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REVIEW

An early history of CCN2/CTGF research: the road to CCN2 via *hcs24*, *ctgf*, ecogenin, and regenerin

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Abstract The principal aim of this historical review is to present the processes by which the different aspects of CCN2/CTGF/Hcs24 were discovered by different groups and how much CCN2/CTGF, by being integrated into CCN family, has contributed to the establishment of the basic concepts regarding the role and functions of this new class of proteins. This review should be particularly useful to new investigators who have recently entered this exciting field of study and also provides a good opportunity to acknowledge the input of those individuals who participated in the development of this scientific field.

Keywords CCN family · CCN proteins · CCN2/CTGF/Hcs24 · Ecogenin · Regenerin

Abbreviations

CCN	**C**ysteine-rich 61, **C**onnective tissue growth factor, **N**ephroblastoma-overexpressed
CCN2	CCN family member 2, Connective tissue growth factor
Hcs24	Hypertrophic chondrocyte-specific gene product 24
CCN3	CCN family member 3, Nephroblastoma-overexpressed
ECM	Extracellular matrix
MMP	Matrix metalloproteinase
TGF-β	Transforming growth factor-beta
BMP	Bone morphogenetic protein
VEGF	Vascular endothelial growth factor
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
EGF	Epidermal growth factor
PDGF	Platelet-derived growth factor
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
OPG	Osteoprotegerin
IGFBP	Insulin-like growth factor-binding protein
VWC	Willebrand factor type C repeat
TSP	Thrombospondin
HUVEC	Human umbilical vein endothelial cells
HCS-2/8	Human chondrosarcoma derived chondrocytic cell line 2/8
CAESAR	*cis*-acting element of structure-anchored repression

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Introduction

In this manuscript I am presenting milestones in the early history of the research on Hcs24/CCN2, which became the prototype of the CCN family of genes/proteins (Bork 1993; Brigstock 1999; Lau and Lam 1999; Perbal 2004; Takigawa 2003; Takigawa et al. 2003) and is now known to be composed of six members. Public international announcement of the CCN nomenclature was made by the Steering Committee (Brigstock et al. 2003) that was constituted by major CCN researchers at the first Workshop on the CCN family of genes and spread worldwide in the early 2000's. Among the family members, CCN2 has been the most investigated with respect

to biological roles and functions. It plays critical roles in normal fundamental biological processes including, cartilage and bone formation, angiogenesis, and wound repair, as well as serves to regulate cell proliferation, differentiation, adhesion, spreading, migration, and survival of cells under normal and pathological conditions (Brigstock 1999; Takigawa 2000, 2003, 2005; Takigawa et al. 2003; Perbal 2001; Perbal and Takigawa 2005). Regarding the regulation of gene expression, that of CCN2 has also been the most investigated among the CCN members (Kubota and Takigawa 2007b).

Discovery – independently discovered by different groups from different aspects

CCN2 was first discovered as a result of an attempt to isolate immediate early genes from serum-stimulated NIH3T3 cells by differential screening of a cDNA library (Almendral et al. 1988). A detailed characterization such as gene structure and predicted protein sequence of one of the immediate early genes named "fibroblast-inducible secreted protein-12" (*fisp12*), which is now defined as mouse CCN2, was subsequently reported in May of 1991 (Rysec et al. 1991). At almost the same time, using a similar screening strategy, Brunner and co-workers also isolated this mouse CCN2 from transforming growth factor (TGF)-β-stimulated mouse ARK-2B cells and named it *bIG-M2* (Brunner et al. 1991). However, these studies involved only gene isolation; and functional studies on the genes that they found were not conducted.

A few months later, human CCN2 was partially purified from the culture medium of human umbilical vein endothelial cells (HUVEC) as a growth factor related to platelet-derived growth factor (PDGF) (Bradham et al. 1991). In that study, human CCN2 was discovered due to the cross-reactivity of a PDGF antiserum with a single polypeptide having a molecular weight of 38 kDa and secreted by cultured HUVEC; although it is now recognized that no structural similarity exists between them. Its cDNA was isolated from a HUVEC cDNA expression library with anti-PDGF and shown to encode a 349-amino acid protein (Bradham et al. 1991). Because the partially purified protein was found to be both mitogenic and chemotactic for fibroblast-like cells in vitro, this protein was named "connective tissue growth factor (CTGF;" Bradham et al. 1991). Fisp12/bIG-M2 encodes 348 amino acids, contains, 39 cysteine residues, and is 91% homologous to human CCN2 (CTGF).

Independent of these studies, my research group was searching for a putative regulatory molecule(s) involved in the whole process of endochondral ossification, which I dubbed "endochondral ossification genetic factor (ecogenin)." The strategy that we took was differential display PCR using chondrocytic cells and osteoblastic cells, and as a result we isolated a sequence tag that was expressed in human chondrocytic cell lines HCS-2/8 and 2/A (Takigawa et al. 1989, 1991) as well as in primary cultures of rabbit growth cartilage cells but not in osteoblastic cell lines or primary cultures of rabbit resting cartilage cells (Kimura et al. 1995). Since the sequence tag (tag no. 24) was highly expressed in hypertrophic chondrocytes, its gene was named hypertrophic chondrocyte-specific gene 24 (*hcs24*). The cDNA that contained this sequence tag (tag no. 24) was cloned; and its gene product, which we named Hcs24, was shown to be identical to that of human CCN2 (Nakanishi et al. 1997). This paper was accepted for publication in March of 1997 and published in May of that year. As we had expected, we also found it to be a regulatory molecule involved in the whole process of endochondral bone formation, which I will describe later. In addition, evidence showing the role of CCN2 in skeletal and vascular growth, development, and regeneration had also started to accumulate.

In August of 1977, Brigstock et al. (1997) reported the purification and characterization of a novel heparin-binding growth factor in porcine uterine secretory fluids. The factor was eluted with 0.8 M NaCl from heparin columns and so termed HBGF-0.8. It was shown to be mitogenic for Balb/c 3 T3 fibroblasts but not for capillary endothelial cells, and was identical to a truncated form (~10 kDa) of CCN2, which corresponds to its CT module. They also reported various isoforms of CCN2 in pig uterine luminal flushings later (Ball et al. 1998).

Very early studies

Because *fisp12*/*bIG-M2* was found as an immediate early gene induced by TGF-β, which is a well-known growth factor involved in fibrosis, and because CTGF/FISP12 was reported to be both mitogenic and chemotactic for fibroblast-like cells (Brigstock et al. 1997; Frazier et al. 1996), most of the early studies focused mainly on the role of CCN2 in fibrosis until Dr. T. Shimo, who was a Ph. D. student in my group, reported its possible angiogenic activity in 1998 (Shimo et al. 1998). In addition, because of the limited availability of CCN2 protein, most studies during that time focused on investigation of the gene expression and localization of CCN2 in various tissues; however, many reports were published on overexpression of CCN2 in fibrotic skin disorders and in fibrotic lesions of various organs and tissues including the lung, kidney, liver, cardiovascular system, pancreas, lens, bowel, and gingiva, as well as in the stroma of various tumors (Takigawa 2003). The involvement of CCN2 in fibrosis is in line with the fact that CCN2 is highly expressed during the process of wound healing (Igarashi et al. 1993). However, few reports appeared on its expression in fibroblasts or fibroblast-like cells in the physiological state. These findings strongly suggested the pathological

significance of CCN2 in these fibrotic disorders (Leask et al. 2002), and this line of studies has become one of main streams of CCN2 study, as I will describe in a later section.

Although CCN2 had been originally identified in the conditioned medium from HUVEC (Bradham et al. 1991), no detectable in vivo expression was found in normal adult human arteries (Igarashi et al. 1996; Oemar and Luscher 1997; Oemar et al. 1997). However, the presence of CCN2 protein and/or its mRNA was detected in endothelial cells during human embryonic development (Surveyor and Brigstock 1999; Wandji et al. 2000) and in actively migrating and proliferating endothelial cells in culture, but not in quiescent ones (Shimo et al. 1998). These findings led us to speculate that CCN2 is a novel angiogenesis factor. Therefore, we tried to suppress the endogenous expression of CCN2 in cultured endothelial cells by the addition of antisense oligomer or by transfection with expression vectors that generated its antisense RNA, because recombinant CCN2 was not available at that time. As a result, we found marked inhibition of proliferation and migration of endothelial cells. These findings indicated that CCN2 is involved in the proliferation and migration of endothelial cells via an autocrine pathway (Shimo et al. 1998). To my knowledge, this was the first report showing that CCN2 has angiogenic activity.

Recombinant protein revealed physiological functions of CCN2 – The way from Hcs24 to ecogenin

In 1996, my research group in collaboration with Japan Tobacco's Research Laboratory succeeded in producing recombinant human CCN2 by introducing a pcDNA3.1(−) vector harboring the coding region of CTGF cDNA into HeLa cells; and we reported its actions on chondrocytes and vascular endothelial cells at the Joint Meeting of Japanese Society for Biochemistry and Japanese Society for Molecular Biology held that year. The recombinant CCN2 (rCCN2) produced by this method showed activity at 1–50 ng/ml, indicating that CCN2, like growth factors, is a signaling molecule. As mentioned above, Dr. T. Shimo found that it promoted the adhesion, proliferation, and migration of vascular endothelial cells and induced tube formation by them (Shimo et al. 1998). In addition, *in ovo* application of rCCN2 to the chicken chorioallantoic membrane (CAM) resulted in a gross angiogenic response; and rCCN2 injected along with collagen gel into the back of mice induced strong angiogenesis (Shimo et al. 1999). Kireeva et al. (1997) also reported that mouse recombinant CCN2 (FISP12) produced by a vaculovirus system directly stimulated the adhesion of fibroblasts to substratum but that much higher concentrations (3–10 μg/ml) were required to show its activity. Using mouse rCCN2, this group also showed that it stimulated endothelial cell adhesion and migration through integrin avb3 and promoted endothelial cell survival, as well as also induced neovascularization in rat corneal micropocket implants (Babic et al. 1999). These findings in conjunction with the in vitro data described above also indicate that CCN2 is a potent angiogenesis factor.

Availabilty of recombinant protein also enabled us to investigate its action on various types of cells. Since we had been interested in endochondral ossification, we first focused on chondrocytes, especially growth-plate chondrocytes. In addition to its effect on endothelial cells, human rCCN2 protein (20–50 ng/ml) promoted the proliferation of cultured chondrocytes in sparse and growing cultures (Nakanishi et al. 2000) and increased the proteoglycan synthesis and gene expression levels of aggrecan and collagen type II, which are typical markers of chondrocyte maturation, in confluent cultures in which they were maturing. Moreover, the rCCN2 effectively stimulated the gene expression of collagen type X, a marker of chondrocyte hypertrophy, in over confluent cultures in which the cells were in the prehypertrophic stage (Nakanishi et al. 2000) and also stimulated alkaline phosphatase activity, a marker of calcification, as well as indeed induced matrix calcification of chondrocytes in culture (Nakanishi et al. 2000). Considering the high expression of CCN2 in hypertrophic chondrocytes, these results indicate that CCN2 produced by hypertrophic chondrocytes promotes the proliferation and differentiation of growth-cartilage cells toward endochondral ossification (Takigawa et al. 2003).

Moreover, Dr. T Nishida, who was one of the Ph. D. students in my laboratory, discovered that rCCN2 promoted the proliferation of osteoblastic cell lines Saos-2 and MC3T3E1 (Nishida et al. 2000), and increased the mRNA expression levels of type I collagen, alkaline phosphatase, osteopontin, and osteocalcin, as well as the activity of alkaline phosphatase, in these cells (Nishida et al. 2000). It also stimulated collagen synthesis in and matrix mineralization by MC3T3-E1 cells (Nishida et al. 2000). These findings indicated that osteoblasts are also target cells for CCN2 and suggested that CCN2 produced by hypertrophic chondrocytes acts on osteoblasts in bone close to the hypertrophic zone of cartilage in a paracrine manner and that in some cases, as described later, CCN2 acts on osteoblasts in an autocrine manner in addition to the paracrine manner.

At the final stage of endochondral ossification, calcifying cartilage is invaded by blood vessels, which leads to the recruitment of perivascular osteoblast-progenitor cells. Therefore, the findings described above, taken together with the high expression level of CCN2 in hypertrophic chondrocytes in vivo and the potent angiogenic activity of CCN2 described above, indicate that CCN2 is a novel, paracrine regulator that promotes the entire process of endochondral ossification by stimulating the proliferation and differentiation of chondrocytes, osteoblasts, and endothelial cells.

Thus CCN2 was revealed to be "ecogenin (endochondral ossification genetic factor)," which we had named as a putative factor 7 years earlier. This conclusion was confirmed later by the results of gene knockout experiments in mice, which showed that *ctgf*-deficient mice displayed skeletal dysmorphisms due to impaired endochondral ossification (Ivkovic et al. 2003).

The availabilty of recombinant protein enabled us to develop various polyclonal and monoclonal antibodies against CCN2. Using these reagents, Dr. T Tamatani of Japan Tobacco' Research Laboratory detected CCN2 in the sera of individuals with biliary atresia (Tamatani et al. 1998). These antibodies and ELISA became useful tools for studies described later. Using an antibody, Dr. H Ohnishi, who was a lecturer of internal medicine at Okayama University Medical School, in collaboration with my laboratory showed increased expression of CCN2 in the infarct zone of experimentally induced myocardial infarction in rats (Ohnishi et al. 1998); and Dr. Y Kondo, who belonged to the Department of Neuroscience, Okayama University, reported the localization of CCN2 in the rat central nervous system (Kondo et al. 1999).

From searching for cell-surface receptors to discovery of multiple types of binding partners

The recombinant protein of CCN2 also enabled us to investigate the receptors of CCN2 on the cell surface. Back in 1998, using iodinated rCCN2/CTGF/HCS24 Dr. T Nishida, who was a graduate student in my laboratory at that time, found 2 classes of binding sites, with Kd values of 18.6 nM and 259 nM, on chondrocytic HCS-2/8 cells (Nishida et al. 1998; Takigawa 2000). To my knowledge, this was the first report of an attempt to identify possible receptors for CCN2. He found similar binding sites on osteoblasts (Takigawa 2000; Nishida et al. 2000). A cross-linking study using chondrocytic cells, osteoblastic cells, and endothelial cells revealed the formation of ^{125}I–CTGF/HCS24-receptor complexes with an apparent molecular weight of 280 kDa (Takigawa 2000; Nishida et al. 2000). This receptor on HCS-2/8 cells was phosphorylated by stimulation of the cells with CCN2, suggesting it to be a signal-transducing receptor (Takigawa 2000). However, this receptor has still not been isolated nor cloned yet.

Until the early 2000's the best-known CCN2 receptors on the cell surface were integrins. Accumulated experimental data showed that integrins a_Mb_2, a_6b_1, and $a_{IIb}b_3$ mediated the cell adhesion of monocytes, fibroblasts, and platelets, respectively, under the direct interaction with CCN2 (Schober et al. 2002; Chen et al. 2001; Jedsadayanmata et al. 1999). Another type of integrin, a_vb_3, was reported to assist CCN2 in inducing angiogenesis (Babic et al. 1999). This a_vb_3 molecule was reported to mediate the adhesion of hepatic stellate cells under the interaction with the CT module (Gao and Brigstock 2004), suggesting that particular types of integrins are supposedly direct receptors of the CT module. In addition to integrins, low-density lipoprotein receptor-like protein 1 (LRP-1) was identified as a binding protein of the CCN2 molecule in 2001 (Segarini et al. 2001). We also confirmed the display of LRP-1 molecules on chondrocytes both in vivo and in vitro, and it is therefore highly possible that LRP-1 plays a significant role in cartilage biology in collaboration with CCN2 (Kawata et al. 2006). Another intriguing candidate is ErbB4, which is also a tyrosine kinase-type receptor for one of the neurotrophins and expressed on chondrocytes (Nawachi et al. 2002).

After the unified name CCN2 was introduced (Brigstock et al. 2003), known receptor molecules such as Trk A, LRP-1, LRP-6, RANK, OPG, FGFRs, and DC-STAMP were reported to bind to CCN2. Details of advances in the search for specific receptors and known receptor molecules were described in later review articles (Kubota and Takigawa 2007b; Takigawa 2013, 2017; Lau 2016).

After CCN2 had been shown to bind BMP4 and TGF-β1 and to modify the action of these growth factors (Abreu et al. 2002), other growth factors such as BMP-2, TGF-β3, VEGF, FGF-2, PDGF-BB, and GDF-5 were also shown to bind to CCN2. Details regarding advances in the search for CCN2-binding growth factors were described in later review articles (Kubota and Takigawa 2007b; Takigawa 2013, 2017).

Because Brigstock's group had purified CCN2 as a heparin-binding growth factor, Dr. Nishida in my group showed the possible binding of CCN2 to perlecan, which may be regarded as a co-receptor of CCN2 in growth-plate cartilage (Nishida et al. 2003). Later, other matrix components such as aggrecan, matrilin, and decorin were found to interact with CCN2 (Kubota and Takigawa 2007b; Takigawa 2013, 2017). Moreover, matrix metalloproteinase has been shown to bind to CCN2. Details on advances in the search for CCN2-binding matrix components were described in later review articles (Kubota and Takigawa 2007b; Takigawa 2013, 2017).

In this way, CCN2 has recently been shown to utilize multiple receptors as well as to modify a growth factor's action and its retention in the extracellular matrix when exerting its multiple functions. Such findings also indicate that CCN2 orchestrates other extracellular signaling molecules in its microenvironment rather than being simply a growth factor, and thus it may be referred to as a "signal conductor" (Takigawa 2013, 1017).

Daybreak of insight into CCN2 intracellular signal transduction

Recombinant protein also enabled us to investigate intracellular signal transduction pathways of CCN2. In 2001, one of my Ph. D students, Gen Yosimichi, reported that two classical MAPKs, p38 MAPK and ERK, mediated the

CCN2-emitted signals to promote differentiation and proliferation of chondrocytes, respectively (Yosimichi et al. 2001). The ERK signaling pathway was also reported to be involved in LRP-1-mediated transmission of CCN2 signals promoting myofibroblastic differentiation (Yang et al. 2004). Although the following were findings made after the establishment of CCN2, these extensive studies performed later also revealed the contribution of Jun N-terminal kinase (JNK) to CCN2 signal transduction for both proliferation and differentiation of chondrocytes (Yosimichi et al. 2006). Moreover, Yoshimichi revealed the involvement of the phosphatidylinositol- 3 kinase (PI3K) - protein kinase B/Akt pathway in transmitting the CCN2 signal to promote chondrocyte hypertrophy. Furthermore, he also showed that protein kinase C (PKC), particularly PKCα, was the major secondary messenger kinase that mediates most of these CCN2 signals described, except for those involving JNK (Yosimichi et al. 2006). In other types of cells CCN2 signal transduction cascades have been uncovered little by little after recombinant CCN2 proteins became commercially available. However, it should be noted that there has been confusion in this research area because some studies were made using a commercially available c-terminal 11.0-kDa protein consisting of 97 amino acid residues, almost corresponding to c-terminal cystine-knot module of CTGF/CCN2, as CTGF/CCN2. CCN researchers should clearly distinguish full-length CCN2 and its independent modules, because each module has its own specific activity; and so signal transduction cascades could be different dependent on the modules used (Takigawa 2015).

History of the search for extracellular factors that regulate CCN2/CTGF gene expression

As mentioned earlier, CCN2 was first described as an immediate-early gene that was induced by TGF-β. Consistent with this initial report, a number of reports have described the involvement of TGF-β in the induction of *ccn*2 gene expression in different types of cells and tissues (Eguchi et al. 2001; Igarashi, 1993; Kikuchi et al. 1995). Because TGF-β had been shown to be highly expressed in the hypertrophic region of cartilage tissue (Joyce et al. 1990) and to induce the expression of CCN2 in chondrocytes, which was shown by one of the instructors in my department (Nakanishi et al. 1997), the predominant expression of CCN2 mRNA in the hypertrophic chondrocytes in their physiological state had been thought to be due to the high level of TGF-β (Takigawa et al. 2003). Because TGF-β is a potent inducer of matrix formation and cartilage contains many matrix components such as collagen type II and proteoglycans, we hypothesized that TGF-β-induced up-regulation of CCN2 would be a naturally occurring physiological phenomenon in cartilage and that its ectopic overexpression in non-skeletal, soft tissues could cause fibrosis. Similarly, Nakanishi et al. (1997) reported that BMP-2, which is involved in bone formation, induced CCN2 gene expression in the chondrocytic cell line HCS-2/8; and the highest immunoreactivity for BMP-2-7 was detected in chondrocytes in the hypertrophic and calcifying zones of the growth plate (Anderson et al. 2000). Therefore, we also hypothesized that the high-level expression of CCN2 in hypertrophic chondrocytes is at least partially due to high levels of BMPs in them and that CCN2 mediates the action of BMP on bone formation, including endochondral ossification.

In the period from the late twentieth century to the very early part of the twenty-first century, at the time when CCN2 recombinant protein was unavailable yet, the search for extracellular factors such as growth factors that control the gene expression of *ccn2* was the most popular aspect of CCN2 research. Although their effect was less than that of TGF-β (Grotendorst et al. 1996; Igarashi et al. 1993; Rysec et al. 1991), PDGF, EGF, and FGF were shown to induce CCN2 gene expression in fibroblasts (Riser et al. 2000); and bFGF and VEGF, to induce CCN2 production by endothelial cells (Shimo et al. 2001b). Moreover, auto-induction by CCN2 was observed in mesangial cells (Riser et al. 2000) and endothelial cells (Shimo et al. 2001b). It is of particular interest that most of these growth factors or their receptors were later revealed to bind to CCN2 (Takigawa 2013, 2017). On the other hand, hepatocyte growth factor (HGF) was shown to counteract TGF-β1 by attenuating CCN2/CTGF induction and thus prevent renal fibrogenesis (Inoue et al. 2003).

Besides growth factors and cytokines, dexamethasone causes a large increase in CCN2 expression in Balb/c 3 T3 cells (Dammeier et al. 1998); and one of my instructors confirmed this phenomenon in chondrocytic HCS-2/8 cells (Kubota et al. 2003).

On the other hand, several inhibitors of CCN2 gene expression have been reported. Examples include cyclic AMP and agents that elevate its level (Duncan et al. 1999; Kothapalli et al. 1998), such as forskolin, choleratoxin, and prostaglandin E2 (Ricupero et al. 1999).

As a physical factor, Dr. T Yamashiro, an Assistant Professor in the Department of Orthodontics and member of my group, found that mechanical force induced CCN2 gene expression in osteoblasts and osteocytes in a mouse model (Yamashiro et al. 2001). Such stress also was shown to induce CCN2 gene expression in chondrocytes (Wong et al. 2003) and fibroblasts (Schild and Trueb 2004). Moreover, hypoxic stress increases both the *ccn2/ctgf* gene expression level and CCN2 secretion by tumor cells (Kondo et al. 2002; Shimo et al. 2001a). The observed hypoxia-elicited increase in the steady-state mRNA level was accounted for not by a transcriptional activation of the *ccn2/ctgf* promoter, but by a post-

transcriptional alteration of the stability of *ccn2/ctgf* mRNA (Kondo et al. 2002; Shimo et al. 2001a).

Based on our better understanding of the mechanisms regulating CCN2 gene expression, the development of positive regulatory molecules and physical treatments should be therapeutically useful for tissue regeneration; whereas that of negative regulatory ones may be therapeutically useful for fibrotic disorders.

History of regulation of CCN2 gene expression

After intracellular signal transduction has occurred, the gene expression of CCN2 is performed at the transcriptional and posttranscriptional levels. The most classical *cis*-element for transcriptional control of *ccn2/ctgf* is the TGF-β response element, which was reported by Grotendorst et al. (1996). The TGF-β-response element/basal control element 1 (TbRE/BCE-1) was found to be a critical element that determines the basal promoter activity in mesangial cells and mediates the gene regulation by TGF-β in fibroblasts (Grotendorst et al. 1996). After confirming the presence of the TbRE/BCE-1 element in chondrocytes in 2001, T. Eguchi, one of my graduate students, also revealed another *cis*-element that is critical for mediating the chondrocyte-specific induction of CCN2 gene expression. This element, designated transcriptional enhancer dominant in chondrocytes (TRENDIC), was discovered in the proximal promoter region of the CCN2 gene, where it forms a cluster with TbRE and SBE (Eguchi et al. 2001, 2002). TRENDIC is required for maintaining the high level CCN2 expression in chondrocytic cell line HCS-2/8 (Eguchi et al. 2002). Although this element acts as an enhancer in several different types of cells, its effect is the strongest in chondrocytic cells among the cells examined (Eguchi et al. 2002).

Gene regulation can be also conducted at the posttranscriptional level. It is well known that the 3′-untranslated region (3′-UTR) plays major roles in mediating post-transcriptional gene regulation (Kubota et al. 1999). In 2000, my research group identified a single RNA *cis* element regulating both mRNA stability and translation of CCN2 and designated this element as a *cis*-acting element of structure-anchored repression (CAESAR) present in both human and mouse *ccn2* genes (Kubota et al. 2000; Kondo et al. 2000). The CAESAR element is also located in the 3′-UTR of human CCN2 mRNA, facing the junction with the open reading frame. It is a secondary-structured RNA element of 84 bases in length. The involvement of CAESAR itself represses the basal gene expression at a low level by interfering with efficient translation, which probably contributes to enable restricted CCN2 expression in vivo; and thus it is considered to be the static function of CAESAR (Kubota et al. 2005). The repressive potential of CAESAR does not depend on the primary nucleotide sequence, but highly depends on the secondary structure, which provided the basis of the nomenclature (Kubota et al. 2000). This static aspect of the CAESAR function appears to be a general event that occurs in most cells in vivo. Details regarding recent advances in transcriptional and posttranscriptional regulation of CCN2 gene expression are described in our review articles (Kubota and Takigawa 2007b, 2011, 2013, 2015).

From ecogenin to regenerin (a regeneration factor)

Just after we proved that CCN2 was ecogenin, evidence that CCN2 has wide physiological functions, especially in skeletal development and regeneration, started to accumulate.

Wound healing is believed to be one of the regeneration processes and to occur by reproducing normal developmental processes. During fracture healing, both endochondral and intramembranous ossification processes occur. Dr. E. Nakata, who was a Ph. D. student in my laboratory, reported that the expression of CCN2 mRNA markedly increases during fracture healing in a mouse rib model (Nakata et al. 2002). He also found that CCN2 mRNA and protein were remarkably detected especially in hypertrophic chondrocytes and in proliferating chondrocytes in the regions of regenerating cartilage, suggesting that proliferating chondrocytes can also express CCN2 in rapidly regenerating cartilage. CCN2 mRNA was also expressed in proliferating periosteal cells in the vicinity of the fracture sites and in cells in the fibrous tissue around the callus. Moreover, CCN2 was also detected in active osteoblasts in the regions of intramembranous ossification (Nakata et al. 2002) and in cells in fibrous tissue, in vascular endothelial cells in the callus, and in periosteal cells around the fracture sites, suggesting the involvement of CCN2 in regeneration of chondrocytes, osteoblasts, and endothelial cells from precursor cells or mesenchymal stem cells.

One year later, Dr. M Kanyama, who was an Assistant Professor in the Department of Oral and Maxillofacial Rehabilitation at our university reported that CCN2 is also involved in alveolar bone regeneration after tooth extraction (Kanyama et al. 2003). His findings suggest that CCN2 produced by residual periodontal cells, endothelial cells, and undifferentiated mesenchymal cells acts as an autocrine/paracrine factor in the extraction sockets, causing the mesenchymal cells to differentiate into osteoblasts or the recruitment of osteoblasts from bone, which then replace the granulation tissue with bone.

To clarify the role of CCN2 in tissue regeneration of bone, Dr. H Kadota, a Ph. D. student in the Department of Orthopedics at Okayama University who joined my research group investigated the localization and expression of CCN2 during distraction osteogenesis, which is a bone regeneration

therapy to expand bone length. He first showed the localization of CCN2 in various cells located in the bone-forming area around the osteotomy site (Kadota et al. 2004). During the distraction phase, CCN2 mRNA was expressed not only in hypertrophic chondrocytes and osteoblasts but also in fibroblast-like cells and mesenchymal cells in sites of endochondral ossification, and not only in osteoblasts but also in pre-osteoblasts and fibroblast-like cells in sites of intramembranous ossification. These results suggest that CCN2 is involved in not only endochondral but also intramembranous ossification and plays important roles in regeneration of bone during distraction osteogenesis. The role of CCN2 in bone regeneration via intramembranous ossification was confirmed by Safadi et al. (2003), who reported that in vivo delivery of rCCN2 into the femoral marrow cavity induced osteogenesis in an animal model. Dr. T Kikuchi, another Ph. D. student in the Department of Orthopedics at Okayama University who joined in my research group also found that administration of CCN2-gelatin hydrogel complex with collagen into an artificial bone defect caused regeneration of the bone (Kikuchi et al. 2008).

Bone fracture, distraction osteogenesis, and tooth extraction are accompanied by bleeding; and then a blood clot forms, which disrupts platelets. Because we (Kubota et al. 2004) and Cicha et al. (2004) found that CCN2 is abundant in platelets and released from activated platelets, it is feasible that CCN2 is supplied by blood clots at the initial step of wound healing.

Unlike growth cartilage, articular cartilage does not show hypertrophic change or calcify in the physiological state. Consistent with their different characteristics, Dr. T. Nishida, who had already become an instructor in my laboratory at that time, found that rCCN2 increased DNA synthesis and stimulated the gene expression of common markers of cartilage such as type II collagen and aggrecan core protein in both growth and articular cartilage cells but that the gene expression of type X collagen, a marker of hypertrophic chondrocytes, was stimulated by rCCN2 in growth cartilage cells, but not in articular cartilage cells (Nishida et al. 2002). Oppositely, the gene expression of tenascin-C, a marker of articular chondrocytes, was stimulated by rCCN2 in articular chondrocytes, but not in growth cartilage cells (Nishida et al. 2002). Moreover, rCCN2 effectively increased both alkaline phosphatase (ALPase) activity and matrix calcification of growth cartilage cells, but not those of articular chondrocytes (Nishida et al. 2002). These findings indicate that CCN2 promotes the proliferation and expression of the specific differentiated phenotypes of various types of chondrocytes.

CCN2 is normally not expressed in articular cartilage cells of adult rodents, although its weak expression is observed in growing, young animals (Nawachi et al. 2002). However, even in adult rodents, a significant increase in the level of CCN2 mRNA was observed in the monoiodoacetic acid (MIA)-induced OA model (Nishida et al. 2004). The important finding was its localization. The clustered chondrocytes, in which clustering indicates an attempt to repair the damaged cartilage, produced CCN2. Therefore, CCN2 was suspected to play critical roles in cartilage regeneration. In fact, an injection of CCN2 incorporated in gelatin hydrogel (CCN2-hydrogel) into the joint cavity of MIA-induced OA model rats repaired their articular cartilage (Nishida et al. 2004). When the CCN2-hydrogel-collagen complex was implanted into the defects on the surface of articular cartilage in situ, new cartilage filled the defect by 4 weeks postoperatively (Nishida et al. 2004). These findings suggest the utility of CCN2 for the regeneration of articular cartilage.

Gene expression of CCN2 in osteoblasts in adult mice is minimal; but it is observed in osteoblasts in the primary spongiosa in embryonic mice, although its level is lower than that in the hypertrophic chondrocytes. In addition, the expression of CCN2 is detected in both ameloblasts and odontoblasts in the developing tooth germ (Shimo et al. 2002; Takigawa et al. 2003; Yamaai et al. 2005). These findings indicate that CCN2 is also involved in tooth development. Because regeneration is thought to recapitulate the process of development, all these findings indicate that CCN2 is a regeneration factor and thus should be named "regenerin" rather than ecogenin (Takigawa 2003, 2005). These names express the physiological function of CCN2 very well. However, since the CCN nomenclature started to be used under the proposal by major scientists in this field (Brigstock et al. 2003), the life of these names was not so long.

After the public announcement of the CCN nomenclature

After the CCN nomenclature was announced worldwide by the Steering Committee composed of major CCN researchers (Brigstock et al. 2003), a number of papers regarding CCN proteins including CCN2 logarithmically increased; and a tremendous number of them have been published so far (Perbal and Perbal 2016). Therefore, I do not describe each of them in this review article. Detailed advances in our understanding of major physiological functions of CCN2, such as in skeletal development, growth, and regeneration, are described in recent review articles (Takigawa 2013, 2017; Kubota and Takigawa 2013, 2015). Other physiological functions of CCN2 are also described in our and other's reviews (Kubota and Takigawa 2013, 2015; Charrier and Brigstock 2013).

Concerning the pathological significance of CCN2, fibrosis has been one of the major targets for CCN research ever since the discovery of CCN2. Detailed advances in this area are described in various reviews (Kubota and Takigawa 2015; Leask 2013; Riser et al. 2015); and various papers have appeared, including those on systemic sclerosis (Leask 2015),

skin fibrosis (Leask et al. 2002; Leask 2017), cardiac fibrosis (Leask 2015), kidney fibrosis (Charrier and Brigstock 2013), hepatic fibrosis (Huang and Brigstock 2012), and gingival outgrowth (Trackman and Kantarci 2015). Before the name "CCN2" was born, my group also reported the role of CCN2 in fibrosis and scleroderma in collaboration with Professor K. Takehara (Mori et al. 1999; Sato et al. 2000; Chujo et al. 2005). In addition, we also reported the role of CCN2 in the increased expression of p53 in liver fibrosis in collaboration with Dr. T. Takehara's group (Kodama et al. 2011). Considering the fact that skeletal tissues are rich in extracellular matrix, the production of which is stimulated by CCN2, fibrosis can be thought to be a result of ectopic overexpression of CCN2 in soft tissues, as described above. In this respect, better understanding of the molecular mechanism of regulation of gene expression under physiological and pathological conditions will be very important for development of CCN2-targeted therapeutics.

Another major target regarding the pathological significance of CCN2 research is tumor development and metastasis. Detailed advances in this field are described in recent review articles (Kubota and Takigawa 2015; Riser et al. 2015; Yeger and Perbal 2016; Wells et al. 2015); and numerous papers have appeared, including those on colorectal cancer (Ubink et al. 2016), breast cancer (Kleer 2016), pancreatic cancer (Banerjee et al. 2016), and blood cancers (Crawford and Irvine 2016). Other pathological events of significance such as inflammation, diabetic nephropathy, and diabetic retinopathy, have been described in a variety of reviews (Kubota and Takigawa 2007a; Kubota and Takigawa 2015; Wang et al. 2015; Klaassen et al. 2015).

Another pivotal advance in CCN2 research has been the uncovering of the molecular mechanisms of CCN2 action. Although specific receptors for CCN2 have not been cloned yet, CCN2 has been shown to bind known receptor molecules such as integrins, TrkA, FRFR1, EGFR, RANK, OPG, DC-STAMP (Segarini et al. 2001; Nishida et al. 2011; Aoyama et al. 2012, 2015; Takigawa 2013, 2017; Lau 2016), and growth factors themselves such as TGF-β, BMP-2 (Abreu et al. 2002), BMP-4 (Maeda et al. 2009), VEGF (Inoki et al. 2002), and PDGF-BB (Khattab et al. 2015) and GDF-5 (Khattab et al. 2015) via its four hands or modules (Takigawa 2013, 2017). We also found that CCN2 binds to itself as well as to CCN3 (Hoshijima et al. 2012). Moreover, it can also bind to extracellular matrix components such as heparin, perlecan, fibronectin, aggrecan, and matrillin-3 (Nishida et al. 2003; Aoyama et al. 2009; Takigawa 2013, 2017) and to their degrading enzymes, MMPs (Hashimoto et al. 2002; Takigawa 2013, 2017). These findings strongly suggest that CCN2 orchestrates various signaling pathways by binding to the above molecules and modify their actions as a "signal conductor," like the conductor of an orchestra. In other words, because the composition of these binding partners is different in the different microenvironments of various types of cells, as well as in their different states of differentiation, it is not surprising that CCN2 would exhibit multi-functionality. Such a molecular mechanism would be a source of CCN2 action to promote harmonized development and regeneration (Takigawa 2013, 2017).

Conclusion and perspectives

In this historical review, I have described the early history of CCN2 research in detail with respect to major physiological functions of CCN2, i.e., its role in skeletal development, growth, and regeneration. This review should be particularly useful to investigators who have recently become engaged in this exciting field and also has provided a good opportunity to acknowledge the input of those who have participated in the development of this scientific field. After the CCN nomenclature was announced worldwide, many investigators have joined this exciting field, resulting in a better understanding of the physiological and pathological functions and their molecular mechanism of CCN2. Because there are many good reviews about recent advances in CCN2 research, I have described the progress of CCN2 research after the worldwide announcement of the CCN nomenclature briefly by citing those reviews; and I would strongly recommend investigators new to the field to read those reviews. Because the molecular basis of CCN2 action has not been fully elucidated, its continuous basic investigation is essential for firmly establishing a new concept for this unique class of proteins. The other exciting trend is development of its medical and clinical applications.

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This review is dedicated to all my colleagues who have helped me and participated actively in this exciting field of research.

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