Mod Rheumatol (2005) 15:391–396
DOI 10.1007/s10165-005-0436-4

REVIEW ARTICLE

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Involvement of cyclic guanosine monophosphate-dependent protein kinase II in chondrocyte hypertrophy during endochondral ossification

Received: June 21, 2005 / Accepted: September 22, 2005

Abstract During vertebrate skeletal development, the appendicular skeleton forms through endochondral ossification, which involves the intricately regulated multistep differentiation of mesenchymal cells. During this process, mesenchymal condensations initially differentiate into chondrocytes. Then chondrocytes in the center further differentiate into hypertrophic chondrocytes. Hypertrophic chondrocytes express a number of osteogenic factors and induce bone formation. Although numerous studies have provided novel insights into the regulation and function of cartilage development, little is known about the intracellular signaling pathways regulating chondrocyte hypertrophy. Recent study revealed that cyclic guanosine monophosphate (cGMP)-dependent protein kinase II (cGKII) coupled the stop of proliferation and the start of hypertrophic differentiation of chondrocytes. Herein, we review the molecular mechanism of regulation of chondrocyte hypertrophy by cGKII and the interaction between cGKII and other signaling pathways.

Key words Chondrocyte hypertrophy · Cyclic guanosine monophosphate (cGMP)-dependent protein kinase · Endochondral ossification · Sox9

Introduction

Skeletal development occurs through two different mechanisms: intramembranous and endochondral ossification.[1,2] Most of the craniofacial bones develop through intramembranous ossification, in which mesenchymal precursor cells directly differentiate into bone-forming osteoblasts. On the other hand, longitudinal growth of long bones and vertebrae is determined by the process of endochondral ossification, during which a cartilage mold is first formed from mesenchymal condensations, and then replaced by bone and bone marrow (Fig. 1).[3] In this process, hypertrophic chondrocytes, which appear in the center of cartilage mold, play essential roles in bone induction. Hypertrophic chondrocytes express a number of osteogenic factors such as bone morphogenetic proteins (BMPs), Wnt, and vascular endothelial growth factor (VEGF), and direct mesenchymal cells in the perichondrium and in the bone marrow to become osteoblasts, which form the bone collar and the primary spongiosa.

Most endochondral growth in length occurs at the growth plate, which is formed as a distinct plate of cells between the bone of the secondary ossification center and the primary spongiosa. The growth plate is composed of the resting, proliferating, and hypertrophic chondrocytes, typically in orderly columnar arrays (Fig. 2). At the top of the growth plate, round chondrocytes proliferate slowly and are called resting or reserve chondrocytes, and probably serve as precursors for the flat proliferating columnar chondrocytes.[4] In the proliferating layer, the chondrocytes are arranged in columns parallel to the longitudinal axis of the bone and are separated from each other by matrix with large amount of type II collagen and aggrecan.[2] Then the chondrocytes stop proliferating, start enlarging, and become hypertrophic. The prehypertrophic chondrocytes express alkaline phosphatase (ALP), Indian hedgehog (Ihh), parathyroid hormone (PTH)/PTH-related peptide (PTHrP) receptor (PPR), and cyclic guanosine monophosphate (cGMP)-dependent protein kinase II (cGKII). The hypertrophic chondrocytes synthesize type X collagen and matrix metalloproteinase-13 (MMP-13), express a number of growth factors, and increase their intracellular volume from 5 to 10 times. Bone lengthening is driven primarily by the rate of production of hypertrophic chondrocytes from these proliferating chondrocytes.[5,6] Disruption of these processes results in many skeletal abnormalities such as

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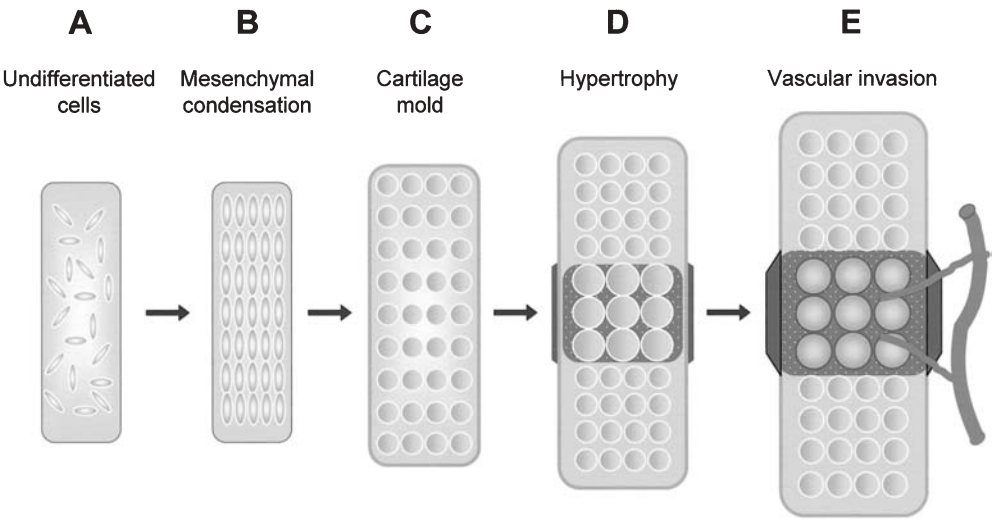

Fig. 1A–E. Endochondral ossification. **A** Undifferentiated cells derived from mesoderm. **B** Mesenchymal cells condense. **C** Cartilage mold is formed from mesenchymal condensation. Chondrocytes proliferate and express extracellular matrix. **D** Hypertrophic chondrocytes appear in the center of cartilage mold and calcify the matrix around them. Bone collar is formed adjacent to hypertrophic chondrocytes. **E** Vascular invasion occurs around hypertrophic chondrocytes. Cells recruited by vascular invasion differentiate into osteoblasts or osteoclasts, and primary spongiosa is formed

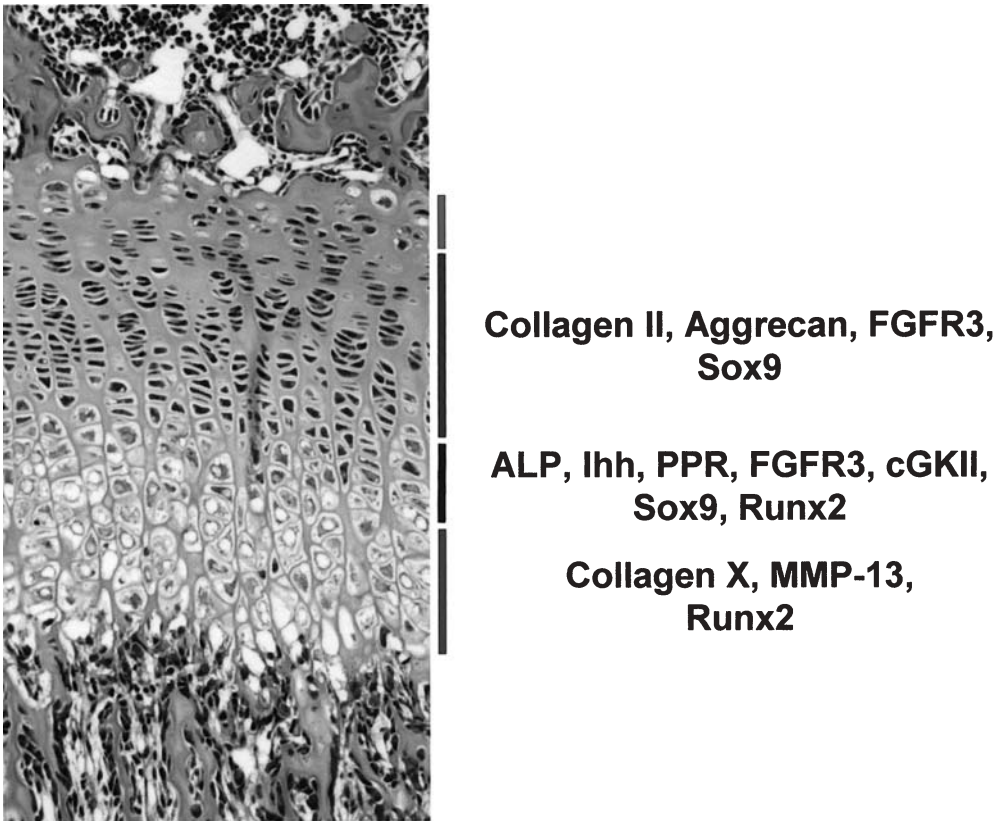

Fig. 2. Morphology of the growth plate. Hematoxylin–eosin staining of the growth plates of the proximal tibia at 3 weeks old. *Lines* show the resting, proliferating, prehypertrophic, and hypertrophic layers from top to bottom. *FGFR3*, fibroblast growth factor receptor-3; *ALP*, alkaline phosphatase; *Ihh*, Indian hedgehog; *PPR*, parathyroid hormone (PTH)/PTH-related peptide (PTHrP) receptor; *cGKII*, cyclic guanosine monophosphate (cGMP)-dependent protein kinase II; *MMP-13*, matrix metalloproteinase-13

chondrodysplasias[7] and chondrogenic tumors[8]. Thorough knowledge of the mechanisms regulating the growth plate physiology is therefore required for the understanding and treatment of theses disorders. Here, we review the molecular mechanism of regulation of chondrocyte hypertrophy by cGKII and the interaction between cGKII and other signaling pathways.

Involvement of cGKII in chondrocyte hypertrophy

cGKII is a serine/threonine kinase with cGMP binding domain and catalytic domain in the C terminus (Fig. 3A). cGKII functions as an effector of cGMP that is activated by nitric oxide and natriuretic peptides (Fig. 3B).[9] The increase in intracellular cGMP concentrations leads to the activation of cGKs.[10,11] In mammalian cells, two known cGK isoforms (cGKI and cGKII) are differently distributed.[12,13] cGKI plays important roles in vascular contraction,[14] platelet

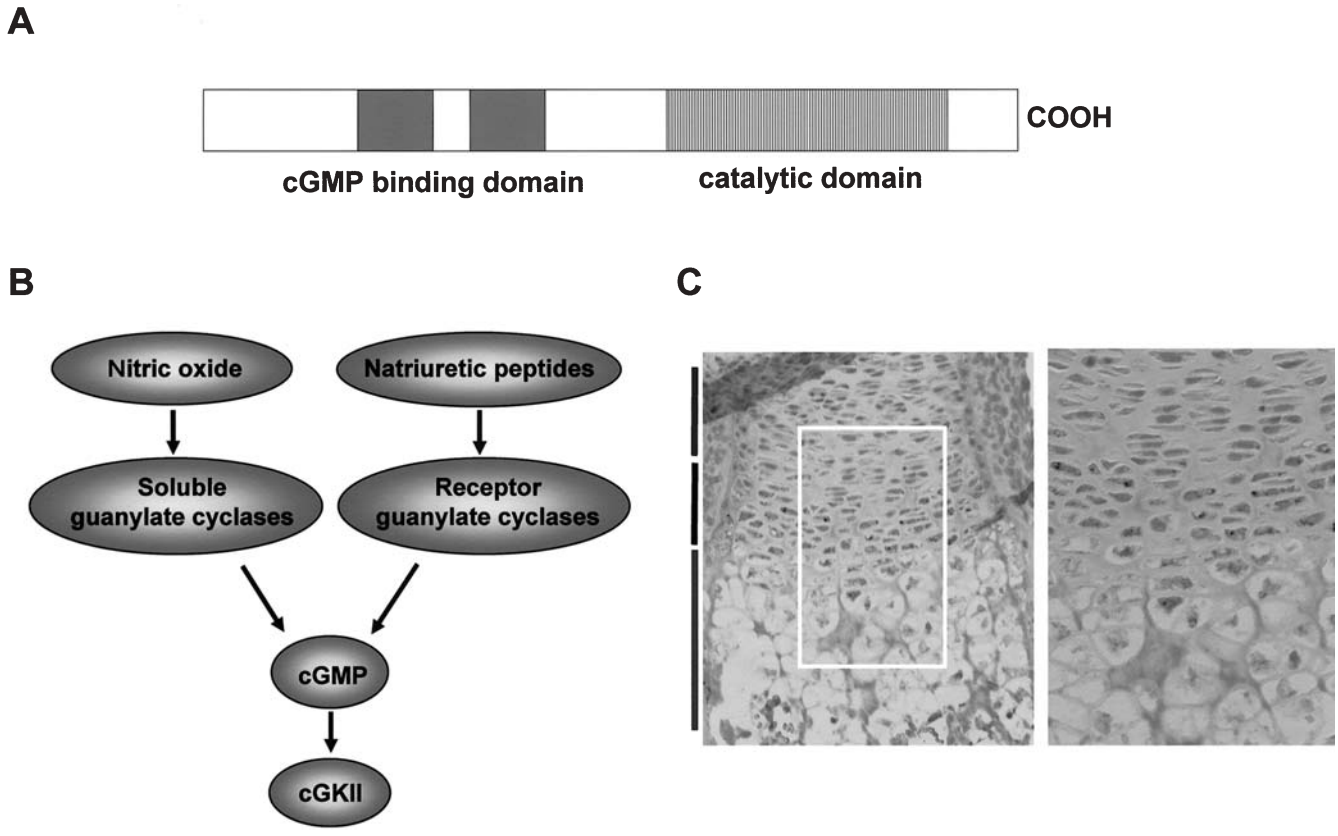

Fig. 3A–C. Structure, signaling pathway, and localization of cyclic guanosine monophosphate (*cGMP*)-dependent protein kinase II (*cGKII*). **A** Structure of cGKII. cGKII has two tandem cGMP-binding domains and a catalytic domain in the C terminus. **B** cGKII signaling pathway. cGMP is synthesized by soluble guanylate cyclases in response to nitric oxide or by receptor guanylate cyclases, which are activated by natriuretic peptides. cGKII is activated by accumulated cGMP. **C** In situ hybridization of cGKII in the distal growth plate of wild-type femur. The *boxed area* in the *left panel* is magnified in the *right panel*. *Lines* show the proliferating, prehypertrophic, and hypertrophic layers from top to bottom

activation,[15] and synaptic plasticity.[16] cGKII is involved in Cl^- and Na^+ transport in the intestine, renin secretion in the kidney,[17,18] and skeletal growth.[12] cGKII is bound to membrane and expressed in the intestinal mucosa and juxtaglomerular cells of kidney, lung, and chondrocytes. In the growth plate, cGKII is expressed predominantly in the prehypertrophic chondrocytes (Fig. 3C). cGKII-deficient mice show postnatal dwarfism, with a 25% reduction in the length of long bones and vertebrae because of impaired endochondral ossification.[12] The growth plate of cGKII-deficient mice is characterized by increased height of the growth plate with proliferating chondrocytes intermingled in the hypertrophic zone.

The Komeda miniature rat Ishikawa (KMI) is a naturally occurring mutant caused by an autosomal recessive mutation *mri*, which exhibits severe longitudinal growth retardation.[19] The positional candidate cloning revealed that the responsible KMI mutation was a 5-kb deletion in the rat gene encoding *cGMP-dependent protein kinase II* (*cGKII*).[20] KMI was born and grew normally until 3–4 weeks of age, when they gradually developed dwarfism.[20] The lengths of long bones of KMI were about 20%–30% shorter than those of wild-type littermates.[21] The height of the KMI growth plate was about 3-fold greater than that of wild type, although the columnar structure was relatively preserved. In the growth plate of KMI, an intermediate layer existed between the proliferative and hypertrophic chondrocytes, which were abnormal cells that had stopped proliferation, but did not start hypertrophic differentiation. Thus, cGKII promotes the hypertrophic differentiation of chondrocytes in the growth plate. The skeletal phenotype of KMI closely resembles that of cGKII deficient mice.

cGKII as an attenuator of Sox9

Recent advances in molecular genetics have uncovered the regulatory mechanism of chondrocyte hypertrophic differentiation. Sox9 has been known as a key transcription factor that is indispensable for chondrogenic differentiation of mesenchymal cells,[22,23] and it upregulates expression of critical cartilaginous matrix genes such as collagen type II, type IX, type XI, and aggrecan. Several genetic approaches have shown that Sox9 positively regulates proliferation and negatively regulates chondrocyte hypertrophy. Heterozygous deletion of Sox9 in mice leads to cartilage hypoplasia and a perinatal lethal osteochondrodysplasia that resembles

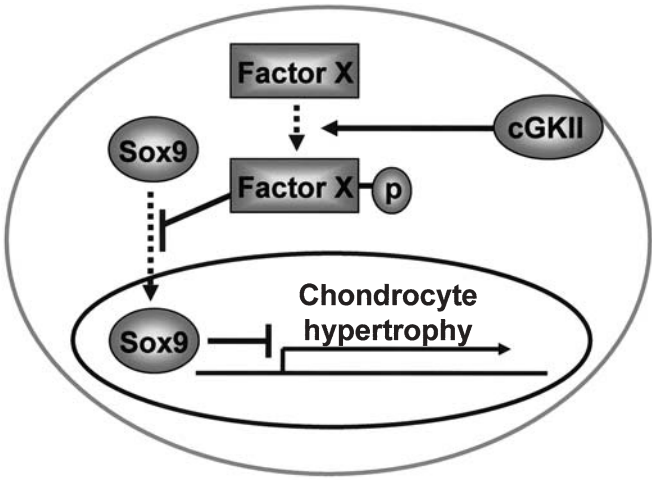

Fig. 4. Regulation of Sox9 subcellular localization by cGMP-dependent protein kinase II (*cGKII*). Hypothetical scheme of cGKII function. cGKII functions as a molecular switch in chondrocyte hypertrophy by attenuating nuclear translocation of Sox9

camplomelic dysplasia, a human syndrome caused by haploinsufficiency of Sox9.[4] These mice have fewer chondrocytes than normal in all bone anlage and malformation of subsequent bones. Furthermore, proliferative chondrocytes seem to convert prematurely to hypertrophic chondrocytes, which then mineralize their matrix prematurely. In contrast, mice overexpressing Sox9 in chondrocytes showed delayed hypertrophic differentiation.[24] Thus, Sox9 is also critical for the conversion of proliferating chondrocytes to hypertrophic chondrocytes. Sox9 is expressed in cells of mesenchymal condensations and in proliferating chondrocytes, but not in hypertrophic chondrocytes. In KMI, expression of Sox9 is persistent in the nuclei of the abnormal postmitotic chondrocytes in the intermediate layer. These data suggest that sustained Sox9 activity might cause the delay of hypertrophic differentiation of chondrocytes.

cGKII attenuates the inhibition of hypertrophic differentiation by Sox9, and the kinase activity of cGKII is indispensable for the attenuation of the Sox9 function. Sox9 reduces type X collagen mRNA expression of mouse chondrogenic ATDC5 cells in the three-dimensional culture. cGKII, but not cGKII mutant lacking the kinase domain, restored the type X collagen expression attenuated by Sox9. Since cGKII affects the subcellular localization of Sox9, cGKII is assumed to promote chondrocyte hypertrophy by attenuating Sox9 nuclear entry (Fig. 4).

Cross-talk with other signals regulating chondrocyte hypertrophy

PTHrP inhibits the rate at which chondrocytes exit the cell cycle and are converted to post-proliferating hypertrophic chondrocytes.[3] In mice lacking the *PPR* gene, the regulation of hypertrophy is disturbed and ectopic hypertrophic chondrocytes appear.[25,26] In transgenic mice expressing the constitutively active PPR under the control of the chondrocyte-specific type II collagen promoter, hypertrophy is suppressed. PPR is expressed mainly in prehypertrophic chondrocytes.[27] Binding of PTHrP to PPR activates protein kinase A (PKA) via accumulation of intracellular cyclic adenosine monophosphate (cAMP). Since cGKII is expressed predominantly in the prehypertrophic chondrocytes and activated by accumulation of intracellular cGMP, cGKII may be involved in PTH/PTHrP signaling. PTH/PTHrP signaling determined by the effect of PTH on cAMP accumulation was not changed in the KMI chondrocyte culture compared to the wild-type culture,[20] indicating that cGKII does not affect PTH/PTHrP signaling in the growth plate.

Runx2 is a member of a family of transcription factors that share the DNA binding domain of *Drosophila* pair rule gene *runt*. Runx2 is also essential for both osteoblast differentiation and chondrocyte hypertrophy. Mice lacking Runx2 have no osteoblasts and also exhibit decreased number of hypertrophic chondrocytes.[28,29] Transgenic overexpression of Runx2 accelerates hypertrophy of chondrocytes.[30] These data demonstrate a pivotal role of Runx2 in the control of hypertrophic differentiation of the growth plate chondrocytes. However, the interaction between cGKII and Runx2 has not been clarified. Expression of Runx2 is controlled by several signaling molecules. Among them, BMPs upregulate Runx2 mRNA level and promote both osteogenic differentiation[31] and terminal differentiation of hypertrophic chondrocytes.[32–35] BMP2 rescued the impaired expression of the hypertophic chondrocyte differentiation markers, such as ALP activity and type X collagen expression, in the KMI chondrocyte culture,[20] indicating that cGKII is not involved in the BMP signaling of chondrocyte hypertrophy.

Putative upstream signaling of cGKII in skeletal development

C-type natriuretic peptide (CNP), a humoral factor that can influence a variety of homeostatic processes by the intracellular accumulation of cGMP through membrane-bound guanylyl cyclase-coupled receptor B (GC-B),[36,37] has also been shown to play important roles in the skeletal growth.[38,39] Recently, it is reported that CNP-deficient mice exhibit dwarfism as a result of impaired endochondral ossification.[40] Targeted expression of CNP in the growth plate chondrocytes has rescued the skeletal defect of CNP-deficient mice.[40] Furthermore, GC-B-deficient mice exhibit impairment of endochondral ossification and an attenuation of longitudinal vertebra or limb-bone growth.[41] These observations indicate that CNP is a local positive regulator of endochondral ossification. Since cGKII is an effecter of CNP/cGMP signaling, cGKII may be essential for the CNP/cGMP-mediated endochondral ossification. However, there is a marked difference between CNP-deficient mice and cGKII-deficient mice. The height of CNP-deficient growth

plates is decreased unlike that of cGKII-deficient mice,[12,42] indicating that cGKII is not the sole mediator of CNP signaling, and the other signaling pathways may be involved in CNP signaling on endochondral ossification.

Fibroblast growth factor receptor-3 (FGFR3) is a membrane-spanning tyrosine kinase receptor and is expressed in the proliferative chondrocytes. The importance of FGF signaling in skeletal development was first revealed by the discovery of the point mutation of FGFR3 in achondroplasia patients. Activating mutations in the *FGFR3* gene lead to dwarf chondroplasia syndromes, including achondroplasia, hypochondroplasia, and thanotophoric dysplasia.[43,44] While several animal models have shown that FGFR3 negatively regulates chondrocyte proliferation, the involvement of FGFR3 signaling in chondrocyte hypertrophy is less clear. Targeted overexpression of CNP in chondrocytes counteracts dwarfism in a mouse model of achondroplasia with activated FGFR3 in the cartilage.[45] Further, the growth plate of these mutant mice resembles that of KMI, raising a possibility that cGKII and FGF signaling may cross-talk.

Conclusion

Many investigators have embarked on searches to understand the novel regulatory mechanisms governing cartilage development. Recently, much progress has been made in identifying the key transcription factors that control cartilage development and uncovering the critical extracellular signals that regulate chondrocyte differentiation. In contrast, little is known about the intracellular signaling pathways regulating chondrocyte hypertrophy. cGKII signaling functions as a molecular switch that couples the cessation of proliferation and the start of hypertrophic differentiation of chondrocytes through attenuation of Sox9 function by inhibiting the nuclear entry. To achieve more understanding of cartilage development at the molecular level, further studies are needed to clarify the molecular interaction of cGKII with other signaling pathways that regulate chondrocyte hypertrophy.

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