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RESEARCH ARTICLE

Bipartite regulation of cellular communication network factor 2 and fibroblast growth factor 1 genes by fibroblast growth factor 1 through histone deacetylase 1 and fork head box protein A1

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Abstract

Fibroblast growth factor 1 (FGF-1) is the first FGF family member, and it induces proliferation of fibroblasts and other types of the cells. However, recent studies are uncovering unexpected functions of this molecule. Our previous study redefined this growth factor as a catabolic molecule produced in cartilage upon metabolic insult. Indeed, FGF-1 was found to repress the gene expression of cellular communication network factor 2 (CCN2), which protects and regenerates cartilage, amplifying its own production through positive feedback regulation. In the present study, we investigated the molecular mechanism of this bipartite *CCN2* repression and *FGF1* activation by FGF-1 in chondrocytes. Repression of *CCN2* and induction of *FGF1* in human chondrocytic cells were both partly abolished by valproic acid, an inhibitor of histone deacetylase 1 (HDAC1), indicating the involvement of chromatin remodeling by histone acetylation in this system. In contrast, RNA degradation analysis suggested no contribution of post-transcriptional regulation of the mRNA stability to the effects conferred by FGF-1. Suspecting a regulation by a specific transcription factor, we next sought a candidate in silico from a large dataset. As a result, we found fork head box protein A1 (FOXA1) as the transcription factor that bound to both *CCN2* and *FGF1* loci. Functional analysis demonstrated that FOXA1 silencing significantly attenuated the *CCN2* repression and *FGF1* induction caused by FGF1. These findings collectively indicate that the bipartite regulation by FGF-1 is enabled by the combination of chromatin remodeling by HDACs and transcriptional modulation by FOXA1 with unknown transcriptional coactivators of opposite functionalities.

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

Introduction

Fibroblast growth factor 1 (FGF-1), which was initially named acidic FGF, is the first member of the FGF family, and it comprises at least 22 members in humans (Ornitz and

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Itoh 2015). As the name suggests, this protein was originally discovered as an extracellular messenger that promotes the proliferation of various types of the cells, including fibroblasts. However, later studies revealed the multiple functionalities of FGF-1, some of which were unexpected based on the original name. Indeed, FGF-1 is known to play significant roles in embryonic development, wound healing, neurogenesis and angiogenesis (Ornitz and Itoh 2015). Unexpectedly, genetic removal of *Fgf1* from mice revealed no apparent deficiency in development, probably due to the compensatory actions of other family members; FGF-2, in particular. Interestingly, although *Fgf1*-null mice showed no apparent phenotypic changes under regular conditions, these mice were found to develop hyperglycemia and insulin resistance upon high fat diet challenge (Suh et al. 2014). Subsequent studies uncovered that this growth factor acts as a metabolic hormone, which normalizes hyperglycemia by improving central glucose sensing and peripheral glucose uptake (Gasser et al. 2017). As such, FGF-1 is now believed to be involved in a variety of physiological and pathological events in human body.

From a pathological point of view, it is widely known that activating mutations in FGF receptor (FGFR) genes cause several skeletal disorders, such as achondroplasia, in humans (Ornitz and Marie 2015). Therefore, FGFR ligands are indicated to play significant roles in skeletal development and diseases. In the case of FGF-1, early studies suggested an active role of this protein in bone fracture repair (Wang et al. 2019). In this context, our recent study uncovered a novel role of FGF-1 in articular cartilage. Namely, we found that FGF-1 was produced in the articular cartilage in a rat experimental osteoarthritis (OA) model induced by a glycolysis inhibitor, monoiodoacetatic acid (El-Seoudi et al. 2017). OA is a common locomotive disorder caused by degenerative changes in synovial joints, particularly in the articular cartilage (van der Kraan and van den Berg 2012). This cartilage degeneration is usually incurred by continual mechanical overload onto the joints; thus, OA is most frequently observed weight-bearing knee and hip joints. Since OA in these joints severely impairs the quality of life of the patients, OA treatment is a critical medical issue, especially in advanced countries with a number of aged OA patients.

Regarding the pathological role of FGF-1 in OA (Fig. 1), we clarified in a previous study that FGF-1 induced the expression of its own gene and matrix metalloproteinase (MMP) -13, which destroyed cartilaginous extracellular matrix (ECM) and repressed the gene expression of cellular communication factor 2 (CCN2), as well as aggrecan and type II collagen, which are major components of the ECM (El-Seoudi et al. 2017). Among these target genes, *FGF1* and *CCN2* are of particular interest; FGF-1 autoactivates *FGF1* to amplify its catabolic actions on cartilage, whereas CCN2 conducts regeneration of damaged articular cartilage

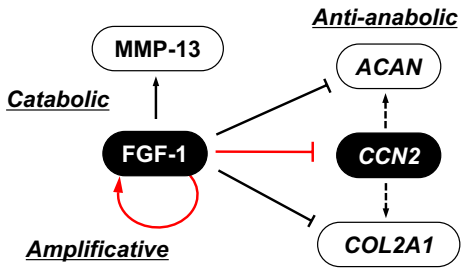


Fig. 1 Summary of catabolic effects of fibroblast growth factor (FGF)-1 on chondrocytes. FGF-1 is involved in osteoarthritis (OA); in which, once FGF-1 is produced, the production is amplified through the auto-induction feedback loop of *FGF1* expression, and activates the matrix metalloproteinase (MMP) -13 gene, which destroys the cartilaginous extracellular matrix (ECM). This could be restored by the supplemental production of cartilaginous ECM components including type II collagen (*COL2A1* product) and aggrecan (*ACAN* product) as well as cellular communication network factor (CCN)2 by articular chondrocytes, which are strongly restrained by FGF-1 at the gene expression level. ECM components and degrading enzyme are shown as white objects, whereas extracellular signaling molecules are in black. Central regulatory actions investigated in this study are described in red

in vitro and in vivo (Nishida et al. 2004; Abd El Kader et al. 2014), supporting energy metabolism (Maeda-Uematsu et al. 2014: Kubota et al. 2015, Akashi et al. 2020). CCN2 is the best-investigated member of the CCN family of six proteins (Perbal 2018) and is known to orchestrate extracellular signaling network via multiple interactions with growth factors (Khattab et al. 2015), cell surface receptors (Lau 2016), and ECM proteins (Kubota and Takigawa 2015). It should be noted that CCN2 overexpressed in the articular cartilage counteracts the development of age-related OA in mice (Itoh et al. 2013). Therefore, the positive and negative regulatory systems of *FGF1* and *CCN2*, respectively, are the central machinery for FGF-1 to exert its catabolic mission in cartilage (Fig. 1). Nevertheless, how *CCN2* and *FGF1* are regulated by FGF-1 is still largely unknown. In this study, we investigated the molecular mechanism of the regulation of *CCN2* and *FGF1* by FGF-1 in chondrocytes and clarified that the same chromatin remodeler and DNA binding transcription factor are mediating the bipartite regulation of these two target genes, in collaboration with unknown transcription coactivators.

Materials and methods

Cell culture

A human chondrocytic cell line HCS-2/8 that originates from a human chondrosarcoma and retains the chondrocytic phenotype (Takigawa et al. 1989; Akashi et al. 2018)

was employed as an in vitro model of human chondrocytes (Maeda-Uematsu et al. 2014; El-Seoudi et al. 2017). These cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2 with Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% fetal bovine serum (FBS).

Evaluation of the effects of valproic acid

Valproic acid (VPA), which is an inhibitor of class I and II histone deacetylases (HDACs), was purchased from Cayman Chemical (Ann Arbor, MI, USA). Before the treatment, HCS-2/8 cells were cultured in D-MEM with 10% FBS and allowed to reach confluence. In order to estimate the cytotoxicity, confluent HCS-2/8 cells in a 96-well multiplate were treated with 0, 0.125, 0.25, 0.5, 1, or 2 μM of VPA for 24 h, and their metabolic activities were evaluated after 2 h by Cell Countng Kit-8 (Dojindo, Kumamoto, Japan). For the evaluation of the effects of FGF-1 in the presence of 0, 0.125, or 0.25 μM of VPA, the cells were prepared in 6-well multiplates. In the beginning, the medium was replaced with DMEM containing 0.5% FBS together with either concentration of VPA and the cells were then incubated for 12 h. Subsequently, FGF-1 was added at a concentration of 25 ng/ml, and the incubation was continued for an additional 12 h. Thereafter, total RNA was harvested and purified as described in the next subsection.

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

Total RNA extraction and purification from the cells were performed by using Isogen (Nippongene, Tokyo, Japan) or an RNeasy kit (Qiagen, Hilden, Germany) as instructed by the manufacturers. Each RNA sample (500 ng) was reverse transcribed by avian myeloblastosis virus (AMV) reverse transcriptase (Takara, Otsu, Japan) for 30 min at 42 °C, as indicated in the manufacturer's instructions. Quantitative real-time PCR was performed with a StepOnePlusTM Real-time PCR System (Applied Biosystems, Basel, Switzerland) by using TOYOBO SYBR Green PCR Master Mix (TOYOBO, Osaka, Japan).

The nucleotide sequences of the DNA primers used for amplification were: 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'-CTA CTA CGC AGA CAC GCA GG-3' (sense) and 5'-CCG CTC GTA GTC ATG GTG TT-3' for human *FOXA1*; 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*.

RNA degradation analysis

The RNA degradation profile was analyzed as previously described (Kubota et al. 2003). Briefly, HCS-2/8 at a density of 4×10^4 cells/cm^2 were seeded into 6-well cell culture plates and were maintained in DMEM containing 10% FBS until the cells reached confluence. Next, the medium was changed to DMEM containing 0.5% FBS, and the cells were then incubated for 12 h. Subsequently, 10 μg/ml of actinomycin D was added to arrest de novo mRNA synthesis, in the presence of 25 ng/ml of recombinant FGF-1. At the same time, total RNA was extracted from cultures without the treatment as 0 h controls. One, two and four hours after the initiation of the treatment, total RNA was isolated as described in the last subsection. RNA degradation profiles were analyzed by quantifying the remaining mRNAs of CCN2 and FGF-1 after the addition of actinomycin D, in comparison with those of 0 h control samples. Four independent cultures were prepared for each experimental condition.

Western blotting analysis

Protein analysis by Western blotting was performed essentially as described in our previous report (Sumiyoshi et al. 2013). HCS-2/8 cells in 6-well cell culture plates were treated with 0, 25, or 50 ng/ml of FGF-1, or the siRNA cocktail against FOXA1, as described in other subsections. Protein concentrations of cell lysates were determined by Pierce™ BCA Protein Assay using bovine serum albumin (BSA) as standards (Thermo Scientific, Rockford, IL, USA). Cell lysate containing equal amounts of total proteins was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). A semi-dry transfer apparatus (Atto Corp., Tokyo, Japan) was used for protein transfer. The membranes were incubated in an anti-FOXA1 (Cat#53,528: Cell Signaling Technology, Danvers, MA, USA) and anti-β-actin (Sigma-Aldrich; Cat# A2228) antibodies overnight at 4 °C. After extensive wash with Tris-buffered saline (TBS) buffers, the blots were incubated for 60 min at room temperature in horseradish peroxidase (HRP)-conjugated secondary antibodies. Thereafter, the membranes were washed again, and the bands were visualized with a chemiluminescence substrate. Images were captured and processed by an image analyzer (LAS-4000 mini, Fuji Film, Tokyo, Japan).

Reporter gene assay

HCS-2/8 cells were seeded into 24-well plates, maintained in DMEM containing 10% FBS, and allowed to reach subconfluence. Then, the medium was changed to DMEM with

1% FBS prior to the addition of FGF-1 at 25 ng/ml and/or plasmid DNA. A reporter gene construct with SV40 promoter-driven firefly luciferase gene containing the entire human CCN2 3'-UTR downstream, pGL3-UTRS, was used to evaluate the post-transcriptional regulation mediated by the 3'-UTR (Kubota et al. 1999). As a negative control, the parental plasmid lacking a promoter was also employed. To standardize the transfection efficiency, the herpes simplex virus *TK* promoter-driven *Renilla* luciferase construct, pRL-TK, was simultaneously introduced. The cells were transfected with the DNA in an optimized concentration of TransIT-IKO transfection reagent (Mirus Bio, Madison, WI, USA). After addition of FGF-1 and/or plasmid DNA, the medium was changed every 12 h with concurrent addition of FGF-1; incubation was continued for 48 h. Then, the cell lysate was extracted with Passive Lysis Buffer (Promega, Fitchburg, WI, USA) following the protocol provided by the manufacturer. Thereafter, the firefly and *Renilla* luciferase activities were measured with the Dual Luciferase system (Promega) by a luminometer (Fluroskan Ascent FL, Labsystems, Helsinki, Finland). Transfection experiments were performed with 3 independent sets of the samples, each in quadruplicate, on 3 different occasions.

Dataset analysis

The datasets of chromatin immunoprecipitation-sequencing (ChIP-seq) experiments targeting H3K4me2 (ENCSR000AMC) and transcription factors (ENCSR000BLE and ENCSR000BMO) in HepG2 cells were downloaded from the ENCODE portal (https://www.encodeproject.org/) (Sloan et al. 2016). The data was analyzed and visualized by the University of California Santa Cruz (UCSC) Genome Browser (http://genome.ucsc.edu).

Gene silencing

In order to silence *FOXA1* via RNAi, we purchased a small interfering RNA (siRNA) cocktail containing 3 distinct synthetic siRNAs targeted to human *FOXA1* (Santa Cruz Biotechnology, Santa Cruz, CA, USA). A non-targeting control siRNA was also obtained from Santa Cruz Biotechnology. HCS-2/8 cells were seeded into 6-well plates and were grown until the cells reach subconfluence. The cells were then transfected with 10 nM of each siRNA using the Lipofectamine® RNAiMAX Reagent (Thermo Fisher Scientific, Carlsbad, CA), according to the protocol supplied by the manufacturer, and the cells were then incubated for 36 h. Next, the medium was changed to DMEM containing 0.5% FBS together with 25 ng/ml of recombinant FGF-1 and the incubation was continued for an additional 12 h. After 48 h of gene silencing, the total cellular RNA was extracted for further analyses.

Statistical analysis

Unless otherwise specified, all of the evaluations were performed at least twice, yielding comparable results. Comparisons between two experimental groups were performed by using Student's *t*-test. Statistical comparison among three groups were performed by Fisher's least significant difference test.

Results

Effects of VPA, a HDAC1 inhibitor, on the regulation of CCN2 and FGF1 by FGF-1

In our previous study, we showed that *CCN2* and *FGF1* mRNA levels were drastically decreased and increased upon FGF-1 stimulation, respectively (El-Seoudi et al. 2017). As a first step to clarify the molecular mechanism of this bipartite regulation, we initially examined the involvement of chromatin remodeling therein, using an HDAC inhibitor, VPA. First, we estimated the cytotoxicity of this compound to HCS-2/8 cells and found that 0.125 µg/ml of VPA had no significant effect on the metabolic activity of these cells, whereas 0.25 µg/ml VPA showed modest reduction (< 15%) therein (Fig. S1). Therefore, we decided to treat HCS-2/8 cells with these two doses.

As clearly observed in Fig. 2a, FGF-1 dramatically repressed the steady state mRNA level of *CCN2* in the absence of VPA, recapitulating our previous finding. However, in the presence of increasing concentrations of VPA, *CCN2* expression without FGF-1 stimulation was strongly inhibited, suggesting that chromatin loosening by histone acetylation led to CCN2 silencing. Interestingly, VPA contrarily increased the CCN2 mRNA level repressed by FGF-1. Collectively, CCN2 repression by FGF-1 was significantly abolished by VPA in a dose-dependent manner, as clearly represented by the data in Fig. 2b

In contrast, VPA did not show a prominent effect on *FGF1* mRNA levels in the absence or presence of FGF-1 at the lower concentration. However, the higher concentration of VPA markedly attenuated the autoinduction of *FGF1*, while the basal level of *FGF1* expression without exogenous FGF-1 was unchanged (Fig. 2a). As such, autoinduction of *FGF1* was also repressed significantly by 0.25 µg/ml of VPA (Fig. 2b). These results indicate that chromatin remodeling is involved in both *CCN2* repression and *FGF1* induction by FGF-1 and that both events depend on HDAC activity.

Evaluation of possible post-transcriptional regulation of CCN2 and FGF1 mRNA by FGF-1

Steady-state mRNA levels are determined under the balance of nascent transcription and mRNA degradation. Therefore,

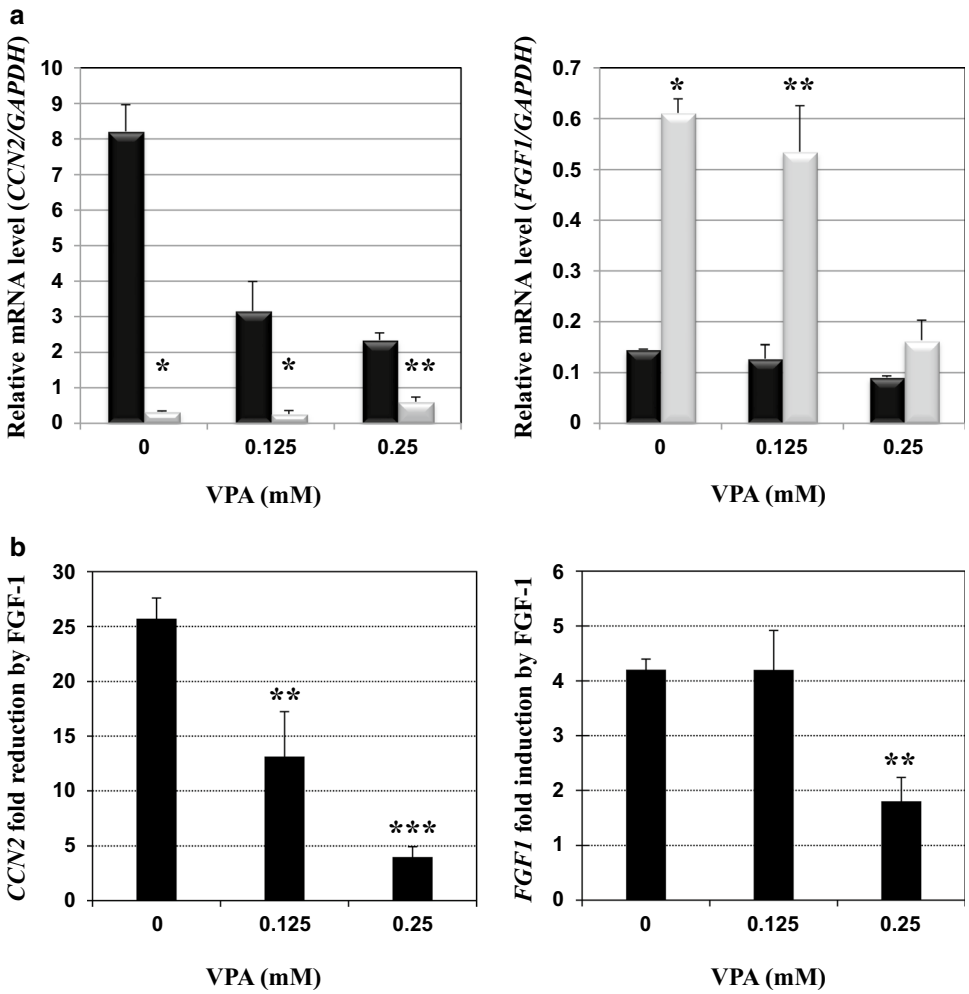


Fig. 2 Effects of valproic acid (VPA), an histone deacetylase (HDAC) 1 inhibitor, on the regulation of *CCN2* and *FGF1* in chondrocytic HCS-2/8 cells. **a** *CCN2* (left panel) and *FGF1* (right panel) mRNA levels with (gray columns) or without (black columns) 25 ng/ml of FGF-1 in the presence of indicated concentrations of VPA. Relative gene expression levels versus the glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*) are presented. Asterisks (*) and (**) denote a significant difference at $p < 0.05$ and 0.01, respectively, between the two groups. **b** Fold repression of *CCN2* expression (left panel) and enhancement of *FGF1* expression (right panel) in the presence of VPA of indicated concentrations. Data represent mean values from 3 independent cell cultures with error bars representing standard deviations. Asterisks (*), (**) and (***) denote significant differences at $p < 0.05$, 0.01 and 0.001, respectively, against the control

the observed decrease in *CCN2* mRNA and increase in *FGF1* mRNA might result from accelerated *CCN2* mRNA degradation and *FGF1 mRNA* stabilization, respectively. To examine these possibilities, we arrested nascent mRNA synthesis by actinomycin D and determined the fate of *CCN2* and *FGF1* mRNAs in HCS-2/8 cells in the presence or absence of exogenous FGF-1. In our previous report, we found relatively rapid degradation of *CCN2* mRNA in the same cells under regular conditions (Kubota et al. 2003), which was also confirmed in this study (Fig. 3a). Surprisingly, in the presence of FGF-1, *CCN2* mRNA was found to be stabilized. Indeed, no appreciable *CCN2* mRNA decay was observed 4 h after transcription, which counteracted the resultant decrease in the steady-state mRNA level caused by FGF-1 (Fig. 2a). Also, to our surprise, FGF-1 mRNA was found to be unusually stable in HCS-2/8 cells, regardless of FGF-1 treatment. These data indicate that *CCN2* and *FGF1* mRNAs are not dominantly regulated by FGF-1 at a post-transcriptional stage.

It is generally recognized that stability of mRNA is usually determined by the cis-elements built in their 3'-UTR. In fact, the 3'-UTR of human *CCN2* mRNA contains a number of post-transcriptional repressive elements, including a cis-acting element for structure-anchored repression (CAESAR) (Kubota et al. 2000; Leask and Abraham 2006) and various miRNA targets (Ohgawara et al. 2009; Kubota and Takigawa 2015). Thus, we evaluated the possible contribution of these cis-repressive elements to the CCN2 repression by FGF-1 with a reporter plasmid (Fig. 3b) that expresses a firefly luciferase mRNA with the 3'-UTR of human CCN2. The reporter gene assay showed, however, that this CCN2 3'-UTR containing luciferase gene did not respond to FGF-1 (Fig. 3c), indicating that the UTR-mediated post-transcriptional regulation did not contribute to the observed repression of *CCN2* expression by FGF-1.

Identification of FOXA1 as an FGF-1 inducible transcription factor that binds to both CCN2 and FGF1 loci

The results shown in Figs. 2 and 3 suggest that the effects of FGF-1 on *CCN2* and *FGF1* are the outcomes

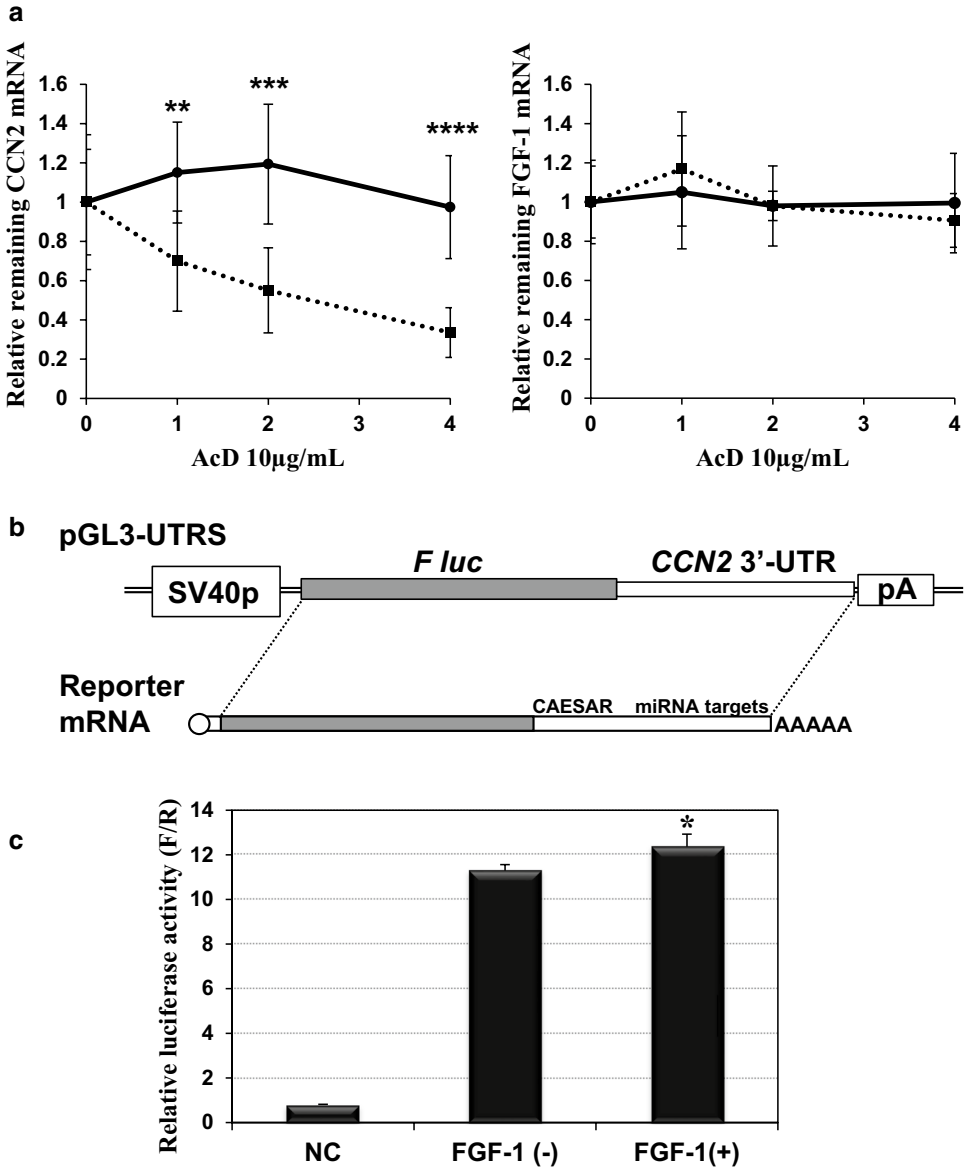


Fig. 3 Post-transcriptional effects of FGF-1 on *CCN2* and *FGF1* mRNAs. **a** RNA degradation profile of *CCN2* (left panel) and *FGF1* (right panel) mRNAs in the absence (dotted lines) or presence (solid lines) of 25 ng/ml FGF-1. Relative levels of remaining mRNAs after the indicated hours of actinomycin D treatment are plotted with error bars of standard deviations. Data were computed from the results obtained with 8 independent cultures for each condition. Asterisks (**), (***) and (****) indicate significant difference at $p<0.01$, $p<0.001$ and $p=0.0001$, respectively, between the two groups at the same time point. **b** Structure of the plasmid reporting the post-transcriptional regulatory functions of *CCN2* 3'-UTR. This plasmid, pGL3-UTRS (shown at the top), produces firefly luciferase (F luc) mRNA with the entire 3'-UTR of *CCN2* mRNA (at the bottom). SV40p and pA represent the SV40 promoter and polyadenylation signal with a t-splice site, respectively. The cis-repressive elements involved in the *CCN2* 3'-UTR are also noted. **c** Expression of the firefly luciferase gene with the CCN2 3'-UTR in the absence or presence of exogenous FGF-1. NC represents the control experiments with a vector containing no promoter (pGL3ΔP). Data are shown as firefly luciferase activities standardized by the internal control (*Renilla* luciferase activities from pRL-TK). Each value represents the average and standard deviation of the data obtained from 3 experiments performed on separate occasions, in which 3 independent sets of samples with 4 quadruplicate cultures were evaluated. Asterisks (*) denote significant differences at $p<0.05$ between the two groups, FGF-1(−) and FGF-1(+)

of collaborative transcriptional regulation of chromatin remodelers and transcription factors that recognize *CCN2* and *FGF1* loci. As a next step, we tried to find a transcription factor candidate which actually binds to these loci. For this objective, we analyzed datasets deposited in the ENCODE portal site. By utilizing chromatin immunoprecipitation (ChIP) sequencing datasets, histone modifications representing chromatin status and

transcription factors binding profiles of any locus in particular cell(s) can be analyzed. Unfortunately, no such data in chondrocytes were available to date. Instead, we analyzed data from another human cell line, in which both *CCN2* and *FGF1* loci are active in transcription, as observed in HCS-2/8 cells. As illustrated in Fig. 4a, these loci are characterized by histone H3 di-methylation on Lys 4 (H3K4me2), representing euchromatin that is open for transcription. Therefore, in these cells, a number of transcription factors are accessible to these loci, and thus transcription factor–ChIP datasets indicated the binding of a number of different transcription factors therein. Among them, we found FOXA1 bound to both loci, *FGF1* and *CCN2* (Fig. 4a, b). Most interestingly, this transcription factor binds to 3 distinct areas within the 5 kb-long proximal promoter area of human *CCN2*. In our previous study, we evaluated the *FGF1* responsiveness of a *CCN2* proximal promoter of < 1 kb in length (El-Seoudi et al. 2017), in which only one FOXA1 binding site was involved. Consistent with its molecular structure, this short proximal promoter fragment showed a significant, but modest, response to FGF-1. Next, we evaluated the effect of exogenous FGF-1 on *FOXA1* expression. As observed in Fig. 4c, the gene expression and production of FOXA1 was enhanced by FGF-1 stimulation in HCS-2/8. Therefore, we chose FOXA1 as a candidate that might regulate both *CCN2* and *FGF1* in an opposite manner upon FGF-1 stimulation, and forwarded this candidate to subsequent analysis.

FOXA1-dependence of the bipartite regulation of CCN2 and FGF1 by FGF-1

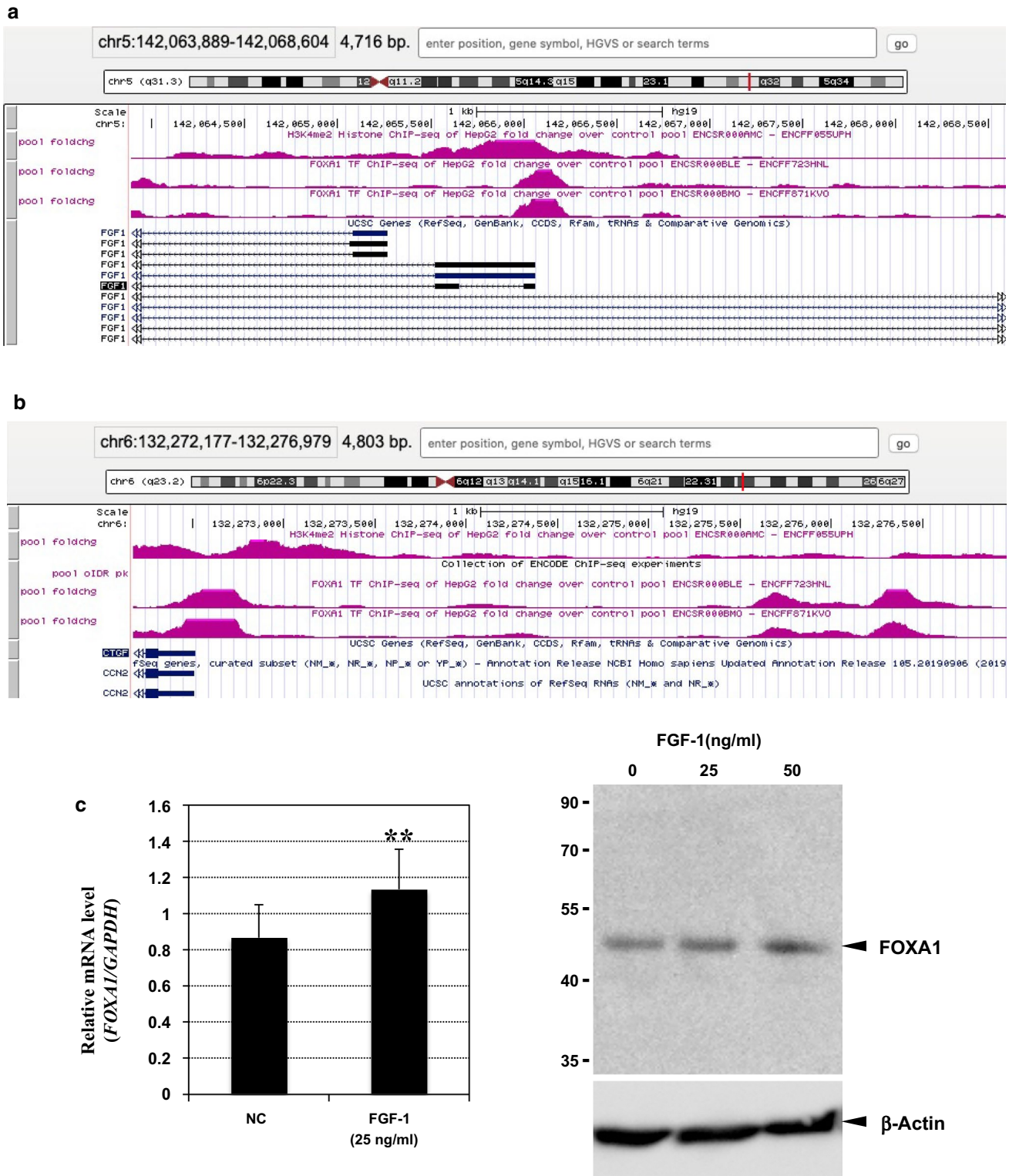
In order to clarify whether FOXA1 mediated the regulation of *CCN2* and *FGF1*, we utilized an RNAi strategy to silence *FOXA1* and evaluated its effects on each mRNA level modified by exogenous FGF-1. As shown in Fig. 5a, transfection of a synthetic siRNA cocktail into HCS-2/8 cells significantly silenced *FOXA1* expression in those cells, compared to that with control siRNAs. Efficient silencing of FOXA1 production was also confirmed at a protein level (Fig. 5b). Under the same conditions, the cells were exposed to FGF-1 in the medium, and the *CCN2* and *FGF1* mRNA levels were compared. As expected, silencing of *FOXA1* significantly de-repressed the *CCN2* expression repressed by FGF-1, whereas it repressed the enhanced *FGF1* expression (Fig. 5c). However, the effects of *FOXA1* silencing were not striking, which is probably due to the limited level of *FOXA1* silencing. This single transcription factor was shown to mediate the regulation of these two genes by FGF-1, at least in part, in chondrocytes.

Discussion

In the present study, we investigated the molecular mechanism of the central regulatory system of FGF-1-mediated cartilage degeneration. Consequently, we found a specific transcription factor, FOXA1, mediates both *CCN2* transcriptional repression and *FGF1* induction by FGF-1, which is dependent on chromatin remodeling complexes, HDACs. Additionally, possible involvement of post-transcriptional regulation in these regulatory processes was experimentally addressed, and this possibility was ruled out.

HDACs are enzyme complexes that catalyze the deacetylation of acetylated lysine residues in histones (Khan and Haqqi 2018). Human HDACs consist of HDAC 1 to 11, classified as classes I, II, and III HDACs and sirtuin (SIRT) 1 to 7, classified as class III. By the removal of the acetyl groups by HDACs, histones redeem the positive charge on the lysine residues and DNA binding ability, which results in chromatin condensation. The requirement of HDAC I or HDAC II activity for the process of *CCN2* and *FGF1* regulation, which conducts the FGF-1-induced cartilage degeneration, is consistent with the fact that HDACs are critical factors in OA development. Indeed, HDACs 1, 2 and 7 were found upregulated in OA chondrocytes, and more interestingly, HDAC gene silencing or enzymatic inhibition could protect from the degeneration of cartilaginous ECM in vitro and showed protective effects in animal OA models in vivo (Khan and Haqqi 2018). Therefore, HDAC inhibitors are currently considered for potential OA therapeutics. In this context, our study adds a novel mechanistic view of the anti-OA actions of HDAC inhibitors through *CCN2* and *FGF1*.

FOXA1 is a member of a huge transcription factor family with 50 members, which are classified into 19 subgroups, FOXA to FOXS (Lam et al. 2013). These members are structurally characterized by the retention of fork head DNA binding domains, and thus are believed to possess the ability to directly bind to DNA. The first subgroup of this family, FOXA, comprises three members, FOXA1, A2 and A3. These transcription factors regulate the temporo-spatial expression of a number of genes during development and at adult stages (Lam et al. 2013). In relation to cartilage biology, FOXA1 and A2 are produced in notochordal cells during vertebral development, regulating Shh expression, and thus contribute to intervertebral disc formation (Nakamichi and Asahara 2020). More importantly, FoxA members including FoxA1 were found to be critically involved in the regulation of chondrocyte hypertrophy in mice (Ionescu et al. 2012). This notion was further confirmed by a recent study with deer antler chondrocytes, which constructs and reconstructs the antlers of deer, revealed that FoxA1, A2 and A3 are all required for the chondrocytes to proceed to



hypertrophy properly, under the regulation of desert hedgehog (Dhh) (Ma et al. 2020). Mechanistically, FOXA members are shown to compete with SOX9 that is an inhibitor of hypertrophy (Tan et al. 2018). It should be noted that hypertrophic changes are frequently observed in osteoarthritic articular chondrocytes (van der Kraan and van den Berg 2012). FGF-1, which induces FOXA1, is a mediator of osteoarthritic changes in articular cartilage in a rat model (El-Seoudi et al. 2017). Therefore, FOXA members could also play critical roles in the OA pathogenesis.

◂**Fig. 4** Binding of FOXA1 to *CCN2* and *FGF1* loci and its induction by FGF-1. **a, b** Preferential binding of FOXA1 to the *FGF1* (**a**) and *CCN2* (**b**) loci. Analysis of the chromatin immunoprecipitation-sequencing (ChIP-seq) datasets in the ENCODE portal site revealed strong binding signals of FOXA1 to the regions characterized by H3K4me2 histone modification (shown in pink). Transcripts from the illustrated region are also summarized (shown in dark blue; boxes and lines represent exons and introns, respectively). **c** Induction of *FOXA1* by exogenous FGF-1 in HCS-2/8 cells. Relative gene expression levels were computed against those of *GAPDH*. Mean values from 3 independent cell cultures are shown with error bars representing standard deviations. Asterisks (**) indicate a statistically significant difference at $p < 0.01$ between the two groups (left). Dose-dependent enhancement of FOXA1 protein production by FGF-1 was confirmed by Western blotting. Positions of molecular weight markers (in kDa) are indicated at the left. Signals of β-actin are also shown as an internal control (right)

In this study, we elucidated the involvement of FOXA1 in the regulation of *CCN2* and *FGF1* by the signal evoked by FGF-1. However, no information is available on the cell surface receptors and intracellular signaling molecule that actually transmit the FGF signal to FOXA1. Since FGF receptor 3 is demonstrated on chondrocytes and emits signals to control the chondrocytic phenotype (Wang et al. 2019), it is the most plausible receptor that may accept FGF-1 and initiate a signaling cascade towards FOXA1. Subsequent studies in the future may clarify the signaling molecules involved in this regulation.

These FOXA members exert highly context-dependent effects on target gene expression. For example, FOXA1 upregulates CDKN1B encoding p27, while it downregulates SLUG in cancer cells (Lam et al. 2013). Similarly, FOXA1 may regulate *CCN2* and *FGF1* in opposite directions, depending upon the nano-environment around each locus. Such a bipartite regulation is supposed to be performed under the collaboration of transcriptional co-activators and co-repressors, which are conditionally recruited to the target loci (Fig. 6).

FOXA1 is not only a DNA binding transcriptional factor, but also a pioneer factor that is able to actively modify chromatin structure. In fact, this factor induces H3K4 methylation and DNA demethylation to open the chromatin

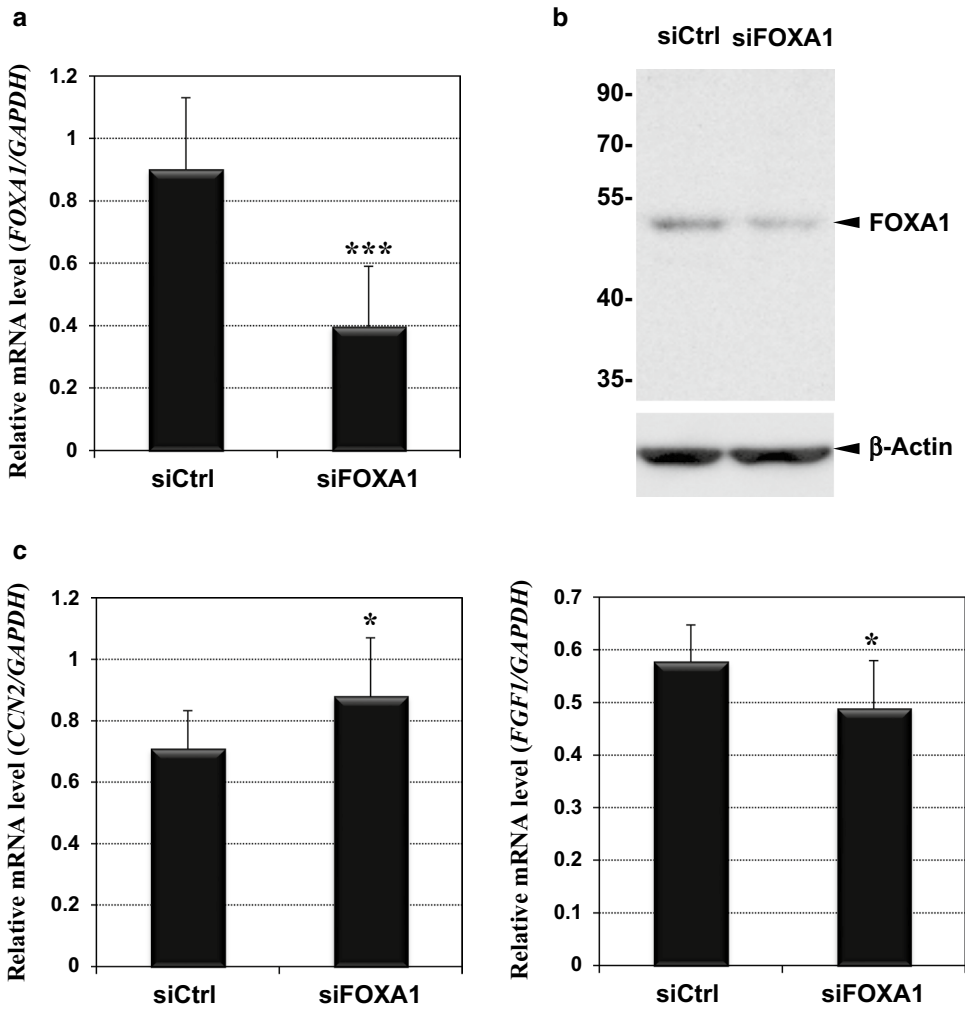


Fig. 5 Reversion of the effects of FGF-1 on *CCN2* and *FGF1* expression by *FOXA1* silencing. **a** *FOXA1* silencing by an siRNA cocktail. Relative *FOXA1* mRNA levels in HCS-2/8 cells were quantified after the transfection with non-targeting control (siCtrl) or siRNAs against FOXA1 (siFOXA1). **b** Confirmation of *FOXA1* silencing by Western blotting. Positions of molecular weight markers (in kDa) are indicated at the left. Signals of β-actin are also shown as an internal control. **c** Effects of *FOXA1* silencing on the FGF-1-modulated expression of *CCN2* (left panel) and *FGF1* (right panel). All of the cells were treated with FGF-1 after the respective siRNA transfection. *CCN2* expression was de-repressed, and *FGF1* induction was suppressed by siFOXA1. Values were standardized against the mRNA levels of *GAPDH* and displayed with standard deviations (error bars). Asterisks (*) and (***) indicate statistically significant differences at $p < 0.05$ and $p < 0.001$, respectively, between the two groups

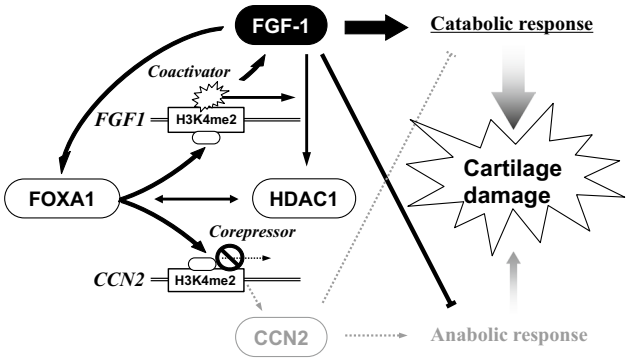


Fig. 6 Schematic representation of the molecular mechanism of the bipartite regulation by FGF-1. Chromatin remodeling by histone methylation and acetylation, in which HDAC1 plays a significant role, is required for this regulation. FGF-1 induces *FOXA1*, which acts as a pioneer factor to open the chromatin structure with H3K4me2 regions in *CCN2* and *FGF1* loci. Thereafter, *FOXA1* binds to the *CCN2* silencer and *FGF1* enhancer to facilitate the access of the coactivator and corepressor, respectively. In consequence, amplified FGF-1 signal incurs catabolic and anti-anabolic responses of chondrocytes, leading to OA development

structure, allowing the access of other transcription factors represented by steroid hormone receptors (Lam et al. 2013). FOXA1 is likely opening the cis-elements in the H3K4me2-marked loci in *CCN2* and *FGF1* for co-activators and co-repressors, which are, however, still unidentified, and thus further investigation is needed to verify this hypothesis.

Considering the HDAC-dependence of the *CCN2* and *FGF1* regulation by FGF-1, it is suspected that the pioneer actions of FOXA1 are exerted under collaboration with HDACs. Consistent with this idea, HDAC7 was reported to form a complex with FOXA1, together with estrogen receptors, to repress Reprimo, which encodes a cell cycle inhibitor (Malik et al. 2010). If similar machinery is utilized for the regulation of *CCN2* and *FGF1*, FOXA1 and HDAACs may synergistically work to yield maximal effects, which may account for the limited effect of partial FOXA1 silencing alone on the *CCN2* and *FGF1* expression in the presence of FGF-1. Alternatively, these findings indicate the utility of anti-FOXA1 molecules as a novel therapeutic tool for OA treatment in combination with HDAC inhibitors.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interests to declare.

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