Basic fibroblast growth factor increases IFT88 expression in chondrocytes

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Abstract. Intraflagellar transport protein 88 (IFT88) is protein crucial for the assembly and maintenance of primary cilia in chondrocytes. Primary cilia regulate mechanical and chemical signals in chondrocytes; however, the effects of cytokines on IFT88 expression and cilia formation and maintenance remain to be elucidated. Therefore, the role of basic fibroblast growth factor (bFGF) on IFT88 expression were examined in the ATDC5 murine chondrocytic line, in order to investigate the signaling pathways involved in this process. bFGF treatment upregulated IFT88 expression in a dose- and time-dependent manner in ATDC5 cells. The effects of bFGF on IFT88 protein expression were suppressed in the presence of the extracellular signal-regulated protein kinase (ERK) inhibitor PD0325901 and the FGF receptor inhibitor BGJ398. In addition, treatment with IFT88-targeting small interfering (si)RNA downregulated the protein expression of IFT88 and ERK, thus suggesting that the ERK signaling pathway may be involved in the regulation of IFT88 expression in ATDC5 cells. bFGF treatment increased the number of ciliated ATDC5 cells and primary cultured chondrocytes. Downregulation of IFT88 expression by PD0325901, BGJ398, or IFT88-targeting siRNA was revealed to reduce the number of ciliated cells. bFGF also upregulated the mRNA and protein expression of IFT88 in primary cultured chondrocytes. In conclusion, the findings of the present study suggested that bFGF may enhance the expression of IFT88, and promote primary cilia development, through the mitogen-activated protein kinase/ERK-mediated pathway in chondrocytes.

Introduction

Primary cilia are microtubule-based organelles that protrude from most eukaryotic cells (1). Primary cilia consist of nine pairs of peripheral microtubules organized in a ring without a central microtubule. The outer peripheral microtubule structure is collectively termed the 9+0 axoneme (2,3). With the exception of cells undergoing division, most eukaryotic cells have a single primary cilium (4,5). Primary cilia were believed to have no important functions in normal cells; however, previous studies have indicated that they can act as sensory receptors for mechanical and chemical stimuli, and they may transmit these signals intracellularly (6-8).

The intraflagellar transport (IFT) complex consists of >20 different IFT proteins that assemble into macromolecular complexes, which can be transported through the action of kinesin or dynein motors along the flagellar axoneme (9,10). IFTs are responsible for the assembly and maintenance of cilia and flagella in eukaryotic cells (9,11). Intraflagellar transport protein 88 (IFT88) is a central component of the IFT-B complex, and is required for ciliogenesis in vertebrates and for flagellar assembly in Chlamydomonas (12,13). IFT88 has a critical role in cilia formation; however, the regulation of IFT88 expression in chondrocytes remains to be fully elucidated.

Basic fibroblast growth factor (bFGF) is a member of the FGF family with mitogenic properties, which may exert numerous functions and a wide range of effects in cells. bFGF may bind heparin and heparin sulfate (14) and regulate the migration, differentiation, proliferation and survival of various types of cells (15-17). bFGF is also involved in the regulation of articular cartilage homeostasis; however, the exact mechanical and biochemical processes involved in cartilage degeneration and the function of bFGF in these processes remain to be fully elucidated. In addition, the effects of bFGF on IFT88 expression and the formation and maintenance of cilia in chondrocytes remain unclear. Therefore, the present
study aimed to identify the effects of bFGF on IFT88 expression and primary cilia formation in chondrocytes in vitro.

Materials and methods

Cells and reagents. Murine ATDC5 chondrogenic cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were plated at a density of 1x10^6 cells/cm^2 and cultured in Dulbecco's modified Eagle's medium/nutrient mixture F12 (DMEM/F12; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 5% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 U/ml). Cells were maintained at 37°C in a 5% CO₂ atmosphere. Media were replaced every other day.

Primary cultured chondrocytes were prepared from the femoral cartilage of 6 male C57BL mice (age: 2 weeks; weight: 8.85±1.07 g). Mice were sacrificed in an environment with a 12 h light/dark cycle and at 23±1°C and with free access to food and water. All mice were supplied by the Experimental Animal Center of Tongji Hospital (Wuhan, China) and the procedures were approved by the Ethics Committee on Animal Experimentation of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). The cartilage was dissected, enzymatically digested with 0.25% trypsin solution (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 37°C for 30 min and 0.1% type I collagenase solution (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C for 6 h. The single cell suspensions that were obtained were resuspended in DMEM/F12 supplemented with 10% FBS following washing in phosphate-buffered saline (PBS).

bFGF was purchased from Cyagen Biosciences Inc. (Santa Clara, CA, USA). PD0325901 and BGJ398 were purchased from Selleck Chemicals (Houston, TX, USA). ATDC5 cells were treated at 37°C with different concentrations of bFGF (0, 2.5, 5, 10, 20 or 50 ng/ml for 24 h) and for different durations (5 ng/ml for 0, 12, 24, 36, 48 or 72 h). The control group received a DMSO vehicle treatment. The ERK and FGFR inhibitors PD0325901 (11 nM) and BGJ398 (1 nM), respectively, were used to treat cells at 37°C for 24 h.

Western blot analysis. ATDC5 cells were lysed in radioimmunoprecipitation assay lysis buffer (Wuhan Boster Biological Technology, Ltd., Wuhan, China) containing a protease inhibitor cocktail. Extracted protein concentrations were measured using a bicinchoninic acid assay and equal quantities (20 µg) were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The proteins were then blocked with 5% BSA (Biosharp, Hefei, China) at 37°C for 1 h. Blots were incubated at 4°C overnight with the following primary antibodies: IFT88 (AP11138b, 1:1,000 dilution; Wuhan Boster Biological Technology, Ltd.), β-actin (BM0627, 1:400 dilution; Wuhan Boster Biological Technology, Ltd.), extracellular signal-regulated protein kinase (ERK, #4695; 1:1,000 dilution), and phosphorylated (p)-ERK (#4370, 1:1,000 dilution) (both from Cell Signaling Technology, Inc., Danvers, MA, USA), followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse immunoglobulin (IgG) (BA1054 and BA1050, 1:5,000 dilution; Wuhan Boster Biological Technology, Ltd.) secondary antibodies at 37°C for 1 h. An enhanced chemiluminescence (ECL) western blot detection kit (Thermo Fisher Scientific, Inc.) was used to visualize the protein bands on an ECL system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Blots were semi-quantified by densitometric analysis using ImageLab software version 4.0.1 (Bio-Rad Laboratories, Inc.).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cells following incubation using the RNeasy Mini kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocol. cDNA was synthesized from total RNA using the SuperScript First-Strand Synthesis system for RT-PCR (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. qPCR was performed on cDNA using a SYBR Green Real-Time PCR Master Mix kit (Toyobo Life Science, Osaka, Japan). The thermocycling conditions were as follows: Pre-denaturation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 15 sec and extension at 72°C for 45 sec. Gene expression levels were normalized to those of β-actin and the method of quantification was 2^ΔΔCt(18). The primer sequences used in the present study were as follows: IFT88 forward, (F) 5'-TGGCCAACGCCTGAGATTACCA-3' and reverse, (R) 5'-ATAGCTGGCGTGGTTGGCAAATTC-3'; and β-actin forward, (F) 5'-CTTCTTTGGGTATTGGAATCTTGG-3' and reverse, (R) 5'-TGTTTGGGCCATAAGGTCTTACG-3'.

Small interfering (si)RNA transfection. ATDC5 cells (1x10^5) were transfected with 50 nM siRNA targeting IFT88 or with a scrambled sequence (negative control siRNA) for 72 h using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) as the transfection reagent. The knockdown efficiency of IFT88 siRNA was assessed using RT-qPCR and western blot analysis, as aforementioned. IFT88 siRNA and negative control siRNA (siG151225113820 and siN05815122147) were synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China).

Immunofluorescence. ATDC5 cells were seeded on coverslips at a density of 5x10^3 cells/cm² and stimulated with the various reagents. The cells were fixed in 4% parafomaldehyde for 15 min following 24 h of treatment, blocked with 0.5% bovine serum albumin (BioSharp) at room temperature for 1 h, and then incubated with a primary acetylated α-tubulin (αTub) antibody (ab24610, 1:500 dilution; Abcam, Cambridge, UK) overnight at 4°C. The cells were then incubated with cyanine 3-conjugated goat anti-mouse IgG secondary antibody (BA1031, 1:200 dilution; Wuhan Boster Biological Technology, Ltd.) at room temperature for 1 h, and the nuclei were stained with 1 µg/µl DAPI at 37°C for 5 min. The cells were washed with PBS 3 times for 10 min after each step. Stained cells were visualized under a fluorescence microscope and photomicrographs were captured using an EVOS FL Auto Cell Imaging System (Thermo Fisher Scientific, Inc.).

Statistical analysis. Each experiment was performed at least 3 times. Data are presented as the mean ± standard deviation.
Figure 1. bFGF treatment enhanced the mRNA and protein expression of IFT88 in a time- and dose-dependent manner in murine ATDC5 chondrocytes in vitro. (A-C) ATDC5 cells were cultured for the indicated times (0, 12, 24, 36, 48 and 72 h) in the presence of bFGF (5 ng/ml) or (D-F) with the indicated concentrations of bFGF (0, 2.5, 5, 10, 20 and 50 ng/ml) for 24 h. (A, B, D, E) IFT88 protein expression levels were assessed using western blot analysis with β-actin as a loading control. (C and F) IFT88 mRNA expression was detected using reverse transcription-quantitative polymerase chain reaction and normalized to β-actin mRNA levels. Data are expressed as the mean ± standard deviation of 3 independent experiments. *P<0.05, **P<0.01, as indicated. bFGF, basic fibroblast growth factor; IFT88, intraflagellar transport protein 88.

Figure 2. Treatment with the ERK inhibitor PD0325901 attenuated the effects of bFGF on the protein expression of IFT88 in murine ATDC5 chondrocytes. ATDC5 cells were treated with bFGF in the presence or absence of 11.0 nM PD0325901. (A) Protein expression of ERK, p-ERK and IFT88 was detected using western blot analysis. β-actin was used as the loading control. (B) Blots were semi-quantified by densitometry. Data are expressed as the mean ± standard deviation. *P<0.05, **P<0.01, as indicated. ERK, extracellular signal-regulated protein kinase; p-ERK, phosphorylated-ERK; bFGF, basic fibroblast growth factor; IFT88, intraflagellar transport protein 88.
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Figure 3. Treatment with the FGFR inhibitor BGJ398 downregulated IFT88 and p-ERK protein expression in murine ATDC5 chondrocytes in vitro. ATDC5 cells were cultured in the presence of 5 ng/ml bFGF or 1 nM BGJ398. (A) The protein expression levels of IFT88, ERK, and p-ERK were detected using western blot analysis with β-actin as a loading control. (B) Blots were semi-quantified by densitometry. Data are expressed as the mean ± standard deviation. *P<0.05, **P<0.01, as indicated. FGF, fibroblast growth factor; FGFR, FGF receptor; IFT88, intraflagellar transport protein 88; ERK, extracellular signal-regulated protein kinase; p-ERK, phosphorylated-ERK; bFGF, basic FGF.

Figure 4. IFT88 knockdown suppresses the mRNA and protein expression of IFT88 in ATDC5 cells. ATDC5 cells were transfected with control siRNA or IFT88-targeting siRNA and cultured for 72 h. (A) The efficacy of transfection was confirmed using reverse transcription-quantitative polymerase chain reaction analysis of IFT88 mRNA expression. (B and C) The protein expression levels of IFT88, p-ERK and ERK were detected using western blot analysis with β-actin as a loading control. Data are expressed as the mean ± standard deviation of 3 independent experiments. *P<0.05, **P<0.01, as indicated. IFT88, intraflagellar transport protein 88; si, small interfering; ERK, extracellular signal-regulated protein kinase; p-ERK, phosphorylated-ERK.
The statistical significance of the differences between groups was assessed using Student’s t-test and two-way analysis of variance followed by a Bonferroni post-hoc test were used. P<0.05 was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS version 20.0 (IBM Corporation, Armonk, NY, USA).

Results

**bFGF treatment increases IFT88 expression in a time- and dose-dependent manner in ATDC5 cells.** To examine the effects of bFGF on IFT88 mRNA and protein expression levels, ATDC5 cells were treated with 5 ng/ml bFGF for up to 72 h. bFGF treatment was demonstrated to upregulate the mRNA and protein expression of IFT88 within 12 h, a maximal effect was evident following 24 h of treatment; the effects of bFGF continued for 72 h (Fig. 1A-C). To investigate whether the effects of bFGF on IFT88 expression were dose-dependent, RT-qPCR and western blot analysis were performed using ATDC5 cells treated with various doses of bFGF. ATDC5 cells were treated with bFGF at concentrations of 0, 2.5, 5, 10, 20, and 50 ng/ml for 24 h. bFGF treatment was revealed to potentiate the protein expression of IFT88 in a dose-dependent manner; this effect was evident at 2.5 ng/ml and peaked at 5 ng/ml (Fig. 1D and E). The effect of bFGF on IFT88 protein expression gradually decreased at concentrations >5 ng/ml, but expression remained above the IFT88 levels in control cells, with the exception of cells treated with 50 ng/ml bFGF (Fig. 1D and E). In addition, bFGF treatment increased the mRNA expression of IFT88 in a dose-dependent manner, and the results were similar to those regarding protein expression (Fig. 1F).

**ERK inhibition counteracts the effect of bFGF on IFT88 protein expression.** To investigate the putative signaling pathways implicated in the regulatory effects of bFGF on IFT88 expression, ATDC5 cells were treated with bFGF in the presence or absence of the ERK inhibitor PD0325901. The
present results demonstrated that bFGF treatment enhanced the phosphorylation of ERK, as indicated by western blot analysis of p-ERK expression (Fig. 2). Conversely, treatment with PD0325901 significantly downregulated the protein expression levels of p-ERK in ATDC5 cells, and suppressed IFT88 protein expression (Fig. 2B), however, no differences
were evident across total ERK expression levels. bFGF treatment in the presence of PD0325901 appeared to upregulate the expression of IFT88; however, the effect was significantly reduced when compared with bFGF treatment alone (Fig. 2B). A similar trend was revealed in p-ERK protein expression levels, suggesting that PD0325901 may reduce the expression of IFT88 by inhibiting ERK phosphorylation. These findings suggested that the ERK signaling pathway may be involved in the regulation of IFT88 expression by bFGF in chondrocytes in vitro.

Inhibition of the FGF receptor (FGFR) suppresses IFT88 protein expression in ATDC5 cells. To investigate the effects of FGFR inhibition on IFT88 expression, ATDC5 cells were treated with the FGFR inhibitor BGJ398. The present findings revealed that BGJ398 downregulated the protein expression levels of IFT88 in ATDC5 cells, which was accompanied by a decrease in p-ERK expression levels (Fig. 3). These findings suggested that FGFR may participate in the molecular mechanisms underlying the regulation of IFT88 expression in chondrocytes.

Knockdown of IFT88 downregulates the protein expression of p-ERK in ATDC5 cells. To investigate the effects of IFT88 downregulation on the mitogen-activated protein kinase (MAPK)/ERK signaling pathway, RNA interference was used to silence IFT88 expression in ATDC5 cells. The efficacy of IFT88 siRNA transfection was confirmed (Fig. 4A), and the protein expression levels of IFT88, p-ERK and ERK were evaluated using western blot analysis. IFT88 knockdown was revealed to suppress the protein expression of IFT88 and p-ERK in ATDC5 cells, whereas it had no effect on ERK protein expression (Fig. 4B and C). These findings suggested that the MAPK/ERK signaling pathway may be involved in the regulation of IFT88 expression in ATDC5 cells.

bFGF exposure increases the number of ATDC5 cells exhibiting primary cilia. To investigate the effects of bFGF on the development of primary cilia, the presence of primary cilia in ATDC5 cells was detected using an acTub antibody. Following staining with the anti-acTub antibody, primary cilia were visualized as red acetylated α-tubulin-positive structures in ATDC5 cells under a fluorescence microscope. bFGF exposure increased the percentage of ciliated cells compared with the control group (Fig. 5). bFGF treatment increased the number of ciliated cells by ~80%, suggesting that the bFGF-mediated regulation of IFT88 expression may be involved in the development of primary cilia in chondrocytes.

PD0325901 or BGJ398 treatment and IFT88 knockdown decreases the number of ciliated ATDC5 cells. To further investigate the effects of IFT88 downregulation on primary cilia development, primary cilia were visualized in ATDC5 cells using an anti-acTub antibody. Treatment with PD0325901 or BGJ398, and transfection with IFT88 siRNA, resulted in fewer ciliated cells compared with the control group (Fig. 6). These findings suggested that the regulation of IFT88 expression, and MAPK/ERK- and FGFR-mediated pathways may be involved in primary cilium maintenance.

bFGF treatment upregulates IFT88 expression and promotes primary cilium development in primary chondrocytes. To investigate whether primary chondrocytes may exhibit a similar response to bFGF to ATDC5 cells in vitro, the mRNA and protein expression of IFT88 was assessed in primary
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Primary chondrocytes were prepared from newborn mice and treated with bFGF at a concentration of 5 ng/ml for 24 h. bFGF treatment upregulated the IFT88 mRNA and protein expression in primary chondrocytes (Fig. 7A-C). An immunofluorescence assay was used to evaluate the effects of exogenous bFGF on cilium development, and results...
revealed that bFGF increased the number of ciliated cells compared with the control group (Fig. 7D and E).

Discussion

The IFT protein complex is essential for the formation and maintenance of primary cilia in eukaryotic cells (9,11,12); however, the molecular mechanisms involved in the effects of IFT complexes on cilia remain to be elucidated. In addition, little is known regarding the regulation of IFT proteins by cytokines in vivo or in vitro, and the effects of cytokines on cilia formation.

In the present study, exogenous bFGF was demonstrated to increase the mRNA and protein expression of IFT88 in ATDC5 chondrocytes in vitro. It is possible that the upregulated expression of IFT88 may potentiate the function of the IFT system in chondrocytes, through the amplification of mechanical stimulation and sensory perception of the extracellular microenvironment (6-8). These processes have been reported to contribute to the regulation of cartilage development (19,20). A depletion of primary cilia has been demonstrated in the articular cartilage of Col2αCre; IFT88^fl/fl mice, which resulted in abnormal articular cartilage development (21). The cartilage of Col2αCre; IFT88^fl/fl mice was thicker, had increased cell density and exhibited enhanced expression of osteoarthritic markers, including matrix metalloproteinase-13, disintegrin-like and metalloprotease with thrombospondin type 1 motif 5, collagen X and runt-related transcription factor 2 (21).

The findings of the present study suggested that bFGF may upregulate the protein expression of IFT88 through the MAPK/ERK signaling pathway in chondrocytes. Following treatment with the ERK inhibitor PD0325901, the FGFR inhibitor BGJ398, or IFT88-targeting siRNA, the protein expression levels of p-ERK appeared to be downregulated. These findings suggested that the IFT88 and MAPK/ERK pathways may be closely associated in chondrocytes; however, further studies are required to investigate the specific targets of the MAPK/ERK pathway and its downstream effects in primary cilia.

Murine chondrocytes express all FGFR subtypes (FGFR1-4); however, bFGF treatment has been reported to significantly induce the expression of FGFR3 (22,23), which exerted anabolic effects in murine chondrocytes. In the present study, the FGFR inhibitor BGJ398 suppressed the expression of IFT88 in murine chondrocytes, thus suggesting that the IFT88 expression regulation process may involve FGFR regulation. It is of note that bFGF in human articular cartilage has been suggested to exert opposite roles compared with in murine cartilage, and bFGF has been reported to activate catabolic processes primarily via FGFR1 signaling in human cartilage (24,25). Considered together, these findings suggested that the effects of bFGF may be species-dependent.

Primary cilia have a microtubule-based infrastructure, which consists of 9 pairs of peripheral microtubules without a central microtubule, and can detect alterations in mechanical and biochemical stimulation from the extracellular milieu (26,27). In addition, it has been suggested that cilia may be essential for the development and progression of tumors (28). In the present study, IFT88 expression was downregulated in ATDC5 chondrocytes in vitro, through the inhibition of ERK and FGFR signaling. Following siRNA-mediated knockdown, IFT88 downregulation resulted in the suppression of primary cilium development in chondrocytes. These findings suggested that IFT88 may be an essential factor for the formation of primary cilia. To the best of our knowledge, the present study demonstrated for the first time that bFGF enhanced the mRNA and protein expression of IFT88, which in turn may promote primary cilia formation in chondrocytes. These findings propose a novel function for bFGF in chondrocytes.

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