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FGFR3 down-regulates PTH/PTHrP receptor gene expression by mediating JAK/STAT signaling in chondrocytic cell line

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Running Title: *Downregulation of PTH/PTHrP receptor by FGFR3*

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Abstract

The signaling axis comprised by the parathyroid hormone (PTH)-related peptide (PTHrP), the PTH/PTHrP receptor and the fibroblast growth factor receptor 3 (FGFR3) plays a central role in chondrocyte proliferation. The indian hedgehog (IHH) gene is normally expressed in early hypertrophic chondrocytes, and its negative feedback loop was shown to regulate PTH/PTHrP receptor signaling. In this study, we examined the regulation of PTH/PTHrP receptor gene expression in a FGFR3-transfected chondrocytic cell line, CFK2. Expression of IHH could not be verified on these cells, with consequent absence of hypertrophic differentiation. Also, expression of the PTH/PTHrP receptor (75% reduction of total mRNA) and the PTHrP (50% reduction) genes was reduced in CFK2 cells transfected with FGFR3 cDNA. Interestingly, we verified significant reduction in cell growth and increased apoptosis in the transfected cells. STAT1 was detected in the nuclei of the CFK2 cells transfected with FGFR3 cDNA, indicating predominance of the JAK/STAT signaling pathway. The reduction in PTH/PTHrP receptor gene in CFK2 cells over-expressing FGFR3 was partially blocked by treatment with an inhibitor of JAK3 (WHI-P131), but not with an inhibitor of MAPK (SB203580) or JAK2 (AG490). Altogether, these findings suggest that FGFR3 down-regulates PTH/PTHrP receptor gene expression via the JAK/STAT signaling in chondrocytic cells.

(201 words)

Key words: *FGFR3, PTH/PTHrP receptor, chondrocyte, PTHrP, cell proliferation*

Introduction

During embryonic growth, elongation of epiphyseal cartilage is achieved by both apposition by chondrocyte precursors and interstitial growth performed by chondrocytes in the resting and proliferative zones, which simultaneously proliferate and synthesize cartilage matrix. The length of the resting and proliferative zones of the epiphyseal cartilage is, therefore, determined by the speed of matrix synthesis and proliferation of these chondrocytes, as well as by the speed at which these cells enter their hypertrophic stage.

Parathyroid hormone-related peptide (PTHrP) was originally discovered in tumors that induced the common syndrome of malignancy-associated hypercalcemia (8, 15, 20, 30, 34, 36, 42). The N-terminal amino acids of PTHrP share sequence homology with the parathyroid hormone (PTH), which allows both peptides to share a common G protein-coupled receptor, the PTH/PTHrP receptor. Unlike the role played by PTH in maintaining systemic calcium and phosphate homeostasis, PTHrP regulates cell proliferation and differentiation primarily at the local level. PTHrP and PTH/PTHrP receptor are expressed in chondrocytes throughout the epiphyseal cartilage, and in osteoblasts from the metaphyseal bone (3). Targeted disruption of PTHrP or PTH/PTHrP receptor in mice resulted in lethal chondrodysplasia, a condition characterized by reduced proliferation and premature hypertrophic differentiation of chondrocytes (2, 18, 21). These studies suggested that PTH/PTHrP receptor signaling stimulates proliferation of chondrocytes and inhibits their passage to a hypertrophic phenotype in developing cartilage.

Fibroblast growth factor receptor 3 (FGFR3) is another critical mediator of chondrocyte function. Ligand-induced activation of this receptor provokes dimerization, autophosphorylation and activation of intracellular signaling through transduction pathways linked to ras-raf-MEKK, phospholipase-C γ (PLC γ) and signal transducer and activator of transcription (STAT) (24, 31). In contrast to PTHrP, FGFR3 has been shown to be a negative regulator of chondrocyte proliferation. Constitutive activation of FGFR3 is caused by point mutations throughout the receptor, and gives rise to chondrodysplastic disorders such as achondroplasia, hypochondroplasia and thanatophoric

dysplasias (25). Targeted expression of FGFR3 carrying the mutations found in human achondroplasia and thanatophoric induced similar abnormalities in the epiphyseal cartilage of newborn mice (9, 16, 17, 22, 26, 28, 40).

Thus, PTHrP and FGFR3 have been shown to play critical roles in cartilage and bone development, and may as well interplay during the processes of bone and cartilage development and growth. Chen *et al.* reported that FGFR3 inhibited PTH/PTHrP receptor expression at the transitional zone that marks the entry point of chondrocytes into a hypertrophic phenotype (10). The molecular regulation was shown to be mediated through indian hedgehog (IHH), and supported the idea of the existence of a negative feedback on PTH/PTHrP receptor signaling by IHH, a gene that is normally expressed in the hypertrophic zone *in vivo*.

For *in vitro* experiments for chondrocyte function, the rat chondrocytic cell line CFK2, established by Bernier *et al.*, is well characterised and broadly used since these cells express PTHrP, PTH/PTHrP receptor and type II collagen (1), and their proliferation was promoted by PTH/PTHrP and epidermal growth factor, but inhibited by dexamethasone and retinoic acid (7). This immortalized cell line, therefore, has properties similar to those of primary proliferating chondrocytes and provides a suitable model for examining the interactions between FGFR3 and PTH/PTHrP signaling.

By employing the CFK2 cell line, in this study, we have examined whether there would be an alternative pathway through which FGFR3 inhibits PTH/PTHrP receptor expression without the mediation of IHH.

Materials and Methods

Animals and CFK2 cell culture and transfection of cDNAs encoding PTH/PTHrP receptor and FGFR3

A rat chondrocytic cell line, CFK2, which has been previously proven to synthesize type II collagen, PTHrP, PTH/PTHrP receptor (1, 7), was cultured in α minimal essential medium (α MEM, Flow Laboratories, Irvine, Scotland) containing 10% fetal calf serum (FCS) until a confluence of 70% before the transfection. Under Niigata University's guidelines for animal experimentation, the tibiae of newborn Wistar rats were extracted and dissected under a binocular microscope in order to obtain the tissue correspondent to the proximal epiphyseal cartilage. The dissected cartilage pieces were immediately frozen in liquid nitrogen for subsequent RT-PCR. CFK2 cells were cultured at approximately 70% confluence in α MEM. As previously described by Amizuka *et al.* (5), these cells were transfected with pSG5 containing rat PTH/PTHrP receptor cDNA, with pcDNA3 carrying full length mouse FGFR3 or with empty vectors (control), using Lipofectamine plus (Invitrogen BV, Groningen, The Netherlands) for 3 hrs without FCS, and then cultured in α MEM containing FCS for 24 hrs prior to paraformaldehyde-fixation, or RNA extraction for RT-PCR and real-time PCR.

Administration of inhibitors for MAPK, JAK2 and JAK3

According to Nielsen *et al.* (27) and Sudbeck *et al.* (33), the transfected CFK2 cells with cDNA encoding FGFR3 were treated with 1, 10 and 100 μ M of α -Cyano-(3,4-dihydroxy)-N-benzylcinnamide (AG490), or with 4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline (WHI-P131), which are selective inhibitors of *Janus* tyrosine kinase 2 (JAK2) and JAK3 signaling, respectively. While WHI-P131 inhibits JAK3, it does not inhibit signaling by JAK1, JAK2, ZAP/SYK family tyrosine kinase BTK, the SRC family tyrosine kinase LYN, and the receptor family tyrosine kinase IRK (33). Additionally, CFK2 cells transfected with FGFR3 cDNA were treated with 1, 10 and 100 μ M of 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)

1H-imidazole, SB203580, an inhibitor of MAPK. Treated and untreated CFK2 cells were subjected to RNA extraction as described below.

Immunolocalization of FGFR3 and STAT1 in transfected CFK2 cells

CFK2 cells transfected with FGFR3 cDNA were fixed with a mixture of acetone and methanol for 10 min. The specimens were pretreated with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS, pH 7.4) for 30 min, and then incubated with goat polyclonal antibody to FGFR3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a dilution of 1:100 with 1% BSA -PBS for 24 hrs at 4 °C. The specimens were rinsed with PBS for 6 hrs, and subsequently incubated with rabbit FITC-conjugated anti-goat F(ab)₂ (Kirkegaard & Perry laboratories Inc. Gaithersburg, MD). After that, the specimens were incubated with mouse monoclonal anti-STAT1 antibody (BD transduction laboratories, Los Angeles, LA) at a dilution of 1:80 with 1% BSA-PBS for 24 hrs at 4 °C, followed by incubation with RITC-conjugated anti-mouse F(ab)₂ (Kirkegaard & Perry laboratories). Nuclear counterstaining was performed with DAPI. As a control experiment for immunostaining, the sections were incubated with non-immune normal goat serum or normal mouse serum (Jackson ImmunoResearch Laboratories, Inc., PA) instead of the immune primary antibodies, and then, were processed as mentioned above. The immunofluorescent reaction of these specimens was observed under a confocal laser microscope (LSM 510; Carl Zeiss, Oberkochen, Germany).

Staining for TUNEL on transfected CFK2 cells

In vitro assessment of cells undergoing apoptosis was performed as described previously, using the ApopTag method (Oncor, Gaithersberg, MD) (6). The total number of positively stained cells was expressed as a percentage of the total number of cells counted per field, and the results expressed as mean \pm SD. Significant differences between control and transfected cells were determined using the unpaired Student's t test.

Statistical analysis for the cell growth of transfected CFK2 cells

CFK2 cells transfected with pcDNA 3 (Invitrogen) carrying FGFR3 cDNA and with the empty vector were counted for statistical analysis on their proliferative profile. After the transfection, these cells were cultured in the same culture medium containing 10% FCS, and their numbers/ dish (ϕ 3.5cm) were counted everyday for 5 days. Statistical analysis was carried out by unpaired Student's *t-test*.

Reverse transcription-polymerase chain reaction (RT-PCR) amplification of PTHrP, IHH, FGFR3, PTH/PTHrP receptor and GAPDH transcripts.

Total RNAs from the proximal epiphyseal cartilage of newborn rats and from transfected CFK2 cells were extracted with Trizol reagent (Invitrogen). Five micrograms of total RNA was subjected to reverse transcription using Superscript II (Invitrogen). PCR reactions (50 μ l) contained 5 μ l of 10X reaction buffer, 1 μ l of 25pmol of each primer, 1 μ l of 10mM of dNTPs mixture, 1U of Taq polymerase, 3 μ l of 25mM MgCl₂ and 2 μ l of RT-DNA and sterile distilled water. Amplification was performed with denaturation of 94 °C for 1 min, annealing at 55°C for 1 min, and extension at 72 °C for 1 min using GeneAmp PCR System 9700 (Applied Biosystem Japan Ltd). Primer constructs for IHH, GAPDH, FGFR3, PTHrP and PTH/PTHrP receptor are presented in **Table 1**.

Real-time PCR for expression of PTH/PTHrP receptor, PTHrP and GAPDH

Total RNA was extracted from CFK2 cells transfected with cDNA encoding FGFR3 or empty vector by Trizol reagent as described above. Five micrograms of total RNA, in the presence of oligo (dT)₁₂₋₁₈ primers, were reverse-transcribed by Superscript II (Invitrogen). Gene expression levels of PTHrP and PTH/PTHrP receptor were comparatively measured by real-time quantitative polymerase chain reaction. Series of cDNA concentrations (1:10:100:1000) of a representative cDNA were used for linear regression analyses of PTH/PTHrP receptor genes. After denaturing for 30 seconds at 95°C, amplification was performed for 45 cycles by denaturation for 5 seconds at 95°C and extension for 30 seconds at 66°C (Smart Cycler, Cepheid, Sunnyvale, CA), using Takara

Ex TaqTM R-PCR Version (Takara, Shiga, Japan). Real time monitoring of the PCR products was done by fluorescence with SYBR Green I (Takara). The gene expression levels of PTH/PTHrP receptor genes were represented as a ratio to that of GAPDH levels from the same master reaction. The PCR primer pairs of PTHrP were same as regular PCR, and GAPDH and the PTH/PTHrP receptor were as follows: GAPDH, 5'-CATCACTGCCACTCAGAAGACTG-3' (sense) and 5'-ACATTGGGGGTAGGAACACG-3' (antisense); PTH/PTHrP receptor, 5'-TTCCCAAGGACGATGGATTC-3' (sense) and 5'-GATAGACAACCAGCCAAACACTG-3' (antisense). For statistical evaluation of PTH/PTHrP receptor expression after treatment with JAK inhibitors, CFK2 cells transfected with FGFR3 cDNA were treated with 1, 10 and 100 μ M of JAK2 inhibitor AG490, JAK3 inhibitor WHI-P131 and MAPK inhibitor, SB203580 (n=6, for each) prior to culture for 24 hrs. Samples were subjected to real-time PCR, and subsequently, the gene expression levels of the PTH/PTHrP receptor genes were represented as a ratio to that of GAPDH levels. The ratio of PTH/PTHrP receptor to GAPDH (mean \pm SD) was analyzed by Fischer's test.

Results

Downregulation of PTH/PTHrP receptor and PTHrP in CFK 2 cells transfected with FGFR3 cDNA

While the tibial epiphyseal cartilage of newborn rats expressed IHH, cultured CFK2 cells did not express IHH, verifying the absence of an IHH negative feedback loop on PTHrP signaling (**Fig. 1A**). CFK2 cells were then transfected either with cDNA encoding PTH/PTHrP receptor or FGFR3. RT-PCR showed marked reduction in the expression of PTH/PTHrP receptor in CFK2 cells transfected with FGFR3 cDNA, while transfection with PTH/PTHrP receptor cDNA did not affect the expression level of FGFR3 in the CFK2 cells (**Fig. 1B**). In addition, lower levels of PTHrP gene were expressed by CFK2 cells transfected with FGFR3 cDNA (**Fig. 1C**). In order to quantify the reduction of PTH/PTHrP receptor and PTHrP gene by FGFR3 over-expression, we performed real-time PCR (**Fig. 1D**). After standardization with GAPDH expression level, the expression level of PTH/PTHrP receptor seen in CFK2 cells transfected with FGFR3 was one-fourth of that observed in control CFK2 cells (transfected with empty vector). In contrast, PTHrP expression levels in FGFR3-transfected cells were reduced to only half of that seen in the control CFK2 cells. Real-time PCR therefore confirmed the substantial reduction in PTH/PTHrP receptor and PTHrP expression in FGFR3-overexpressing CFK2 cells.

Reduced proliferation and increased apoptosis of CFK2 cells transfected with FGFR3 cDNA

The decrease in PTHrP and PTH/PTHrP receptor expression was accompanied by a significant decrease in the proliferative profile of the transfected cells compared with the control, as evidenced by decreased cell numbers at day 4 after the transfection (29.25 ± 5.34 vs 63.50 ± 15.96 , $p < 0.05$; **Fig. 2A**). In addition to decreased proliferative capacity, many transfected CFK2 cells showed TUNEL-positivity compared with the control (**Fig. 2B, C**). Statistical analysis demonstrated significant increase of TUNEL-positive cells when transfected with FGFR3 gene after day 3 (**Fig. 2D**).

Reduction of PTH/PTHrP receptor gene is mediated by JAK/STAT pathway in CFK 2 cells

Since FGFR3 activation is linked to JAK/STATs and leads to autophosphorylation and translocation of STAT into the nucleus, we have performed a double detection of FGFR3 and STAT1, one of major STATs, in control and FGFR3-expressing CFK2 cells (**Fig. 3**). As expected, FGFR3-expressing CFK2 cells showed intranuclear localization of STAT1, while control CFK2 cells failed to show STAT1, consistent with the idea that nuclear translocation of STAT1 is involved in FGFR3 signal transduction. No immunopositivity was seen for control experiments using non-immune sera (data not shown).

We further examined whether the activation of JAKs linked to FGFR3 was related to the suppression of PTH/PTHrP receptor expression seen in transfected CFK2 cells. CFK2 cells overexpressing FGFR3 were treated with JAK2 inhibitor AG490, JAK3 inhibitor WHI-P131 or MAPK inhibitor, SB203580. Inhibition of PTH/PTHrP receptor expression was seen in an inverse dose-dependent manner of WHI-P131 (**Fig. 4A**), but not with AG490 or the MAPK inhibitor, SB203580 (data not shown). Depending on the concentration of WHI-P131, there was a recovery of the expression levels of PTH/PTHrP receptor (**Fig. 4B**). The ratio of the PTH/PTHrP receptor to GAPDH without inhibitor was 0.09 ± 0.02 , whereas it significantly increased to 0.16 ± 0.02 ($p < 0.05$) at $10 \mu\text{M}$ and 0.43 ± 0.10 ($p < 0.05$) at $100 \mu\text{M}$ of WHI-P131 ($n=6$).

Discussion

Our study indicates that proliferating chondrocytes that do not express IHH could also be affected by FGFR3, possibly through PTH/PTHrP receptor signaling. It seems likely that 1) inhibition of expression of PTH/PTHrP receptor in a chondrocytic cell may, in part, lower such cell's proliferative capability, and 2) signaling linkage to JAK/STATs may be a candidate pathway leading to inhibition of the PTH/PTHrP receptor. Using the chondrocytic cell line CFK2, FGFR3 overexpression was shown to reduce PTHrP gene expression by almost 50% and that of the PTH/PTHrP receptor by more than 75%. Our findings are not only consistent with the report by Yamanaka *et al.* that showed diminished expression of PTHrP when there are FGFR3 mutations (41), but also provide evidence of an association between FGFR3 overexpression and suppression of PTH/PTHrP receptor.

In the developing cartilage, PTH/PTHrP receptor is expressed in both the proliferative and the upper region of hypertrophic zones (4), while FGFR3 is found in the resting and proliferating zones (26). Although PTHrP regulates the entry into the chondrocytic hypertrophic phase via a negative feedback loop that involves IHH (39), PTHrP and IHH have been reported to regulate chondrocyte proliferation independently (19, 29). Alternatively, signaling from FGFR3 inhibits chondrocyte proliferation: Ornitz and colleagues demonstrated that FGFR3 inhibits chondrocyte replication in the resting and proliferative zones (26) and Chen *et al.* reported that FGFR3 down-regulates IHH expression, inhibiting chondrocyte proliferation in a process independent of the signaling through the PTH/PTHrP receptor (10). However, our study indicates the interplay between FGFR3 and PTH/PTHrP receptor signaling in an *in vitro* system that does not include hypertrophic chondrocytes. Therefore, CFK2 chondrocytic cell line without expressing IHH, when compared to primary cultured chondrocytes that possess various pathways of FGFR3 signaling for chondrocyte proliferation, appears to be the useful cell source in order to clarify the interplay between FGFR3 and PTH/PTHrP receptor signaling. Using this cell line, we postulate that there are multiple pathways through which FGFR3 affects chondrocyte proliferation and

differentiation, with or without mediation by IHH. We verified the inhibition of PTH/PTHrP receptor expression as a consequence of the JAK/STATs signaling link to FGFR3, but it seems necessary to examine whether other pathways that are associated with FGFR3, *e.g.*, ras-raf-MEKK-PLC γ are also involved in such inhibition.

Overexpression of FGFR3 through transfection in CFK2 cells also resulted in decreased cell numbers after 4 days (See **Fig. 2**). Previous work has demonstrated that PTHrP stimulates CFK2 proliferation by a PTH/PTHrP-mediated mechanism (7, 12, 13) and that expression of a constitutively active FGFR3 inhibited these cells' proliferation via an integrin-mediated pathway (14). It is interesting that not only retarded proliferation, but also TUNEL-positive apoptosis were increased after FGFR3 cDNA transfection into CFK2 cells. Our previous report demonstrated increased apoptosis of chondrocytes in PTHrP deficiency (2, 6), and this study showed reduced expression of PTHrP by means of FGFR3-overexpression. Taken together, it seems likely that PTHrP is a negative regulator of apoptosis and that the reduced expression of PTHrP by FGFR3 overexpression may increase chondrocytic apoptosis.

STAT1 was translocated into the nucleus of FGFR3-transfected CFK2 cells, but not in cells transfected with an empty vector. Furthermore, down-regulation of PTH/PTHrP receptor expression in these cells was partially recovered by treatment with the JAK3 inhibitor WHI-P131, but not with JAK2 and MAPK inhibitors. Although JAK3 may be one of main components of FGFR3-driven signal transduction, other pathways may exist since WHI-P131 did not completely recover the inhibitory effect on the PTH/PTHrP receptor expression. In addition, while we examined localization of STAT1, it is possible that other kinds of STATs take part in FGFR3 function. It seems difficult to identify which STAT is the predominant transducer linked to FGFR3, since the congenital absence of STAT1 in mice did not cause obvious abnormalities on skeletal tissues, suggesting a degree of redundancy in this signaling pathway (23). The embryonic lethality of the STAT3-deficient mouse precludes further study until a mouse with conditional inactivation of the gene is created (35). Thus, the molecular interactions related to STATs-driven signal transduction in skeletal development appear complex and deserve further studies. However, our

findings demonstrated, at least in part, that the JAK/STAT signaling linked to FGFR3 inhibits the expression of the PTH/PTHrP receptor in CFK2 cells.

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References

1. Aarts M, Davidson D, Corluka A, Petroulakis E, Guo J, Bringhurst F, Galipeau J and Henderson JE (2001) PTHrP promotes quiescence and survival of serum deprived chondrocytes by inhibiting rRNA synthesis. *J Biol Chem* 276: 37934-37943.
2. Amizuka N, Warshawsky H, Henderson JE, Goltzman D and Karaplis AC (1994) Parathyroid hormone-related peptide (PTHrP)-depleted mice show abnormal epiphyseal cartilage development and altered endochondral bone formation. *J Cell Biol* 126: 1611-1623.
3. Amizuka N, Karaplis AC, Henderson JE, Warshawsky H, Lipman ML, Matsuki Y, Ejiri S, Tanaka M, Izumi N and Ozawa H (1995) Haploinsufficiency of parathyroid hormone-related peptide (PTHrP) results in abnormal post-natal bone development. *Dev Biol* 175: 166-176.
4. Amizuka N, Henderson JE, Hoshi K, Warshawsky H, Ozawa H, Goltzman D and Karaplis AC (1996) Programmed cell death of chondrocytes and aberrant chondrogenesis in mice homozygous for parathyroid hormone-related peptide gene deletion. *Endocrinology* 137: 5055-5067.
5. Amizuka N, Fukushi-Irie M, Sasaki T, Oda K and Ozawa H (2000) Inefficient function of signal sequence of parathyroid hormone-related peptide (PTHrP) for targeting to the secretory pathway. *Biochem Biophys Res Commun* 273: 621-629.
6. Amizuka N, Davidson D, Liu H, Valverde-Franco G, Chai S, Maeda T, Ozawa H, Hammond V, Ornitz DM, Goltzman D and Henderson JE (2004) Signaling by fibroblast growth factor receptor 3 and parathyroid hormone-related peptide coordinate cartilage and bone development. *Bone* 34: 13-25.

7. Bernier S and Goltzman D (1993) Regulation of expression of the chondrocytic phenotype in a skeletal cell line (CFK2) in vitro. *J Bone Miner Res* 8: 475-484.
8. Burtis WJ, Wu T, Bunch C, Wysolmerski JJ, Isogna KL, Weir EC, Broadus AE and Stewart AF (1987) Identification of a novel 17,000-dalton parathyroid hormone-like adenylate cyclase-stimulating protein from a tumor associated with humoral hypercalcemia of malignancy. *J Biol Chem* 161: 7151-7156.
9. Chen L, Adar R, Yang X, Monsenago E, Li C, Hauschka P, Yayon A and Deng CX (1999) Gly369Cys mutation in mouse FGFR3 causes achondroplasia by affecting chondrogenesis and osteogenesis. *J Clin Invest* 104: 1517-1525.
10. Chen L, Li C, Qiao W, Xu X and Deng C (2001) A Ser 365→Cys mutation of fibroblast growth factor receptor 3 in mouse downregulates Ihh/PTHrP signals and causes severe achondroplasia. *Hum Mol Genet* 10: 457-465.
11. Hebrok M, Kim S, St-Jacques B, McMahon A and Melton D (2000) Regulation of pancreas development by hedgehog signaling. *Development* 127: 4905-4913.
12. Henderson JE, Amizuka N, Warshawsky H, Biasotto D, Lanske BMK, Goltzman D and Karaplis AC (1995) Nucleolar targeting of PTHrP enhances survival of chondrocytes under conditions that promote cell death by apoptosis. *Mol Cell Biol* 15: 4064-4075.
13. Henderson JE, He B, Goltzman D and Karaplis AC (1996) Constitutive expression of parathyroid hormone-related peptide (PTHrP) stimulates growth and inhibits differentiation of CFK2 chondrocytes. *J Cell Physiol* 169:33-41.

14. Henderson JE, Naski MC, Aarts MM, Wang D, Cheng L and Ornitz DM (2000) Expression of FGFR3 with the G380R achondroplasia mutation inhibits proliferation and maturation of CFK2 chondrocytic cells. *J Bone Miner Res* 15: 155-165.
15. Horiuchi N, Caulfield MP, Fisher JE, Goldman ME, McKee GR, Reagan JE, Levy JJ, Nutt RF, Rodan SB, Clemens TL and Rosenblatt M (1987) Similarity of synthetic peptide from human tumor to parathyroid hormone in vivo and in vitro. *Science* 238: 1566-1568.
16. Iwata T, Chen L, Li CL, Ovchinnikov D, Behringer R, Francomani C and Deng CX (2000) A neonatal lethal mutation in FGFR3 uncouples proliferation and differentiation of growth plate chondrocytes in embryos. *Hum Mol Genet* 9: 1603-1613.
17. Iwata T, Li CL, Deng CX and Francomano C (2001) Highly activated FGFR3 with the K644M mutation causes prolonged survival in severe dwarf mice. *Hum Mol Genet* 10: 1255-1264.
18. Karaplis AC, Luz A, Glowacki J, Bronson RT, Tybulewicz VLJ, Kronenberg HM and Mulligan RC (1994) Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide (PTHrP) gene. *Genes Dev* 8: 277-289.
19. Karp SJ, Schipani E, St-Jacques B, Hunzelman J, Kronenberg H and McMahon AP (2000) Indian hedgehog coordinates endochondral bone growth and morphogenesis via parathyroid hormone related protein dependent and independent pathways. *Development* 127: 543-548.
20. Kemp BE, Moseley JM, Rodda CP, Ebeling PR, Wettenhall REH, Stapleton D, Diefenbach-Jagger N, Ure F, Michlangeli VP, Simmons HA, Raisz LG and Martin TJ (1987) Parathyroid hormone-related protein of malignancy: active synthetic fragments. *Science* 238: 1568-1570.

21. Lanske B, Karaplis AC, Lee K, Luz A, Vortkamp A, Pirro A, Karperien M, Defize LHK, Ho C and Mulligan RC (1996) PTH/PTHrP receptor in early development and indian hedgehog-regulated bone growth. *Science* 273: 663-666.
22. Li C, Chen L, Iwata T, Kitagawa M, Fu XY and Deng CX (1999) A Lys644Arg substitution in fibroblast growth factor receptor 3 (FGFR3) causes dwarfism in mice by activation of STATs and Ink4 cell cycle inhibitors. *Hum Mol Genet* 8: 35-44.
23. Meraz MA, White JM, Sheehan KCF, Bach EA, Rodig SJ, Dighe AS, Kaplan DH, Riley JK, Greeblund AC, Campbell D, Carver-Moore K, DuBois RN, Clark R, Aguet M and Schreiber RD (1996) Targeted disruption of the stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. *Cell* 84: 431-442.
24. Mohammadi M, Dionne CA, Li W, Li N, Spivak T, Honegger AM, Jaye M and Schlessinger J (1992) Point mutation in FGF receptor eliminates phosphatidylinositol hydrolysis without affecting mitogenesis. *Nature* 358: 681-684.
25. Muenke M and Schell U (1995) Fibroblast-growth-factor receptor mutations in human skeletal disorders. *Trends in Genetics* 11: 308-313.
26. Naski MC, Colvin J, Coffin J and Ornitz DM (1998) Repression of hedgehog signaling and BMP4 expression in growth plate cartilage by fibroblast growth factor receptor 3. *Development* 125: 4977-4988.
27. Nielsen M, Kaltoft K, Nordahl M, Ropke C, Mustelin T, Dobson P, Svejgaard A and Odum N (1997) Constitutive activation of a slowly migrating isoform of Stat3 in mycosis fungoides:

tyrphostin AG490 inhibits Stat3 activation and growth of mycosis fungoides tumor cell lines. *Proc Natl Acad Sci USA* 94: 6764-6769.

28. Segev G, Chumakov I, Nevo Z, Givol D, Madar-Shapiro L, Sheinin Y, Weinreb M and Yayon A (2000) Restrained chondrocyte proliferation and maturation with abnormal growth plate vascularization in human FGFR3G380R transgenic mice. *Hum Mol Genet* 9: 249-258.
29. St-Jacques B, Hammerschmidt M and MacMahon A (1999) Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. *Genes Dev* 13: 2072-2086.
30. Strewler G, Stern JPH, Jacob JW, Eveloff J, Klein RF, Leung SC, Rosenblatt M and Nissenson RA (1987) Parathyroid hormonelike protein from human renal carcinoma cells: structural and functional homology with parathyroid hormone. *J Clin Invest* 80: 1803-1807.
31. Su WCS, Kitagawa M, Xue N, Xie B, Garofalo S, Cho J, Deng C, Horton WA and Fu XY (1997) Activation of STAT-1 by mutant fibroblast growth factor receptor in thanatophoric dysplasia type II dwarfism. *Nature* 386: 288-292.
32. Suda N, Gillespie MT, Traianedes K, Zhou H, Ho PWM, Hards DK, Allan EH, Martin TJ and Moseley JM (1996) Expression of parathyroid hormone-related protein in cells of osteoblast lineage. *J Cell Physiol* 166: 94-104.
33. Sudbeck EA, Liu XP, Narla RK, Mahajan S, Ghosh S, Mao C and Uckun FM (1999) Structure-based design of specific inhibitors of Janus kinase 3 as apoptosis-inducing antileukemic agents. *Clinical Cancer Res* 5: 1569-1582.

34. Suva LJ, Winslow GA, Wettenhall REH, Hammond RG, Moseley JM, Diefenbach-Jaggard H, Rodda CP, Kemp BE, Rodriguez H, Chen EY, Hudson PJ, Martin TJ and Wood WI (1987) A parathyroid hormone-related protein implicated malignant hypercalcemia: cloning and expression. *Science* 237: 893-896.
35. Takeda K, Noguchi L, Shi W, Tanaka T, Matsumoto M, Yoshida N, Kishimoto T and Akira S (1997) Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. *Proc Natl Acad Sci USA* 94: 3801-3804.
36. Thompson DD, Seedor JG, Fisher JE, Rosenblatt M and Rodan GA (1988) Direct action of the parathyroid hormone-like human hypercalcemic factor on bone. *Proc Natl Acad Sci USA* 85: 5673-5677.
37. Tso JY, Sun XH, Kao T, Reece KS and Wu R (1985) Isolation and characterization of rat and human glyceraldehydes-3-phosphate dehydrogenase cDNA: Genomic complexity and molecular evolution of the gene. *Nucleic Acid Res* 13: 2485-2502.
38. Van der Eerden BCJ, Karperien M, Gevers EF, Löwik C and Wit JM (2000) Expression of indian hedgehog, parathyroid hormone-related protein, and their receptors in the postnatal growth plate of the rat: evidence for a locally acting growth restraining feedback loop after birth. *J Bone Miner Res* 15: 1045-1055.
39. Vortkamp A, Lee K, Lanske B, Segre GV, Kronenberg HM and Tabin CJ (1996) Regulation of rate of cartilage differentiation by indian hedgehog and PTH-related protein. *Science* 273: 613-622.

40. Wang Y, Spatz M, Kannan K, Hayk H, Avivi A, Gorivodsky M, Pines M, Yayon A, Lonai P and Givol D (1999) A mouse model for achondroplasia produced by targeting fibroblast growth factor receptor 3. *Proc Natl Acad Sci USA* 96: 4455-4460.
41. Yamanaka Y, Tanaka H, Koike M, Nishimura R and Seino Y (1999) Mutations of fibroblast growth factor receptor 3 (FGFR3) that causes short-limb dwarfism induced apoptosis of chondrocytes by down-regulating PTHrP expression: Overexpression of PTHrP rescued apoptosis. *J Bone Miner Res* 14 suppl: S175.
42. Yasuda T, Banville D, Rabbani SA, Hendy GN and Goltzman D (1989) Rat parathyroid hormone-like peptide: comparison with the human homologue and expression in malignant and normal tissue. *Mol Endocrinol* 3: 518-525.

Figure legends

Fig. 1

Down-regulation of PTH/PTHrP receptor and PTHrP expression in CFK2 cells transfected with FGFR3 cDNA

A: RT-PCR reveals the expression of IHH using two different sets of primers (IHH-1, IHH-2; please refer to Table 1) in the tibial epiphyseal cartilage of newborn rats, while expression of IHH is not verifiable in the cultured CFK2 cells.

B: Left and right panels show the levels of expression for FGFR3 (left) and for PTH/PTHrP receptor (right) after transfection with FGFR3, PTH-R (PTH/PTHrP receptor) or control (empty vector). The expression of PTH/PTHrP receptor was markedly-reduced in the FGFR3 cDNA-transfected cells (right panel). In contrast, the transfection with PTH/PTHrP receptor cDNA does not show any alteration on the expression of FGFR3 in CFK2 cells (left panel).

C: The expression of PTHrP in CFK2 cells transfected with FGFR3 cDNA. PCR product representative for PTHrP is weakly seen in the FGFR3 cDNA-transfected CFK 2 cells compared with the control CFK2 cells. GAPDH expression is similar between groups.

D: Relative quantification of PTH/PTHrP receptor and PTHrP expressions in transfected CFK2 cells. Quantitative analysis on the reduction of PTH/PTHrP receptor and PTHrP is evaluated as the ratio to GAPDH in FGFR3-transfected CFK 2 cells. There is roughly a 75% reduction of the PTH/PTHrP receptor expression ratio compared to the one verified for control CFK2 cells. The expression of PTHrP is diminished to half of that found in control cells.

Fig. 2

Growth curve and apoptosis of CFK2 cells transfected with vectors carrying FGFR3 cDNA

A: FGFR3-transfected (blue line) or control (red dotted line) CFK2 cells were cultured for 5 days with cell number/ dish (ϕ 3.5cm) being counted everyday. Significant differences were detected between the number of the control and transfected CFK2 cells on day 4 and day 5. * $P < 0.05$, ** $P < 0.005$

B, C: TUNEL detection (black dots) on CFK2 cells after transfection. Many CFK2 cells show TUNEL-positivity when transfected with cDNA encoding FGFR3 (**C**) compared with the control cells (**B**).

D: Statistical analysis on the percentage of TUNEL-positive cells on CFK2 cells with vector carrying FGFR3 cDNA (dotted bar) or with empty vector (black bar). Significant differences were detected between the control and transfected CFK2 cells after day 3.

Fig. 3

Immunolocalization of FGFR3 and STAT 1 in CFK2 cells transfected with FGFR3 cDNA

A: Single immunodetection for FGFR3 (green) in the transfected cells followed by counterstaining with DAPI (blue).

B: Single detection for STAT1 (red) in the transfected cells followed by counterstaining with DAPI (blue).

C: Merged image of FGFR3 (green) and STAT1 (red) without DAPI staining.

D: Merged image of FGFR3 (green) and STAT1 (red) with DAPI staining.

In the cell transfected with FGFR3 cDNA (a white arrow), an intense STAT1-immunoreactivity can be seen in nucleus of the cell (red color; B-D). Note the absence of immunoreactivity for STAT1 in cells that do not express FGFR3 (an arrowhead).

Fig. 4

Rescue of the reduced expression of PTH/PTHrP receptor found in transfected CFK2 cells by treatment with JAK3 inhibitor

A: Without treatment with WHI-P131 (inhibitor free), weaker expression of the PTH/PTHrP receptor is detected. When treated with 1, 10 and 100 μ M of WHI-P131, however, the PTH/PTHrP receptor expression gradually becomes more intense. Note that GAPDH expression levels are consistent no matter the treatment.

B: Quantitative analysis of on the ratio of the PTH/PTHrP receptor to GAPDH without inhibitor was 0.09 ± 0.02 , whereas it significantly increased to 0.16 ± 0.02 ($p < 0.05$) at 10 μ M and 0.43 ± 0.10 ($p < 0.05$) at 100 μ M of WHI-P131 (n=6).

Table 1. Primer design for PCR (See Materials and Methods)

rat/mouse PTHrP	sense 5'-TGGTGTTCCCTGCTCAGCTA-3' antisense 5'-CCTCGTCGTCTGACCCAAA-3'	Yasuda et al(42)
rat PTH/PTHrP receptor	sense 5'-TGGTGAGGTGCAGGCAGAGTATAG-3' antisense 5'-AAACACTGGCTTCTTGGTCCATC-3'	Suda et al (32)
rat/mouse GAPDH	sense 5'-CATGGAGAAGGCTGGGGCTC-3' antisense 5'-AACGGATACATTGGGGGTAG-3'	Tso et al (37)
IHH-1	sense 5'-TCCTATGCTCCTCTCACAAG-3' antisense 5'-TCTCTTCTAGCAAGAGACGC-3'	Hebrok et al(11)
IHH-2	sence 5'-TGGATATCACCCACCTCAGAC-3' antisense 5'-GATTGTCCGCAATGAAGAGC-3'	Van der Eerden et al (38)
rat/mouse FGFR3	sense 5'-AGAAGGCTGCTTTGGACA-3' antisense 5'-TGAGCTGTTCCCTCTGGCA-3'	Henderson et al (14)

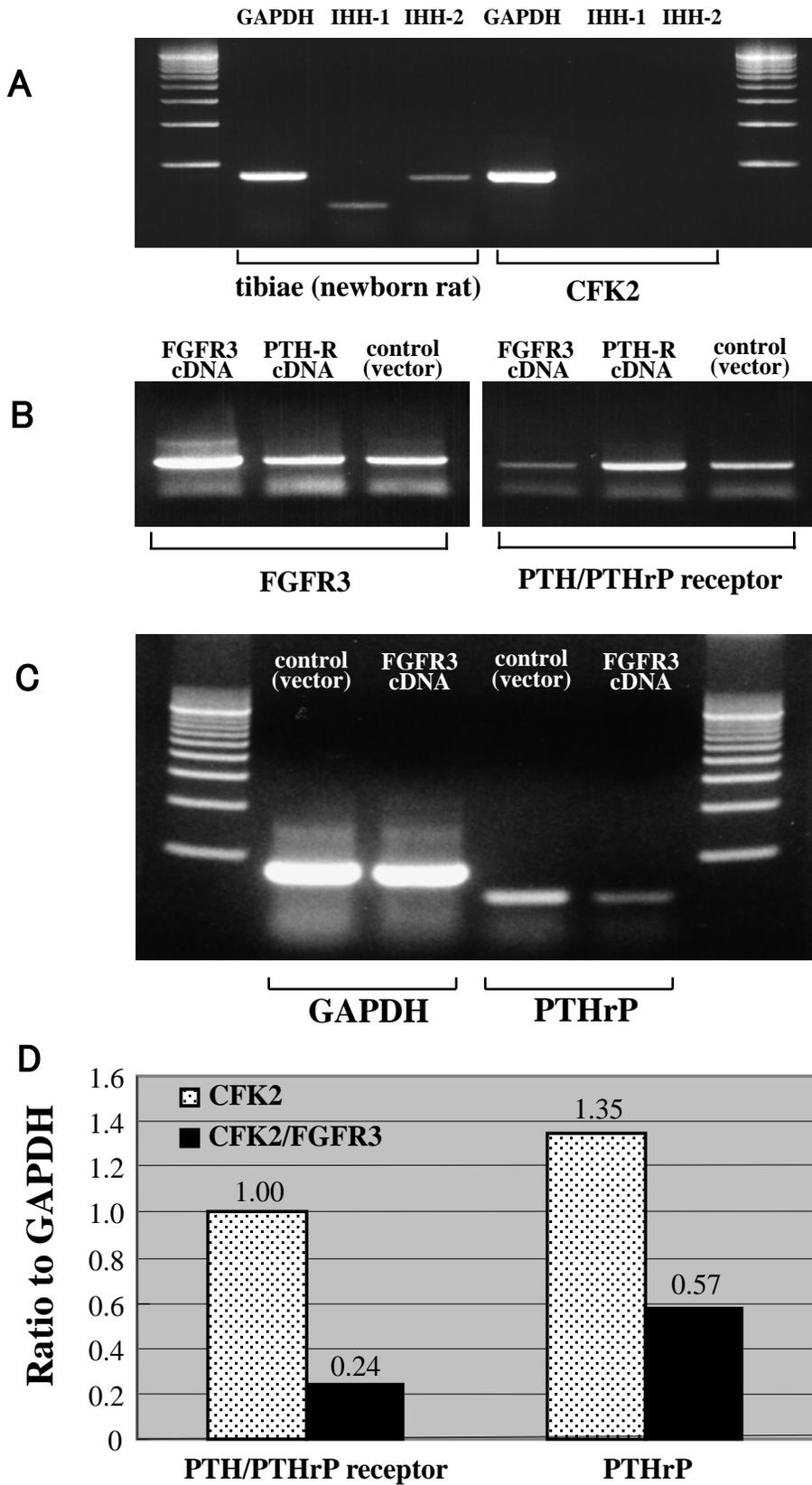


Fig.1

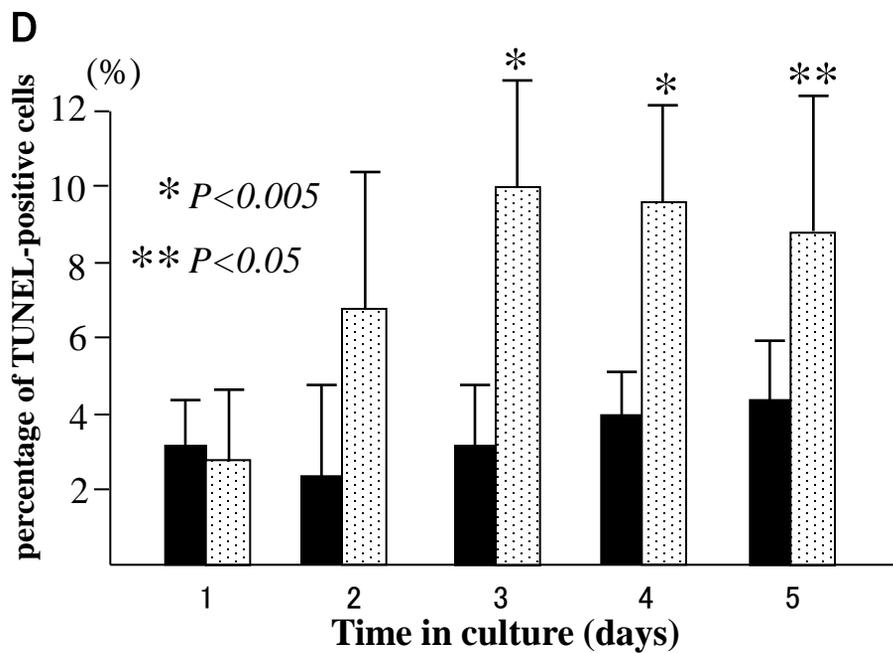
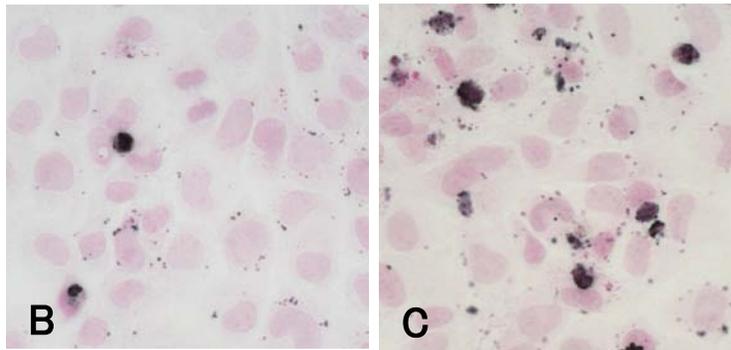
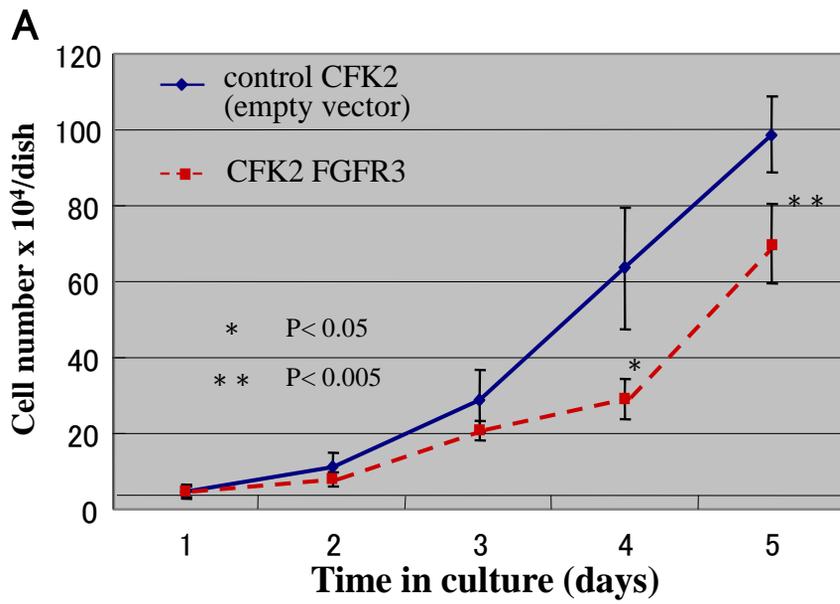


Fig.2

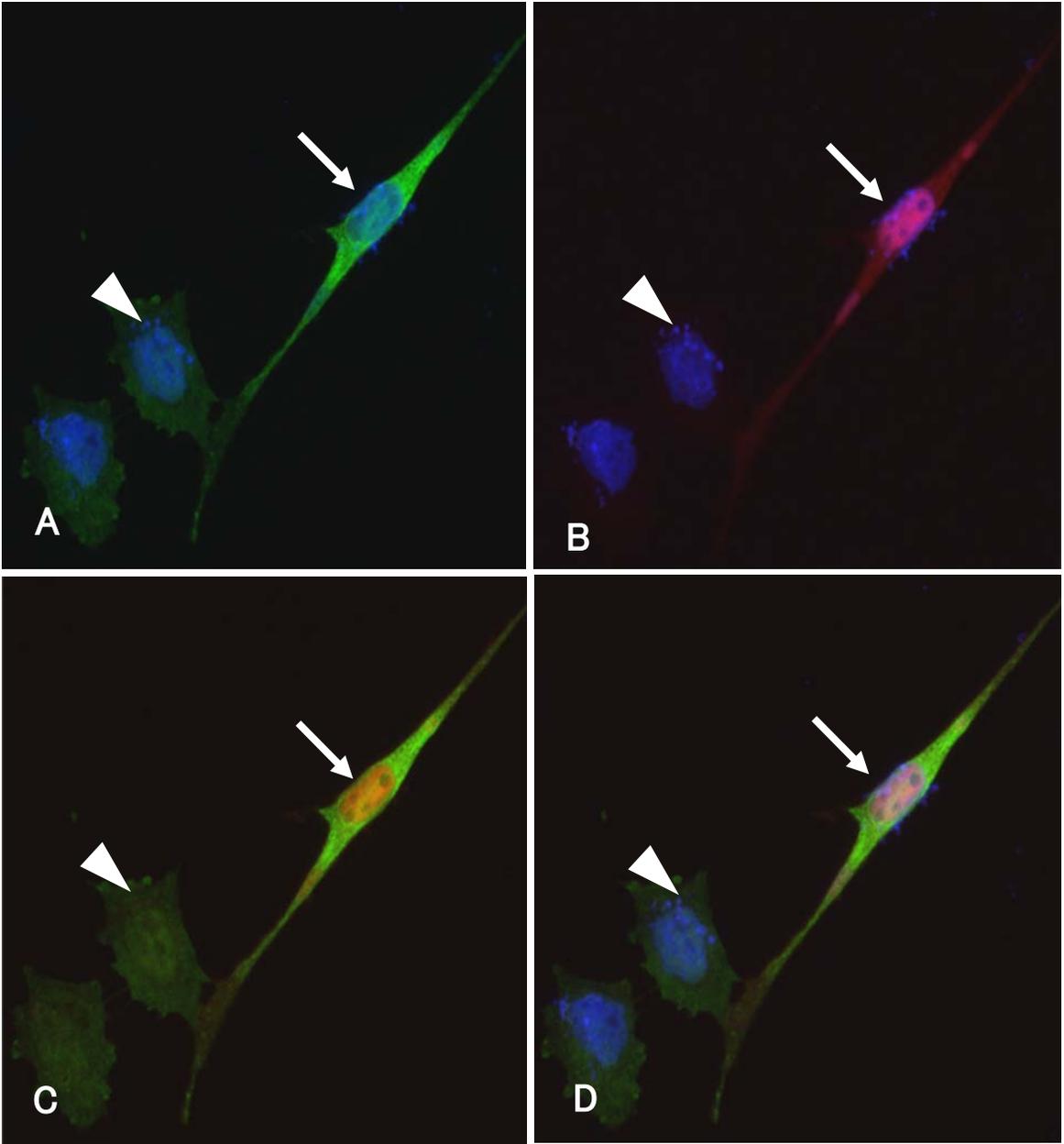


Fig.3

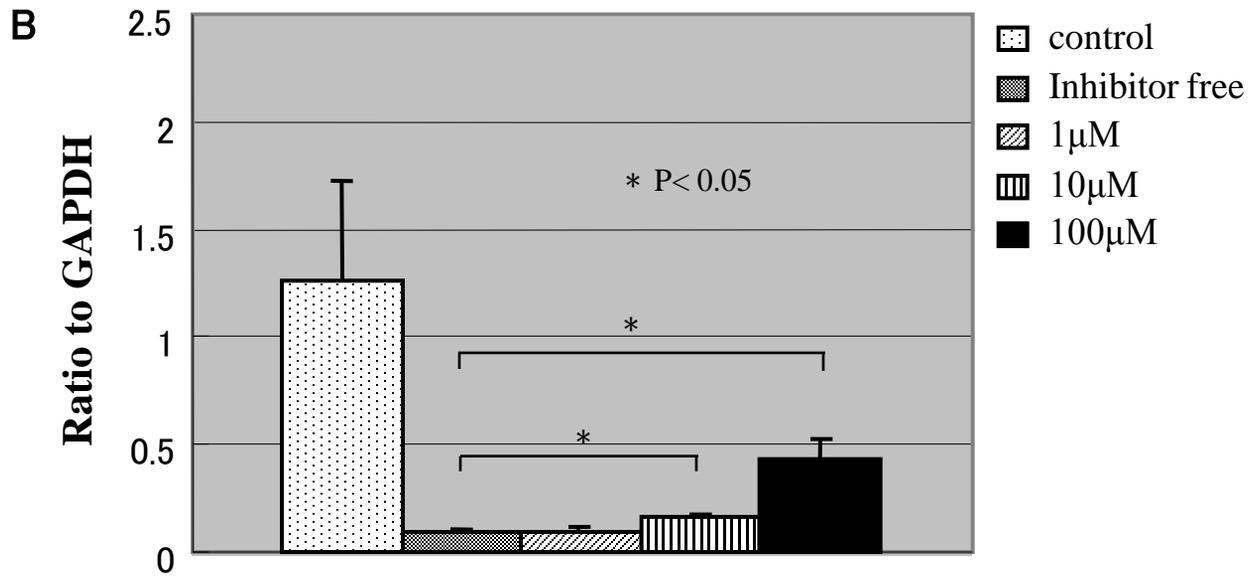
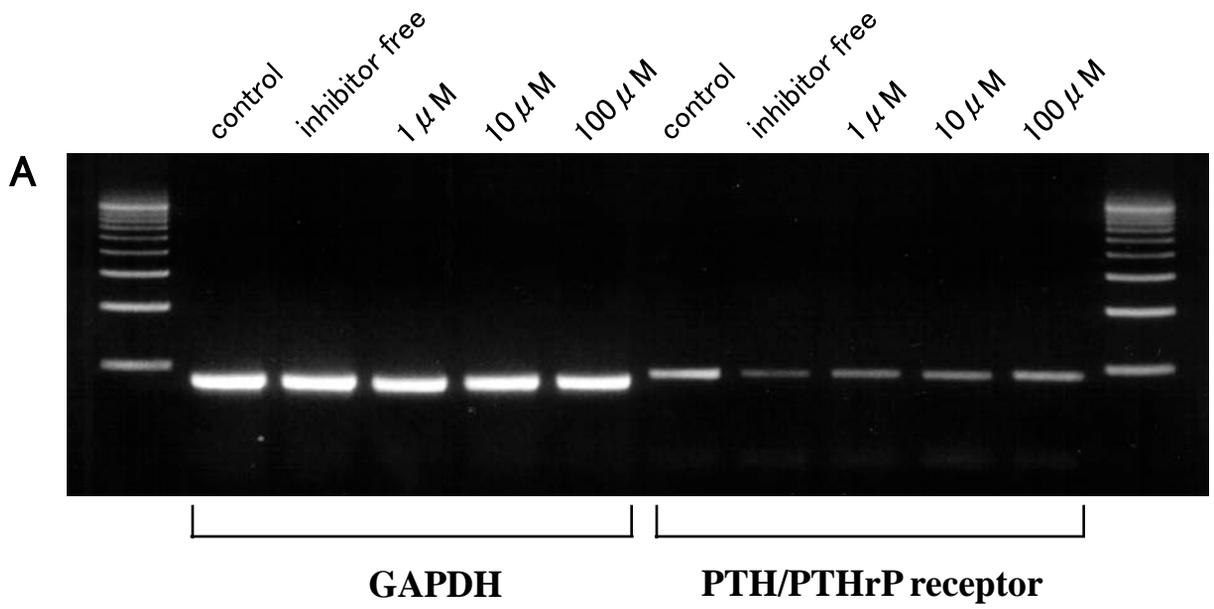


Fig.4