RESEARCH ARTICLE

Metabolic regulation of the CCN family genes by glycolysis in chondrocytes

Sho Akashi^{1,2} · Takashi Nishida¹ · Abdellatif El-Seoudi¹ · Masaharu Takigawa³ · Seiji Iida² · Satoshi Kubota^{1,3}

Received: 12 October 2017 / Accepted: 13 October 2017 / Published online: 11 November 2017
© The International CCN Society 2017

Abstract The CCN family consists of 6 genes in the mammalian genome and produces multifunctional proteins involved in a variety of biological processes. Recent reports indicate the profound roles of CCN2 in energy metabolism in chondrocytes, and *Ccn2* deficiency is known to alter the expression of 2 other family members including *Ccn3*. However, almost nothing is known concerning the regulation of the CCN family genes by energy metabolism. In order to gain insight into this critical issue, we initially and comprehensively evaluated the effect of inhibition of glycolysis on the expression of all of the CCN family genes in chondrocytic cells. Upon the inhibition of a glycolytic enzyme, repression of *CCN2* expression was observed, whereas *CCN3* expression was conversely induced. Similar repression of *CCN2* was conferred by the inhibition of aerobic ATP production, which, however, did not induce *CCN3* expression. In contrast, glucose starvation significantly enhanced the expression of *CCN3* in those cells. The results of a reporter gene assay using

a molecular construct containing a *CCN3* proximal promoter revealed a dose-dependent induction of the *CCN3* promoter activity by the glycolytic inhibitor in chondrocytic cells. These results unveiled a critical role of glycolytic activity in the regulation of *CCN2* and *CCN3*, which activity mediated the mutual regulation of these 2 major CCN family members in chondrocytes.

Keywords CCN2 · CCN3 · Glycolysis · Metabolism · Cartilage

Introduction

Mammalian skeletal growth is achieved mainly by the biological process known as endochondral ossification. In this process, chondrocytes differentiate from mesenchymal stem cells, proliferate and mature to produce cartilaginous extracellular matrix (ECM), which enables the growth of long bones initially as growth-plate cartilage (Kubota and Takigawa 2011). Thereafter, chondrocytes undergo hypertrophic differentiation, followed by their removal and replacement of the cartilage with bone tissue. As such, chondrocytes are the central players of skeletal growth, although these cells eventually disappear. Endochondral ossification is under the regulation of a number of systemic and local factors represented by hormones and growth factors. Among them, it is of particular note that all of the CCN family members are produced and play significant roles in this process (Kawaki et al. 2008).

The CCN family of matricellular proteins comprises 6 members in mammals, which members are structurally characterized by 3 or 4 conserved modules connected in tandem immediately after the signal peptide, which functions for secretion (Perbal and Takigawa 2005; Jun and Lau 2011). These modules are designated as insulin-like growth factor binding protein-like (IGFBP)

✉ Masaharu Takigawa
takigawa@md.okayama-u.ac.jp

✉ Satoshi Kubota
kubota1@md.okayama-u.ac.jp

¹ Department of Biochemistry and Molecular Dentistry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8525, Japan

² Department of Oral and Maxillofacial Reconstructive Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8525, Japan

³ Advanced Research Center for Oral and Craniofacial Sciences, Okayama University Dental School, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8525, Japan

module, von Willebrand factor type C repeat (VWC) module, thrombospondin type 1 repeat (TSP1) module and C-terminal cystine knot (CT) module, this last one not being present in CCN5. These 4 modules are known to interact with a variety of extracellular molecules, such as growth factors including bone morphogenetic proteins (BMPs), cell-surface receptors represented by integrins, ECM components that include heparan sulfate proteoglycans (Rachfal and Brigstock 2005), and even with another CCN family member (Hoshijima et al. 2012). Under multiple interactions with these molecules, CCN family proteins manipulate the extracellular signaling network to yield a variety of biological outcomes (Kubota and Takigawa 2013). Thus, all of these members display quite diverse physiological and pathological functions, depending upon their microenvironment (Leask and Abraham 2006; Kubota and Takigawa 2013).

Among the CCN family members produced during endochondral ossification, CCN2 is the one best recognized as being critically required for this process. Earlier studies in vitro indicate that CCN2 promotes the proliferation, maturation and hypertrophic differentiation of growth-plate chondrocytes (Nakanishi et al. 2000; Kubota and Takigawa 2015). Consistent with these findings, *Ccn2*-null mice show severe skeletal deformity caused by impaired endochondral ossification (Ivkovic et al. 2003; Kawaki et al. 2008). It should be also noted that overexpression of *Ccn2* counteracts osteoarthritis (Nishida et al. 2002; Itoh et al. 2013) and that CCN2 and its derivatives enhance the regeneration of damaged articular cartilage (Nishida et al. 2004; Abd El Kader et al. 2014). Interestingly, *Ccn2* deficiency affects the expression of a few other CCN family member genes in chondrocytes. Particularly, strong induction of *Ccn3* by *Ccn2* depletion is striking (Fig. 1; Kawaki et al. 2008). However, the mechanism responsible for this up-regulation of *Ccn3* by *Ccn2* depletion has been unclear.

Recently, novel metabolic effects of CCN2, which may account for the versatile functions of CCN2 in endochondral ossification, were uncovered. Since cartilage is an avascular tissue without a sufficient oxygen supply, glycolysis is the

principal metabolic pathway to obtain biological energy. CCN2 was found to support the activity of glycolysis by enhancing the gene expression of glycolytic enzymes in growth-plate chondrocytes (Maeda-Uematsu et al. 2014). Indeed, the cellular ATP levels in *Ccn2*-null chondrocytes were found less than the half of those in wild-type chondrocytes. As such, CCN2 is indicated to support all of its biological activity through the enhancement of energy supply.

In spite of such profound contribution of CCN2 to energy metabolism in chondrocytes, the regulation of CCN2 expression by energy metabolism has not been investigated. Also, we suspected that the metabolic impact of CCN2 might be related to the distinct induction of *Ccn3* observed in *Ccn2*-null chondrocytes. Furthermore, no information is available concerning the interplay between the CCN family members other than CCN2 and energy metabolism in chondrocytes. Therefore, we started this study by performing comprehensive analysis of the gene expression of all of the 6 CCN family members under a glycolysis-deficient condition. Our subsequent analyses revealed the critical involvement of glycolysis in the regulation of 2 classical CCN family member genes, *CCN2* and *CCN3*.

Materials and methods

Cell culture

Human chondrocytic cell line HCS-2/8 was established from a human chondrosarcoma and is known to retain chondrocytic properties, such as gene expression and production of type II collagen and aggrecan (Takigawa et al. 1989). Therefore, this cell line was employed as a representative of human chondrocytes. 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.

Inhibition of energy metabolism

For the inhibition of glycolysis, monoiodoacetic acid (MIA), which inhibits the activity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a glycolytic enzyme, was employed. HCS-2/8 cells were seeded into 6-well plates and allowed to reach confluence. Thereafter, MIA (Sigma-Aldrich, St. Louis, MO, USA) dissolved in phosphate-buffered saline (PBS) was added at a final concentration of 0, 2 or 4 µg/ml; and the cells were then incubated for 12 h. Finally, total RNAs were extracted and purified from the cells as described in another subsection.

In order to selectively inhibit aerobic ATP production, we utilized oligomycin, which inhibit ATP synthase by interfering with proton transport. The cells were seeded into 6-well or 24-well plates and allowed to reach confluence. Subsequently, oligomycin (Abcam, Cambridge, UK) dissolved in dimethyl

Fig. 1 Impacts of *Ccn2* deficiency on energy metabolism and CCN family expression. Metabolic impacts (left panel: Maeda-Uematsu et al. 2014) and effects on CCN family expression (right panel: Kawaki et al. 2008) by *Ccn2* deletion in mouse chondrocytes are summarized. Possible link between the right and left is suspected

sulfoxide (DMSO) was added at a final concentration of 0, 10 or 100 ng/ml; and the cells were thereafter incubated for 6 h, as described previously (Hao et al. 2010). Then, total RNAs were extracted and purified from the cells as described in another subsection, or ATP was extracted with boiling hot 100 mM Tris-HCl containing 4 mM EDTA, as described previously (Maeda-Uematsu et al. 2014).

Glucose starvation

After having been seeded onto 6-well plates, HCS-2/8 cells were grown until they reached confluence. Thereafter, the medium was changed to D-MEM with or without D-glucose; and the cells were further incubated therein for 24 h or 48 h. Total RNA extraction from those cells was then performed as described in another subsection.

ATP quantification

Intracellular ATP levels were measured by use of an ATP Bioluminescence Assay Kit CLS II (Roche, Basel, Switzerland) according to manufacturer's recommendations. The cells were collected and intracellular ATP was extracted by the boiling method mentioned above. After the extract was mixed with luciferase reagent in 96-well plates, the bioluminescence was measured with a luminometer (Fluoroskan Ascent™ FL, Thermo Labsystems, Helsinki, Finland). The ATP levels were normalized to the total protein concentration, which was determined by using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

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

Total RNAs were extracted and purified from the cells by use of ISOGEN (Nippon Gene, Tokyo, Japan) or an RNeasy® Mini Kit (Qiagen, Hilden, Germany) by following the manufacturers' instructions, and then they were reverse-transcribed to cDNA by use of avian myeloblastosis virus reverse transcriptase with oligo dT as the primer (PrimeScript™ RT reagent Kit, Takara Bio, Shiga, Japan). Subsequent quantitative real-time PCR was performed by using SYBR® Green Realtime 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 *CCN2*; 5'-GGA GCG CGC TAT AAA ACC TG -3' (sense) and 5'-TCC CCT CTC GCT TTT ACC AA -3' (antisense) for *CCN3*; and 5'-GAT CAT TGC TCC TCC TGA GC -3' (sense) and 5'-ACT CCT GCT TGC TGA TCC AC -3' (antisense) for *ACTB* (β -actin).

Plasmid construction

A reporter gene construct to evaluate the promoter activity of human *CCN3* was constructed by using pGL3-control (Promega, Madison, WI, USA) as a backbone. The genomic DNA, which was isolated from HCS-2/8 cells, was used as template, and specific segment of the *CCN3* 5'-flanking region was prepared by using nested-PCR. The nucleotide sequences of the primers used were as follows: 5'-CCG CAA GCT GCC TTT GAT TT-3' (sense) and 5'-GGC TTT CCC CTC TCG CTT TT-3' (antisense) for outer primers; and 5'-TGC TGG GGA CAG ATG AGG TA-3' (sense) and 5'-TGT AGA TTG GCA CTG CTC GC-3' (antisense) for nested primers. The amplified fragment was subcloned into the pGEM-T Easy vector (Promega), and digested with both *KpnI* and *SacI*. Then, a 1.4-kb *KpnI-SacI* fragment lacking the translation initiation site was ligated into the *KpnI-SacI* sites of pGL3-control lacking the simian virus 40 (SV40) promoter (pGL3ΔP: Kubota et al. 1999). The reporter construct contained the human *CCN3* gene from -1403 to -45, which was confirmed by DNA sequencing.

CCN3 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 and were allowed to reach 60–70% confluence in D-MEM with 10% FBS. Thereafter, the medium was exchanged for a fresh one with the same composition (10% FBS) immediately before the plasmid DNA transfection. The human *CCN3* promoter reporter construct described in the previous subsection was used for transfection of the HCS-2/8 cells, in combination with a herpes simplex virus *TK* promoter-driven *Renilla* luciferase construct (pRL-TK: an internal control). The cells were transfected with the aid of an optimized amount of FuGENE 6 (Roche). At 24 h after DNA transfection, MIA of different concentrations (0.5, 1, 2 and 4 μg/mL) was added, and the cells were further incubated for 12 h. Then, the cells were collected after being lysed by Passive Lysis Buffer (Promega) with gentle rocking for 10–15 min. Thereafter, the Dual Luciferase Reporter Assay System (Promega) was used for the measurement of firefly and *Renilla* luciferase activities, and calculation of relative ratios were carried out with a luminometer (Fluoroskan Ascent FL, Thermo Labsystems). All transfection experiments were performed 4 times in quadruplicate (El-Seoudi et al. 2017).

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 glycolysis inhibition by MIA on the expression of CCN family genes

Our previous study indicated that CCN2 deficiency impaired glycolysis, resulting in a severe decrease in cellular ATP level in chondrocytes (Maeda-Uematsu et al. 2014). At the same time, gene expression of a few other CCN family genes were affected (Fig. 1), suggesting possible regulation of CCN family members through glycolysis. Therefore, initially we comprehensively analyzed the impact of glycolytic inhibition on the gene expression of all of the CCN family members by use of MIA, which is an inhibitor of GAPDH (Fig. 2a). Quantitative real-time RT-PCR analysis of the RNA from HCS-2/8 cells revealed that treatment with MIA significantly decreased the

expression of CCN2. Interestingly, CCN3 was contrarily induced by MIA in a dose-dependent manner (Fig. 2b).

Although CCN5 was also found to be induced by MIA, this result was not consistent with the finding observed in *Ccn2*-null chondrocytes (Fig. 1). As such, further investigation was performed on CCN2 and CCN3 in this study.

Regulation of CCN2 by aerobic ATP production

Since the effect of MIA suggested the involvement of cellular ATP shortage in the regulation of CCN2 and CCN3, we next induced ATP shortage via a different mechanism. Aerobic ATP production in mitochondria of the HCS-2/8 cells was blocked by oligomycin (Fig. 3a), and its effect on CCN2 and CCN3 was evaluated. As expected, treatment with oligomycin decreased the cellular ATP level (Fig. 3b); and the expression

Fig. 2 Effects of MIA treatment on the expression of CCN family members. **a** Mechanism of MIA action to inhibit glycolysis. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is the target. **b** Gene expression of all of the CCN family members in the presence of the indicated concentrations of MIA in human chondrocytic HCS-2/8 cells. Relative expression levels standardized against those of β -actin mRNA are displayed. Mean values from 4 independent cell cultures are shown with error bars representing standard deviations. Asterisks (*) and (**) denote significant differences at $p < 0.05$ and $p < 0.01$, respectively, from the control (0 μ g/ml of MIA), which were evaluated by Student's *t*-test

Fig. 3 No effect of oligomycin treatment on the expression of *CCN2* and *CCN3*. **a** Mechanism of action of oligomycin. ATP synthesis on the inner membrane of a mitochondrion is illustrated. V : membrane potential formed by the respiratory chain. **b** Reduction of cellular ATP level by oligomycin treatment. ATP contents in 1 mg of total cellular protein are shown. **c** Effect on *CCN2* expression. **d** Effect on *CCN3* expression. Relative expression levels standardized against those of β -actin mRNA are displayed. Mean values from 4 independent cell cultures are shown with error bars of standard deviations. Asterisks (*) and (***) denote significant differences at $p < 0.05$ and $p < 0.01$, respectively, from the control (0 ng/ml of oligomycin), which were evaluated by Student's *t*-test

level of *CCN2* in the cells (Fig. 3c) was concomitantly repressed. However, *CCN3* expression was not affected by oligomycin (Fig. 3d). These results indicate that *CCN2* expression was under the control of the cellular ATP level, whereas neither the activity of oxidative phosphorylation in the mitochondria nor the intracellular ATP level regulated the expression of *CCN3*.

Induction of *CCN3* by glucose starvation in chondrocytic cells

The data in Fig. 2 and Fig. 3 indicated that *CCN3* was not regulated by the ATP produced through glycolysis, but was under the direct control of glycolytic activity. In order to confirm this notion, we evaluated the effect of glucose starvation on the expression of *CCN3* in HCS-2/8 cells, following the method summarized in Fig. 4a. Compared to the cells cultured in medium containing glucose, the cells incubated in medium lacking it showed the induction of *CCN3* at 48 h; although the induction did not reach a statistically significant level at 24 h of glucose starvation (Fig. 4b). There was no significant difference in the expression of *CCN2* even after 48 h of glucose starvation (data not shown). These findings firmly indicated a proper regulatory system of *CCN3* tightly linked to glycolysis, one distinct from that of *CCN2*.

Activation of the *CCN3* promoter by glycolytic inhibition

Finally, to clarify the mechanism of *CCN3* regulation by glycolysis, we constructed a reporter plasmid to evaluate the transcriptional activity of a *CCN3* proximal promoter. This plasmid contained a firefly luciferase gene driven by a *CCN3* fragment (1359 bp in length, as explained in Materials and methods) including the transcription initiation site (Fig. 5a). Using this plasmid, the *CCN3* promoter activity in HCS-2/8 cells was comparatively analyzed in the presence or absence of MIA. As shown in Fig. 5b, the *CCN3* promoter activity was increased by the MIA treatment in a dose-dependent manner. This result suggested that *CCN3* was regulated via the proximal promoter by the inhibition of glycolysis, presumably at the transcriptional level.

Discussion

In this study, metabolic regulation of 2 major CCN family members by glycolysis in chondrocytes was uncovered. First, *CCN2* was positively regulated by the intracellular ATP level, which in turn promotes glycolysis, as previously described (Maeda-Uematsu et al. 2014). Second, *CCN3* expression was contrarily repressed by glycolytic activity. This

Fig. 4 Induction of *CCN3* by glucose starvation in HCS-2/8 cells. **a** Experimental procedure of glucose starvation. Solid and open arrows denote incubation with and without glucose, respectively. **b** *CCN3* expression after 24 h (left) and 48 h (right) of glucose starvation. Relative gene expression levels versus β -actin are shown. Mean values from 3 independent cell cultures are shown with error bars representing standard deviations. The asterisk (*) represents a significant difference at $p < 0.01$ from the control, which was evaluated by Student's *t*-test

regulation by glycolysis accounted for the drastic reduction in *CCN3* expression observed in *Ccn2*-null chondrocytes and also suggested profound roles of these 2 CCN family

members in the energy metabolism in chondrocytes. In *Ccn2*-null chondrocytes, *Ccn6* expression was reported to be repressed (Kawaki et al. 2008), whereas no effect was

Fig. 5 Induction of *CCN3* promoter activity by MIA in HCS-2/8 cells. **a** Structure of the plasmids constructed and utilized. The plasmid phCCN3 contained a human *CCN3* proximal promoter fragment fused to a firefly luciferase gene. **b** Luciferase gene expression after 12 h of MIA treatment at indicated doses. Relative firefly luciferase activity standardized against *Renilla* luciferase activity (internal control) is shown. Mean values from 3 independent cell cultures are shown with error bars of standard deviations. The asterisk (*) denotes a significant difference at $p < 0.05$ from the control, which was evaluated by Student's *t*-test

Fig. 6 Schematic representation of the metabolic regulation of CCN family members in chondrocytes. Arrows and T-bar represent positive and negative regulations, respectively. The dotted arrow indicates a relatively indirect effect

observed by the pharmacological inhibition of glycolysis (Figs. 1 and 2). Thus, *Ccn6* regulation by *Ccn2* was not mediated by its metabolic impact, but by an unknown independent molecular mechanism associated with CCN2 function. In contrast, although no change was observed by *Ccn2* depletion, *CCN5* gene expression was significantly induced by MIA treatment in this study. According to a previous report, GAPDH, the target of MIA, is not only an enzyme engaged in glycolysis, but also binds to mRNA and regulates target gene expression (Chang et al. 2013). Therefore, *CCN5* may be under the direct regulation of GAPDH protein rather than the glycolytic activity. In order to clarify these points further investigation is underway.

As clearly illustrated in Fig. 6, *CCN2* gene and glycolysis are activating each other via the cellular ATP level, forming a feedback loop. This system enables autonomous, simultaneous and constant increase in CCN2 production and energy production during endochondral ossification. In fact, expression of CCN2 continuously increases along with the differentiation of growth-plate chondrocytes until hypertrophic onset, which give rise to increasing ATP production (Kawaki et al. 2008). Using this ATP, chondrocytes are able to grow long bones under the regulation of extracellular signaling molecules. As such, impaired endochondral ossification found in *Ccn2*-null mice may be ascribed to the deficiency in this energy supply system, at least in part. In addition, it is of great interest to determine if this system is at work in malignant tumor cells. As widely recognized, constitutive activation of glycolysis is frequently observed in such cells, which activation is designated as the Warburg effect. Considering the involvement of this molecule in a variety of malignancies (Kubota and Takigawa 2015), CCN2 is possibly involved in this pathological process as a critical mediator. Evaluation of other cell lines derived from a variety of malignancies may uncover a novel pathological aspect of CCN2 metabolic function.

The prominent induction of *CCN3* by glycolytic inhibition and glucose starvation represents an important metabolic role of CCN3 in chondrocytes. It may be assumed that the observed CCN3 induction is a protective response against energy or nutrition shortage. Interestingly, application of MIA to articular cartilage in vivo results in immediate expenditure of CCN3 in articular cartilage and production of CCN3 in clustering chondrocytes, suggesting its role in cartilage protection (Janune et al. 2017). CCN3 is shown to maintain the

differentiated phenotype of articular chondrocytes, repressing their proliferation to save energy consumption (Janune et al. 2011). Therefore, this metabolic regulation of CCN3 may be of critical significance in the maintenance of articular cartilage, although its precise molecular mechanism is still unclear. Of note, recent studies suggest a related metabolic function of CCN3 in cells other than chondrocytes. In adipocytes, CCN3 may be delimiting the energy consumption at the genetic level. In fact, a strong correlation between plasma CCN3 level and fat mass was observed in human cases (Escoté et al. 2017). Similar CCN3 function is suspected in fibroblasts as well, since CCN3 is reported to repress fibrosis (Riser et al. 2009). Interestingly, overexpression of CCN3 in fibroblasts down regulates CCN2, which may be mediated by metabolic activities (Abd El Kader et al. 2013). Considering these metabolic functions of CCN3 in mesenchymal cells, extensive studies on CCN3 from the viewpoint of energy metabolism should uncover novel metabolic roles of this matricellular protein expressed throughout the human body.

Acknowledgments The authors thank Drs. Takako Hattori, Kazumi Kawata, Eriko Aoyama for their helpful support in experiments, as well as Ms. Yoshiko Miyake for her secretarial assistance. This study was supported by grants from the program Grants-in-aid for Scientific Research (B) [JP15H0514 to M.T.], and for Challenging Research (Exploratory) [JP17K19757 to M.T. and JP17K19756 to S.K.] from the Japan Society for the Promotion of Science.

References

- Abd El Kader T, Kubota S, Janune D, Nishida T, Hattori T, Aoyama E, Perbal B, Kuboki T, Takigawa M (2013) Anti-fibrotic effect of CCN3 accompanied by altered gene expression profile of the CCN family. *J Cell Commun Signal* 7:11–18
- Abd El Kader T, Kubota S, Nishida T, Hattori T, Aoyama E, Janune D, Hara ES, Ono M, Tabata Y, Kuboki T, Takigawa M (2014) The regenerative effects of CCN2 independent modules on chondrocytes in vitro and osteoarthritis models in vivo. *Bone* 59:180–188
- Chang CH, Curtis JD, Maggi LB Jr, Faubert B, Villarino AV, O'Sullivan D, Huang SC, van der Windt GJ, Blagih J, Qiu J, Weber JD, Pearce EJ, Jones RG, Pearce EL. (2013) Posttranscriptional control of T cell effector function by aerobic glycolysis. *Cell* 153:1239–1251
- El-Seoudi A, Abd El Kader T, Nishida T, Eguchi T, Aoyama E, Takigawa M, Kubota S (2017) Catabolic effects of FGF-1 on chondrocytes and its possible role in osteoarthritis. *J Cell Commun Signal*. <https://doi.org/10.1007/s12079-017-0384-8>
- Escoté X, Gómez-Zorita S, López-Yoldi M, Milton-Laskibar I, Fernández-Quintela A, Martínez JA, Moreno-Aliaga MJ, Portillo MP (2017) Role of omentin, vaspin, cardiotrophin-1, TWEAK and NOV/CCN3 in obesity and diabetes development. *Int J Mol Sci* 18. <https://doi.org/10.3390/ijms18081770>
- Hao W, Chang CP, Tsao CC, Xu J (2010) Oligomycin-induced bioenergetic adaptation in cancer cells with heterogeneous bioenergetic organization. *J Biol Chem* 285:12647–12654
- Hoshijima M, Hattori T, Aoyama E, Nishida T, Yamashiro T, Takigawa M (2012) Roles of heterotypic CCN2/CTGF-CCN3/NOV and homotypic CCN2-CCN2 interactions in expression of the differentiated phenotype of chondrocytes. *FEBS J* 279:3584–3597

- Itoh S, Hattori T, Tomita N, Aoyama E, Yutani Y, Yamashiro T, Takigawa M (2013) CCN family member 2/connective tissue growth factor (CCN2/CTGF) has anti-aging effects that protect articular cartilage from age-related degenerative changes. *PLoS One* 8:e71156
- Ivkovic S, Yoon BS, Popoff SN, Safadi FF, Libuda DE, Stephenson RC, Daluiski A, Lyons KM (2003) Connective tissue growth factor coordinates chondrogenesis and angiogenesis during skeletal development. *Development* 130:2779–2791
- Janune D, Kubota S, Lazar N, Perbal B, Iida S, Takigawa M (2011) CCN3-mediated promotion of sulfated proteoglycan synthesis in rat chondrocytes from developing joint heads. *J Cell Commun Signal* 5:167–171
- Janune D, Abd El Kader T, Aoyama E, Nishida T, Tabata Y, Kubota S, Takigawa M (2017) Novel role of CCN3 that maintains the differentiated phenotype of articular cartilage. *J Bone Miner Metab.* <https://doi.org/10.1007/s00774-016-0793-4>
- Jun JJ, Lau LF (2011) Taking aim at the extracellular matrix: CCN proteins as emerging therapeutic targets. *Nat Rev Drug Discov* 10: 9459–9463
- Kawaki H, Kubota S, Suzuki A, Lazar N, Yamada T, Matsumura T, Ohgawara T, Maeda T, Perbal B, Lyons KM, Takigawa M (2008) Cooperative regulation of chondrocyte differentiation by CCN2 and CCN3 shown by a comprehensive analysis of the CCN family proteins in cartilage. *J Bone Miner Res* 23:1751–1764
- Kubota S, Takigawa M (2011) The role of CCN2 in cartilage and bone development. *J Cell Commun Signal* 5:209–217
- Kubota S, Takigawa M (2013) The CCN family acting throughout the body: recent research developments. *Biomol Concepts* 4:477–494
- Kubota S, Takigawa M (2015) Cellular and molecular actions of CCN2/CTGF and its role under physiological and pathological conditions. *Clin Sci (Lond)* 128:181–196
- Kubota S, Hattori T, Nakanishi T, Takigawa M (1999) Involvement of cis-acting repressive element(s) in the 3'-untranslated region of human connective tissue growth factor gene. *FEBS Lett* 450:84–88
- Leask A, Abraham DJ (2006) All in the CCN family: essential matricellular signaling modulators emerge from the bunker. *J Cell Sci* 119:4803–4810
- Maeda-Uematsu A, Kubota S, Kawaki H, Kawata K, Miyake Y, Hattori T, Nishida T, Moritani N, Lyons KM, Iida S, Takigawa M (2014) CCN2 as a novel molecule supporting energy metabolism of chondrocytes. *J Cell Biochem* 115:854–865
- Nakanishi T, Nishida T, Shimo T, Kobayashi K, Kubo T, Tamatani T, Tezuka K, Takigawa M (2000) Effects of CTGF/Hcs24, a product of a hypertrophic chondrocyte-specific gene, on the proliferation and differentiation of chondrocytes in culture. *Endocrinology* 141:264–273
- Nishida T, Kubota S, Nakanishi T, Kuboki T, Yosimichi G, Kondo S, Takigawa M (2002) CTGF/Hcs24, a hypertrophic chondrocyte-specific gene product, stimulates proliferation and differentiation, but not hypertrophy, of cultured articular chondrocytes. *J Cell Physiol* 192:55–63
- Nishida T, Kubota S, Kojima S, Kuboki T, Nakao K, Kushibiki T, Tabata Y, Takigawa M (2004) Regeneration of defects in articular cartilage in rat knee joints by CCN2 (connective tissue growth factors). *J Bone Miner Res* 19:1308–1319
- Perbal B, Takigawa M (2005) CCN protein -a new family of cell growth and differentiation regulators. Imperial College Press, London, pp 1–311
- Rachfal AW, Brigstock DR (2005) Structural and functional properties of CCN proteins. *Vitam Horm* 70:69–103
- Riser BL, Najmabadi F, Perbal B, Peterson DR, Rambow JA, Riser ML, Sukowski E, Yeger H, Riser SC (2009) CCN3 (NOV) is a negative regulator of CCN2 (CTGF) and a novel endogenous inhibitor of the fibrotic pathway in an in vitro model of renal disease. *Am J Pathol* 174:1725–1734
- Takigawa M, Tajima K, Pan HO, Enomoto M, Kinoshita A, Suzuki F, Takano Y, Mori Y (1989) Establishment of a clonal human chondrosarcoma cell line with cartilage phenotypes. *Cancer Res* 49:3996–4002