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CCN2/CTGF binds to fibroblast growth factor receptor 2 and modulates its signaling

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ABSTRACT

CCN2 plays a critical role in the development of mesenchymal tissues such as cartilage and bone, and the binding of CCN2 to various cytokines and receptors regulates their signaling. By screening a protein array, we found that CCN2 could bind to fibroblast growth factor receptors (FGFRs) 2 and 3, with a higher affinity toward FGFR2. We ascertained that FGFR2 bound to CCN2 and that the binding of FGFR2 to FGF2 and FGF4 was enhanced by CCN2. CCN2 and FGF2 had a collaborative effect on the phosphorylation of ERK and the differentiation of osteoblastic cells. The present results indicate the biological significance of the binding of CCN2 to FGFR2 in bone metabolism.

Structured summary of protein interactions:
FGFR2 binds to **CCN2** by protein array (View interaction)
FGFR1OP binds to **CCN2** by protein array (View interaction)
FGFR3 binds to **CCN2** by protein array (View interaction)

1. Introduction

CCN2 is a member of the CCN family of proteins [1,2], and CCN2-null mice show remarkable abnormalities in skeletal development, as represented by bent costal cartilage and long bones with impaired endochondral ossification [3]. Also, *in vitro* studies have shown that CCN2 is a stimulator of both the proliferation and differentiation of chondrocytes, osteoblasts, vascular endothelial cells, and mesenchymal stem cells [1,2]. These findings indicate that CCN2 plays a critical role in the metabolism of cartilage and in bone formation.

Nevertheless, the molecular behavior determining the effect of CCN2 has not been totally elucidated. CCN2 is known as a binding partner of many types of cytokines and extracellular matrix proteins, such as bone morphogenetic protein (BMP) 2, fibronectin, and aggrecan [4–6]. Also, CCN2 was reported to be a modulator of signaling by BMP2, BMP4, and TGF-β [4,7]. Therefore, we assumed that proteins binding to CCN2 define the various effects of CCN2 in certain circumstances. Searching for new factors binding to CCN2, we performed a protein-array experiment with recombinant CCN2 as a probe and found fibroblast growth factor (FGF) receptor 2 (FGFR2) to be among the candidates as partners of CCN2.

FGFR2 is a member of the FGF receptor family and is expressed in condensed mesenchyme and later in sites of endochondral and intramembranous ossification [8]. *In vitro* assays show that FGFR2 regulates certain steps of skeletal development [9–11]. In humans, mutations of FGFR2 cause endochondral diseases such as Apert and Crouzon syndromes [12–14]. FGFR2-deficient mice die at 10–11 days of gestation because of failure in the formation of their placenta and limb buds [15]. These mice, deficient in FGFR2 specifically in the condensed mesenchyme, indicate that FGFR2 is essential for the normal proliferation of osteoblasts and osteogenic gene expression during postnatal bone development [16].

Based on this background, we subsequently confirmed the binding of CCN2 to FGFR2 and assessed its effects on FGFR2 signaling in osteoblastic cell line MC3T3-E1. Finally, we found that CCN2 enhanced the signaling effected by FGF2-induced activation of extracellular regulated kinase (ERK), which action resulted in the collaborative enhancement of the differentiation of the osteoblastic cells.

2. Materials and methods

2.1. Screening of CCN2-binding proteins

Proteins binding to CCN2 were screened with a ProtoArray® Human Protein Microarray ver. 4 (Invitrogen, Carlsbad, CA, USA). Experiments were professionally performed by Filgen Incorporated

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(Nagoya, Japan) by using an oligohistidine-tagged recombinant human CCN2 (BioVendor Laboratory Medicine, Brno, Czech Republic) as a probe.

2.2. Solid-phase binding assay

For the examination of the direct interaction between CCN2 and FGFR-Fc, a solid-phase binding assay was carried out, as described previously [5,6]. Briefly, wells of ELISA plates previously coated with 1 μg/ml of rCCN2 (BioVendor Laboratory Medicine) or bovine serum albumin (BSA) were blocked with blocking buffer. Diluted recombinant human FGFR2a (IIIc)/Fc (FGFR2-Fc) or recombinant human FGFR3a (IIIc)/Fc (FGFR3-Fc; R&D Systems, Minneapolis, MN) was then added to the wells, and incubation was conducted for 2 h at 37 °C. Thereafter, the wells were washed and incubated with horseradish peroxidase (HRP)-conjugated anti-human Fc antibody (Sigma Aldrich, St Louis, MO); and the bound HRP was then monitored by using 3,3,5,5-tetramethylbenzidine (TMB) peroxidase substrate (Sigma–Aldrich). The effect of CCN2 on the binding of FGFR2-Fc to FGF2 or FGF4 was assayed on ELISA plates coated with 1 μg/ml of FGF2 or FGF4 (R&D Systems). FGFR2-Fc was incubated with CCN2 for 30 min before being added to the wells, and detection of signals was performed as described above.

2.3. SPR (surface plasmon resonance) measurements

For the calculation of dissociation constant (Kd) values, SPR measurements were performed, as described previously [4,6]. Briefly, FGFR2-Fc was diluted to concentrations of 1.6, 8, 40, 200, and 1000 nM with HBS-EP (GE Healthcare UK, Ltd, Little Chalfout, UK, Little Chalfout, UK); and each was reacted with rCCN2 immobilized on CM5 sensor chips (GE Healthcare) according to the standard amine coupling procedure recommended by the manufacturer. Binding and dissociation of these molecules were monitored with a Biacore X (GE Healthcare) and the data were fitted using the BIAevaluation software version 4.1 (GE Healthcare) with the single cycle kinetics support package (GE Healthcare). The sensorgrams were corrected by subtracting the signal of the reference cell and were forwarded for Kd computation.

2.4. Cell culture

Cells of the mouse osteoblastic cell line MC3T3-E1 were cultured in a humidified incubator (5% CO_2 in air) at 37 °C and maintained in 10% FCS/DMEM supplemented with 50 μg/ml streptomycin and 100 units/ml penicillin.

2.5. Immunoprecipitation and western blotting

The cell lysate mixed with oligohistidine-tagged rCCN2 was incubated for 2 h at 4 °C and reacted with Protein G Sepharose 4 Fast Flow beads (GE Healthcare) having adsorbed anti-FGFR2 (Santa Cruz Biotechnology, Santa Cruz, CA) or control goat IgG for 1 h at 4 °C. The precipitated protein was assayed by western blotting analysis using rabbit anti-6-His Antibody Affinity Purified (Bethyl Laboratories, Inc., Montgomery, TX, USA).

2.6. Evaluation of ERK protein phosphorylation

Cells lysates were collected in RIPA buffer after stimulation of the cells with FGF2, rCCN2 or FGFR2-Fc. Western blotting was carried out with Anti-ACTIVE® MAPK pAb, Rabbit pTEpY (Promega Corporation, Madison, Wl) or p44/42 MAPK antibody (Cell Signaling Technology, Danvers, MA). The density of each band was quantified by Multi Gauge Ver3.0 (FUJIFILM, Japan).

2.7. Quantitative real-time PCR analysis

Total RNA was isolated by using an RNeasy Mini Kit (Qiagen, Hilden, Germany) and was reverse-transcribed to cDNA by using a Takara RNA PCR kit (AMV), Version 3.0 (Takara Shuzo, Tokyo, Japan). Amplification reactions were performed with a SYBR® Green Real-time PCR Master Mix (Toyobo; Tokyo, Japan) by using StepOne™ Software v2.1 (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences of the primers used were as follow: 5′-GCCAAAAGGGTCATCATCTC-3′ (forward) and reverse 5′-GTCT TCTGGGTGGCAGTGAT-3′ (reverse) for *Gapdh*; 5′-CTCACAGATGCCAAGCCCA-3′ (forward) and reverse 5′-CCAAGGTAGCGCCGGAG TCT-3′ (reverse) for *Osteocalcin*, as reported earlier [17,23].

2.8. Statistical analysis

Statistical analyses were performed by using Student's t-test, if necessary. $^{*}p < 0.05$, and $^{**}p < 0.01$, as compared with the values for control wells.

3. Results

3.1. FGFR2 and 3 as candidate CCN2-binding proteins

First, we used a protein array to screen for proteins binding to CCN2. Fig. 1 shows the CCN2 binding profile of the FGF-associated molecules included in the protein array. Among the top 3 candidates, we focused on FGFR2 and 3, because their mutants are known to result in genetic disorders of bone formation, in which CCN2 plays a critical role. Thus, these 2 molecules were forwarded for subsequent investigation.

3.2. Specific binding of CCN2 to FGFR2 and FGFR3

To confirm the binding of CCN2 to FGFR2 and 3, we initially employed a solid-phase binding assay, which results are displayed in Fig. 2(A) and (B). Though FGFR2-Fc and FGFR3-Fc increasingly bound to CCN2 depending upon the doses, the bound FGFR2-Fc is more than twice as much as FGFR3-Fc in each dose. Both FGFR2 and 3 bound to CCN2 hardly bound to wells coated with BSA, which protein was used as a negative control for CCN2 protein. Similarly, human IgG used as a negative control for FGFR fused to human IgG Fc domain did not bind to CCN2 at all (data not shown). These results mean that the binding of FGFR2 and 3 to CCN2 was specific and the binding of FGFR2 appeared to be stronger than that of FGFR3. On the basis of these results, we decided to further investigate the mode and functional significance of the binding of FGFR2 to CCN2.

First we analyzed the affinity of CCN2 to FGFR2 by performing SPR analysis (Fig. 2(C)). SPR assay indicated the Kd value for the binding of CCN2 to FGFR2 to be 7.71 nM. According to previous studies, the Kd values for FGF1, 2 and 4, which are known as ligands of FGFR2, were reported to be 0.791 nM, 0.490 nM, and 0.412 nM, respectively [17]. These data taken together indicate that the binding affinity of CCN2 for FGFR2 was not equivalent, but at least comparable, to that of known FGFR2 ligands.

Next, the binding of FGFR2 *in vivo* to CCN2 was ascertained by co-immunoprecipitation of FGFR2 on MC3T3-E1 cells with rCCN2 (Fig. 2(D)). Much more CCN2 protein was detected in the immunoprecipitant with anti-FGFR2 Ab than with control Ab. These data represent CCN2 bound to FGFR2 expressed on MC3T3-E1.

3.3. Effect of CCN2–FGFR2 interaction on the binding of FGFs to FGFR2

The major function of FGFR2 is to transmit the signals from its authentic ligands. Therefore, we verified whether or not this binding of CCN2 to FGFR2 could influence the binding of FGFR2 to its