Catabolic effects of FGF-1 on chondrocytes and its possible role in osteoarthritis

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Abstract Fibroblast growth factor 1 (FGF-1) is a classical member of the FGF family and is produced by chondrocytes cultured from osteoarthritic patients. Also, this growth factor was shown to bind to CCN family protein 2 (CCN2), which regenerates damaged articular cartilage and counteracts osteoarthritis (OA) in an animal model. However, the pathophysiological role of FGF-1 in cartilage has not been well investigated. In this study, we evaluated the effects of FGF-1 in vitro and its production in vivo by use of an OA model. Treatment of human chondrocytic cells with FGF-1 resulted in marked repression of genes for cartilaginous extracellular matrix components, whereas it strongly induced matrix metalloproteinase 13 (MMP-13), representing its catabolic effects on cartilage. Interestingly, expression of the CCN2 gene was dramatically repressed by FGF-1, which repression eventually caused the reduced production of CCN2 protein from the chondrocytic cells. The results of a reporter gene assay revealed that this repression could be ascribed, at least in part, to transcriptional regulation. In contrast, the gene expression of FGF-1 was enhanced by exogenous FGF-1, indicating a positive feedback

system in these cells. Of note, induction of FGF-1 was observed in the articular cartilage of a rat OA model. These results collectively indicate a pathological role of FGF-1 in OA development, which includes an insufficient cartilage regeneration response caused by CCN2 down regulation.

Keywords FGF-1 · CCN2 · Osteoarthritis · Chondrocytes · Cartilage

Introduction

Osteoarthritis (OA) is known to be a degenerative disease of the joints, which is caused by cartilage damage with the involvement of a group of catabolic factors such as inflammatory cytokines and matrix metalloproteinases (Berenbaum 2013). Recent studies attempted to examine the role of growth factors, including fibroblast growth factors (FGFs), and their effects on articular cartilage as well as on the progression of OA. The family of fibroblast growth factors (FGFs) is widely known to be involved in a variety of developmental and pathological processes in the human body, which family comprises 22 members including FGF-1 to FGF-23; although FGF-15 has not been identified in humans (Itoh and Ornitz 2011). Among the 22 members of this large family of growth factors, their molecular function and biological role in cartilage have been relatively well characterized in only 2 members, FGF-2 and FGF-18 (Ellman et al. 2008). Indeed, a significant number of previous studies indicate that FGF-2 enhances the proliferation of chondrocytes, whereas extracellular matrix (ECM) degradation is accelerated by this molecule. In contrast, FGF-18 was shown to act as an anabolic factor for chondrocytes, increasing proteoglycan synthesis and type II collagen production (Ellman et al. 2008). In addition to these 2 FGF family members, a few other members were shown to play certain roles in cartilaginous

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tissues (Weksler et al. 1999). FGF-1, previously also known as acidic FGF, is also suspected to play some roles in the biology of articular cartilage, since FGF-1 is structurally and functionally similar to FGF-2 (Ornitz and Itoh 2015). However, only limited information on the functionality of FGF-1 in cartilage is currently available, with study results appearing to be contradictory. For example, a few reports indicated a proliferative effect on sheep and rabbit chondrocytes (Froger-Gaillard et al. 1989; Acosta et al. 2006), whereas another suggested the opposite outcome in rodent chondrocytes, occurring via a mechanism involving the STAT pathway (Sahni et al. 1999). The discrepancy between these results may have resulted from differences in the molecular background between different species. Therefore, investigation on FGF-1 conducted with human cells is of particular interest from the medical point of view.

Recently we found that the FGF-1 gene is actually expressed in chondrocytes obtained from the articular cartilage taken from osteoarthritis patients (Abd El Kader et al. 2014a). In the same report, we described that FGF-1 directly binds to CCN2, which plays a critical role in cartilage development, defense and regeneration. CCN2 is the founding member of the CCN family of proteins, with 6 members found in mammals. These proteins are commonly characterized by a conserved modular structure and matricellular actions, interacting with various molecular counterparts (Perbal and Takigawa 2005; Rachfal and Brigstock 2005; Leask and Abraham 2006; Jun and Lau 2011; Khattab et al. 2015). Particularly, CCN2 is known to be involved in the development of a wide variety of tissues including bone, cartilage, olfactory bulb, pancreas, and eye (Takigawa 2013; Kubota and Takigawa 2015). Indeed, *Ccn2*-null mice are characterized by delayed endochondral ossification leading to skeletal defects (Ivkovic et al. 2003; Kawaki et al. 2008). Concerning articular cartilage, CCN2 enhances both proliferation and maturation of articular chondrocytes without promoting ectopic hypertrophic differentiation (Nishida et al. 2002). Consistent with this finding, CCN2 was shown to regenerate damaged articular cartilage in experimental rat osteoarthritis (OA) models in vivo (Nishida et al. 2004; Abd El Kader et al. 2014b). Furthermore, cartilage-specific overexpression of CCN2 provides resistance to OA-like changes caused by continuous mechanical loading during aging in mice without showing no appreciable adverse effects (Itoh et al. 2013). These findings together indicate CCN2 as a critical molecule to maintain and recover the integrity of articular cartilage.

Although we found earlier that FGF-1 is produced by human chondrocytes and physically interacts with CCN2 (Abd El Kader et al. 2014a), the pathobiological effects of FGF-1 itself on human chondrocytic cells are still unclear. Therefore, in this present study, we investigated this scientific issue and clarified the genetic interaction between these factors and its pathological significance, which was supported by findings obtained in vivo as well.

Materials and methods

Cell culture

The human chondrocytic cell line HCS-2/8, which was established from a human chondrosarcoma, retains chondrocytic properties represented by the gene expression and production of type II collagen and aggrecan (Takigawa et al. 1989). Previously, chondrocytes from sheep and mice revealed almost opposite responses to FGF-1 (Acosta et al. 2006; Sahni et al. 1999). Therefore, we selected this human cell line for in vitro experiments in relation to OA, which is a human joint disorder. 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.

Enzyme-linked immunosorbent assay (ELISA)

For performing an enzyme-linked immunosorbent assay (ELISA), HCS-2/8 were seeded into 6-well plates at a density of 4×10^4 cells/cm², maintained in DMEM with 10% FBS, and allowed to reach confluence. Thereafter, the medium was changed to DMEM with 0.5% FBS; and the cells were incubated in it for 12 h. Next, recombinant CCN2 (rCCN2) was added at a concentration of 50 ng/ml, after which FGF-1 was added at a concentration of 25 ng/ml; and the cultures were continued for another 12 h. Lastly, the cell lysates were collected and evaluated by using a sandwich ELISA system with 2 different anti-human CCN2 monoclonal antibodies, one for capture and the other for detection, as described previously (Tamatani et al. 1998; Kubota et al. 2004).

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

For the analysis of RNA, the cells were cultured in D-MEM with 10% FBS and allowed to reach confluence. Thereafter, the medium was replaced with DMEM containing 0.5% FBS; and the cells were then incubated for 24 h. Subsequently, FGF-1 was added at a concentration of 25 ng/ml, and incubation was continued for an additional 12 h. Then, total RNAs were extracted and purified from the cells by use of Isogen (Nippongene, Tokyo, Japan) or an RNeasy kit (Qiagen, Hilden, Germany) by following the manufacturers' protocols. Total RNA (500 ng) was reverse transcribed by avian myeloblastosis virus (AMV) reverse transcriptase (Takara, Otsu, Japan) at 42 °C for 30 min, following the manufacturer's instructions. Quantitative real-time PCR was performed by using TOYOBO SYBR Green PCR Master Mix (TOYOBO, Osaka, Japan) with a StepOnePlus™ Real-time PCR System (Applied Biosystems, Basel, Switzerland).

The nucleotide sequences of the primers used were as follows: 5'- GCA GGC TAG AGA AGC AGA GC -3' (sense) and

5'- ATG TCT TCA TGC TGG TGC AG -3' (antisense) for human *CCN2*; 5'-ACA AGG GAC AGG AGC GAC-3' (sense) and 5'-TCC AGC CTT TCC AGG AAC A-3' (antisense) for human *FGF1*; 5'-TTC GGG CAG AAG AAG GAC-3' (sense) and 5'-CGT GAG CTC CGC TTC TGT-3' (antisense) for human *ACAN*; 5'-GAG GGC AAT AGC AGG TTC ACG TA-3' (sense) and 5'-TGG GTG CAA TGT CAA TGA TGG-3' (antisense) for human *COL2A1*; 5'-TGG TGG TGA TGA AGA TGA TTT GTC T-3' (sense) and 5'-AGT TAC ATC GGA CCA AAC TTT GAA G-3' (antisense) for human *MMP13*; and 5'-GCC AAA AGG GTC ATC ATC TC-3' (sense) and 5'-GTC TTC TGG GTG GCA GTG AT-3' (antisense) for human *GAPDH*.

CCN2 promoter reporter gene assay

For the reporter gene assay, HCS-2/8 cells were seeded into 24-well plates at a density of 1.5×10^5 cells/cm², maintained

in DMEM with 10% FBS, and allowed to reach 60–70% confluence. Thereafter, the medium was changed to DMEM with 1% FBS immediately before the addition of FGF-1 at 25 ng/ml and/or plasmid DNA. A human *CCN2* promoter (from −802 bp to +22 bp around the transcription initiation site-driven firefly luciferase reporter construct, pTS589, was used for transfection studies (Eguchi et al. 2001). A herpes simplex virus *TK* promoter-driven *Renilla* luciferase construct, pRL-TK, was also utilized as an internal control. The cells were transfected with the aid of an optimized amount of FuGENE 6 (Roche). After addition of FGF-1 and/or plasmid DNA, the medium was changed every 12 h with concurrent addition of FGF-1; and the cells were further incubated for 48 h. Then, the cells were collected after having been lysed with Passive Lysis Buffer (Promega, Fitchburg, WI, USA) with gentle rocking for 10–15 min. Thereafter, the Dual Luciferase system (Promega) was used for measurement of firefly and *Renilla* luciferase activities, and calculation of relative ratios were carried out with a luminometer (Fluoroskan

Fig. 1 Effects of FGF-1 on the chondrocytic phenotype of HCS-2/8 cells. **a** Effect on the gene expression of cartilaginous extracellular matrix (ECM) components. Gene expression of aggrecan core protein (*ACAN*) and type II collagen (*COL2A1*) was comparatively analyzed in the presence (FGF-1) and absence (C) of 25 ng/ml of FGF-1. Relative gene expression levels versus glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*) are presented. Mean values from 3 independent cell cultures are shown with error bars representing standard deviations. Asterisks (**) denote a significant difference at $p < 0.01$ between the 2 groups. **b** Effect on the gene expression of matrix metalloproteinase-13 (*MMP13*), an enzyme that degrades cartilaginous ECM components. Experiments and representation of the results were performed as indicated for panel A

Ascent FL, LabSystems, Helsinki, Finland). All transfection experiments were performed 4 times, each in quadruplicate.

Animal model

A typical OA model chemically induced by monoiodoacetic acid (MIA) was employed. Sixteen rats (7 weeks old) were injected in the right knee with MIA in sterile PBS (60 mg/ml) intraarticularly through the patellar ligament at a dose of 6 mg on day 0. The control (left knee joint) was injected with PBS only. The rats were anesthetized again at 14 days after the injection with MIA and were re-injected with 1 μ g rCCN2 in gelatin hydrogel (Wako, Osaka, Japan), as described previously (Nishida et al. 2004; Abd El Kader et al. 2014b). The rats were sacrificed 21 days after the injection with MIA, and knee joints were obtained and prepared as mentioned above. The Animal Committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences approved these procedures.

Immunohistochemistry

The knee joints from the rats were fixed in a 4% paraformaldehyde (PFA) solution overnight. After that procedure, the tissues were thoroughly decalcified in Morse's solution and then embedded in paraffin blocks. Sagittal sections were prepared, using a microtome, at a thickness of 5 μ m with a microtome and mounted on silane-coated glass slides. For immunostaining, the sections were deparaffinized and rehydrated according to standard protocols and were then treated with 3% hydrogen peroxide for 10 min. Afterwards, Antibody Diluent (Zymed, South San Francisco, CA, USA) was applied to the sections as a blocking buffer for 30 min. The sections were then incubated overnight at 4 °C with an anti-FGF-1 (Anti-FGF1 mouse- poly, Abnova), or anti-MMP-13 (Goat anti-MMP-13 polyclonal, Millipore) in the blocking buffer (1:100 dilution). A polymer conjugate (Max-PoM-17 ml, Invitrogen, Carlsbad, CA, USA) was next added to the sections, followed by a 1-h incubation. DAB staining was then performed as described in the manufacturer's protocol (Invitrogen, PCNA kit reagent). Thereafter, the sections were dehydrated, mounted (Permount, FALMA, Tokyo, Japan), and examined under an Olympus microscope (magnification: $\times 100$; Olympus Corporation, Tokyo, Japan).

Statistical analysis

Unless otherwise specified, all of the evaluations were performed at least twice, yielding comparable results. Statistical comparisons between 2 experimental groups were performed by using Student's *t*-test.

Results

Effects of FGF-1 on the expression of anabolic and catabolic genes in human chondrocytic cells

Since we had found earlier that the FGF-1 is expressed in chondrocytes from OA patients (Abd El Kader et al. 2014a), we evaluated the effects of FGF-1 on chondrocyte marker gene expression in the human chondrocytic HCS-2/8 cell line

Fig. 2 **a** Auto-induction of the FGF-1 gene (*FGF1*) expression in HCS-2/8 cells by exogenous FGF-1 at a concentration of 25 ng/ml. **b** Striking repression of CCN2 gene (*CCN2*) expression in the same cells by 25 ng/ml of FGF-1. Relative gene expression levels versus glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*) are presented. Mean values from 3 independent cell cultures are shown with error bars representing standard deviations. Asterisks (**) denote a significant difference at $p < 0.01$ between the 2 groups

to estimate the role of this factor in OA development. As FGF-1 is known to exhibit sufficient biological effects at 25 ng/ml (Byrd et al. 1999), its effect was examined at this dose. Quantitative real-time PCR analysis of the RNA from HCS-2/8 cells treated with recombinant FGF-1 revealed that the expression of genes encoding 2 major cartilaginous ECM components, aggrecan and type II collagen, was markedly repressed (Fig. 1a). In contrast, treatment of HCS-2/8 cells with FGF-1 dramatically induced the gene expression of MMP-13, which enzyme degrades ECM (Fig. 1b). These results strongly indicate that FGF-1 may have acted as a catabolic factor in articular cartilage, inhibiting the production and promoting the degradation of ECM towards the development of OA.

Auto-induction and CCN2 repression by FGF-1 in chondrocytic cells

In addition to that on basic cartilaginous marker genes, the impact of FGF-1 on the production of signaling molecules, which play critical roles in cartilage biology, had to be evaluated. Firstly, the effect of recombinant FGF-1 on the endogenous FGF-1 was evaluated. Quantitative RNA analysis indicated that FGF-1 up-regulated the expression of FGF-1 itself, suggesting a positive-feedback system to enhance the action of this molecule in cartilage (Fig. 2a). Next, the effect of FGF-1 on CCN2, which promotes the regeneration of articular cartilage by enhancing the cellular activities of chondrocytes, was evaluated. Interestingly, FGF-1 drastically down-

Fig. 3 **a** Reduced production of the CCN2 protein in HCS-2/8 cells by exogenous FGF-1 treatment. HCS-2/8 cells were treated with 25 ng/ml of FGF-1 or an equal volume of PBS (C), and then cell-associated CCN2 was quantified by ELISA. Data are presented as relative protein levels versus the control without FGF-1 treatment. **b.** Effect of FGF-1 on the transcriptional activity of a CCN2 proximal promoter. Structures of the reporter plasmids used for the evaluation are illustrated at the top. The plasmid pTS-589 contains a firefly luciferase gene (*F luc*) driven by a *CCN2* promoter fragment (from -802 to +22 bp around the transcription initiation site) followed by an SV40 polyadenylation signal sequence, whereas pGL3 Δ P lacks the promoter (negative control). Results with these plasmids are shown at the bottom. Promoter activities are shown as firefly luciferase activities standardized by the internal control (*Renilla* luciferase activities from pRL-TK). Asterisks (**) denote significant difference at $p < 0.01$ between the 2 groups

regulated CCN2 expression (Fig. 2b). Since this FGF-1 action is quite important, we examined whether the production of CCN2 protein was affected by FGF-1 or not. As shown in Fig. 3a, ELISA results consistently revealed that the addition of FGF-1 dramatically decreased the protein level of CCN2 in the chondrocytic cells. These results firmly indicate the strong repressive effect of FGF-1 on CCN2 production by these chondrocytic cells.

Effect of FGF-1 on the CCN2 promoter activity in chondrocytic cells

The findings obtained for both mRNA and protein of CCN2 suggested that FGF-1 down-regulated CCN2 production at the transcriptional level. Therefore, in order to analyze the regulatory mechanism of CCN2 down-regulation by FGF-1, we utilized a proximal *CCN2* promoter reporter construct. The effect of FGF-1 on the transcriptional activity of the CCN2 promoter was examined by using pTS589, which is a reporter plasmid that contains a firefly luciferase gene driven by an 800-bp CCN2 promoter segment. The results of this assay revealed that FGF-1 significantly decreased the luciferase production under the control of the CCN2 promoter segment (Fig. 3b), suggesting negative transcriptional regulation of *CCN2* by FGF-1.

Effect of CCN2 on the gene expression of FGF-1

We also evaluated the effect of CCN2 on the gene expression of FGF-1 at a concentration optimized in a previous study (Nishida et al. 2002). Unlike with FGF-1 treatment, CCN2 treatment did not yield significant difference in the mRNA level of FGF-1 in HCS-2/8 cells with (Fig. 4b) or without (Fig. 4a) FGF-1 stimulation, despite showing a stable tendency to decrease it.

Production of FGF-1 and MMP-13 in the osteoarthritic articular cartilage in a rat model in vivo

Finally, we performed an in vivo experiment using a rat model to confirm that FGF-1 is actually produced and involved in the development of OA. For this objective, an established OA model, in which OA-like changes can be induced by the local application of glycolysis-inhibiting MIA, was employed. After the MIA application, the loss of the integrity of cartilaginous ECM was confirmed by Toluidine blue staining (data shown in Abd El Kader et al. 2014b). Indeed, damaged nuclear/cellular remnant without distinct structure is observed in the MIA treated articular cartilage (Fig. 5). Immuno-histochemical analysis of the affected joints revealed significant signals indicating FGF-1, whereas control joints injected with PBS showed no such signals (Fig. 5). Subsequent injection of CCN2 in gelatin hydrogel, which could regenerate damaged articular cartilage in this OA model, has decreased the amount of FGF-1 in the articular cartilage. Furthermore, enhanced MMP-13 production

Fig. 4 Effects of CCN2 at a concentration of 50 ng/ml on the gene expression of FGF-1 in the absence (a) or presence (b) of 25 ng/ml of FGF-1. Relative gene expression levels versus glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*) were standardized against the control in each panel. Mean values from 3 independent cell cultures are shown with error bars representing standard deviations.

was observed as well in the OA articular cartilage, confirming the effect of FGF-1 on MMP-13 gene expression (Fig. 6). In contrast to articular cartilage, no specific signals were induced by MIA treatment in joint tissues surrounding it (Fig. 6).

Discussion

For a long time, the role of FGF-1 in OA development remained controversial. However, our present study using a

Fig. 5 Immunohistochemical analysis of FGF-1 in an experimentally induced OA model of rat articular cartilage. Distinct FGF-1 signals (brown) are observed in monoiodoacetic acid (MIA)-induced OA articular cartilage (MIA), whereas no clear signal is visible in normal articular cartilage (Normal). Faint nuclear staining in purple observed in the normal articular cartilage is a background, which appears even without the primary antibody (Normal without α -FGF1). Induction of FGF-1 by MIA is attenuated by CCN2 treatment after the cartilage damage with MIA (MIA treated with rCCN2). All of the joints examined in each group revealed comparable findings, and representative images are shown. Scale bars: 0.1 mm

human chondrocytic cell line provided a comprehensive view on the pathogenic role of this growth factor in OA development (Fig. 7). First, FGF-1 can be induced by initial cartilage damage leading to OA, which is well represented by the observed induction of FGF-1 after MIA exposure in the rat

model. Second, once FGF-1 is produced, the production is amplified through the auto-induction feedback loop of *FGF1* expression. The FGF-1 molecule overproduced in articular cartilage strongly activates the *MMP13* gene, which encodes a proteinase to destroy cartilaginous ECM. The destroyed

Fig. 6 Immunohistochemical analysis of MMP-13 in the articular cartilage of the rat OA model. Enhanced MMP-13 signals (brown) are observed in the MIA-induced OA cartilage (MIA), in comparison with the control (Normal). Areas magnified in the right panels are shown in yellow rectangles in the left panels under a low power magnification. All of the joints examined in each group revealed comparable findings, and representative images are shown. Scale bars: 0.1 mm

Fig. 7 Schematic representation of the role of FGF-1 in the development of OA. FGF-1 is produced upon initial cartilage damage and enhances the degradation of cartilaginous ECM by the induction of MMP-13. At the same time, this molecule strongly prevents the regeneration of articular cartilage by repressing the gene expression of aggrecan core protein and type II collagen. Production of CCN2, a cartilage regenerator that encourage cartilaginous ECM formation, is also blocked by FGF-1, which leads to progressive degradation of the articular cartilage

ECM could be restored by the supplemental production of cartilaginous ECM components including type II collagen and aggrecan by articular chondrocytes, which is, however, strongly restrained by FGF-1 at the gene expression level. Furthermore, FGF-1 also inhibits the gene expression and protein production of CCN2, a molecule that promotes cartilage repair. These findings taken together thus suggest a critical role for FGF-1 in the development of OA.

Interestingly, such a repressive effect of FGF-1 on CCN2 production was strongly implicated by the results of a recent study on lung fibrosis as well (Shimbori et al. 2016). According to that study, overexpression of FGF-1 by an adenoviral vector strongly attenuated the TGF- β 1-induced lung fibrosis in a rat model in vivo. In that same study, evaluation in vitro with human normal lung fibroblasts also indicated the repression of TGF- β 1-induced α -smooth muscle actin (α -SMA) gene expression by FGF-1. Considering that CCN2 is induced by TGF- β 1 and induces α -SMA production (Abd El Kader et al. 2013), leading to lung fibrosis, it is clear that FGF-1 also represses CCN2 in those cells. Therefore, the FGF-1-TGF- β 1 axis may constitute the central machinery that regulates a variety of TGF- β 1-CCN2-associated physiological and pathological events. Examination of the effect of FGF-1 on articular cartilage by direct intraarticular injection may provide further evidence to support this notion, and thus is now under planning.

The regenerative effects of CCN2 are widely recognized, as was summarized in Introduction. In fact, CCN2 was shown to enhance the regenerative response in articular chondrocytes, promoting the gene expression of type II collagen and aggrecan genes per se (Nishida et al. 2002). Therefore, the observed strong effect of FGF-1 on these anabolic genes can be, at least in part, mediated by the repression of CCN2 expression. By using a reporter gene assay system, we found that the resultant decrease in CCN2 production by

FGF-1 could be partly ascribed to transcriptional repression through the *CCN2* proximal promoter. It should be noted that the activation of the Smad signaling pathway was attenuated upon treatment of lung fibroblasts with FGF-1 (Shimbori et al. 2016), suggesting the possible involvement of this Smad pathway also in our case. However, the negative effect of FGF-1 on CCN2 promoter activity appears to have been too weak to account for the observed decrease at mRNA and protein levels. We strongly suspect the additional involvement of chromatin regulation and post-transcriptional events. In this scenario, the involvement of multiple post-transcriptional regulatory elements in the 3'-untranslated region of the *CCN2* gene should be particularly noted (Kubota et al. 2000; Ohgawara et al. 2009). Extensive investigation to clarify the regulatory mechanism of CCN2 by FGF-1 is still underway. In contrast to such a prominent effect of FGF-1 on CCN2, no significant effect on *FGF1* was observed after CCN2 treatment of HCS-2/8 cells (Fig. 4). Nevertheless, less FGF-1 in the MIA-damaged cartilage could be observed after CCN2 treatment (Fig. 5) in vitro, which may have resulted from amelioration of damage due to the regenerative effects of CCN2. Although CCN2 may not have directly repressed *FGF1* expression, this molecule could attenuate *FGF1* expression indirectly by regenerating normal cartilage after 14 days of application in vivo. It is also possible that long term exposure to CCN2 gradually released in vivo may directly affect the induced FGF-1 production, since even the short term CCN2 treatment showed a stable tendency to repress FGF1 expression (Fig. 4). Of note, CCN2 in the gelatin hydrogel enables gradual release of CCN2 in vivo up to 14 days (Kikuchi et al. 2008).

Our previous report demonstrated the direct interaction between FGF-1 and CCN2 (Abd El Kader et al. 2014a), but its pathological significance remains unclear. Of note, an earlier study of ours revealed the direct interaction between CCN2 and FGF receptors (FGFRs; Aoyama et al. 2012). In the case of another FGF member, FGF-2, complex formation among FGF-2, FGFR, and CCN2 was indicated, altering the intracellular signaling and resultant biological effects of FGF-2 (Nishida et al. 2011; Aoyama et al. 2012). Future studies examining similar complex formation may provide more insights into the molecular behavior of FGF-1 in cartilage and its role in OA. From a clinical point of view, similar evaluation with another in vivo system that better models human OA is necessary, since MIA treatment induces acute damage, which may not happen in human OA cases. Since the locomotive ability of the MIA-treated limbs is rapidly impaired, it may also affect contralateral limbs. As a next step, employment of anterior crucial ligament transection model is considered in our future study, together with the evaluation of human OA samples.

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