Phosphorylation of GSK-3β by cGMP-dependent protein kinase II promotes hypertrophic differentiation of murine chondrocytes

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Nonstandard abbreviations used: ALP, alkaline phosphatase; Bad, BCL2-antagonist of cell death; cdk25, cell division cycle 25 homolog; cGK, cGMP-dependent protein kinase; cGKI, kinase truncated cGKII protein that lacks the kinase domain; CNP, C-type natriuretic peptide; COL10, type X collagen; GC-B, guanylyl cyclase–coupled receptor B; GSK-3β, glycogen synthase kinase–3β (encoded by Gsk3b); Gsk3b–/–, phosphorylation-deficient mutant of GSK-3β; H19, insulin-like growth factor II mRNA; Hoxa, homeobox A; IGF-I, insulin-like growth factor–I; IGF2, insulin-like growth factor–II; JAM-A, junctional adhesion molecule A; JASPR, junctional adhesion molecule-related protein; Mdr1, multidrug resistance 1; Nppc, natriuretic peptide receptor C; Npr2, natriuretic peptide receptor 2; PLK, polo-like kinase; p90RSK, 90-kDa ribosomal protein S6 kinase; PTH, parathyroid hormone; PTHrP, parathyroid hormone–related protein; TCF, T cell factor; VASP, vasodilator-stimu-
lation mutant of GSK-3β; Δ-Catenin, truncated β-catenin levels decreased in mice known as acromesomelic dysplasia, skeletal growth (2, 3). Loss-of-function mutations in both genes result in impaired skeletal growth, because mice deficient in either gene exhibit impaired skeletal growth (2, 3). Loss-of-function mutations in Npr2 also show dwarfism in patients known as acromesomelic dysplasia, type Maroteaux (4), demonstrating the importance of CNP/GC-B signaling in the skeletal growth of humans as well. This signaling causes the intracellular accumulation of cGMP, which then activates cGMP-dependent protein kinases (cGKs) (5). In mammalian cells, there are 2 cGK isoforms, cGKI and cGKII (encoded by Prkg1 and Prkg2, respectively), which show distinct distributions and functions (6, 7). Although both are expressed in the growth plate cartilage, Prkg2–/– mice show postnatal dwarfism with about 20%–30% reduction in the length of limbs and trunk (6), while Prkg1–/– mice show a normal skeleton (8), indicating that only cGKI is indispensable for skeletal growth.

Introduction

Skeletal growth is achieved by endochondral ossification in the growth plate cartilage, with orderly columnar arrays of resting, proliferative, and hypertrophic zones of chondrocytes. During the process, chondrocytes undergo proliferation, hypertrophic differentiation, and apoptosis, each of which is regulated by distinct molecular signaling systems (1). Among them, C-type natriuretic peptide (CNP; encoded by Prkg2) increases the accumulation and transactivation function of cGKII. In cultured mouse chondrocytes, phosphorylation-mediated inactivation of GSK-3β was associated with enhanced hypertrophic differentiation. Furthermore, cGKII induction of chondrocyte hypertrophy was suppressed by cotransfection with a phosphorylation-deficient mutant of GSK-3β.

Analyses of mice with compound deficiencies in both protein kinases (Prkg2–/–, Gsk3b–/–) demonstrated that the growth retardation and elongated growth plate associated with cGKII deficiency were partially rescued by haploinsufficiency of Gsk3b. We found that β-catenin levels decreased in Prkg2–/– mice, while overexpression of cGKI increased the accumulation and transactivation function of β-catenin in mouse chondroprogenitor ATDC5 cells. This effect was blocked by coexpression of phosphorylation-deficient GSK-3β. These data indicate that hypertrophic differentiation of growth plate chondrocytes during skeletal growth is promoted by phosphorylation and inactivation of GSK-3β by cGKII.

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cGKII is a membrane-bound serine/threonine kinase with a cGMP-binding domain and a catalytic domain in the C terminus (7). In addition to growth retardation resulting from cGKII deficiency in mice, our previous positional cloning analysis identified a deletion in Prkg2, the rat gene encoding cGKII, in the Komeda miniature rat Ishikawa (KMI), a naturally occurring mutant rat, which also exhibited dwarfism with 20%–30% shorter long bones and vertebrae (9). The deletion resulted in a frame shift and a premature stop codon, predicting a truncated cGKI protein that lacks the kinase domain (cGKI-Akinase). KMI rats show an elongated growth plate, whose height is about 2.5-fold that of WT littermates. This is caused by the existence of an abnormal intermediate layer between the proliferative and hypertrophic zones with accumulation of few proliferative or hypertrophic chondrocytes, which indicates that the kinase activity of cGKII is necessary for

Conflict of interest: The authors have declared that no conflict of interest exists.

hypertrophic differentiation of growth plate chondrocytes (9). To investigate the mechanism underlying cGKII kinase activity in chondrocyte hypertrophy, in the present study we performed a screen of its potential phosphorylation targets and identified glycogen synthase kinase–3β (GSK-3β; encoded by Gsk3b) as a significant phosphorylation target of cGKII. Because the phosphorylation of GSK-3β at Ser9 is known to cause its inactivation (10), we further examined the functional involvement of GSK-3β in the cGKII-induced hypertrophic differentiation of chondrocytes and investigated the underlying mechanism. Our results demonstrated that cGKII promotes chondrocyte hypertrophy and skeletal growth through phosphorylation and inactivation of GSK-3β.

Figure 1
Skeletal abnormality in Prkg2−/− mice. (A) Gross appearance and radiographs of femurs, tibias, lumbar vertebrae, and skulls of WT and Prkg2−/− littermates at 8 weeks of age. (B) Time course of H&E staining of the growth plates in proximal tibias of the 2 genotypes from 0–8 weeks of age. Vertical black bars indicate the height of the growth plates. (C) H&E staining, BrdU labeling, and in situ hybridization of COL10 of the tibial growth plates in 2-week-old littermates. (D) H&E and Safranin-O stainings, BrdU labeling, and COL10 immunostaining of the growth plates in the fourth lumbar vertebra of 2-week-old littermates. (C and D) Blue, red, green, and yellow bars indicate proliferative zone, abnormal intermediate layer, hypertrophic zone, and primary spongiosa, respectively. Boxed regions in H&E and COL10 panels are shown at higher magnification to the right. Scale bars: 50 μm.
Results
Growth plate abnormality in Prkg2−/− mice. Prkg2−/− mice showed postnatal dwarfism with short limbs and trunk compared with WT littermates (Figure 1A), as previously reported (6). Radiographic analysis at 8 weeks of age revealed that the lengths of femur, tibia, and vertebra, which are known to be primarily formed through endochondral ossification, were shorter in Prkg2−/− mice. The longitudinal length of the Prkg2−/− skull was also shorter, while the width was comparable to WT. This finding is probably attributable to 2 types of the skull growth via endochondral ossification and intramembranous ossification (11), although this needs to be further investigated. The time course of histological observation of the tibial growth plate revealed that the height was greater in Prkg2−/− than WT mice from 2 to 4 weeks after birth but was restored to a level comparable to that of WT mice by 8 weeks of age (Figure 1B). As previously observed in KMI rats (9), growth plate elongation during these ages was caused by an abnormal intermediate layer between the proliferative and hypertrophic zones, with accumulation of few proliferative or hypertrophic chondrocytes, as determined by BrdU uptake and expression of type X collagen (COL10), respectively (Figure 1C). The growth plate of the Prkg2−/− vertebral bodies also contained the abnormal intermediate layer, which was intermittently focal in the elongated growth plate (Figure 1D). These results indicate that cGKII is necessary for hypertrophic differentiation of growth plate chondrocytes during endochondral ossification for longitudinal growth of limbs and trunk not only in rats, but also in mice.

Phosphorylation targets of cGKII in chondrocyte hypertrophy. To investigate the mechanism underlying cGKII activity in hypertrophic differentiation of chondrocytes, we performed a screen of its phosphorylation targets using the same antibody as the screening array above, and the phosphorylation was confirmed by in vitro kinase assay and phospho-specific stainings were confirmed by immunohistochemistry (Figure 2). We identified 8 substrates that were most strongly phosphorylated by cGKII: caspase-9, BCL2-associated agonist of cell death (Bad), poloid-like kinase (PLK), 90-kDa ribosomal protein S6 kinase (p90RSK), eNOS, GSK-3β (p-GSK-3β), 90-kDa ribosomal protein S6 kinase (p90RSK), eNOS, GSK-3β, vaso-endothelial growth factor (VEGF), and cell division cycle 25 homolog (cDC25). All of these molecules were confirmed to be expressed in mouse chondrogenic ATDC5 cells in the prehypertrophic or hypertrophic differentiation stage (Figure 2A). However, a luciferase reporter assay revealed that GSK-3β markedly suppressed COL10 promoter activity, while none of the other candidates had a significant effect (Figure 2B). These data suggest that GSK-3β might be functionally involved in chondrocyte hypertrophy, although involvement of the other factors cannot be ruled out. Direct phosphorylation of recombinant GSK-3β at Ser9, the crucial site for inactivation of GSK-3β (10), by recombinant cGKII protein was confirmed by in vitro kinase assay using the same antibody as the screening array above, and the phospho-specific stainings were confirmed by immunohistochemistry.
Phosphorylation was enhanced by the addition of cGMP (Figure 2C). Furthermore, endogenous GSK-3β in cell lysates of ATDC5 cells was phosphorylated at Ser9 by recombinant cGKII protein, which was further enhanced by the addition of cGMP. On the other hand, GSK-3α, the closely related isoform of GSK-3β, was not phosphorylated by cGKII, nor were protein levels of GSK-3β and GSK-3α altered by cGKII or cGMP (Figure 2D). Immunohistochemistry revealed that cGKII, total GSK-3β, and Ser9-phosphorylated GSK-3β were colocalized in prehypertrophic chondrocytes of the growth plate, implicating the interaction of these molecules in vivo as well (Figure 2E). Compared with the respective nonimmune controls, the localization of Ser9-phosphorylated GSK-3β appeared to be restricted to those cells that also produced cGKII, whereas total GSK-3β was more broadly distributed, which supports the notion that cGKII is an important regulator of GSK-3β phosphorylation.

**Regulation of chondrocyte hypertrophy by GSK-3β.** In the 3-dimensional cultures of ATDC5 cells and primary costal chondrocytes in alginate beads, LiCl, a selective inhibitor of GSK-3β, stimulated the expression of chondrocyte hypertrophic differentiation markers COL10, alkaline phosphatase (ALP), and MMP-13 and induced morphological hypertrophy of the cells (Figure 3, A and B). COL10 expression also increased in cultured primary costal chondrocytes in alginate beads, LiCl, a selective inhibitor of GSK-3β substituted mutant of GSK-3β cells stimulated expression of hypertrophic markers, and this was attenuated by the cointraduction of a phosphorylation-deficient mutant of GSK-3β with a serine-to-alanine substitution (GSK-3βS9A), which is known to have constitutive activity (Figure 3D). These data demonstrated that Ser9 phosphorylation of GSK-3β is necessary for the induction of chondrocyte hypertrophy by cGKII. The GSK-3βS9A introduction alone altered none of the 3 markers (Figure 3D), which indicates that an endogenous GSK-3β level was sufficient for the suppression of chondrocyte hypertrophy in this culture system.

**Mechanism underlying cGKII/GSK-3β signaling in chondrocyte hypertrophy.** We further examined the molecular mechanism whereby GSK-3β phosphorylation by cGKII regulates hypertrophic differentiation of chondrocytes. Because GSK-3β is known to be a negative regulator of β-catenin through its phosphorylation and degradation (10), we compared the localization of β-catenin, Ser9-phosphorylated GSK-3β, and total GSK-3β in the growth plates of WT and Prkg2−/− littermates. In the WT growth plate, β-catenin as well as both GSK-3β proteins were localized mainly in the cytoplasm of prehypertrophic chondrocytes (Figure 4A). cGKII deficiency caused similar decreases in β-catenin and Ser9-phosphorylated GSK-3β levels with little effect on the total GSK-3β level in the abnormal intermediate layer. In cultured ATDC5 cells, cGKII induced cytosolic accumulation of β-catenin after stimulation by 8-bromo-cGMP, while cGKII-Δkinase had a minimal effect (Figure 4B). Overexpression of constitutively active cGKII enhanced the promoter activity of the β-catenin target T cell factor (TCF), which was markedly suppressed by cotransfection of GSK-3βS9A (Figure 4C). Again, GSK-3βS9A alone did not have an effect, which indicates that an endogenous GSK-3β level is sufficient for β-catenin suppression. We next examined the involvement of a scaffolding peptide, Axin, which is known to associate with GSK-3β and promotes effective phosphorylation and degradation of β-catenin under conditions of Wnt stimulation (10). IP/IB analysis using HEK293 cells transfected with Myc-tagged Axin and cGKII revealed that cGKII formed a complex with Axin and phosphorylated GSK-3β not only in the whole-cell lysates, but also in the IP with Axin, suggesting some interaction between Ser9 phosphorylation and coupling with Axin in regulation of GSK-3β by cGKII (Figure 4D).

In our previous study, we showed that cGKII caused attenuation of Sox9 transcriptional function through inhibition of nuclear entry (9). Because Sox9 is known not only to induce chondrogenic differentiation of mesenchymal cells, but also to prevent hypertrophic differentiation of chondrocytes (12), this may contribute...
to the mechanism whereby cGKII promotes chondrocyte hypertrophy. However, inhibition of Sox9 nuclear entry by cGKII was independent of phosphorylation of Sox9 itself, because cGKII inhibited not only the nuclear entry of the WT Sox9, but also that of the phosphorylation-deficient Sox9 mutants with serine-to-alanine substitutions at putative phosphorylation sites Ser64 and Ser181 (Figure 5A), which suggests that other phosphorylation targets of cGKII are important. We therefore examined the involvement of GSK-3β phosphorylation in the inhibition of Sox9 nuclear entry by cGKII. Neither addition of the GSK-3β inhibitor LiCl nor overexpression of GSK-3βS9A altered cGKII-dependent inhibition of Sox9 nuclear entry, which indicates that the inhibitory effect of cGKII was independent of GSK-3β phosphorylation by cGKII (Figure 5B).

We next assessed involvement of other putative signaling systems in cGKII action on chondrocyte hypertrophy. Although Runx2 has previously been shown to be an important transcription factor that induces hypertrophic differentiation of chondrocytes (13), its expression was visible in the abnormal intermediate layer of the Prkg2−/− growth plate (Supplemental Figure 1A). In addition, neither the mRNA level nor the subcellular localization of Runx2 was altered by cGKII overexpression in cultured ATDC5 cells (Supplemental Figure 1, B and C).

FGF signaling has also been shown to be important for chondrocyte differentiation and endochondral ossification in mice and humans (14). Considering that targeted overexpression of CNP in chondrocytes counteracts dwarfism in a mouse model of achondroplasia with activated FGF receptor 3 (15) and that the mutant mice exhibit an elongated growth plate similar to that of Prkg2−/− mice (16), there might be cross-talk between cGKII and FGF signaling. Because FGF signaling stimulates MAPK or STAT-1 signaling pathways, we examined the interaction of phosphorylation of Erk1, Erk2, p38 MAPK, JNK1, JNK2/3, and STAT-1. Among these, FGF-2 most strongly phosphorylated Erk1 and Erk2; however, overexpression of cGKII affected none of these in the presence or absence of FGF-2, indicating no apparent interaction between cGKII and FGF, MAPK, or STAT-1 signaling (Supplemental Figure 2A).

Partial reversal of the skeletal abnormality in Prkg2−/− mice by GSK-3β insufficiency. To test whether GSK-3β plays a role in mediating the effect of cGKII-induced skeletal growth changes in vivo, we examined the effect of genetic insufficiency of GSK-3β on the skeletal abnormality apparent in Prkg2−/− mice. Although Gsk3b−/− mice were embryonically lethal (17), Gsk3b−/− mice developed and grew normally (Supplemental Figure 3). We therefore crossed Prkg2−/− mice with Gsk3b−/− mice to generate compound Prkg2−/−Gsk3b−/− mice. Radiographic analysis and total axial length measurement showed that Prkg2−/−Gsk3b−/− mice exhibited partial, but significant, restoration (about 30%-40%) of the impaired skeletal growth of Prkg2−/− mice at 8, 12, and 16 weeks after birth (Figure 6, A and B). Measurement of skeletal length confirmed that the endochondral ossification of femur, tibia, humerus, ulna, vertebra, and skull were decreased by cGKII deficiency, while skull width and clavicle length — which are known to develop by endochondral and intra-

**Figure 4**

**Mechanism underlying cGKII/GSK-3β signaling in chondrocyte hypertrophy.** (A) Localization of β-catenin, Ser9-phosphorylated GSK-3β, and total GSK-3β, as assessed by immunohistochemistry in the growth plates of the proximal tibias of WT and Prkg2−/− mice at 2 weeks of age. Blue, red, green, and yellow bars indicate proliferative zone, abnormal intermediate layer, hypertrophic zone, and primary spongiosa, respectively. Scale bars: 50 μm. (B) Time course of β-catenin protein level after stimulation by 8-bromo-cGMP, as assessed by IB in the cytosolic fraction of ATDC5 cells with retroviral introduction of cGKII or cGKII-Δkinase. (C) Promoter activity of the β-catenin target TCF, as assessed by luciferase (Luc) assay using TOPflash and FOPflash reporter plasmids in HEK293 cells transfected with constitutively active cGKII, GSK-3βS9A, or the control GFP (−). Data are mean ± SD fold change compared with control (−/−). *P < 0.01 versus control. **P < 0.01 versus constitutively active cGKII alone. (D) Physical association of cGKII and GSK-3β with Axin by IP/IB analysis. HEK293 cells were transfected with Myc-tagged Axin (Myc-Axin) and/or cGKII, and an aliquot of the cell lysates underwent IP with the high-affinity anti-c-Myc antibody–coupled agarose as described in Methods. The IP (Myc) or the whole-cell lysates underwent IB with an antibody to cGKII, Ser9-phosphorylated GSK-3β, GSK-3β, or Myc.
membranous ossification (11, 18) — were comparable to those of WT littermates (Figure 6C). The genetic insufficiency of GSK-3β in the Prkg2−/− Gsk3b+/− mice partially, but significantly, restored the impaired skeletal growth (about 20%–40%). These findings indicate that sufficient GSK-3β function is needed for skeletal growth and endochondral ossification to be impaired by cGKII deficiency.

Further histological analyses revealed that the elongated growth plate and decreased COL10 expression in Prkg2−/− mice were also partially restored in the Prkg2−/− Gsk3b+/− mice (Figure 7, A and B). In contrast, GSK-3β insufficiency did not alter skeletal growth or growth plate parameters in WT or Prkg2+/− mice, as shown in Gsk3b−/− and compound Prkg2−/− Gsk3b−/− mice, respectively (Supplemental Figure 3). GSK-3β may therefore function specifically as a mediator of cGKII signaling, rather than generally in the regulation of chondrocyte hypertrophy and endochondral ossification.

**Discussion**

Based on our previous finding that cGKII activity is essential for the promotion of skeletal growth through hypertrophic differentiation of growth plate chondrocytes (9), the results of our present study initially identified GSK-3β as a likely substrate of this protein kinase. Figure 7C summarizes the mechanism underlying chondrocyte hypertrophy by cGKII/GSK-3β signaling based on the present and previous studies. cGKII phosphorylates GSK-3β at Ser9 and inactivates it, which may contribute to the suppression of β-catenin degradation, as previously reported (10). We and others have reported that β-catenin/TCF signaling causes stimulation of hypertrophic differentiation of chondrocytes in vitro (19–22). In addition, chondrocyte-specific inactivation of β-catenin in mice results in dwarfism with delayed hypertrophic differentiation of chondrocytes (23). Hence, the stabilization and accumulation of β-catenin by cGKII/GSK-3β signaling in chondrocytes may lead to hypertrophic differentiation, although the underlying molecular mechanism is still controversial.

Genetic rescue of impaired skeletal growth in Prkg2−/− mice by suppression of GSK-3β was significant, but incomplete (Figures 6 and 7). This might be because GSK-3β haploinsufficiency was inadequate to fully overcome the deficiency of cGKII. Indeed, cultured Gsk3b−/− chondrocytes showed higher COL10 expression, but similar ALP and MMP-13 expression, compared with WT cells, while LiCl clearly increased all hypertrophic markers in the ATDC5 cell culture (Figure 3, A and C). We cannot exclude the possibil-
Chondrocyte hypertrophy in the growth plate is a rate-limiting step for longitudinal skeletal growth (25), because this step is responsible for 40%–60% of endochondral ossification, with the remainder caused by chondrocyte proliferation and matrix synthesis (26). Sox9 is a representative regulator of this step, as are Runx2 (1, 13) and parathyroid hormone/parathyroid hormone–related protein (PTH/PTHrP) (1, 27), uncovered downstream mediators, such as cGKI, cyclic nucleotide phosphodiesterases, and cGMP-regulated ion channels (5, 28). Although the fact that CNP was unable to affect chondrocyte differentiation in the absence of cGKII either in vitro or in vivo indicates a crucial role of cGKII in CNP signaling pathways in CNP/GC-B–mediated endochondral ossification. In fact, the intracellular accumulation of cGMP caused by CNP/GC-B signaling activates not only cGKII, but also other downstream mediators, such as cGKI, cyclic nucleotide phosphodiesterases, and cGMP-regulated ion channels (5, 28). Although no skeletal abnormality has been reported in Prkg1–/– mice (8), it would be helpful to investigate whether mice doubly deficient for cGKI and cGKII mimic the phenotype of Nppc–/– or Npr2–/– mice. In addition, targeted overexpression of CNP in growth plate chondrocytes was reported to restore the achondroplastic bone with FGF receptor 3 mutation through inhibition of the MAPK pathway (15), which we found in the present study to be unrelated to cGKII (Supplemental Figure 2A). Furthermore, cGKII functions as an effector of cGMP that is activated not only by CNP, but also by nitric oxide and other types of natriuretic peptides (5, 7, 28). Although the fact that CNP was unable to affect chondrocyte differentiation of skeletal growth in the absence of cGKII either in vitro or in vivo indicates a crucial role of cGKII in CNP signaling (29), CNP and cGKII are unlikely to function with a one-to-one correspondence during endochondral ossification.

The abnormal elongation of the Prkg2–/– mouse growth plate was apparent from 2 to 4 weeks after birth, but not before or after these ages (Figure 1B). This observation suggests some compensatory mechanisms for cGKII deficiency. Besides signaling via Runx2, PTH/PTHrP, and the CNP-related factors described above, GSK-3β (the other GSK-3 in mammals) might substitute for GSK-3β signal transduction associated with chondrocyte hypertrophy. In addition, because Sox9 has previously been reported to physically interact with β-catenin and to compete with its binding to TCF (23), the downstream pathways of cGKII through GSK-3β and Sox9 might interact at the level of β-catenin during chondrocyte hypertrophy. Chondrocyte hypertrophy in the growth plate is a rate-limiting step for longitudinal skeletal growth (25), because this step has been shown to be responsible for 40%–60% of endochondral ossification, with the remainder caused by chondrocyte proliferation and matrix synthesis (26). Sox9 is a representative regulator of this step, as are Runx2 (1, 13) and parathyroid hormone/parathyroid hormone–related protein (PTH/PTHrP) (1, 27), uncovered via recent advances in molecular genetics. The present study failed to find interaction between cGKII and Runx2 (Supplemental Figure 1). Although PTH/PTHrP has previously been shown to be a potent inhibitor of chondrocyte hypertrophy by the findings in deficient and transgenic mice (1, 27), our previous study revealed that neither expression levels of PTHrP and PTH/PTHrP receptor nor cAMP accumulation by PTH stimulation was altered by cGKII deficiency in chondrocytes (9). Hence, cGKII may regulate chondrocyte hypertrophy by a mechanism independent of those of Runx2 and PTH/PTHrP.

In line with the view that cGKII is a downstream mediator of CNP, Nppc–/– and Npr2–/– mice also exhibit dwarfism (2). However, unlike Prkg2–/– mice, which have an elongated growth plate with an abnormal intermediate layer, both Nppc–/– mice and Npr2–/– mice showed thinned growth plates with chondrocytes arranged in a regular columnar array. This may indicate the involvement of other signaling pathways in CNP/GC-B–mediated endochondral ossification.

Prkg2–/– and Prkg2–/–Gsk3b–/– littermates at 9 weeks of age. (B) Time course of total axial length (from nose to tail end) of the 3 genotypes from 3 to 16 weeks of age. The recovery by the GSK-3β–/– mice was 43.2%, 31.4%, and 41.9% at 8, 12, and 16 weeks, respectively. (C) Length of bones of the 3 genotypes at 8 weeks of age. Percent recovery was 21.7%, 18.3%, 24.3%, 16.2%, 24.3%, and 42.6% in femur, tibia, humerus, ulna, vertebra, and skull length, respectively. Data are mean ± SD for 4–9 mice per genotype. *P < 0.05 versus WT. **P < 0.05 versus Prkg2–/–.
GSK-3β is known to be active under resting conditions and inactivated upon stimulation by several signaling pathways, such as Wnt and insulin/Akt; however, the role of phosphorylation of GSK-3β remains controversial (32, 33). Our present results led us to propose cGKII as a novel regulator of GSK-3β and showed that the β-catenin activity enhanced by cGKII was suppressed by GSK-3β phosphorylation at Ser9 in cGKII/β-catenin signaling (Figure 4C). Conversely, several reports showed that GSK-3β inactivation causing β-catenin induction by Wnt stimulation depends not on Ser9 phosphorylation, but rather on coupling with the scaffolding protein, such as Axin (34, 35). The present study, however, showed that cGKII formed a complex with Axin and further phosphorylated GSK-3β that bound to Axin (Figure 4D), suggesting some interaction between Ser9 phosphorylation and coupling with Axin in the regulation of GSK-3β by cGKII. In fact, a previous report proposed that Wnt signaling, similar to insulin/Akt signaling, induces GSK-3β phosphorylation via the interaction between the signaling pathways both in neuronal PC12 cells and in human embryonic kidney 293T cells (36). While these findings imply a possible link between cGKII/β-catenin and canonical Wnt/β-catenin signaling, we note that there is no direct evidence of cGKII being involved in the canonical Wnt pathway. We therefore believe that cGKII/β-catenin signaling, which is dependent on GSK-3β phosphorylation, may have a mechanism that is, at least in part, distinct from that of Wnt/β-catenin signaling. Further studies will be needed to clarify the details of GSK-3β-related signaling not only in chondrocytes, but also in other cells.

We conclude that cGKII promotes chondrocyte hypertrophy and skeletal growth through phosphorylation and inactivation of GSK-3β. For the application of this intracellular signaling to yield novel therapeutics for skeletal disorders, we are now developing a gene transfer system using biocompatible polyplex nanomicelles (37, 38). Further understanding of the molecular signaling related to the cGKII/GSK-3β axis, in combination with other putative signaling systems, will greatly assist in unraveling the molecular network that modulates endochondral ossification and skeletal growth.

**Methods**

*Animals.* The Prkg2−/− mice and Gsk3b−/− mice were maintained in a C57BL/6 background. To generate Prkg2−/− Gsk3b−/− mice, Gsk3b−/− mice were mated with the homozygous Prkg2−/− mice to obtain Prkg2−/− Gsk3b−/− mice, which were then mated with each other. All experiments were performed on male mice and were approved by the Animal Care and Use Committee of the University of Tokyo.
Radiological and histological analyses. Plain radiographs were taken using a soft X-ray apparatus (Softex CMB-2; Softex). For histological analyses, skeletons were fixed in 10% EDTA, embedded in paraffin, sectioned in 5-μm slices, and stained with H&E or Safranin-O, according to standard procedures. For BrdU labeling experiments, mice were injected intraperitoneally with BrdU (25 μg/g body weight) 2 h prior to sacrifice, and the sections were stained with a BrdU staining kit (Zymed Laboratories) according to the manufacturer’s instructions. In situ hybridization with nonradioactive probes was performed as previously described (39). For immunohistochemistry, antibodies to cGKI, Ser9-phosphorylated GSK-3β, MMP-13, Runx2 (1:50; Santa Cruz Biotechnology Inc.), GSK-3β (1:200; Chemicon), COL10 (1:1000; LSL), β-catenin (1:100; Cell Signaling Technology), and respective nonimmune serum standards were used, and the signal was detected with an HRP-conjugated secondary antibody. For fluorescent visualization, a secondary antibody conjugated with Alexa Fluor 488 (Invitrogen) was used.

Cell cultures. ATDC5 cells were grown and maintained in DMEM and F12 at a 1:1 ratio with 5% FBS. To induce hypertrophic differentiation, the ATDC5 cells were cultured in the presence of insulin, transferrin and sodium selenite (ITS) supplement (Sigma-Aldrich) for 21 d as described previously (40). We confirmed COL10 expression by real-time RT-PCR and used the cells whose stage of differentiation was assumed to be prehypertrophic or hypertrophic. Primary chondrocytes were isolated by digestion of E18.5 costal cartilage. Primary chondrocytes, HuH-7 cells, HEK293 cells, and HeLa cells were cultured in high-glucose DMEM with 10% FBS. Three-dimensional alginate bead cultures of primary costal chondrocytes and ATDC5 cells were performed with or without LiCl (8 mM) for 72 h, and the cells were analyzed as described previously (21). For immunocytochemistry of primary costal chondrocytes, the cell colonies were fixed with 4% paraformaldehyde, embedded in paraffin, sectioned in 5-μm slices, and underwent immunostaining for COL10 and MMP-13 as described above. For ALP staining, sections were fixed in 70% ethanol and stained for 10 min with a solution containing 0.01% Naphthol AS-MX phosphate disodium salt (Sigma-Aldrich), 1% N, N-dimethylformamide (Wako Pure Chemical Industries Ltd.), and 0.06% fast blue BB (Sigma-Aldrich).

In vitro kinase assay. ATDC5 cells were cultured in the presence of ITS for 21 d to differentiate into prehypertrophic or hypertrophic chondrocytes, as described above. The whole-cell lysate of the differentiated cells was prepared using Cell Lysis Buffer (Cell Signaling Technology). The cell lysate or recombinant GSK-3β (Upstate Biotechnology Inc.) was incubated with recombinant cGKI (Sigma-Aldrich) in a reaction buffer (Cell Signaling Technology) containing 1.6 mM ATP and 100 μM 8-bromo-cGMP (Biomol) at 30°C for 30 min. An equal amount of protein (15 μg) was subjected to SDS–PAGE and transferred onto nitrocellulose membranes. IB was then performed using primary antibodies to Ser9-phosphorylated GSK-3β (Cell Signaling Technology), GSK-3β (Chemicon), Ser21-phosphorylated GSK-3α and GSK-3β (Cell Signaling Technology), and β-actin (Sigma-Aldrich). The membrane was incubated with HRP-conjugated antibody (Promega), and the immunoreactive proteins were visualized with ECL Plus (Amersham Biosciences).

Plasmids and viral vectors. cDNA of caspase-9 (GenBank accession no. NM_001229.1), Bad (NM_007522.2), PLK (NM_011121.3), p90RSK (NM_009097.4), eNOS (NM_000603.3), GSK-3β (NM_002093.2), VASP (NM_009499.1), cd25 (NM_008860.2), and caveolin- and glycine-rich protein 2 (CSRP2; NM_007792.3) was used to construct the cGKII-Δkinase plasmid. cGKII, cGKII-Δkinase, GSK-3β, MMP-13, Runx2 (NM_009820.3) was ligated into pEGFP-C1 (Clontech) to generate GFP-tagged plasmids. To create phosphorylation-deficient mutants, GFP-tagged Sox9 plasmid and GSK-3β plasmid were subjected to site-directed mutagenesis using the inverse PCR technique. All constructs were verified by sequencing. cGKII, cGKI-Akinase, GSK-3β, GSK-3β, and control GFP retrovirus vectors were constructed using pMX vector and plat-E cells as described previously (41).

Gene transfection. For the transient transfection, a total of 1 μg plasmid DNA was transfected using FuGene6 (Roche). For cotransfection, all plasmids were added in an equal ratio. Total RNA was isolated 72 h after the transfection and used for the subsequent assays. For fluorescent detection, HeLa cells were transiently transfected, and fluorescent images were taken 24 h after transfection. To investigate the interaction of cGKI and MAPK/STAT signaling, ATDC5 cells were transfected with cGKI or the empty vector, and pEGFP-C1 was added 72 h after transfection. IB was then carried out using primary antibodies to p-Erk1/2, Erk1/2, p-p38MAPK, p38MAPK, p-JNK2/3, p-JNK1, p-STAT1, and STAT1 (Cell Signaling Technology) as described above.

Real-time RT-PCR. Total RNA was reverse-transcribed with Multi-Scribe RT (Applied Biosystems Inc.). Semiquantitative RT-PCR was performed within an exponential phase of the amplification, with the following primer sequences: caspase-9 forward, 5′-CGATGCAAGGAGCTGCTCATGTA-3′; caspase-9 reverse, 5′-TGACAGCTGCTGCGCTCTGATC-3′; Bad forward, 5′-CCAGGTCTCTCGGGAGCAACTATCC-3′; Bad reverse, 5′-AGCTCCTCCTCATCTCCTATCC-3′; PLK forward, 5′-TGGCACTCTTCACTACATAGCTCCTGAGG-3′; PLK reverse, 5′-CGGAGGTAGCTTCTTTTTAGGCAAGC-3′; p90RSK forward, 5′-GATCTTCTGCGGCTGATCAGGCA-3′; p90RSK reverse, 5′-TGCGCTAGATCTATGCTTGGC-3′; eNOS forward, 5′-CTCACTAGTGGTTCTTTCAGG-3′; eNOS reverse, 5′-GGCCATGTCCATATCAGTCTGA-3′; eNOS forward, 5′-GGCGGAACTCCTGAAGGAGG-3′; eNOS reverse, 5′-GGCGGAACTCCTGAAGGAGG-3′; eNOS forward, 5′-GGCGGAACTCCTGAAGGAGG-3′; eNOS reverse, 5′-GGCGGAACTCCTGAAGGAGG-3′; eNOS forward, 5′-GGCGGAACTCCTGAAGGAGG-3′; eNOS reverse, 5′-GGCGGAACTCCTGAAGGAGG-3′.

For the transient transfection, a total of 1 μg plasmid DNA was transfected using FuGene6 (Roche). For cotransfection, all plasmids were added in an equal ratio. Total RNA was isolated 72 h after the transfection and used for the subsequent assays. For fluorescent detection, HeLa cells were transiently transfected, and fluorescent images were taken 24 h after transfection. To investigate the interaction of cGKI and MAPK/STAT signaling, ATDC5 cells were transfected with cGKI or the empty vector, and pEGFP-C1 was added 72 h after transfection. IB was then carried out using primary antibodies to p-Erk1/2, Erk1/2, p-p38MAPK, p38MAPK, p-JNK2/3, p-JNK1, p-STAT1, and STAT1 (Cell Signaling Technology) as described above.

Luciferase reporter gene assay. The human COL10 promoter regions from –4,459 bp relative to the transcriptional start site were cloned into the pGL3-Basic vector (Promega). The TOPFlash system (Upstate Biotechnology Inc.) was used according to the manufacturer’s protocol. The luciferase assay was performed with a dual–luciferase reporter assay system (Promega) using a GloMax 96 Microplate Luminometer (Promega).
at 4 °C overnight. Immunocomplexes were washed 3 times with cold wash solution. c-Myc-tagged proteins were eluted, and an equal amount of each eluted sample (15 μg) was subjected to SDS-PAGE, transferred onto nitrocellulose membranes, and subjected to IB using primary antibodies to cGKI (Santa Cruz Biotechnology Inc.), GSK-3β and Ser9-phosphorylated GSK-3β (Chemicon), and Myc tag (Upstate Biotechnology Inc.). Immunoreactive proteins were visualized as described above.

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