was noticeably stimulated with the addition of FGF2 (10ng/ml) and FGF18 (10ng/ml) in ATDC5 cells. Treatment of quiescent ATDC5 cells with CNP (10^{-7}-10^{-6}M) for one hour prior to the addition of FGF2 and FGF18 reduced the phosphorylation of ERK1/2 in a dose-dependent manner. 10^{-6}M CNP completely eliminated the FGF-stimulated phosphorylation of ERK1/2 (Fig.3 A, B), while 10^{-4}M 8-bromo cGMP also inhibited ERK1/2 phosphorylation of FGF2 (Fig.3 C).

No change in STAT-1 phosphorylation in ATDC5 cells by FGFs or CNP

STAT-1 phosphorylation was already detectable at the basal level in ATDC5 cells. The level of phosphorylated STAT-1 remained unchanged when treated with FGF2 or FGF18 for 3, 10, 15, 30, or 60 minutes. Treatment of quiescent ATDC5 cells with CNP (10^{-7}-10^{-6}M) prior to the addition of FGF2 (10ng/ml) and FGF18 (10ng/ml) did not alter the amount of phosphorylated STAT-1 (Fig.4 A, B), nor did CNP itself (10^{-6}M) affect the basal level of phosphorylated STAT-1 in ATDC5 cells (Fig.4 C), or treatment of quiescent ATDC5 cells with 10^{-4}M 8-bromo cGMP prior to the addition of FGF2 (10ng/ml) cause no change in the amount of phosphorylated STAT-1 (Fig.4 A).

No effect of CNP on FGFR-3 expression in ATDC5 cells

FGFR3 mRNA levels in ATDC5 cells treated with the vehicle and pretreated with CNP (10^{-6}M) were 1.00±0.06 and 1.04±0.12 in arbitrary units, respectively, and this difference was not significant.

Effect of CNP and FGF18 on mouse fetal tibia organ culture

The organ culture of fetal mouse tibias provides a unique *in vitro* experimental model system of endochondral ossification. For our study, we used cultured tibias prepared from ICR mice, to examine the effects of CNP and FGF18. Treatment with 10^{-7}M CNP for 5 days produced a 12% increase in the total length of tibial explants compared with vehicle treated explants (vehicle treated: 3.56±0.05mm vs. CNP treated: 3.99±0.07mm). On the other hand, treatment with 10ng/ml FGF18 for 5 days resulted in a 6% decrease in the total length of tibial explants compared with vehicle treated explants (vehicle treated: 3.57±0.03mm vs. FGF18 treated: 3.37±0.04mm). Treatment with a combination of 10^{-7}M CNP and 10ng/ml FGF18 increased the total length of tibial explants for 8% compared with vehicle treated explants (vehicle treated: 3.55±0.04mm vs. CNP and FGF18 treated: 3.84±0.06mm). These differences were all statistically significant ($p<0.01$) (Fig.5).

Effect of CNP and FGF18 on cell size and cell numbers of the growth plate chondrocytes

Microscopic examination disclosed elongation of the growth plate in mouse tibias cultured with 10^{-7}M CNP. Higher magnification of Alcian blue H&E staining (Fig.6 A,B,E,F) and immunohistochemical staining for type X collagen (Fig.6 I,J) showed that the hypertrophic chondrocyte layer increased after treatment with 10^{-7}M CNP compared with treatment with the vehicle. The mean size of hypertrophic chondrocytes was markedly increased (vehicle treated: 497.85±19.2 vs. CNP treated:1071.42±53.5μm^2) (Fig.7 A), and the number of cells in the hypertrophic chondrocyte layer was reduced (vehicle treated: 152.67±4.1 vs. CNP treated: 118±3.61 cells) (Fig.7 B). In contrast, culturing with 10ng/ml FGF18 caused shortening of the growth plate in cultured mouse tibias compared with that in vehicle treated ones. Higher magnification of Alcian blue H&E staining (Fig.6 A,C,E,G) and immunohistochemical staining for type X collagen (Fig.6 I,K) demonstrated that the hypertrophic chondrocyte layer was reduced. Not only the cell size (vehicle treated: 497.85±19.2 vs. FGF18 treated: 314.01±23.67μm^2) (Fig.7 A), but also the number of

hypertrophic chondrocytes was reduced by the treatment with 10ng/ml FGF18 (vehicle treated:152.67±4.1 vs. FGF18 treated:89.3±1.45 cells) (Fig.7 B). Culturing with both 10^{-7}M CNP and 10ng/ml FGF18 resulted in recovery by CNP of shortening of the growth plate in cultured mouse tibias by FGF18. Higher magnification of Alcian blue H&E staining (Fig.6 C,D,G,H) and immunohistochemical staining for type X collagen (Fig.6 K,L) showed an increase in the hypertrophic chondrocyte layer compared with that of the FGF18 treated ones. The mean size of hypertrophic chondrocytes also increased compared with that of FGF18 treated ones (FGF18 treated: 314.01±23.67 vs. CNP and FGF18 treated: 751.16±41.6μm^2) (Fig.7 A), and the reduction in number was also undone by CNP (FGF18 treated:89.3±1.45 vs. CNP and FGF18 treated:145.3±4.41 cells) (Fig.7 B).

DISCUSSION

The study reported here used two different experimental designs to examine the interaction of the CNP/GC-B and FGFR-3 pathways in mouse chondrogenic ATDC5 cells and in organ-cultured tibias. In this study we were able to show that: 1) ATDC5 cells express GC-B, 2) FGF2 and FGF18 reduce CNP-dependent cGMP production in a dose-dependent manner without changing the amount of GC-B, 3) MAPK inhibitors attenuate the FGF inhibition of CNP-dependent cGMP production, 4) both CNP and cGMP inhibit the MAPK pathway but not the STAT-1 pathway of FGFR-3 activation without changing the amount of FGFR-3, and 5) CNP and FGF18 counteract longitudinal bone growth in organ cultured tibias.

Substantial evidence exists that CNP is an antagonist of mitogenic action in many cell types. Activation of the CNP/GC-B pathway in the vascular smooth muscle cells was found to attenuate the onset of DNA synthesis, diminish cell proliferation, and inhibit chemotaxis (18). As for the interaction of CNP with FGF signaling, marked elevation of cGMP induced by CNP had been reported to block the activation of the MAPK cascade induced by FGFs in fibroblast (19). These indicate that significant

antagonistic interplay may also occur between the CNP/GC-B pathway and growth factor-regulated pathways in growth plate chondrocytes.

Constitutive activation of FGFR-3 has been reported to inhibit the proliferation and differentiation of the growth plate chondrocytes (20). Our study clarified that CNP can undo the reduction in the size and numbers of chondrocytes of the hypertrophic zone of the growth plate of mouse tibias expressing the type X collagen, and compensate for the shortening of the growth plate resulting from the treatment with FGF18. These results are consistent with the findings of previous *in situ* hybridization studies which have shown that the mouse *fgfr-3* gene is widely expressed in the proliferative and prehypertrophic chondrocyte zones of cartilage (20), thus overlapping the CNP/GC-B expression described in a previous study of ours (3). Therefore, the interaction between the CNP/GC-B and FGFR-3 pathways in these cells results the change of the hypertrophic chondrocytes during the endochondral ossification in the growth plate.

These findings, together with the fact that the specific MAPK inhibitors eliminated the FGF2-inhibition of CNP-dependent cGMP production in ATDC5 cells,

suggest that the CNP/GC-B pathway and MAPK pathway counteracts through FGFR-3 in the regulation of growth plate chondrocytes.

Alternatively, one can hypothesize that the MAPK pathway is not the only FGFR-3 signaling pathway in chondrocytes. It has been suggested that the inhibitory effects of FGFs on bone growth are mediated by the STAT-1 pathway of FGFR-3 signaling (21), while a highly controlled balance between the MAPK and STAT-1 pathways has been demonstrated recently in growth factor-stimulated cells. The observation that the *Stat-1* null mice with overexpressing FGF2 can overcome apoptosis and the reduction in chondrocyte proliferation gives support to the view that the Stat-1 pathway may also have a key function in growth retardation (22). In keeping with these findings, however, our observation of a significant ligand-independent STAT-1 phosphorylation in ATDC5 cells and no change in the level of STAT-1 phosphorylation as a result of stimulation by FGFs, CNP, or cGMP, leads us to conclude that the mechanism by which CNP undoes the shortening of bone length treated by FGFs consists of overcoming the MAPK-mediated pathway, not the STAT-1-mediated pathway.

Constitutive activation of ERK1 in chondrocytes reportedly induces a condition resembling achondroplasia (23). Many studies have shown that activating mutations in FGFR-3 inhibit bone growth in patients with achondroplasia and thanatophoric dysplasia (24, 25) while overexpression of FGF2 in mice slows longitudinal growth (26) and inactivating knockout mutations in FGFR-3 increase longitudinal long bone growth in mice (5). On the other hand, CNP null mice as well as GC-B null mice are characterized by short stature with a phenotype histologically similar to that of achondroplasia (27). Recently we have shown that achondroplasia model mice, expressing the constitutive active mutant form of FGFR-3 (G380R) in cartilage (5), recovered from abnormal growth plate development and dwarfing phenotype as a result of the overexpression of CNP in their cartilage (28), suggesting that interaction between the CNP/GC-B and FGFR-3 pathways also occurs *in vivo*. Although two pathways are antagonistic, the effect of the CNP/GC-B pathway can overcome the effect of FGFR-3 pathway, partly because the activation level of downstream signaling of FGFR-3 (G380R) mutant is relatively weak (26), compared to the overexpressed CNP. During the course of this study, mutations in the human

GC-B gene were reported to cause acromesomelic dysplasia, type Maroteaus, a type of skeletal dysplasia (29). Therefore, the CNP/GC-B pathway appears critical for the proper progression of endochondral ossification also in human bone.

The downstream of the interaction between CNP/GC-B/cGMP pathway and FGFR-3/MAPK pathway remains unexplored. We recently reported that cGMP-dependent kinase depleted mice (*Prkg2*$^{-/-}$) showed abnormal growth plate development and dwarfing phenotype, that these changes were not affected by the overexpression of CNP in cartilage (30) and that the growth plate chondrocyte differentiation was disorganized, which is different from what has been observed in CNP depleted mice.

GC-B is constitutively phosphorylated while receptor phosphorylation is absolutely essential for hormonal activation. On the other hand, the dephosphorylation of GC-B in response to hormone binding has been shown to correlate with the declining activity of these receptors in whole cells, suggesting that receptor dephosphorylation mediates the homologous desensitization of the receptor (31). As the expression level of GC-B was not changed by FGF18 stimulation in our

study, this dephosphorylation process may be involved in the desensitization of GC-B after FGFR-3 stimulation.

The clearance receptor of the natriuretic peptide reportedly mediates antimitogeic action of CNP in some but not all cell lines (32). To determine if the effects of CNP were mediated by the clearance receptor in the ATDC5 cells, C-ANF, a selective ligand for the clearance receptor, was tested on ATDC5 cells and did not inhibit basal or FGF-stimulated ERK1/2 phosphorylation (data not shown).

We have demonstrated that the CNP/GC-B pathway engages in negative cross talks with FGFR pathways, that FGFs reduce CNP-dependent intracellular cGMP production and that CNP and cGMP markedly diminish the FGF-induced phosphorylation of ERK1/2 in chondrocytes. We also showed that CNP does not affect the amount of phosphorylated STAT-1 in chondrocytes. The results of our study show that FGFs and the activity of MAPK play an important role in the growth of chondrocytes, and negatively interact with the CNP/GC-B pathway and explain one of the molecular mechanisms of the growth stimulating action of CNP, suggesting that activation of the CNP/GC-B pathway may be effective for the treatment of

achondroplasia.

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FIGURE LEGENDS

Fig.1 The effects of FGF2 on CNP dependent cGMP production in ATDC5 cells. **(A)**, Controls (open columns) show CNP stimulation of the intracellular cGMP production in a dose-dependent manner (10^{-9}-10^{-7}M CNP), and this increase was inhibited by pretreatment with 10ng/ml FGF2 (closed columns). Columns represent means ±SE (n=6, each), *p<0.01 vs. control **(B)**, MEK inhibitor, U0126 (dotted columns) resulted in recovery from the reduction of cGMP by 10ng/ml FGF2 (closed columns). Columns represent means ±SE (n=6, each), *p<0.01 vs. control (DMSO 0.0025%) (open columns), **p<0.01 vs. FGF2.

Inset, 20μM U0126 completely blocked the phosphorylation of ERK1/2 in ATDC5 cells. 1: control (DMSO 0.0025%), 2: treatment with 10ng/ml FGF2, 3: pretreatment with 20μM PD098059.

Fig.2 The effects of FGF18 on CNP dependent cGMP production in ATDC5 cells.

As in the case of FGF2, FGF18 inhibited the increase in CNP induced cGMP in a dose-dependent manner (0.3-100ng/ml). Columns represent means ±SE (n=6, each), *p<0.01 vs. control.

Inset, expression of GC-B in ATDC5 cells (Western blot analysis).

1: vehicle, 2: treatment with 1ng/ml FGF18, 3: treatment with 10ng/ml FGF18.

Fig.3 CNP and cGMP reduced FGF-stimulated elevation of phosphorylated ERK1/2. ATDC5 cell extracts were examined with Western blotting for phosphorylated ERK1/2 (pERK1/2) and total ERK1/2 (ERK1/2) as described under "Materials and Methods". **(A)**, CNP diminished FGF2-stimulated elevation of phosphorylated ERK1/2. 1: vehicle, 2, 3, 4: stimulation with 10ng/ml FGF2, 3: preincubation with 10^{-7}M CNP, 4: preincubation with 10^{-6}M CNP. **(B)**, CNP attenuated FGF18-stimulated elevation of phosphorylated ERK1/2. 1: vehicle, 2, 3, 4: stimulation with 10ng/ml FGF18, 3: preincubation with 10^{-7}M CNP, 4: preincubation with 10^{-6}M CNP. **(C)**, 8-bromo cGMP decreased FGF2-stimulated elevation of phosphorylated ERK1/2. 1: vehicle, 2, 3: stimulation with 10ng/ml FGF2, 3: preincubation with 10^{-4}M 8-Bromo cGMP. Representative blots are shown, and the relative levels of proteins were measured as phospho-ERK1/2 / ERK1/2 density values. Each experiment was repeated 3 times, and data from a representative experiment are shown.

Fig.4 Effects of CNP on the STAT-1 pathways of FGFs. ATDC5 cell extracts were

examined with Western blotting for phosphorylated STAT-1 (pSTAT-1) and total STAT-1 (STAT-1) as described under "Materials and Methods". **(A)**, CNP and 8-bromo cGMP did not alter the level of phosphorylated STAT-1 after FGF2 stimulation. 1: vehicle, 2, 3, 4, 5: stimulation with 10ng/ml FGF2, 3: preincubation with 10^{-7}M CNP, 4: preincubation with 10^{-6}M CNP, 5: preincubation with 10^{-4}M 8-bromo cGMP. **(B)**, CNP did not alter the level of phosphorylated STAT-1 after FGF18 stimulation. 1: vehicle, 2 3, 4: stimulation with 10ng/ml FGF18, 3: preincubation with 10^{-7}M CNP, 4: preincubation with 10^{-6}M CNP. **(C)**, CNP did not alter the basal expression of phosphorylated STAT-1. 1: vehicle, 2: 10^{-6}M CNP alone. Representative blots are shown, and the relative levels of proteins were measured as the phospho-STAT-1/STAT-1 density values. Each experiment was repeated 3 times, and the data from a representative experiment are shown.

Fig.5 Percent change in total length of fetal mouse tibias cultured with CNP and/or FGF18 for 5 days. Open columns show the percent change in total length of one tibia from mouse fetus treated with the vehicle. Hatched columns show the percent change in total length of the other tibia from the same mouse treated with 10^{-7}M CNP, 10ng/ml

FGF18, or with a combination of 10^{-7}M CNP and 10ng/ml FGF18. Columns represent means ±SE (n=5, each), *p<0.01 vs. vehicle.

Fig.6 Alcian blue and hematoxylin-eosin staining (upper and middle panels), and immunohistochemical staining for type X collagen (bottom panel) of cultured tibiae with CNP and/or FGF18. A, E, I: control; B, F, J: 10^{-7}M CNP; C, G, K: 10ng/ml FGF18; D, H, L: 10ng/ml FGF18 and 10^{-7}M CNP. Arrows indicate the hypertrophic chondrocyte zone. (A-D: magnification ×4, E-H: magnification ×20, I-L: magnification ×10).

Fig.7 (A), Effect of CNP and FGF18 on the size of hypertrophic chondrocytes (n=10). Columns represent means ±SE (n=10, each), *p<0.01 vs. vehicle, ** p<0.01 vs. FGF18.

(B), Effect of CNP and FGF18 on the numbers of hypertrophic chondrocytes (n=5). Columns represent means ±SE (n=5, each), *p<0.01 vs. vehicle, ** p<0.01 vs. FGF18.