CCN Proteins in Health and Disease
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An Overview of the Fifth International Workshop on the CCN Family of Genes
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Chapter 1
A Recent Breakthrough in the CCN Field: Functional Interactions Between CCN2 and CCN3 are Uncovered

Bernard Perbal

Abstract As an introduction to the 2008 Toronto CCN meeting Proceedings, we provide in this chapter a brief discussion of new findings regarding the functional interactions of CCN2 and CCN3 that will certainly have an impact on our understanding of the functions of CCN proteins and the regulatory processes that control and govern their biological roles.

1.1 Introduction

The CCN family of proteins contains six members (CCN1–6) that are known to play key roles in fundamental biological processes (Perbal and Takigawa, 2005). The CCN acronym was originally coined by P. Bork (1993) who realized that the three founding members of this family, CYR61 (CCN1), CTGF (CCN2), and NOV (CCN3), shared a striking mosaic organization, with four modules exhibiting a high degree of conservation with large families of regulatory proteins. Hence the naming of these modules, IGFBP, VWC1, TSP1, and CT, was based on the fact that they contained stretches of sequences highly similar to the amino-terminal portion of IGFBPs (insulin-like growth factor binding proteins), to the Von Willebrand type C repeat (VWC), to the thrombospondin type 1 repeat (TSP1) and to a series of matrix proteins and growth factors that contain a cystine-knot motif (C-terminal module: CT) (Perbal and Takigawa, 2005). The high degree of homology, that is observed both at the level of primary and secondary structure (Holbourn et al., 2008) first suggested that these proteins might have similar or redundant functions. However, a handful of experimental data quickly established that the various CCN proteins play specific roles in normal and pathological conditions (Perbal and Takigawa, 2005; Leask and Abraham, 2006).
Considering that the expression of CCN proteins is subject to a tight spatio-temporal regulation, as first established in chicken (Joliot et al., 1992), it was proposed that their distinct biological roles might be dependent upon the bioavailability of the ligands and proteins with which they physically interact (Perbal, 2001). The tetramodular structure of the CCN proteins together with their complex expression pattern provided the basis for a wide variety of combinatorial events that would, in the end, dictate the biological properties of these proteins (Perbal, 2001). Along the same line, it is now obvious that the biological properties of the CCN proteins cannot result solely from their interactions with integrins, and it was proposed that the great variety of partners that physically interact with the CCN proteins might allow for the coordination of signaling pathways in which these partners are involved (Perbal, 2003).

Because the CCN proteins are composed of four modules whose sequences are partially represented in other large families of regulatory proteins, which also interact with several factors and ligands, it was proposed that functional interactions (positive or negative) between these proteins (CCNs, IGFBPs, VWC, TSP, and all other proteins interacting with them) might constitute the basis of a higher ordered level of regulation involving complex interdependent systems (Perbal, 2001, 2003). Furthermore, the identification of CCN proteins variants (for a recent review see Perbal, 2008) raised the possibility that such rearranged proteins lacking one or more structural modules, can compete with the full length proteins for an interaction with a specific target, that could either be a regulatory factor or a receptor (Perbal, 2001, 2004; Planque and Perbal, 2003). In light of this model, the growth arrest properties of CCN5, which lacks the entire CT module, are quite interesting as they suggest that CCN5 might act as a dominant negative regulator for cell proliferation, via its interaction with either other CCN proteins or their ligands, in a way similar to IkappaB which regulates the activity of NFKappaB (see recent review by Sun and Zhang, 2007).

A physical interaction between CCN2 and CCN3 has been reported to occur in vitro (Perbal et al., 1999). Because CCN2 is encoded by an immediate early gene, whereas CCN3 is a growth arrest/tumor suppressor type of protein (Perbal and Takigawa, 2005), we hypothesized that they might functionally interact in some way. In spite of our observation that the expression levels of CCN2 and CCN3 were inversely correlated in many cell lines (C.L. Li and B. Perbal, unpublished observations), the evidence for a functional interaction has been lacking. Two series of results presented at the 5th International Workshop on the CCN family of genes held in Toronto, Oct 2008, confirmed that CCN2 and CCN3 functionally interact, therefore reinforcing the fascinating possibility that other members of the CCN family might also be involved in such cross-regulations.

In the first example (Takigawa et al., in this volume), it was found that CCN3 expression was dramatically up-regulated in CCN2-null cartilage and chondrocytes (Kawaki et al., 2008) suggesting that the expression of these two proteins were inversely proportional to each other. In this model, recombinant CCN3 protein did not affect the proliferation of CCN2 K/O chondrocytes whereas it was reported to inhibit the proliferation of wild type chondrocytes, while recombinant CCN2
induced proteoglycan synthesis and calcification in long-term chondrocyte cultures, recombinant CCN3 dramatically inhibited both maturation and calcification. Along the same line, recombinant CCN2 induced formation of cartilage nodules, whereas recombinant CCN3 significantly repressed their formation. Taken together, these observations indicated that CCN3 and CCN2 exhibit opposite effects both on the proliferation and the differentiation of chondrocytes. Further studies established that CCN2 induces chondrocyte differentiation by stimulating the expression of chondrocyte associated genes, and that CCN3 counteracted these effects. Interestingly, cross-regulation of the two genes was also observed. Indeed, addition of recombinant CCN3 to the chondrocyte cultures, resulted in a marked decrease of CCN2 expression, whereas addition of recombinant CCN2 protein resulted in a rapid stimulation of CCN2 expression.

In the second example (Riser et al., in this volume), studies performed with rat kidney mesangial cells also established that CCN3 is a negative regulator of CCN2 profibrotic activity. In this system, TGF-beta induces the expression of CCN2 and production of extracellular matrix leading to fibrosis in pathological cases. In contrast, TGF-beta treatment of mesangial cells results in a dramatic reduction of CCN3 expression levels.

Moreover, either the addition of CCN3 or targeted CCN3 overexpression resulted in a marked down-regulation of CCN2 expression followed by efficient down-regulation of collagen type I transcription. The action of CCN3 was independent of smad signaling or cell proliferation.

Little is known about the processes involved in the cross regulation of these two genes.

As suggested elsewhere (Leask, 2009) the identification of precise elements in the type I collagen promoter which respond to CCN3 would be extremely informative. In any case, these examples provide the first two demonstrations that the biologically activities of CCN proteins are highly interdependent and that functional regulation occurs at both the transcriptional and translational levels.

The Yin/Yang roles of CCN2 and CCN3 which are uncovered in these studies also open a new era in the CCN field, as they point out the complex regulatory processes that might govern the biological properties of CCN proteins. Deciphering the structure–function relationship that underlies these mechanisms is certainly one of the most exciting challenges in the field.

Acknowledgments I am grateful to Dr. Herman Yeger for critical reading of the manuscript and Annick Perbal for her support and editorial help.

References


Chapter 2
Report on the Fifth International Workshop on the CCN Family of Genes

A. E. Irvine, B. Perbal, and H. Yeger

Abstract  The Fifth International Workshop on the CCN Family of Genes was held in October 2008. This bi-annual meeting provides a unique forum for researchers in the CCN field to present and exchange ideas. The CCN family of regulatory proteins play key roles in both normal cell development and a wide range of pathologies. This was reflected in the breadth of basic cell biology and translational studies presented at the conference. Exciting new in vitro and in vivo model systems are providing new insights into the functional complexity of the CCN family and promise much more to come.

Keywords  CCN1 · CCN2 · CCN3 · CCN4 · CCN5 · CCN6 · Cyr61 · CTGF · NOV · Wisp-1 · Wisp-2 · Wisp-3

The Fifth International Workshop on the CCN Family of Genes was held in Toronto, October 18–22, 2008. The meeting was organised by Herman Yeger, Bernard and Annick Perbal and provided an excellent environment for discussion and interaction amongst a diverse and enthusiastic group of researchers.

The Journal of Communication and Cell Signalling has now been launched as the official journal of the International CCN Society. The publishers, Springer Science & Business Media, sponsored the opening plenary session of the conference and also three scholarships for young investigators to attend the meeting. The first Springer Award for outstanding research in this field was presented by Peter Butler (Springer) to Professor Paul Bornstein by for his work on matricellular proteins. Professor Bornstein gave a stimulating talk focussed on the concept of ‘dynamic reciprocity’ and presented his studies using thrombospondin (TSP-1 and TSP-2) null mice. Opening remarks by Professor Perbal coupled with Professor Bornstein’s lecture set the stage nicely for the meeting to follow.
The first session of the meeting on CCN Structure/Function and Expression opened with a presentation by Dr. Ravi Acharya (Bath, England). Dr. Acharya examined the protein structure of the CCN family and discussed how this might contribute to the functional differences between family members. By modelling the three dimensional structure of the domains he provided insight as to how this might influence interaction with other key molecules. This was followed by a series of presentations on CCN2 gene regulation lead by Dr. Satoshi Kubota (Okayama, Japan) who described the characterisation of Nucleophosmin/B23 as a regulator of CCN2 in chicken chondrocytes. Nucleophosmin has the ability to shuttle between the nucleus and the cytoplasm and Satoshi presented extensive experiments demonstrating both transcriptional (nuclear) and post-transcriptional (cytoplasmic) regulation of CCN2. Further work from this group, presented by Dr. Ogawara, demonstrated that mi-RNA 18a acts on CCN2 via the 3′-UTR and regulates human chondrocytic differentiation. This work now introduces a new level of CCN regulation that should get increasing attention. In a change of cellular context, Dr. Cabello-Verrugio (Santiago, Chile) presented work on the regulation of CCN2 by TGF-b and LPA in skeletal muscle cells in parallel with other studies from his lab suggesting dependency on decorin and involvement of the endocytic receptor LRP-1. He used the C2C12 myoblast cell line to show a dose-dependent induction of CCN2 where the induction is mediated classically through TGFbR1 and SMAD2/3.

The final paper in this session was given by Dr. Yasuda (Boston, USA) and described the use of ChIP on CHIP analysis to identify a SOX9 binding site in the promoter region of CCN2.

Brahim Chaqour (New York, USA) described elegant studies to examine the mechanical regulation of CCN1 in smooth muscle cells. Myocardin related transcription factor (MRTF-A) was shown to shuttle between the nucleus and the cytoplasm being localised in the cytoplasm in unstimulated cells and accumulating in the nucleus of mechanically stimulated cells. He showed that nuclear MRTF-A acts in concert with P300/CBP to regulate CCN1. Periostin is not a member of the CCN family but is a novel secreted matricellular protein with a similar expression pattern to CCN2. Douglas Hamilton (Ontario, Canada) described experiments showing that periostin expression was increased in mechanically stressed skin fibroblasts and was also increased in cutaneous wound repair. Periostin seems to become extracellular in various pathologies and its mode of action and expression suggest that it may have similarities to the CCN family. Interestingly, periostin binds to CCN3. Lan Wei (Boston, USA) constructed a series of plasmids expressing each domain of CCN5 alone and in combinations both with and without the secretory peptide. By using fluorescently tagged constructs they were able to show that non-secreted forms of the Von Willebrand type C (V) domain alone, Thrombospondin type-I domain (T) alone and IV, VT and IVT domains were imported into the nucleus; the same constructs containing an N-terminal signal peptide were not detected. They are currently applying monoclonal antibodies against specific domains of CCN5 to further elucidate its mode of action. This session finished with a return to the topic of CCN2 regulation. Hirokazu Okada (Saitama, Japan) identified a 20 bp region in the mouse CCN2 promoter which is bound by PolyADP-Ribose Polymerase-1.
(PARP-1) and named it PARP Binding Element (PBE). Since PARP-1 is a higher order modifier of histones and CCN2 is the main mediator of the pro-fibrotic effects of TGF-β in fibrotic kidneys, this may open up new anti-fibrotic therapeutic targets for this disorder.

The session on Osteogenesis and Chondrogenesis was opened by Professor Masaharu Takigawa (Okayama, Japan) who described a transgenic mouse model in which the CCN2 gene was overexpressed in cartilage under the control of the type II collagen promoter. He showed that this lead to accelerated endochondral ossification by promoting proliferation and differentiation of chondrocytes. He also compared wild-type and CCN2 null mice and showed that CCN2 is also required for normal intramembranous bone development. This theme was carried on by a presentation from Fayez Safadi (Philadelphia, USA) who has developed a transgenic mouse model in which CCN2 is overexpressed in cells of the osteoblast lineage. By comparing moderate to high level overexpressing mice he was able to show that moderate levels of CCN2 promote bone formation whilst high levels have an opposite effect and promote osteoclast formation. Faith Hall-Glenn (Los Angeles, USA) described a further mouse model in which CCN1 and CCN2 were specifically knocked out in cartilage. Cartilage specific loss of CCN1 is perinatal lethal although the skeletons of the mice have only minor alterations whilst the double knockout animals have a much more severe phenotype. The phenotypes of the animals developed by Takigawa and Hall-Glenn were quite different reflecting the different strategies used to control gene expression. These animal models provide exciting new tools to investigate the functional roles of the CCN proteins. Ken-ichi Katsube (Tokyo, Japan) constructed CCN3 deletion mutants lacking the CT domain (del CT) and used these to study NOTCH and BMP signalling in the mouse osteogenic cell line, Kusa-A1. The CT domain is responsible for binding NOTCH yet the delCT mutant was still able to activate downstream NOTCH signalling pathways and was able to bind BMP. These results suggest that the inhibitory effect of CCN3 on osteogenesis is mediated by both NOTCH and BMP signalling pathways. Andrew Leask (Ontario, Canada) grew mesenchymal cells in a micromass model culture system for his studies on chondrogenesis. FAK/src signalling mediates cell adhesive properties and inhibition of this pathway lead to increased expression of CCN2 and chondrogenic matrix associated genes in wild type cells; CCN2 null cells did not respond. These results suggest that CCN2 operates downstream of FAK/src and that loss of FAK/src is critical for chondrocytic differentiation.

Kruppel-like factor 15 (KLF-15) is expressed in both cardiomyocytes and fibroblasts and is reduced by pro-hypertrophic stimuli both in vitro and in vivo. Mukesh Jain (Cleveland, USA) used a rat ventricular fibroblast cell model to show that KLF-15 inhibits CCN2 by preventing recruitment of P/CAF to the promoter region. He also generated KLF-15 null mice and found increased levels of CCN2 and collagen in the heart as a result of mechanical stress. These results suggest that KLF-15 plays a key role in the regulation of cardiac stress. Mark Erwin (Toronto, Canada) presented some fascinating studies using canine models to explore biological therapies for degenerative disc disease. Mongrel dogs which do not get disc problems retain a notochordal cell rich region, and these cells secrete CCN2 which can then
upregulate aggrecan expression in chondrocytes. In contrast, Beagles are prone to disc degeneration similar to ageing humans. This is likely a promising non-rodent animal model for the CCN field. Karen Lyons (Los Angeles, USA) used a CCN2−/− mouse model and mice with CCN2 specifically knocked out in endothelial cells to look for effects on angiogenesis. She showed that the vascular system develops normally in these mice initially but that vascular remodelling is defective, probably due to defective basement membrane assembly. Of further relevance here is the disruption in pericyte/endothelial cell interactions with broader implications for stem cell biology. Ursula Kees (Perth, Australia) has found markedly elevated levels of CCN2 in paediatric acute lymphoblastic leukaemia (ALL). She presented data showing that CCN2 may be involved in the interaction of the ALL cells with the bone marrow microenvironment and is currently investigating the underlying mechanisms involved. Gene enrichment analysis suggested multiple genes upregulated in ALL as in other cancers.

The first of the pathobiology sessions started with a series of presentations examining the role of CCN proteins in normal and wounded skin. Laure Rittie (Michigan, USA) used laser capture micro-dissection coupled with real time PCR and immunocytochemistry to study CCN expression in normal skin and following wounding by thermal ablation. She showed very clearly that the CCN proteins are expressed in a cell type and stage specific manner during wound healing. She also observed CCN expression in nuclei during injury and a marked decrease in CCN3 after wounding and return to normal levels in the healing phase thus supporting the notion of CCN proteins operating alternately and in balance. Following on from this Andrew Leask specifically examined CCN2 using a novel transgenic knock-in mouse model with a GFP gene inserted between the endogenous CCN2 promoter and gene. Using this strategy Andrew was able to show that, on wounding, CCN2 induction parallels the appearance of myofibroblasts and that pericytes also express CCN2 and contribute to myofibroblast activity. This data further begs the question of whether reactive mesenchymal phenotypes all exploit CCN2 for repair processes. Taihao Quan (Michigan, USA) presented studies on UV and chronologically aged skin showing that CCN1 plays a role in aberrant dermal collagen homeostasis. The thinking is that changes in CCN1 modify the TGFβ mediated effects on collagen synthesis and thereby structurally change skin. Interestingly, he also found that retinol had the potential to reverse many of these effects consistent with its application in anti-ageing skin care products. Kirsten Bielefeld (Toronto, Canada) gave an excellent presentation on her doctoral studies of β-catenin during wound healing. She used primary dermal fibroblasts to show that the extracellular matrix acts as a feedback loop to regulate β-catenin during wound repair. DEL1, a further matrix-cellular protein with an expression pattern similar to CCN1 and CCN2, was shown to enhance bone fracture healing (Yang, Stanford, USA). In contrast to the CCN1 and CCN2 mutants, DEL1 knockout mice have normal skeletons but heal fractures with less bone. This may reflect a role for DEL1 in preventing premature apoptosis of the hypertrophic cartilage during endochondral ossification. Cutler (Maryland, USA) used a mouse mammary epithelial cell line (HC-11) to examine the role of CCN2 during lactogenic differentiation. She showed that CCN2 levels increased
more than ten fold during differentiation, CCN2 enhances HC-11 differentiation, blocked by CCN2 siRNA, and that effects are mediated via activation of the β-1 integrin mediated adhesion complexes and integrin dependent signalling pathways. Shakil Ahmed (Oslo) used CCN2 transgenic mice to investigate the role of CCN2 in heart disease. CCN2 was shown to act as a survival factor in these mice resulting in reduced infarct size and improved recovery. Apparently the cardioprotection exhibited by CCN2 is mediated by a complex regulatory network. Stephen Twigg (Sidney, Australia) followed on from this with studies of CCN2 in diabetic cardiomyopathy. In contrast to the previous study, CCN2 was found to mediate the adverse effects of high glucose and free fatty acids in the H9C2 cardiomyocyte cell line. Treatment with a specific trkA inhibitor, K252a, blocked the effects of CCN2 on hypertrophy and apoptosis, an observation supporting the previous report by Wahab et al. (2005), suggesting that CCN2 is acting via the trkA pathway. The search for new biomarkers in systemic sclerosis was explored by the Robert Lafyatis (Boston, USA) who found that TGF-β regulated genes, including CCN2, are increased in the skin of patients with scleroderma and that some of these genes (COMP and TSP1) correlated with a modified Rodnan skin score. Using the tight skin mouse (Tsk) model he also found that CCN3 was highly expressed and postulated that it counter-regulates fibrillin matrix fibre assembly and deposition.

Takako Hattori (Okayama, Japan) used a combination of in vitro and in vivo studies to show that SOX9 binds to the enhancer region of the CCN2 gene. She then used mouse models in which CCN2 was specifically overexpressed in skin or cartilage to demonstrate a stimulatory feedback loop involving CCN2, SOX9 and aggrecan. CCN2 colocalized with aggrecan on the cell surface. Joshua Russo (Boston, USA) described a new model system they have developed to study leiomyoma in vivo. Fresh human fibroid tissue is broken up and resuspended in a matrigel/collagen I mixture before subcutaneous injection into mice. Following hormone supplementation, human smooth muscle cells grow out of the injection site where they acquire a blood supply through angiogenesis. These mice provide an excellent system in which to study the effects of CCN5 in fibroids. In addition, in tumor xenograft models he showed that CCN5 slowed down tumor growth with reduction in the tumor vascular architecture. Shiwen (London, England) investigated the mechanisms linking microvascular damage to the fibrogenic system in patients with scleroderma (SSc). He found that endothelin-1 stimulated pericytes (a multipotent phenotype) and fibroblasts to produce CCN2 and collagen, via ERK1/2 mediated signalling, and that pericytes acquire fibroblast markers on long term culture. This suggests that pericytes may contribute to the fibrosis observed in SSc and must be considered when developing new treatment strategies. Enrique Brandan (Chile) used a mouse model for Duchenne muscular dystrophy in which exercise protocols induce fibrosis and also lead to an increase in CCN2. He used this system to show that the proteoglycan, decorin, can interact with CCN2 inhibiting the fibrotic action. Gingival fibrosis is a clinical problem which often occurs as a side effect of medication such as cyclosporin. In the first of two presentations from Boston, Alpdogan Kantarci showed that CCN2 is increased at both the mRNA and protein levels in drug induced gingival fibrosis and examined the role of CCN2 in promoting
fibrotic lesions. Philip Trackman then described some beautiful studies in which he delineated the mechanisms underlying the problem and designed a dual pronged treatment strategy using lovastatin and forskolin to reduce TGF-β stimulated CCN2 levels in gingival cells. Bruce Riser (Chicago, USA) used an in vitro model of renal fibrosis to look for endogenous inhibitors of CCN2 and explore the possibility of interaction with other CCN family members. He found that CCN3 (either provided exogenously or overexpressed) downregulates CCN2 activity in mesangial cells and blocks ECM overaccumulation stimulated by TGFβ thus providing an opportunity for therapeutic intervention. This inverse relationship between CCN3 and CCN2 is in agreement with the recently published results by Kawaki et al. (2008). David Brigstock (Columbus, Ohio) has developed an exciting therapeutic strategy to target fibrosis in a mouse model of hepatic fibrosis and evaluated anti-CCN2 therapy in both a preventative and curative setting (before or after onset of collagen deposition). Liposomes containing CCN2 siRNA, when coated with a synthetic peptide to ensure they homed to activated hepatic stellate cells, proved to be effective as an antifibrotic agent. The ready delivery of CCN2 siRNA across multiple tissue barriers opens up possibilities for translational studies in the CCN field. The final presentation of this session was from Margarete Gopelt-Strube (Erlangen, Germany) who investigated the hypoxia-induced regulation of CCN2. Hypoxia was induced by the DMOG inhibitor of PHD2 and thereby activation of HIF1a. She demonstrated that regulation of CCN2 by hypoxia is cell type dependent and involves the FoxO family of transcription factors. Interestingly, she showed an additive effect of DMOG and TGFβ a more complex regulatory situation during injury.

In the spirit of promoting the bridging of two fields, CCN and Matrix, a new feature of the workshop was a special session entitled ‘From Matricellular to Extracellular’ (see http://ccnsociety.com/award.html). Three eminent clinician-scientists from Toronto put the entirety of the conference into context by presenting the human consequences of dysregulation in the extracellular matrix system. Aleksander Hinek described how defective production of components involved in elastin microfibril assembly contributes to numerous skeletal and vascular disorders. William Cole focused on how studies of rare genetic disorders have led to greater understanding of the genes required for normal development of bone and cartilage. Ren-Ke Li gave an inspirational talk on the potential of myocardial cell therapy. Through detailed studies with animal models he is teasing apart the underlying mechanisms for cell based therapy as an approach to matrix remodelling. Katherine Sodek has just completed her PhD and presented the work she carried out on ovarian cancer. She used a novel 3-D culture system and showed that MT1-MMP and MMP2 contribute to cell motility and matrix degradation whilst treatment with TGFβ stimulated spheroid formation and was associated with increased invasive capacity. This was an excellent session and set the clinical framework of matricellular disorders.

CCN3 came to the fore in the second pathobiology session of the meeting. Vivianna Vallachi (Milan, Italy) found that increased CCN3 expression was associated with poor prognosis in metastatic melanoma. Analysis of CCN3 in cultures of cells from melanoma lesions showed heterogeneous expression of the 46 kDa (mostly cytoplasmic) and 32 kDa (nuclear) proteins but this was not associated
with specific CCN3 gene mutations; however, CCN3 polymorphisms were noted. Xenotransplantation studies in immunodeficient mice showed a higher metastatic potential in CCN3 overexpressing cells and a greater resistance to induction of apoptosis by cancer chemotherapeutic drugs. In contrast, CCN3 expression is down-regulated as a result of BCR-ABL kinase activity in Chronic Myeloid Leukaemia (CML; Mc Callum, Belfast, Ireland). Increased CCN3 expression levels resulted in decreasing levels of phosphorylated ERK reducing cell proliferation whilst also increasing levels of cleaved caspase 3 and restoring induction of apoptosis. Primary human CML cells demonstrated growth inhibition in response to recombinant CCN3 which may be important for developing additional therapeutic strategies.

Perbal et al. (Paris, France and Bologna, Italy) examined CCN1-3 to evaluate their prognostic value in osteosarcoma and Ewings sarcoma. They found that CCN3 expression was associated with increased attachment, migration and an aggressive phenotype and with an increased risk of recurrence and metastases. A high number of cases expressed a CCN3 variant, lacking the NH3 domain which conferred worse prognosis for patients receiving chemotherapy and radiotherapy. Studies of CCN1 in osteosarcoma (A. Sabile, Zurich, Switzerland) showed it was upregulated in metastatic osteosarcoma cell lines and also in primary tissues from patients. CCN1 induced phosphorylation of Akt and GSK-3β and coincided with localization of p21 in the cytoplasm. This mechanism effectively activates both pro-survival and pro-proliferative pathways. CCN3 co-localises with a core component of gap junction complexes, connexin 43 (Cx43). These observations are in agreement with previously published work reporting the co-localisation of CCN3 and Cx43 (Fu et al., 2004; Gellhaus et al., 2004). Wun-Chey Sin (Vancouver, Canada) found Cx43 is down-regulated in aggressive breast tumours and Cx43 levels positively regulate expression of CCN3. Overexpression of CCN3 in breast cancer cells inhibited cell growth and was involved in reorganization of the actin cytoskeleton and redistribution of focal adhesions. Further work on breast cancer was discussed by Ruth Lupu (Rochester, USA). She found that CCN1 expression is correlated with advanced disease in breast cancer and induces a taxol resistant phenotype. CCN1 upregulates expression of the αvβ3 integrin; functional blockade of αvβ3 with a synthetic chemical peptidomimetic of the RGD motif is cytotoxic for CCN1 expressing breast cancer cells. Disrupting the interaction of CCN1 and αvβ3 regained sensitivity to taxol. Zoledronic acid, an aminobisphosphonate, inhibits CCN1 expression and reduces anchorage independent cell growth as well as disrupting vimentin distribution. These findings present a novel therapeutic strategy for targeting metastatic breast cancer.

Kallikreins (KLKs) are a multi-gene family of secreted serine proteases. KLKs and kallikrein related peptidases have important implications in regulating cancer cell growth, angiogenesis, invasion and metastasis. Yves Courty (Tours, France) found that KLK12 could cleave CCN1 from the surface of a tumour cell line. He went on to show that all members of the CCN family can be digested with KLKs, notably CCN1 and CCN5 by KLK12 and KLK14, CCN3 by KLK5, 12 and 14. Further work will ascertain the functional CCN properties before and after degradation. The fact that KLK activity released smaller CCN fragments opens up new possible roles for CCN proteins in the multiple disease states where KLKs
figure prominently. Finally Sushanta Banerjee (Kansas, USA) presented his investigations of the role of mi-RNA-10b in metastatic breast cancer. CCN5 is expressed in non-invasive breast cancer cell lines whilst it is not detected in invasive breast cancer. Silencing CCN5 expression in MCF7 cells, increased miR-10b expression and increased cell migration and invasiveness. The mechanism driving invasive capacity is thought to involve down-regulation of CCN5 expression causing increased levels of miR-10b and the transcription factor, twist, leading to increased levels of HIF1α and enhanced motility.

The final session of the meeting featured presentations by the Springer Scholarship awardees. Wei Huang (Michigan, USA) constructed a CCN6 deficient human mammary epithelial cell line model and used this to look at the effects on E-cadherin. CCN6 inhibition was associated with decreased E-cadherin expression which was shown to be mediated by upregulation of SNAIL and ZEB1. These observations are important since loss of E-cadherin is associated with de-differentiation, invasion and metastasis. Since CCN3 is associated with Cx43 perhaps CCN proteins play distinct and important roles in organization of cell junction complexes not yet fully appreciated. Further studies in breast cancer were presented by Ingrid Espinoza (Chicago, USA) who examined the role of CCN1 in the hormonal response. She used a mutant CCN1 construct with substitutions in the α6β1 binding domain to support a possible role for CCN1 as a co-activator of ER, involved in the transcriptional activation of proliferative and survival ERE-genes in breast cancer cells. Of relevance here was the data supporting the posited nuclear role for CCN proteins in transcriptional events. Takanori Eguchi (Okayama, Japan) described a novel role for matrix metalloproteinase (MMP)-3 as a promoter of extra-cellular matrix production through CCN2 trans-activation. MMPs are conventionally regarded as extracellular acting proteases but Takanori showed that MMP-3 can be translocated into the nucleus and bind the Transcription Enhancer Dominant In Chondrocytes (TRENDIC) to regulate CCN2. All three presentations were of an extremely high standard and the recipients were to be congratulated.

Lester Lau concluded the meeting with a masterful exposition on CCNs and inflammation, pulling together many strands of interest that permeated the meeting, particularly the exciting new model systems which have been developed and progress towards translational applications. With much to challenge and stimulate us, and after closing remarks by Dr. Yeger and Professor Perbal, Dr. S. Irvine (Belfast, Ireland) extended an enthusiastic invitation to the 6th ICCNS workshop to be held in Belfast, 2010.

References

Chapter 3
Asking the Right Questions: What Can the Structure of the CCN Protein Domains Tell Us?

Kenneth P. Holbourn, Bernard Perbal, and K. Ravi Acharya

Abstract CCN proteins are modular multifunctional proteins comprised of four discrete domains: (1) an IGFBP domain, (2) a VWC domain, (3) a TSP domain, and (4) a CT domain. These domains give a broad repertoire of functions to the CCN family including angiogenesis, skeletal development tumourigenesis, wound healing, cell proliferation, adhesion and survival. These wide variety of biological functions are enabled due to the multi-domain nature of the protein allowing interactions with an extensive range of ligands and effectors and the possibility of multiple domains acting in concert. A closer look at each domain from a biological and structural perspective could give insights into their function.

Keywords CCN family of proteins · Domain structure · Biology · Angiogenesis · ECM proteins · CCN1 · CCN2 · CCN3 · CCN4 · CCN5 · CCN6

3.1 Introduction

The CCN proteins comprise a family of secreted extra-cellular matrix (ECM) associated proteins that play a role in a diverse array of biological functions including: adhesion; mitogenesis; migration and chemotaxis; cell survival; differentiation; angiogenesis; chondrogenesis; tumourigenesis and wound healing. The CCN acronym stems from the names of the first three members of the family to be characterised: Cyr61 (Cysteine rich protein 61); CTGF (Connective tissue growth factor); and NOV (Nephroblastoma overexpressed gene) (Bork, 1993). Due to their role in many different aspects of biology and similarity to other families of proteins they have multiple names. The official nomenclature of the CCN family in humans is detailed below in Table 3.1 (Brigstock, et al., 2003).

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Table 3.1  Nomenclature of the CCN family of proteins

<table>
<thead>
<tr>
<th>CCN family member</th>
<th>Alternative names</th>
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<tbody>
<tr>
<td>CCN1</td>
<td>Cyr61, CTGF-2, IGFBP10, IGFBP-rP4</td>
</tr>
<tr>
<td>CCN2</td>
<td>CTGF, IGFBP8, IGFBP-rP2, HBGF-0.8 ecogenin</td>
</tr>
<tr>
<td>CCN3</td>
<td>NOV, NOVH, IGFBP9, IGFBP-rP3</td>
</tr>
<tr>
<td>CCN4</td>
<td>Wisp-1, Elm-1</td>
</tr>
<tr>
<td>CCN5</td>
<td>Wisp-2, CTGF-L, CTGF-3, HICP, Cop-1</td>
</tr>
<tr>
<td>CCN6</td>
<td>Wisp-3</td>
</tr>
</tbody>
</table>

For a comprehensive review of the biological function there are many reviews available (Lau and Lam, 1999; Brigstock, 1999; Perbal, 2001a, 2004; Perbal and Takigawa, 2005; Leask and Abraham, 2006).

3.2 The “Building Blocks” of the CCN Proteins

ECM proteins are commonly constructed from a library of commonly used domains (Hohenester and Engel, 2002). In the case of CCN proteins the domains have been classified through recognisable motifs and sequence alignment though the exact role of each domain in CCN biology is not fully understood. Below is a short summary of what is known about each domain and how the predicted structure of the domain might influence this.

The schematic diagram of a prototypic CCN protein in Fig. 3.1 shows the arrangement of the four domains which, following an N-terminal secretory signal peptide, are (i) an insulin-like growth factor binding protein-like domain (IGFBP); (ii) a Von Willebrand factor type C repeat module (VWC); (iii) a thrombospondin type-1 repeat module (TSP1); and (iv) a cysteine knot containing module (CT). These domains are conserved in all family members, except CCN5 that lacks the CT domain, and include a total of 38 conserved cysteine residues. These cysteine residues that spread across the four distinct structural domains represent almost 10% of the CCN molecule by mass. The molecule can also be split into N and C-terminal components, the N-terminal comprising the IGFBP and VWC domains and the C-terminal the TSP and CT domains, separated by a flexible linker region (Bork, 1993; Lau and Lam, 1999). This linker region varies greatly in both composition and length between the CCN family members and contains several sites that may be targeted by cellular proteases. Similar protease sites may also be found in the short linker regions between domains 1 and 2 and domains 3 and 4 (Brigstock et al., 1997; Ball et al., 1998). It has been shown that a wide variety of proteases target these sites with several matrix metalloproteases (MMPs) (MMP-1, 2, 3, 7, 9, 13) targeting the central linker and additional proteases such as elastase and plasmin targeting the small linkers between domains 1 and 2 and domains 3 and 4 (de Winter et al., 2008; Hashimoto et al., 2002). It has been postulated that cleavage at these sites may be responsible for the production of truncated molecules.
Fig. 3.1  Schematic view of the CCN domain architecture. A diagram showing the signal peptide (SP), insulin-like growth factor binding domain (IGFBP), Von Willebrand factor C repeat (VWC), thrombospondin type-1 repeat (TSP1) and cysteine knot (CT). The protein is split into two halves separated by a variable “hinge” region. Some of the known binding partners of each module are also listed: insulin like growth factors (IGFs); bone morphogenic protein-4 (BMP-4); transforming growth factor-β (TGF-β); LDL receptor protein-1 (LRP-1); vascular endothelial growth factor and; heparin sulphated proteoglycans (HSPs)
and individual modules (Brigstock, 1999) that show distinct biological properties and might constitute an additional process for the regulation of the biological activity of the CCN proteins and may be necessary for some functions (Perbal, 2001b, 2004; Tong and Brigstock, 2006). Bioactive truncated components have also been seen to act as markers in some types of disease with N-terminal fragments implicated in some fibrotic diseases (Gao and Brigstock, 2004) and C-terminal fragments in some types of pancreatic cancer (Gao and Brigstock, 2006).

The CCN proteins are closely related on a primary amino acid level sharing ~30–50% identity (and 40–60% similarity) (Brigstock, 2003). CCN6 lacks four conserved cysteine residues in the VWC domain and the entire CT domain is missing from CCN5, but the biological consequences of these differences are not fully understood. The CCN proteins are also similar on the DNA level sharing a common and distinct intron/exon pattern. The first exon maps to the signal sequence and each of the remaining four exons codes for each of the four conserved domains. The use of exons as “building blocks” of domains is common to many large multi-modular human proteins and may come about through evolutionary shuffling (Bork, 1993; Bornstein, 1995; Kireeva et al., 1996). Sequence alignments of the CCN proteins using the T-Coffee program (Notredame et al., 2000) highlighting the conserved cysteine residues and other notable parts of each domain are shown below in Figs. 3.2–3.5 (Holbourn et al., 2008).

Whilst there may be some functions of the CCN proteins directly related to each module there is some evidence that many of the functional effects of CCN proteins result from modules acting in concert. Production of truncated proteins, or proteins missing internal modules, have been shown to possess different biological activities, and in some cases be associated to pathological situations (Perbal, 2001b). This mixture of individual and multi-domain specificity has been demonstrated for CCN2 where individual modules gave rise to some effects but some biological functions where only seen with the full length protein or a cocktail containing the four individual domains (Kubota et al., 2006) strongly suggesting that the modules of CCN2 must act in concert to activate the p38 MAPK pathway (Kubota et al., 2006).

### 3.3 The IGFBP Domain

The human insulin-like growth factor binding proteins (IGFBP) are a family of six closely related IGFBPs (1–6) that bind to insulin-like growth factors (IGFs), that are small ~7 kDa peptides, with high affinity ($K_D \approx 0.1$ nM). The IGFBPs have four main biological roles, all intrinsically linked to the biological functions modulated by IGFs: (a) to act as transport proteins for the IGFs, (b) to regulate the metabolic breakdown of IGFs and extend their biological lifespan, (c) to enable localisation of IGF availability, and (d) to directly affect the interaction between IGFs and their receptors on the cell surface and in doing so indirectly control IGF function (Jones and Clemmons, 1995). The indirect control of IGF function means the IGFBPs play
3 What Can the Structure of the CCN Protein Domains Tell Us?

Fig. 3.2 The IGFBP domain. (a) A sequence alignment of the IGFBP domain from the CCN proteins highlighting the N-terminal thumb region and cysteine rich motif. (b) The structure of the IGFBP domain from IGFBP4 [PDB 1DSP] (Sitar et al., 2006) illustrating the disulphide ladder that forms the palm, the thumb region, the structural cysteine rich motif and the IGF binding site. (c) The models of the CCN IGFBP domains generated by the CPH model server (O. Lund, 2002). This part of the figure is reproduced with permission from Holbourn et al. (2008).

an important part in many cellular functions including: cell cycle progression, cell proliferation, cell death, cell differentiation, amino acid and glucose uptake, hormone and neurotransmitter secretion, chemotaxis, and parts of the immune response (Jones and Clemmons, 1995). This modulation of IGF function is also now observed to play a major role in many forms of cancer but especially breast cancers (Helle, 2004). This could also implicate a role for the IGFBP domain of the CCN proteins as CCNs 4–6 are all “major players” in inflammatory breast cancer and exert their influence via control and manipulation of IGF-1 (Zhang et al., 2005; Davies et al., 2007).
The IGFBP protein family, apart from strong sequence similarity, has several distinguishing features that have helped to classify it as a distinct protein family. They are multidomain proteins with a cysteine rich N- and C-terminal domain linked by a short variable region. The linker region varies between IGFBPs and similar to interdomain linkers in the CCN family has several sites vulnerable to protease degradation (Hwa et al., 1999; Firth and Baxter, 2002). The N-terminal domain is a globular domain that contains 12 conserved cysteine residues that form six disulphide bridges and the C-terminal domain contains a further six conserved cysteine residues (and three disulphide bridges) (Hwa et al., 1999). Although the N and C terminals can bind IGF independently in some of the IGFBPs, and the main binding site for IGFs is located in the N-terminal domain (Kalus et al., 1998) they do so with a much lower affinity (Stndker et al., 2000). The high affinity binding is a result of the N and C terminus working together in concert, like a set of jaws to contain the entire IGF molecule.

The IGFBP domain of the CCN proteins has a strong sequence homology with the N-terminal domain of traditional IGFBP’s (Bork, 1993) and this similarity has led to CCN proteins being classified by some as additional IGFBPs or as IGFBP-related proteins (IGFBP-rPs) (Hwa et al., 1999). Though the known CCN proteins have a high degree of homology with the N-terminal domains they have very low IGF binding strengths by comparison, in the order of a hundred fold lower than the traditional IGFBPs. This low binding affinity likely comes about from the lack of the IGFBP C-terminal like domain (Kim et al., 1997). Though this level of IGF affinity is approximately the same as that for traditional IGFBP truncates that are missing their C-terminal domain (Yamanaka et al., 1997). But in some experiments the IGF binding of the CCN IGFBP domain has been found to be lacking and chimeras with the C-terminal domain of IGFBP3 fused to the IGFBP domain of CCN3 have shown a lack of binding (Yan et al., 2006). This lack of strong binding has led to the exact biological function of the CCN proteins in relation to IGF confused. It has been suggested that there are two classes of IGFBPs; high and low affinity IGFBPs. The six traditional IGFBPs are considered high affinity and the CCN proteins and other proteins that only contain an N-terminal IGFBP domain are considered low affinity binders (Hwa et al., 1999; Kim et al., 1997). Though the biological relevance of IGFBP binding in these proteins is, as yet, still not fully understood. It also may mean that the influence of CCN4–6 on IGF biology may be as a result of more indirect actions.

In the CCN family there is little information on the exact role played by the IGFBP domain in CCN function. While its binding and interactions with IGF are for the most part unknown it has been shown that the independent IGFBP domain is biologically active in other cellular pathways (Kubota et al., 2006). It was reported that the IGFBP domain of CCN2 is capable of stimulating JNK mediated proliferation, in contrast to the other domains promoting differentiation, and it is the only independent domain that was unable to promote ERK signalling (Kubota et al., 2006). It is possible however that the IGF interactions can play a role in some types of tumourigenesis. In 80% of cases of aggressive inflammatory breast cancer the CCN6 gene has been observed to be knocked out. This lack of CCN6 results in uncontrolled