

Direct interaction between CCN family protein 2 and fibroblast growth factor 1

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Abstract In an attempt to find out a new molecular counterpart of CCN family protein 2 (CCN2), a matricellular protein with multiple functions, we performed an interactome analysis and found fibroblast growth factor (FGF) -1 as one of the candidates. Solid-phase binding assay indicated specific binding between CCN2 and FGF-1. This binding was also confirmed by surface plasmon resonance (SPR) analysis that revealed a dissociation constant (K_d) of 3.98 nM indicating strong molecular interaction between the two. RNA analysis suggested that both FGF-1 and CCN2 could be produced by chondrocytes and thus their interaction in the cartilage is possible. These findings for the first time indicate the direct interaction of CCN2 and FGF-1 and suggest the co-presence of these

molecules in the cartilage microenvironment. CCN2 is a well-known promoter of cartilage development and regeneration, whereas the physiological and pathological role of FGF-1 in cartilage mostly remains unclear. Biological role of FGF-1 itself in cartilage is also suspected.

Keywords FGF-1 · CCN2 · CTGF · Chondrocyte · Cartilage

Introduction

Most of the bones constructing our body develop from cartilaginous prototypes, which grow and mineralize through endochondral ossification, leaving permanent articular cartilage at the sites of joints (Takigawa, 2013). Even after growth, articular cartilage remains as an indispensable locomotive element supporting our physical activities with flexible movement. Thus, cartilage is a critical tissue for both development and maintenance of our skeleton. Cartilage development and maintenance are executed under the collaboration of multiple extracellular signaling molecules.

During endochondral ossification, chondrocytes undergo proliferative, maturing and hypertrophic stages towards programmed cell death, in order to enable the growth and gradual mineralization of the bone (Takigawa, 2013). Typically, proliferation and maturation of chondrocytes are accelerated by fibroblast growth factor (FGF)-2 and insulin-like growth factor (IGF)-I & 2 or parathyroid hormone (PTH), respectively (Takigawa, 2013; Weksler et al., 1999; Takigawa et al. 1981, 1997). Among such molecules, CCN family protein 2 (CCN2), formerly designated as connective tissue growth factor, was re-discovered as a factor that promotes all of the stages of cartilage development and

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endochondral ossification (Takigawa, 2013; Nakanishi et al., 2000).

CCN2 is a classical member of the CCN family characterized by the conserved modular structure and matricellular actions (Perbal and Takigawa, 2005; Rachfal and Brigstock, 2005; Leask and Abraham, 2006; Jun and Lau, 2011). Indeed, CCN2 promotes proliferation, maturation and even hypertrophic differentiation of growth plate chondrocytes that conduct endochondral ossification (Kubota and Takigawa, 2007; Takigawa, 2013). Of note, CCN2 also enhances the proliferation and maturation of articular chondrocytes without promoting ectopic hypertrophic differentiation (Nishida et al., 2002). These molecular functions of CCN2 are also confirmed in vivo. Namely, *Ccn2*-null mice are characterized by delayed endochondral ossification leading to major skeletal defects (Ivkovic et al., 2003; Kawaki et al., 2008). Moreover, CCN2 is known to regenerate damaged articular cartilage in experimental rat osteoarthritis (OA) models in vivo (Nishida et al., 2004; Abd El Kader et al., 2014). The multiple functionality of CCN2 in cartilage is enabled by the interaction with multiple counterparts present in the microenvironment (Kubota and Takigawa, 2007; Takigawa, 2013).

Until today, a significant number of CCN2 counterparts have been identified. For example; growth factors including bone morphogenetic proteins (BMPs) (Kubota and Takigawa, 2007; Maeda et al., 2009), vascular endothelial growth factor (VEGF) (Inoki et al., 2002), FGF-2 (Nishida et al., 2011) and transforming growth factor (TGF) β (Kubota and Takigawa, 2007); cell surface receptors as typically represented by various integrins (Perbal and Takigawa, 2005); and extracellular matrix (ECM) molecules, such as fibronectin (Hoshijima et al., 2006) and heparan sulfate proteoglycans (Nishida et al., 2003; Gao and Brigstock, 2004; Jun and Lau, 2011). During the course of searching for additional cofactors of CCN2, we found FGF-1 as one of the candidates. Here, our subsequent study uncovered novel molecular action of FGF-1 in the presence of CCN2.

Materials and methods

Cell culture

The human chondrocytic cell line HCS-2/8, established from a human chondrosarcoma, retains chondrocytic properties exemplified by the gene expression and production of type II collagen and aggrecan (Takigawa et al., 1989). The human breast cancer cell line MCF7 and human cervical carcinoma cell line HeLa were also utilized. Human chondrocytes were isolated from the apparently intact regions of articular cartilage obtained upon total knee replacement of OA patients under the approval of the Ethical Committee for Human Research of Okayama University Graduate School of

Medicine, Dentistry and Pharmaceutical Sciences. These cells were maintained at 37 °C in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10 % fetal bovine serum (FBS) in 5 % CO₂. The human chondrocytes were used for experiments within 3 passages in culture.

Interactome analysis

Interaction between CCN2 and more than 9,000 human proteins was comprehensively analyzed with a Proto Array Human Protein Microarray ver. 5.0 (Life Technologies, Carlsbad, CA). Experiments were professionally performed by Filgen incorporated (Nogoya, Japan) by using an oligohistidine-tagged recombinant human CCN2 fragment as a probe. This fragment was produced by a secretory production system with *Brevibacillus choshinensis* and contains the entire insulin-like growth factor binding protein-like module of CCN2, as described previously (Kubota et al. 2006). Data were analyzed by Proto Array Prospector (Filgen) software specifically designed for this array system.

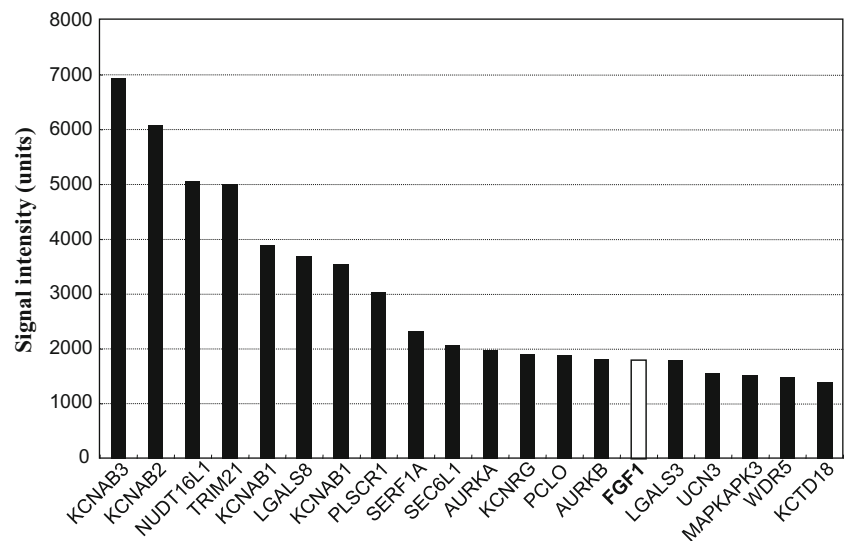
Solid phase binding assay

Evaluation of direct interaction between CCN2 and FGF-1 on ELISA plates was performed as described previously (Aoyama, Kubota and Takigawa, 2012). Briefly, wells of ELISA plates coated with 1 μ g/ml of recombinant human CCN2 (BioVendor Laboratory Medicine) or 2 % bovine serum albumin (BSA), and were blocked with 100 μ l of binding buffer for 2 h at room temperature. Diluted recombinant human FGF-1 (PeproTech, Rocky Hill, NJ) was then added to the wells, and incubation was conducted for 2 h at 37 °C. Thereafter, the wells were washed and incubated primary with an anti-human FGF-1 antibody (Abnova, Taipei, Taiwan) and then with a horseradish peroxidase (HRP)-conjugated anti-human IgG 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).

Surface plasmon resonance (SPR) analysis

Kinetic analysis of the interaction between CCN2 and FGF-1 was analyzed by using a BIAcore X (GE HealthCare, Little Chalfont, UK). As a ligand, CCN2 was immobilized onto a CM5 sensor chip according to the manufacturer's protocol. FGF-1 diluted in HBS-EP buffer (10 mM HEPES, 0.15 M NaCl, 3 mM EDTA and 0.005 % Tween 20; pH 7.4) at 6.25, 12.5, 25, 50 or 100 nM was perfused over the control surface or a surface bearing CCN2, and the resonance changes were monitored. The response was standardized by subtracting the one on the

Fig. 1 Twenty candidates for CCN2 cofactors that may be related to cell signaling. Interactomic analysis with a protein array revealed more than 100 proteins that may directly bind to human CCN2. Among them, 20 proteins related to cellular signaling are shown as gene symbols with mean signal intensities from 2 independent spots. FGF-1 is highlighted in bold letters with an open column



control from the one on the CCN2-conjugated surface. Data analysis and computation of dissociation constant (Kd) were performed by the BIAevaluation software version 4.1 with the single cycle kinetics support package (GE Healthcare)(Aoyama, Kubota and Takigawa, 2012).

RNA extraction and quantitative real-time reverse transcription polymerase chain reaction (RT-PCR)

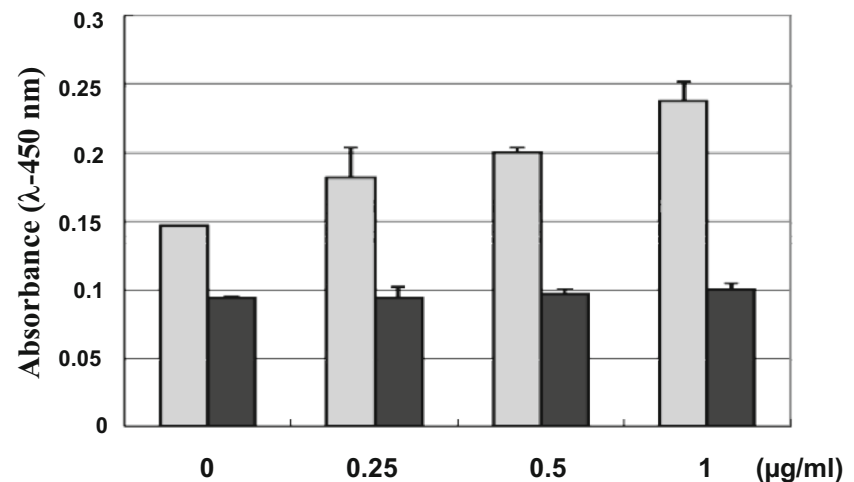
The cells were cultured at 37 °C with 5 % CO₂ in the air and were allowed to reach confluence. Then, total RNA was extracted and purified from the cells with Isogen (Nippongene, Tokyo, Japan) or RNeasy kit (Qiagen, Hilden, Germany), following the manufacturers' instructions. Total RNA (500 ng) was reverse transcribed by avian myeloblastosis virus (AMV) reverse transcriptase (Takara, Otsu, Japan) at 42 °C for 30 min, according to the

manufacturer's protocol. Real-time PCR was performed by using TOYOBO SYBR Green PCR Master Mix (TOYOBO, Osaka, Japan) in a LightCycler™ system (Roche, Basel, Switzerland) as described previously (Moritani et al. 2005). The nucleotide sequences of the primers used are as follows: 5'-GCA GGC TAG AGA AGC AGA GC -3' (sense) and 5'-ATG TCT TCA TGC TGG TGC AG -3' (antisense) for *CCN2*; 5'-ACA AGG GAC AGG AGC GAC-3' (sense) and 5'-TCC AGC CTT TCC AGG AAC A-3' (antisense) for *FGF1*; 5'-GCC AAA AGG GTC ATC ATC TC-3' (sense) and 5'-GTC TTC TGG GTG GCA GTG AT-3' (antisense) for *GAPDH*.

General

Unless otherwise specified, all of the evaluations were performed at least twice, yielding comparable results.

Fig. 2 Direct binding of FGF-1 to CCN2 as evaluated by solid phase binding assay. Binding of FGF-1 to CCN2 (gray columns) and BSA (black columns) coated onto an ELISA plate surface was colorimetrically evaluated. Concentrations of FGF-1 applied onto the ELISA plate are indicated on the abscissa



Results

CCN2 partner candidates as revealed by interactome analysis

In order to find out possible counterparts of CCN2, we screened more than 9,000 proteins on a microarray and found 139 proteins giving significant binding signal to the CCN2 probe in duplicate. From these candidates, proteins that appear to be related to extracellular signaling were further selected. Figure 1 shows the summary of the signals yielded by these candidates. These proteins include several lectins and ion channels, but only a few growth factors were involved therein. Among them, FGF-1 showed the highest signals for CCN2, and thus was selected for further analysis in this study.

Direct and strong binding of FGF-1 to CCN2

To confirm the specific binding of FGF-1 to CCN2, a conventional solid phase binding assay was initially employed. As a result, significant binding of FGF-1 to CCN2 coated on the ELISA plate was observed in a dose dependent manner, whereas that onto BSA showed only a background signal without dose response (Fig. 2), indicating specific interaction between FGF1 and CCN2. Next, molecular interaction of these 2 molecules was kinetically analyzed by an SPR methodology. As shown in Fig. 3, increasing concentrations of FGF-1 revealed dose-dependent response in the resonance units representing molecular interaction, as observed in the solid-phase binding assay. Based on the sensorgram obtained, we computed the K_d of this FGF-1-CCN2 interaction. The K_d value was 3.98 nM, which is indeed comparable to the affinity of interactions between

FGFs and their canonical receptors (Aoyama, Kubota and Takigawa, 2012).

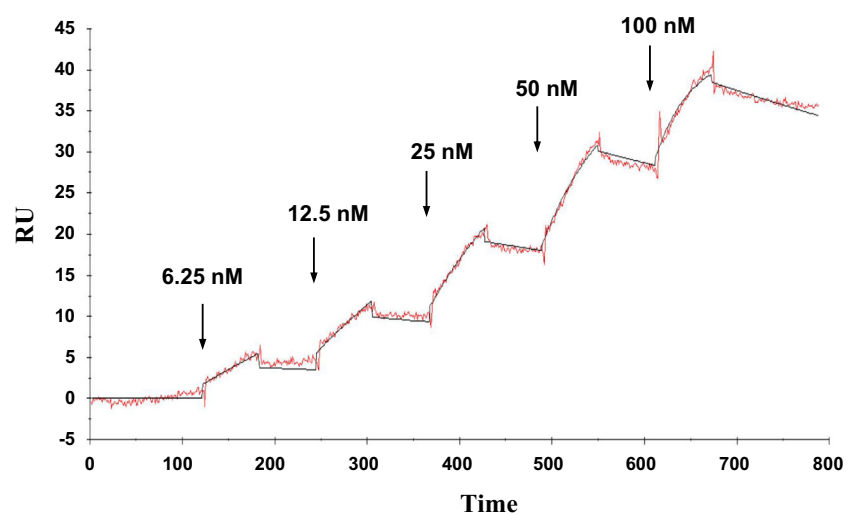
Specific expression of CCN2 and FGF-1 in chondrocytic cells

Although FGF-1 was capable of binding to CCN2, it is necessary for these molecules to actually coexist in biological microenvironments in order to have interactions. In our previous studies, vigorous production of CCN2 by a human chondrocytic cell line, HCS-2/8, was observed; however, it remained to be examined whether FGF-1 is produced therein. Suspecting possible co-existence and molecular interaction of FGF-1 and CCN2, we evaluated the gene expression of FGF-1 in those cells. Quantitative real-time RT-PCR analysis indicated strong gene expression of both CCN2 and FGF-1 in HCS-2/8 cells, whereas human cell lines from different origins showed much lower expression (Fig. 4). These results suggest specific production of FGF-1 by chondrocytes and possible interaction between FGF-1 and CCN2 in cartilage.

Co-expression of CCN2 and FGF-1 in human chondrocytes from OA patients

Osteoarthritis (OA) is a major locomotive disorder of joints that seriously affects the quality of life of patients. Although CCN2 is known to be produced in OA cartilage, no report described the co-expression of *CCN2* and *FGF1* in chondrocytes from OA patients. As a final step of our present research, we performed the same analysis as we employed for Fig. 4 with OA chondrocytes. As displayed in Fig. 5, expression of these genes was distinctly observed in chondrocytes

Fig. 3 Kinetic analysis of direct binding between FGF-1 and CCN2 by SPR methodology. Interaction of FGF-1 as an analyte and CCN2 immobilized to the sensor chip was kinetically analyzed with Biacore X system. The experiment and analysis were performed following the single kinetics protocol with sequential injection of increasing concentrations of the analyte, as shown on the graph. RU: resonance units



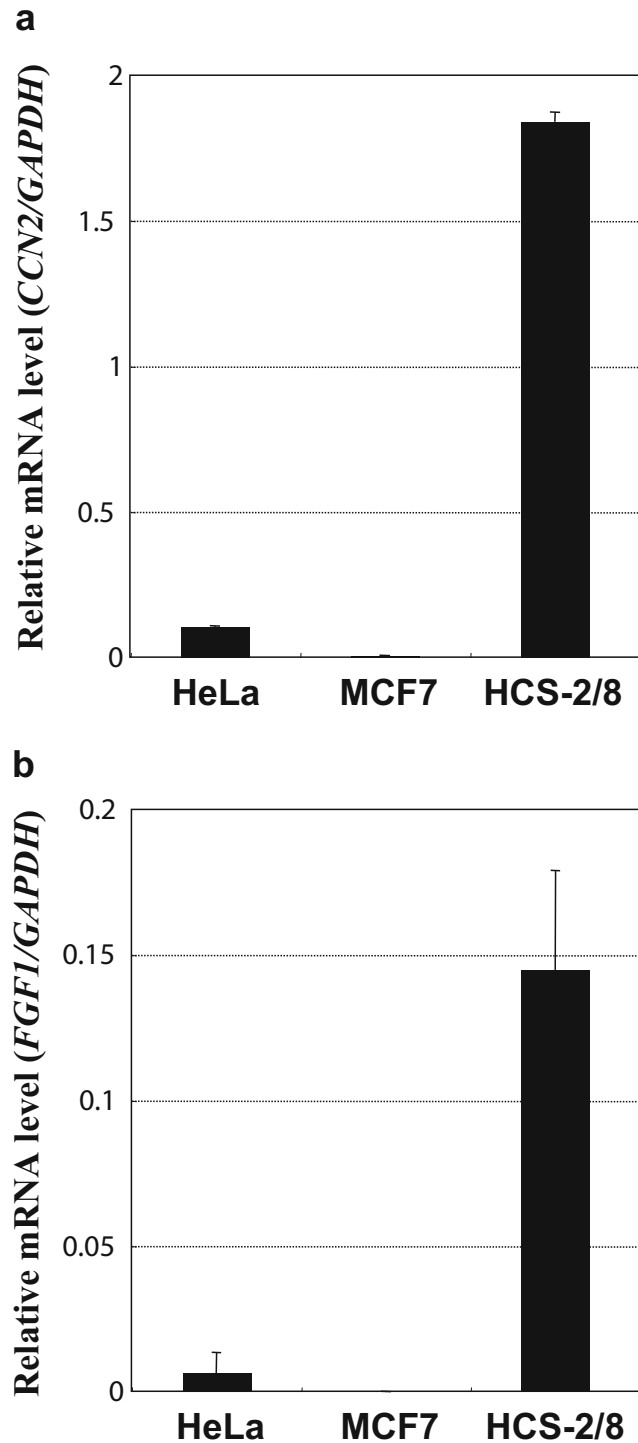


Fig. 4 Specific expression of *FGF-1* and *CCN2* in human chondrocytic HCS-2/8 cells. Distinct gene expression of *CCN2* **a** and *FGF-1* **b** was observed in chondrocytic HCS-2/8 cells, whereas the other non-chondrocytic cell lines showed almost no expression of those genes. Gene expression levels are represented by relative values against those of GAPDH with error bars of standard deviations

from the articular cartilage of OA patients, suggesting the presence of FGF-1 and CCN2-FGF-1 interaction in articular cartilage.

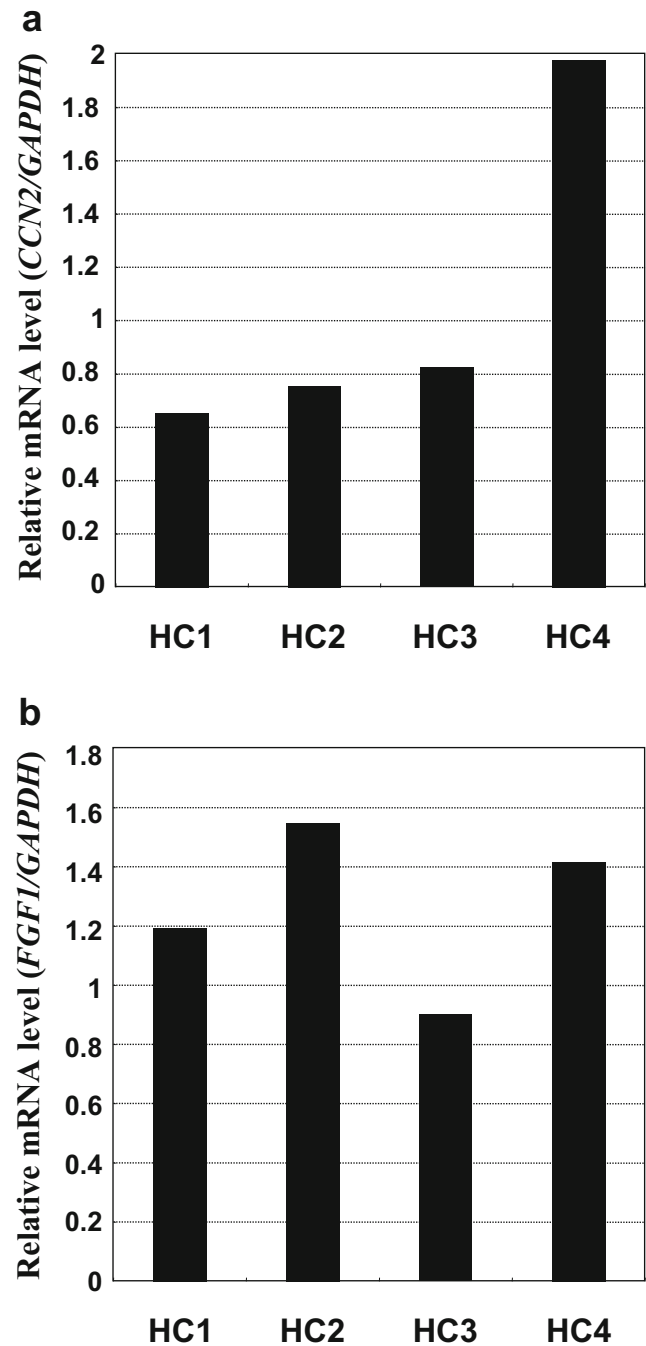


Fig. 5 Concomitant expression of FGF-1 and CCN2 in human articular chondrocytes from OA patients. Expression of *CCN2* **a** and *FGF-1* **b** in human chondrocytes obtained from knee joints upon total knee replacement therapy of OA. Values obtained from each individual (HC1 - 4) are shown. Gene expression levels are represented by relative values against those of GAPDH

Discussion

In this study, we, for the first time, found that FGF-1 and CCN2 specifically and directly interact with high affinity. Recently, interaction of FGF signaling and CCN2 has been attracting the interest of the scientists in the relevant fields. In

fact, FGF-2, another classical member of the FGF family, binds to CCN2 and thus affects the phenotype of chondrocytes (Nishida et al., 2011). Moreover, direct interaction between FGF receptor 2 (FGFR2) and CCN2 is also indicated, which results in the modification of intracellular signaling in osteoblastic cells (Aoyama, Kubota and Takigawa, 2012). Together with the present data, these findings suggest an intimate relationship between CCN family and FGF signaling. Therefore, mutual interaction has to be considered, whenever the functionality of each member of CCN and FGF families is characterized.

In relation to cartilage biology, molecular function and biological role have been extensively characterized, particularly in FGF-2 and FGF-18 (Ellman, et al., 2008). In the case of FGF-18, its anabolic effects on chondrocytes are firmly supported by a number of experimental data including increased proteoglycan synthesis and type II collagen production by FGF-18 (Ellman, et al., 2008). On the contrary, a number of previous publications indicate that FGF-2 enhances proliferation of chondrocytes, whereas ECM degradation is accelerated simultaneously (Ellman, et al., 2008). Although much less information is available, the functionality of FGF-1 in cartilage described in a few previous studies appears contradictory. One report shows the induction of proliferation of sheep chondrocytes (Acosta et al., 2006), while another indicates its repression in rodent chondrocytes mediated by the STAT pathway (Sahni et al., 1999). The discrepancy between these results is anticipated to result from the difference in the molecular background of the experimental systems used, in which CCN2 may be involved.

Since FGF-1 is structurally and functionally similar to FGF-2, FGF-1 itself and CCN2-FGF-1 interaction are suspected to play some roles in the biology of articular cartilage. Investigation to clarify this topic is currently in progress.

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