chondrocytes involved the intracellular adenosine 5′-triphosphate synthesis mediated by type III Na^+/Pi cotransporter Pit1.

Wang et al. [10] examined the expression of Na^+/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^+/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

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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 grand 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^+/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.

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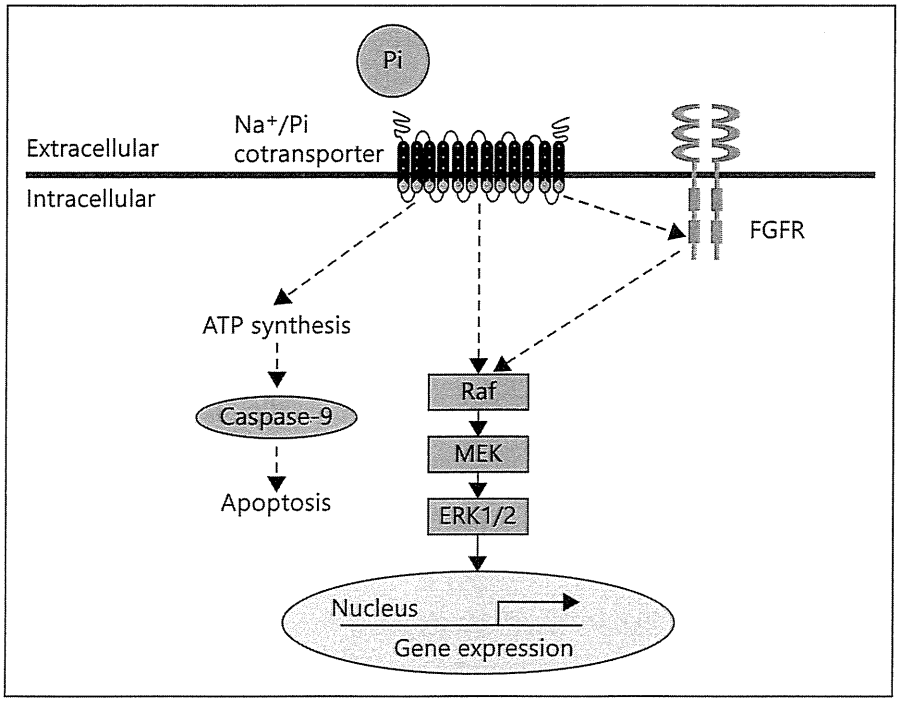

Fig. 1. Transduction of the signal triggered by extracellular Pi. In several cell types such as osteoblastic MC3T3-E1 cells and chondrocytic ATDC5 cells, increased extracellular Pi induces the activation of Raf/MEK/ERK1/2 pathway and regulates gene expression, a process that is mediated by the Na^+/Pi 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 2α (FRS2α) 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 FRS2α 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

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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 dephophorylated, 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

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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^+)-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-

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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^+/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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Cellular and Molecular Life Sciences

REVIEW

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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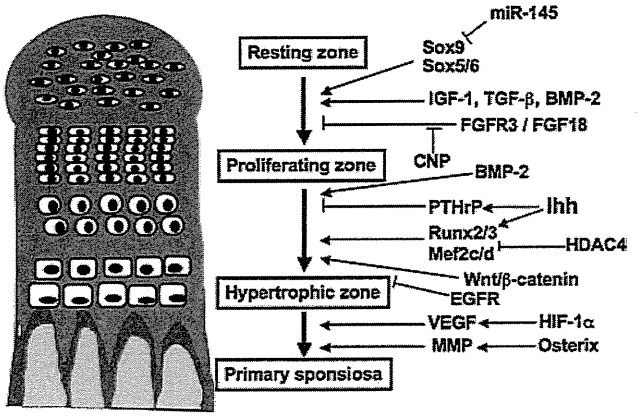

Fig. 1 Schematic representation of molecules involved in the development of growth plate cartilage

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 β-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-1α suggests its role in chondrocyte survival and the regulation of *Vegf* expression [38]. Conditional overexpression of VEGF164 in chondrocytes lacking HIF-1α rescued the phenotype of HIF-1α-deficient growth plate only partially, indicating VEGF-independent functions of HIF-1α in developing growth plate cartilage [39]. It is also reported that HIF-1α 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 acromesolic 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-β), 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 α subunit and a β subunit, and chondrocytes express several integrin subunits including fibronectin receptors (α5β1, αnβ3, αnβ5), a laminin receptor (α6β1) and collagen receptors (α1β1, α2β1, α10β1) [89–92]. The importance of β1 integrin-mediated signaling in chondrogenesis was demonstrated by the chondrodysplasia-like phenotype of chondrocyte-specific β1 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 α10 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 α1 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 β1 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 *dtd* 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-β is produced by chondrocytes as a high molecular weight macromolecule in association with latent TGF-β binding protein (LTBP), which functions in the storage of TGF-β in the ECM [103, 104]. Proteoglycans such as decorin, biglycan, and fibromodulin also regulate TGF-β activity by sequestering TGF-β in the ECM [105].

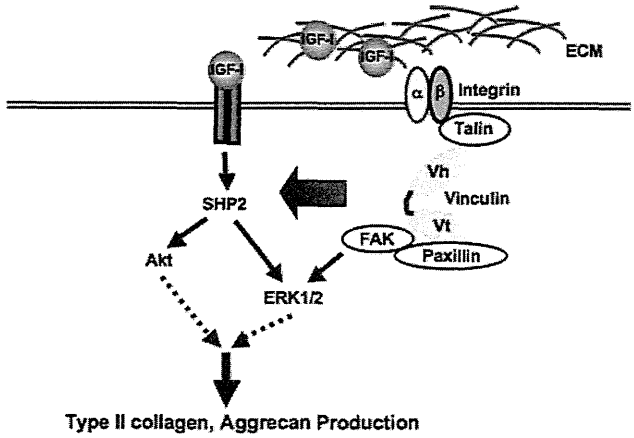

Fig. 2 Vinculin regulates the production of type II collagen and aggrecan by orchestrating the signal of extracellular matrix and that of IGF-1

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^+-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-2α 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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Cell Biology:

Sympathetic Activation Induces Skeletal *Fgf23* Expression in a Circadian Rhythm-dependent Manner

Masanobu Kawai, Saori Kinoshita, Shigeki Shimba, Keiichi Ozono and Toshimi Michigami

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Sympathetic Activation Induces Skeletal *Fgf23* Expression in a Circadian Rhythm-dependent Manner*[S]

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Background: The mechanism whereby the circadian clock regulates phosphate metabolism remains elusive.
Results: *Fgf23* expression is regulated by the time of food intake which involves the alteration in circadian profile of sympathetic activity.
Conclusion: The circadian network plays important roles in phosphate metabolism.
Significance: The sympathetic regulation of *Fgf23* expression may shed light on new regulatory networks that could be important for phosphate homeostasis.

The circadian clock network is well known to link food intake and metabolic outputs. Phosphorus is a pivotal nutritional factor involved in energy and skeletal metabolisms and possesses a circadian profile in the circulation; however, the precise mechanisms whereby phosphate metabolism is regulated by the circadian clock network remain largely unknown. Because sympathetic tone, which displays a circadian profile, is activated by food intake, we tested the hypothesis that phosphate metabolism was regulated by the circadian clock network through the modification of food intake-associated sympathetic activation. Skeletal *Fgf23* expression showed higher expression during the dark phase (DP) associated with elevated circulating FGF23 levels and enhanced phosphate excretion in the urine. The peaks in skeletal *Fgf23* expression and urine epinephrine levels, a marker for sympathetic tone, shifted from DP to the light phase (LP) when mice were fed during LP. Interestingly, β-adrenergic agonist, isoproterenol (ISO), induced skeletal *Fgf23* expression when administered at ZT12, but this was not observed in *Bmal1*-deficient mice. *In vitro* reporter assays revealed that ISO trans-activated *Fgf23* promoter through a cAMP responsive element in osteoblastic UMR-106 cells. The mechanism of circadian regulation of *Fgf23* induction by ISO *in vivo* was partly explained by the suppressive effect of Cryptochrome1 (Cry1) on ISO signaling. These results indicate that the regulation of skeletal *Fgf23* expression by sympathetic activity is dependent on the circadian clock system and may shed light on new regulatory networks of FGF23 that could be important for understanding the physiology of phosphate metabolism.

Phosphorus is an indispensable nutritional element involved in numerous biological processes such as cell signaling, energy homeostasis, and bone metabolism (1–4). The regulation of phosphate metabolism is an integrated process involving multiple organs and accumulating evidence has demonstrated the pivotal roles of fibroblast growth factor 23 (FGF23)[2] in phosphate metabolism (4–9). FGF23 is produced mainly by osteoblastic cells, including osteocytes, and functions as an endocrine factor to regulate genes involved in phosphate and vitamin D metabolism (4). The nodal point of the regulation of phosphate metabolism by FGF23 seems to primarily reside in the suppression of NaP_i-IIa/c expression and 1,25-dihydroxyvitamin D production in the kidney (4). Clinical evidence from genetic disorders in which mutations in the *FGF23* gene or mutations causing aberrant FGF23 signaling are associated with dysregulated phosphate metabolism has placed bone-derived FGF23 in the center of regulatory networks of phosphate metabolism (10–12). Hence, it is critical to understand the mode of the regulation of FGF23 expression in the skeleton to fully understand the physiological and pathological functions of FGF23 in phosphate metabolism. Although previous studies have revealed that 1,25-dihydroxyvitamin D can stimulate *Fgf23* expression in bone in part by directly activating the *Fgf23* gene promoter (13–15), the precise mechanisms by which skeletal *Fgf23* expression is regulated remain largely elusive. Because serum phosphate levels have been shown to exhibit circadian profile in humans, it is likely that phosphate metabolism is under the regulation of the circadian clock system (16–18); however, the precise mechanism by which the circadian clock network regulates phosphate homeostasis is still largely unknown.

The circadian clock network is an evolutionarily conserved process by which organisms adapt to environmental cues such as the availability of nutrients (19–21). For example, when food access is restricted in mice in the daytime (light phase) only, the expression profiles of circadian clock genes and circadian-reg-

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[2] The abbreviations used are: FGF23, fibroblast growth factor 23; AL, *ad libitum*; CRE, cAMP responsive element; CREB, CRE-binding protein; DP, dark phase; IBMX, 3-isobutyl-1-methylxanthine; ISO, isoproterenol; LP, light phase; PRO, propranolol; PTH, parathyroid hormone; RF, restricted feeding; ZT, zeitgeber time.

TABLE 1
Primer sequences for real-time RT-PCR

Gene	Forward Primer	Reverse Primer
Rev-erbα	5′-cccaacgacaacaacctttg-3′	5′-ccctggcgtagaccattcag-3′
Dbp	5′-caccgtggaggtgctaatga-3′	5′-gcttgacagggcgagatca-3′
Bmal1	5′-aggcccacagtcagattgaaa-3′	5′-ccaaagaagccaattcatcaatg-3′

ulated genes related to metabolic outputs have been shown to exhibit a phase shift so that the organisms can utilize ingested nutrients in a timely manner (19, 22–24). The central pacemaker of the circadian clock system is located at the suprachiasmatic nucleus in the hypothalamus and is integrated by multiple steps including transcriptional, translational, and post-translational mechanisms (20). Briefly, Clock (circadian locomotor output cycles protein kaput) heterodimerizes with Bmal1 (brain and muscle ARNT-like 1; also known as ARNTL) and induces the expression of *PER* (period circadian protein) and *CRY* (cryptochrome), which in turn suppresses Clock/Bmal1 transcriptional activity, thereby forming a 24-h feedback loop (20).

The mechanisms by which nutrient availability affects the circadian clock network still need to be determined; however both central and peripheral networks have been implicated as functional in this regulation (20). Centrally, the food-entrainable oscillator, which is anatomically different from the suprachiasmatic nucleus, has been considered to determine food-anticipatory behavior (20). Changes in the circadian profile of sympathetic activity may be one of the central mechanisms connecting food intake and metabolic outputs because food intake has been shown to be associated with enhanced sympathetic activity (25–28). In addition to central regulation, peripheral tissues also possess an oscillator that is synchronized with the central circadian system through retinal, hormonal, nutritional, and neuronal signals (29, 30). Recent advances in our understanding regarding the role of the peripheral oscillator have emphasized its importance in metabolic regulation (21). Furthermore, it has been well established that the circadian clock system in peripheral tissues is entrained by nutritional cues (21, 23). Taken together, these findings led us to hypothesize that phosphate metabolism was regulated by the circadian clock network through the modification of food intake-associated sympathetic activation, which may involve the action of the peripheral clock system.

In the current study, we tested our hypothesis that the circadian profile of circulating phosphate and FGF23 levels is determined by the time of nutrient availability by analyzing the circadian profile of skeletal *Fgf23* expression in mice where the timing of food intake was restricted during the light phase and found that light phase-restricted feeding altered the circadian expression profile of skeletal *Fgf23*, which was in part caused by changes in the circadian profile of sympathetic activity. In addition, we demonstrated that stimulation with a β-adrenergic receptor agonist induced *Fgf23* expression, which was suppressed by the overexpression of Cry1. These results underline the important roles of the circadian clock system in the regulation of phosphate metabolism.

EXPERIMENTAL PROCEDURES

Mice—C57BL/6J mice were purchased from CLEA Japan, Inc., and *Bmal1* knock-out mice on a C57BL/6J background were generated as reported previously (31). Mice were maintained on a 12-h:12-h light dark cycle (lights on at zeitgeber time (ZT) 0) in a pathogen-free animal facility with free access to water and standard chow (CE-2; CLEA Japan, Inc.), unless otherwise mentioned. The light phase restricted feeding regimen was carried out by allowing mice access to food for 6 h between ZT2 and ZT8. A control diet containing 0.6% phosphate and 1.0% calcium and a high phosphate diet containing 1.65% phosphate and 1.0% calcium were purchased from CLEA Japan, Inc. All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of Osaka Medical Center and Research Institute for Maternal and Child Health.

Reagents and Cell Lines—Isoproterenol hydrochloride, propranolol hydrochloride, and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Human parathyroid hormone (PTH)(1–34) was obtained from the Peptide Institute, Inc. (Osaka, Japan). UMR-106 cells were obtained from ATCC (Manassas, VA) and maintained in DMEM supplemented with 10% fetal bovine serum and 1% insulin-transferrin-selenium-G supplement (Invitrogen). Cells were cultured at 37 °C in a 5% CO_2 atmosphere.

Real-time RT-PCR—Total RNA was prepared using TRIzol (Invitrogen) and treated with DNase I (Qiagen). cDNA was generated using a random hexamer and reverse transcriptase (Superscript II; Invitrogen) according to the manufacturer's instructions. The quantification of mRNA expression was carried out using a 7300 Real-time PCR system or a StepOnePlus™ Real-time PCR system (Applied Biosystems). TaqMan Gene Expression Assays for *Fgf23*, *Cryptochrome1*, *Rankl*, *Sost*, *Slc34a1*, *Slc34a3*, *Cyp27b1*, *Cyp24a1*, and *Gapdh* were purchased from Applied Biosystems. Primer sequences for *Rev-erbα*, *Dbp*, and *Bmal1* are described in Table 1. *Gapdh* was used as an internal standard control gene for all quantifications.

Western Blot Analysis—To prepare whole cell lysates, cells were solubilized in radioimmunoprecipitation assay buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM Tris-Cl (pH 7.4), 5 mM EDTA, 1 mM orthovanadate, and protease inhibitor mixture (Complete™; Roche Diagnostics). Equal amounts of protein were separated by SDS-PAGE and transferred electrophoretically to PVDF membranes. Membranes were blocked in BlockAce reagent (Dainippon Pharmaceuticals, Osaka, Japan) or Blocking-one P reagent (Nacalai Tesque, Kyoto, Japan), immunoblotted with anti-CREB (1:1000, 9192; Cell Signaling, Beverly, MA), anti-pCREB (1:1000, 9191; Cell Signaling), anti-V5 (1:5000, 46-0705; Invitrogen), or anti-β-actin (1:2000, sc-47778; Santa Cruz Bio-