic chondrocytes, and mineralization. This process is tightly regulated by various factors including transcription factors, soluble mediators, extracellular matrices, and cell–cell and cell–matrix interactions. Defects of these factors often lead to skeletal dysplasias and short stature. Moreover, there is growing evidence that epigenetic and microRNA-mediated mechanisms also play critical roles in chondrogenesis. This review provides an overview of our current understanding of the regulators for the development of growth plate cartilage and their molecular mechanisms of action. A knowledge of the regulatory mechanisms underlying the proliferation and differentiation of chondrocytes will provide insights into future therapeutic options for skeletal disorders.

**Keywords** Chondrocyte · Transcription factors · Growth factors · Extracellular matrix · Differentiation

## Introduction

Most of the skeleton, including the long bones of the limbs and the vertebral columns, is formed through endochondral ossification, involving a cartilaginous intermediate [1–3].

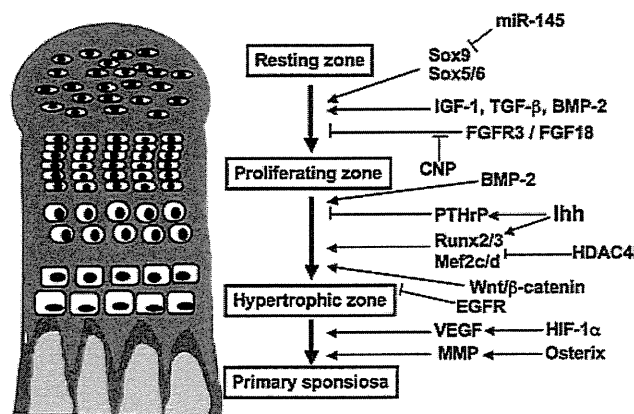
Endochondral bone formation starts with the condensation of mesenchymal cells, which differentiate into chondrocytes characterized by the production of specific extracellular matrix (ECM) proteins such as type II collagen (Col II) and aggrecan. The chondrocytes proliferate unidirectionally to form orderly parallel columns which accumulate a cartilaginous matrix [2]. These cells then exit the cell cycle, differentiate further to become hypertrophic, and produce type X collagen (Col X) [4]. In growth plates, maturing chondrocytes are organized into zones, including a resting zone, a proliferating zone, a prehypertrophic zone, and a hypertrophic zone [5]. Once the hypertrophic chondrocytes have terminally differentiated, the cartilaginous matrix is mineralized and the cells undergo apoptosis. These mature chondrocytes express vascular endothelial growth factor (VEGF) to induce blood vessel invasion and matrix metalloproteinases (MMPs) to aid in the degradation of the cartilaginous matrix by chondroclasts, and the primary ossification center is developed [6, 7] (Fig. 1).

The process of chondrocyte proliferation and differentiation is regulated by various transcription factors, growth factors, ECMs, and cell–matrix interactions [2, 8–10]. In addition, recent studies have revealed the importance of epigenetic and microRNA-mediated control in cartilage development. Defects in the factors involved in the development of growth plate cartilage are often associated with skeletal dysplasias and short stature [11]. In this review article, I will mainly address the mechanisms regulating the development of growth plate cartilage.

## Transcriptional control of chondrogenesis

Sox9 is a member of the Sox family of transcription factors characterized by a high-mobility-group-box DNA binding

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**Fig. 1** Schematic representation of molecules involved in the development of growth plate cartilage

motif related to that of the sex determining factor SRY, and plays a central role in chondrogenesis. In chondrocyte lineage cells, the expression of Sox9 starts at the mesenchymal osteochondroprogenitor stage and remains high during differentiation. Mutations in the human *SOX9* gene result in camptomelic dysplasia characterized by severe skeletal malformation, indicating the critical role of SOX9 in skeletogenesis [12, 13]. Accumulating evidence in mice also has revealed that Sox9 is indispensable for chondrocyte differentiation [3, 14]. Sox9 transcriptionally controls the expression of cartilage-specific genes such as *Col2a1* encoding Col II [15, 16]. Two other Sox family members, Sox5 (L-Sox5) and Sox6, cooperate with Sox9 to activate the chondrocyte-specific enhancers in the genes for ECM components [15, 17, 18]. Other transcription factors, such as members of the activating transcription factor (ATF)/cyclic AMP response element binding protein (CREB) family and the AP1 family member c-Fos, are required to maintain the proliferative capacity of early chondrocytes [19–21]. RhoA is a Rho GTPase, which functions as a regulator of cytoskeletal dynamics. RhoA signaling through its main effector ROCK inhibits chondrogenesis by suppressing the expression of Sox9 [22, 23].

A decrease in the expression and/or activity of the Sox proteins is required for the hypertrophic maturation of chondrocytes. In addition to the negative regulation by Sox proteins, other transcription factors, such as the Runt domain family members Runx2 and Runx3, function to promote chondrocyte hypertrophy [24]. Mice lacking both Runx2 and Runx3 lack hypertrophic chondrocytes [24]. Runx2 directly binds and activates the genes *Ihh* (*Indian hedgehog*), *Col10a1* encoding Col X, and *MMP13* [25–27]. A recent study using a doxycycline-inducible conditional knockout of *Sox9* has revealed that Sox9 suppresses the expression of Runx2 and  $\beta$ -catenin signaling and thereby inhibits the progression from proliferation to

prehypertrophy of chondrocytes [28]. Twist-1 is a basic helix-loop-helix-type transcription factor, which represses the expression of Runx2 in the perichondrium. Runx2 enhances the expression of *fibroblast growth factor 18* (*Fgf18*) and exerts an indirect negative effect on chondrocyte maturation [29]. Osterix regulates the calcification and degradation of cartilaginous matrix through MMP13 expression in association with Runx2 [30].

MADS-box transcription factors Mef2c and Mef2d (myocyte enhancer factor 2c and 2d) are also involved in chondrocyte hypertrophy. Genetic deletion of Mef2c in endochondral cartilage impairs hypertrophic maturation, while the forced expression of a superactivating form of Mef2c resulted in precocious chondrocyte hypertrophy [31]. The activity of Runx2/3 and Mef2c/d is inhibited by the histone deacetylase HDAC4 [31–33]. Other transcription factors, such as Msx2, the AP1 family member Fra2, and FoxA family transcription factors, also positively control chondrocyte hypertrophy [34–37].

The developmental growth plate is hypoxic, especially in its interior. The transcription factor hypoxia-inducible factor I (HIF-1) is one of the major regulators of the hypoxic response in mammals. Genetic evidence obtained from mice lacking HIF-1 $\alpha$  suggests its role in chondrocyte survival and the regulation of *Vegf* expression [38]. Conditional overexpression of VEGF164 in chondrocytes lacking HIF-1 $\alpha$  rescued the phenotype of HIF-1 $\alpha$ -deficient growth plate only partially, indicating VEGF-independent functions of HIF-1 $\alpha$  in developing growth plate cartilage [39]. It is also reported that HIF-1 $\alpha$  regulates collagen hydroxylation and secretion in developing cartilage [40].

### Soluble mediators involved in chondrogenesis

*Ihh*, a member of the hedgehog family of signaling molecules, is expressed in prehypertrophic chondrocytes, and regulates the onset of hypertrophic differentiation through a negative feedback loop with parathyroid hormone-related protein (PTHrP). *Ihh* increases the expression of PTHrP in perichondrial cells and chondrocytes at the ends of long bones, which inhibits chondrocyte hypertrophy through its cognate receptor expressed in proliferating chondrocytes and keeps the cells in the proliferating stage [41]. Moreover, it is also reported that *Ihh* stimulates the proliferation and maturation of chondrocytes independently of PTHrP [42, 43]. Activation of Wnt and bone morphogenetic protein (BMP) signaling is suggested to be involved in the PTHrP-independent role of *Ihh* to regulate chondrocyte hypertrophy [44].

Fibroblast growth factors (FGFs) also play important roles in skeletogenesis by activating signaling through FGF receptors (FGFRs) [45]. Gain-of-function mutations

in human FGFR3 result in chondrodysplasias and dwarfism [46–49]. As to FGF ligands, *Fgf2* was first identified to be expressed in chondrocytes [50], and is also expressed in periosteal cells and osteoblasts [51, 52]. However, *Fgf2*-knockout mice demonstrate no defects in chondrogenesis [52, 53]. *Fgf9* is also expressed in immature chondrocytes in mesenchymal condensation. In the perichondrium, the expression of *Fgf7*, *Fgf8*, *Fgf9*, *Fgf17*, and *Fgf18* has been reported [54–58]. Evidence obtained from mouse models indicates profound role for *Fgf18* in chondrogenesis [56, 57]. *Fgf9* was also proven to regulate early hypertrophic chondrocyte differentiation and skeletal vascularization by the defects in chondrogenesis in *Fgf9*-knockout mice [59]. Among the FGFRs, *Fgfr3* is expressed in chondrocytes undergoing mesenchymal condensation and proliferating chondrocytes, whereas *Fgfr1* is expressed in prehypertrophic and hypertrophic chondrocytes [60–62]. Genetic and functional studies demonstrated that the signaling through FGFR3 negatively regulates the chondrocyte proliferation and differentiation [63–66]. The effects of FGFR3 in chondrogenesis are partly exerted by direct signaling in chondrocytes, and in part indirectly through the regulation of *Ihh*/PTHrP/BMP signaling [67]. In achondroplasia, constitutive activation of FGFR3 results in the activation of downstream pathways including STAT1 and ERK signaling [45]. The growth plates of mice lacking *Fgf18* have a similar histology to those of *Fgfr3*-knockout mice, suggesting that FGF18 is a physiological ligand for FGFR3 in chondrocytes [56, 57].

C-type natriuretic peptide (CNP) controls cell behavior through the activation of two transmembrane receptors, NPR1 and NPR2 [68–70]. Since these receptors synthesize cyclic GMP in response to ligand binding, NPR1 and NPR2 are also called guanylyl cyclase A and B (GC-A and GC-B), respectively. CNP exerts its signal mainly through NPR2/GC-B. The importance of CNP signaling in chondrogenesis was shown by the severe dwarfism of CNP-knockout mice and the finding that CNP stimulated the longitudinal growth of cartilage in organ cultures [71, 72]. NPR2-null mice display a similar phenotype to CNP-knockout mice [73]. CNP promotes endochondral bone growth through several mechanisms, including the stimulation of chondrocyte proliferation, an acceleration of chondrocyte hypertrophy, and an increase of ECM production. In humans, loss-of-function mutations in the *NPR2* gene cause acromesomic dysplasia, type Maroteaux, characterized by severe dwarfism [74]. We have recently identified a novel gain-of-function type mutation of the *NPR2* gene in a family with overgrowth [75]. Such evidence indicates the critical role of CNP/NPR2 signaling in chondrogenesis both in humans and in mice.

The skeletal phenotype in CNP-deficient mice resembles that in cases of achondroplasia. Overexpression of CNP in

cartilage rescued the skeletal phenotypes in a mouse model of achondroplasia, suggesting an intimate link between the FGF and CNP signaling [73]. CNP signaling inhibited the activation of the ERK pathway induced by FGF signaling, while FGF signaling blocked CNP-induced cGMP production in a MAPK-dependent manner [73]. In addition to the ERK pathway, recent studies demonstrated the possible involvement of the p38MAPK and PI3K/Akt pathways in the regulation of chondrocyte development by CNP [76]. CNP analogues are promising as new drugs for the dwarfism associated with skeletal dysplasias [77].

Studies have established the involvement of signaling mediated by epidermal growth factor receptor (EGFR) in chondrogenesis. Delayed primary endochondral ossification associated with defective osteoclast recruitment was reported in mice lacking EGFR [78]. Ubiquitous overexpression of betacellulin, a ligand for EGFR, resulted in defects in growth plates characterized by a smaller zone of hypertrophic chondrocytes in mice [79]. In addition, cartilage-specific inactivation of EGFR in mice as well as the administration of an EGFR-specific small-molecule inhibitor, gefitinib, into rats caused hypertrophic cartilage enlargement [80].

Other growth factors such as Wnts, BMPs, transforming growth factor-beta (TGF- $\beta$ ), insulin-like growth factors (IGFs), thyroid hormone, and connective tissue growth factor (CTGF) also play roles in chondrogenesis. There are several excellent review articles on their actions [81–83].

### Regulation of chondrogenesis by the ECM

In the early stages of chondrogenesis, cell–cell interaction via adhesion molecules such as N-cadherin and N-CAM plays a role in cellular condensation and the subsequent chondrogenesis [84, 85]. As chondrocytes mature, they produce abundant matrix proteins, and the cell–matrix interactions come to have important roles. Integrins bind various extracellular components such as ECMs and other cell surface proteins [86]. The binding of ligands to integrins leads to the formation of focal adhesion complexes, and transduces the signaling from the ECM to intracellular effectors such as cytoskeleton [87, 88]. Integrins exist as dimers of an  $\alpha$  subunit and a  $\beta$  subunit, and chondrocytes express several integrin subunits including fibronectin receptors ( $\alpha5\beta1$ ,  $\alpha\text{v}\beta3$ ,  $\alpha\text{v}\beta5$ ), a laminin receptor ( $\alpha6\beta1$ ) and collagen receptors ( $\alpha1\beta1$ ,  $\alpha2\beta1$ ,  $\alpha10\beta1$ ) [89–92]. The importance of  $\beta1$  integrin-mediated signaling in chondrogenesis was demonstrated by the chondrodysplasia-like phenotype of chondrocyte-specific  $\beta1$  integrin-knockout mice [93]. Growth plates of these mice exhibited unorganized proliferative columns and an abnormal cell shape due to the loss of adhesion to Col II. The chondrocytes isolated

from these mice displayed reduced proliferation caused by a defect in G1/S transition and cytokinesis.

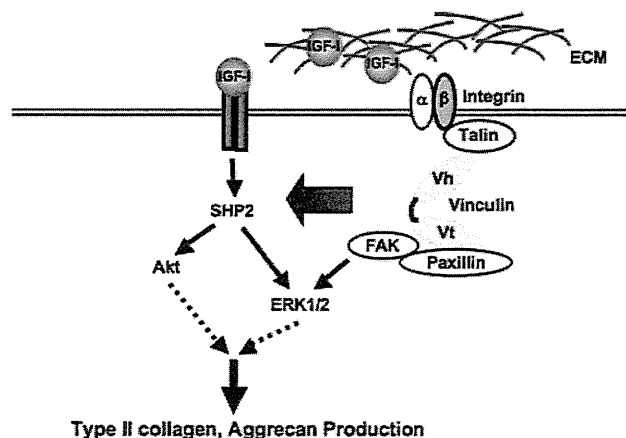
Inactivation of the  $\alpha 10$  integrin gene also resulted in growth plate dysfunction, which was associated with an abnormal cell shape and increased apoptosis of chondrocytes [94]. On the other hand, knockout of the gene for  $\alpha 1$  integrin resulted in osteoarthritis but no abnormalities in the growth plates, despite this gene's predominant expression in hypertrophic chondrocytes [95].

Activation of integrin-mediated signaling triggers the formation of a complex consisting of multiple proteins, which regulate various cellular processes. Integrin-linked kinase is one of the components, and the knockout of its gene caused a chondrodysplasia-like phenotype resembling that of chondrocyte-specific  $\beta 1$  integrin-knockout mice [96]. In these mice, reduced proliferation of chondrocytes was the main cause for the skeletal phenotype, and the expression of chondrocyte-specific genes such as *Col2a1* was comparable with that in wild-type mice.

CD44 is a cell surface glycoprotein that functions as a receptor for collagens and hyaluronan. It was reported that the blocking of CD44-hyaluronan binding on chondrocytes resulted in degradation of the cartilage matrix, suggesting a role for CD44 in cartilage homeostasis [97]. Annexin V acts as a receptor for collagen, specifically for a fragment of Col II in articular chondrocytes [98]. Antibodies against annexin V inhibited the binding of chondrocytes to Col II [99]. It is also suggested that annexin V is involved in regulating the apoptosis of growth plate chondrocytes [100].

Cartilage contains abundant proteoglycans. The sulfate transporter SLC26A2 is responsible for sulfate uptake by chondrocytes, and mutations in its gene lead to undersulfation of cartilaginous proteoglycans, resulting in a chondrodysplasia called diastrophic dysplasia. In *ddd* mice with a knock-in *Slc26a2* mutation, the resulting undersulfation of glycosaminoglycans such as chondroitin destroys the articular surface and correlates with the rate of chondroitin synthesis across epiphyseal cartilage [101]. Chondroitin sulfate *N*-acetylgalactosaminyltransferase 1 (CSGalNAcT-1) is an enzyme that participates in the initiation of the biosynthesis of chondroitin sulfate. Mice lacking the gene encoding CSGalNAcT-1 exhibit shorter, disorganized chondrocyte columns in the growth plates with a rapid catabolism of aggrecan [102].

In addition to providing signals to cells by binding to integrins and other ECM receptors, ECM proteins regulate chondrogenesis through the binding, storage, and release of soluble factors. TGF- $\beta$  is produced by chondrocytes as a high molecular weight macromolecule in association with latent TGF- $\beta$  binding protein (LTBP), which functions in the storage of TGF- $\beta$  in the ECM [103, 104]. Proteoglycans such as decorin, biglycan, and fibromodulin also regulate TGF- $\beta$  activity by sequestering TGF- $\beta$  in the ECM [105].



**Fig. 2** Vinculin regulates the production of type II collagen and aggrecan by orchestrating the signal of extracellular matrix and that of IGF-1

Most FGFs bind to heparan sulfate proteoglycans. They bind to cognate receptors in the context of heparan sulfate proteoglycans and evoke signaling into the cells. Genetic evidence obtained with mice lacking sulfate-modifying factor 1 (Sumf1) has suggested that the desulfation of proteoglycans regulates chondrocyte proliferation and differentiation by limiting FGF signaling [106].

Signals from the ECM itself and those triggered by soluble mediators appear to interplay to regulate chondrogenesis. We have demonstrated that vinculin plays a role in chondrogenesis [107]. Vinculin is a component of multimolecular complexes which function in adhesion and/or signaling between the extracellular microenvironment and the cell, via integrins and cadherins. Impaired functioning of vinculin by knockdown in primary chondrocytes and organ cultures of metatarsal explants resulted in the reduced expression of *Col2a1*, *aggrecan*, *Col10a1*, and *Runx2*. In addition, knockdown of *vinculin* in the metatarsals abrogated IGF-I-induced growth, and inhibited the up-regulation of *Col2a1* and *aggrecan* expression by IGF-I. These results suggest that vinculin regulates the expression of chondrocyte-specific genes via the integration of signaling from the ECM and soluble factors such as IGF- I (Fig. 2). It is also reported that cell adhesion via integrin regulates the activation of growth factor receptors. The orchestration of the signaling of soluble factors and the ECM should be considered a factor in the regeneration of cartilage [107].

### Epigenetic and microRNA-mediated regulation of chondrogenesis

There is growing evidence that epigenetic and microRNA-mediated mechanisms play roles in chondrogenesis as well

as in pathogenesis of osteoarthritis [108]. Histone modifications involving acetylation and deacetylation have an impact on the phenotype of chondrocytes. Among the histone deacetylases (HDACs), HDAC4 has been suggested to prevent premature chondrocyte hypertrophy by blocking the activity of Runx2, as described above [32]. HDAC1 and HDAC2 were shown to repress the expression of some cartilage-specific genes including *Col2a1*, and the Snail transcription factor was identified as a mediator of the repression [109]. The up-regulation of HDAC7 expression was suggested to contribute to the cartilage degradation by promoting the expression of MMP13 [110].

SIRT1 is a NAD<sup>+</sup>-dependent histone deacetylase, and enhances the expression of cartilage-specific ECM genes, such as *Col2a1*, by recruiting co-activators to the enhancer and promoter and facilitating Sox9-mediated transcription [111]. In addition, SIRT1 has been suggested to regulate chondrocyte apoptosis [112].

DNA methylation at CpG dinucleotides is commonly associated with gene repression. An in vitro study demonstrated that the induction of *COL10A1* during the chondrogenesis of mesenchymal stem cells correlated with the demethylation of 2 CpG sites in the *COL10A1* promoter [113]. It was reported that DNA methylation inversely correlated with the expression of cartilage-specific genes including *COL9A1*, but not catabolic genes such as *MMP13*, during fetal femur development in human [114]. Moreover, recent reports have suggested that the methylation of a specific CpG site inhibits the transactivation of *MMP13* by transcription factors HIF-2 $\alpha$  and CREB [115, 116].

MicroRNAs (miRNAs) are a class of ~22 nucleotide noncoding RNAs that regulate the expression of other genes at the posttranscriptional level. Knockout of Dicer, an enzyme required for miRNA synthesis, led to severe skeletal growth defects caused by decreased chondrocyte proliferation and accelerated differentiation in mice, indicating the critical roles of miRNAs in chondrogenesis [117]. Specific miRNAs have been identified to have roles in chondrocyte differentiation. miR-199a was shown to be responsive to BMP and to regulate chondrogenesis by directly targeting Smad1 [118]. Mice lacking miR-140 showed a mild skeletal phenotype with a short stature and age-related OA-like changes associated with the elevated expression of ADAMTS-5, suggesting that miR-140 regulates cartilage development and homeostasis [119]. miR-145 was reported to directly target Sox9 and regulate chondrogenic differentiation of mesenchymal stem cells [120]. miR-675, whose production is positively regulated by Sox9, increases the expression of *COL2A1* in human articular chondrocytes [121]. These findings have established the importance of miRNA-mediated regulation in cartilage development.

## Conclusion

The development of growth plate cartilage is a complex process, regulated by transcription factors, soluble factors, cell–cell and cell–matrix interactions, and epigenetic factors. These factors interplay to control the proliferation and differentiation of chondrocytes. Failure in the development of growth plate cartilage is often associated with skeletal dysplasias, for which currently there is no effective treatment. Understanding the mechanisms regulating chondrogenesis may lead to new therapeutic drugs for these diseases, such as CNP analogues.

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