REVIEW

Report on the second international workshop on the CCN family of genes

B Perbal, D R Brigstock, L F Lau

J Clin Pathol: Mol Pathol 2003;**56**:80–85

For the second time, researchers from leading laboratories in the CCN field gathered in Saint-Malo, France, to participate in the Second International Workshop on the CCN family of genes. In addition to the regular research communications, meeting highlights included the inauguration of the first CCN newsletter (http://ccnnewsletter.free.fr) and the recognition of the International CCN Society (http://www.ccnsociety.jussieu.fr) as an important medium for the exchange of scientific knowledge and resources in the CCN field. Once more, the high quality of scientific communications and individual interactions set the stage for an extremely fruitful meeting.

It is now widely accepted that CCN proteins are secreted signalling molecules that regulate disparate fundamental biological processes, including cell adhesion, growth, differentiation, embryogenesis, vascular diseases, and cancer (for reviews, see Brigstock,[1] Lau and Lam,[2] and Perbal[3]). In this meeting, several new findings and conceptual frameworks have come to the forefront. Gene targeting studies in mice have established the essential nature of CCN1 and CCN2 in mammalian development,[4] although the disruption of CCN6 results in more subtle phenotypes. Structure–function analysis reaffirmed that the four conserved domains of CCN proteins, three of which are homologous to proteins of the extracellular matrix (ECM), can act both independently and interdependently. Thus, functioning as matricellular proteins[5] rather than classic growth factors, CCN proteins can interact with multiple signal transduction pathways. Whereas many activities of CCN proteins are mediated through their direct binding to integrin receptors,[2] new data indicate their interactions with, and regulation of, other signalling molecules, including transforming growth factor β (TGF-β), vascular endothelial growth factor (VEGF), Notch, and voltage independent calcium channels.[6–9] Whereas the interaction of CCN proteins with membrane bound proteoglycans may serve a signalling function, the bioavailability of CCN proteins may be regulated through binding to proteoglycan and salvage receptors.[10–12] Although various CCN proteins clearly have distinct functions, the regulation of angiogenesis emerges as a common theme among their actions. Thus, CCN proteins may regulate angiogenesis in distinct organs or tissues, and their deregulation may contribute to various disease states, including cancer. In this context, investigations into the role of CCN proteins in fibrosis, cancer, and other diseases have pointed to their usefulness in disease diagnosis and/or prognosis, and as potential targets of treatment.

REGULATION OF CCN GENE EXPRESSION

Mechanical strain in smooth muscle cells results in cellular differentiation and hypertrophy, a process that involves both the activation of the RhoA signalling pathway and enhanced CCN1 expression. A functional link between RhoA and CCN1 was reported by Brahim Chaqour, who showed that CCN1 was expressed in cells containing active RhoA. Chloramphenicol acetyl transferase reporter studies in cells expressing RhoA demonstrated that Rho induced CCN1 expression involved CREB (cAMP response element)/JUN and activator protein 1 (AP-1) sequences in the CCN1 promoter. In addition, it was shown that the RhoA pathway is involved in regulating CCN1 expression by JNK or p38. Collectively, these data suggest that RhoA plays a central role in the expression of CCN1, by acting as a "portal" for Janus kinase and p38 signalling, in addition to regulating AP-1 and CREB transcription factors. Similar studies by Margarette Goppelt-Struebe also suggested that CCN2 gene expression is induced by the activation of RhoA. In renal mesangial cells and fibroblasts, the induction of CCN2 by TGF-β was dependent on an intact actin cytoskeleton, as shown by pretreatment with cytochalasin D or latrunclulin. However, cytochalasin D mediated disruption of actin stress fibres itself caused a transient increase in CCN2 mRNA expression, a phenomenon that was inhibited by actinomycin D. CCN2 mRNA was also induced by nocodazole and

Professor B Perbal, Laboratoire d'Oncologie Virale et Moléculaire, UFR de Biochimie, Université Paris 7-D. Diderot, 2 Place Jussieu, 75005 Paris, France; perbal@ccr.jussieu.fr

Accepted for publication 27 January 2003

Abbreviations: AP-1, activator 1 protein; bFGF, basic fibroblast growth factor; CAESAR, cis acting element for structure anchored represion; CML, chronic myeloid leukaemia; CREB, cAMP response element; Cx, connexin; ECM, extracellular matrix; EWS, Ewing's sarcoma; GJIC, gap junctional intercellular communication; HSC, hepatic stellate cell; IBC, inflammatory breast cancer; IL, interleukin; LRP, low density lipoprotein receptor related protein; MAPK, mitogen activated protein kinase; MC, mesangial cell; MMP, matrix metalloproteinase; MRP, myeloid related protein; STZ, streptozotocin; TGF-β, transforming growth factor β; TRENDIC, transcriptional enhancer dominant in chondrocyte; VEGF, vascular endothelial growth factor; VSMC, vascular smooth muscle cell; xCCN1, xenopus CCN1

colchicine, showing that CCN2 gene expression is linked to the microtubular system. Colchicine mediated disruption of the microtubular system was associated with the activation of RhoA, suggesting that RhoA is a molecular switch that translates changes in cell morphology into the expression of CCN2.

The ovary undergoes cyclical remodelling throughout adult life and appears to involve the action of CCN2. Chris Harlow reported data from in vitro studies using rat granulosa cells from diethylstilbestrol treated rats, which showed that TGF-β, dihydroxytestosterone, growth differentiation factor 9, and activin-A stimulated CCN2 expression. This effect was inhibited by follicle stimulating hormone, which itself inhibited basal CCN2 expression. Because CCN2 expression was upregulated by conditions favouring cell replication and downregulated by conditions favouring differentiation, CCN2 probably plays a role in ECM deposition and tissue remodelling during follicular development.

Molecular studies reported by Satoshi Kubota have led to the discovery of two cis acting regulatory elements in the CCN2 gene. One is the transcriptional enhancer dominant in chondrocyte (TRENDIC), located in the promoter region, whereas the other is a cis acting element for structure anchored repression (CAESAR), found in the 3′ untranslated region. CAESAR appears to repress CCN2 gene expression by affecting mRNA translation efficiency, whereas TRENDIC appears to bind certain nuclear transcription factors in a chrondroctye specific manner. As a part of the complex gene regulatory system, both elements are thought to play crucial roles in enabling the differential expression of CCN2 under a variety of biological conditions. Data reported by Tsuyoshi Shimo also highlighted the regulation of CCN2 expression in chondroctyes. During chondrocyte maturation, CCN2 was stimulated by retinoid signalling and p42/p44 mitogen activated protein kinase (MAPK), but inhibited by p38 MAPK, and it was thus proposed that the balance between these pathways plays a crucial role in CCN2 expression and action.

Norbert Schutze reported patterns of expression of CCN genes during the differentiation of primary human mesenchymal stem cells. mRNA for CCN genes was present in the undifferentiated stem cells but expression changed as a function of differentiation. CCN1 appeared to have a potentially important role in the maintenance of the stem cell phenotype because it was highly expressed in non-differentiated cells, but there was a pronounced decrease in its expression during osteogenesis, chrondrogenesis, and adipogenesis. CCN6 expression was enhanced during osteogenesis but decreased during chondrogenesis and adipogenesis. Collectively, the data supported a role for CCN proteins in the function of mesenchymal stem cells and their pathways of differentiation.

David Brigstock presented work on the binding interactions between CCN2 and hepatic stellate cells (HSCs). HSCs exhibit increased CCN2 expression as a function of their activation during liver fibrosis and demonstrate enhanced proliferation, migration, and collagen production in response to CCN2. Studies of CCN2 mediated cell adhesion showed an absolute requirement for cell surface heparin-like molecules, a property that probably resides exclusively in module 4. In addition, low density lipoprotein receptor related protein (LRP) was shown to function as an adhesion coreceptor that fully depends on heparin sulfate binding. Based on the relative binding characteristics of several CCN2 isoforms, CCN2–LRP interactions appeared to be mediated principally by module 3.

INTERACTION OF CCN PROTEINS WITH CELL SURFACE RECEPTORS AND SIGNALLING PATHWAYS

CCN proteins are known to bind integrin receptors on the surface of a variety of cell types, leading to adhesive signalling and other integrin mediated functions. Tatiana Grzeszkiewicz summarised evidence that CCN1 interacts with distinct integrins in a cell type and function specific manner to elicit disparate biological activities. Thus, CCN1 stimulates cell adhesion, migration, and mitogenesis in fibroblasts through interaction with integrins $\alpha_6\beta_1$, $\alpha_v\beta_5$, and $\alpha_v\beta_3$, respectively. In contrast, integrin $\alpha_6\beta_1$ supports cell adhesion and tubule formation in endothelial cells where integrins have not been activated, whereas integrin $\alpha_v\beta_3$ mediates CCN1 stimulated cell adhesion, migration, proliferation, survival, and tubule formation in endothelial cells where the integrins have been activated. Furthermore, the interaction of CCN1 and CCN2 with integrin $\alpha_6\beta_1$ requires concomitant interaction with cell surface heparan sulfate proteoglycans. Mutational analysis identified the heparin binding sites in the C-domain, which is dispensable for CCN1 stimulated cell migration and DNA synthesis.

> "CCN3 and CCN2 may participate in cellular communication, proliferation, and differentiation through calcium signalling"

Both CCN1 and CCN2 have been found in atherosclerotic lesions, restenosed blood vessel walls, and cutaneous wounds. Monocytes are known to play roles in atherosclerosis, inflammation, and wound healing. Joseph Schober showed that CCN1 and CCN2 can both support the adhesion of monocytes through direct interaction with integrin $\alpha_M\beta_2$. Furthermore, binding of CCN1 to monocytes stimulates the expression of inflammatory mediators, including interleukin 1β (IL-1β), IL-8, monocyte chemoattractant protein 1, myeloid related protein 8 (MRP8), and MRP14. The I domain of the α_M integrin subunit is sufficient to bind CCN1. Using synthetic peptides that encompass the entire CCN1 sequence, a unique 13 residue sequence in CCN1 was identified as the integrin $\alpha_M\beta_2$ binding site. This is the first identification of a specific integrin binding site on a CCN protein.

Novel evidence is coming to light that CCN proteins activate complex signalling pathways. Bernard Perbal reported that using a yeast two hybrid system, CCN3 interacts with the S100A4 calcium binding protein. Intracellular calcium concentration and localisation is tightly controlled by several interconnected mechanisms. The addition of CCN3 and CCN2 to G59 glioblastoma and SK-N-SH neuroblastoma cells caused a pronounced but transient increase of intracellular calcium, resulting from both the entry of extracellular calcium and the mobilisation of intracellular stores (in G59) or activation of voltage independent channels (in SK-N-SH cells). These observations indicate that CCN3 and CCN2 may participate in cellular communication, proliferation, and differentiation through calcium signalling. Additional evidence for CCN3 interacting with ion channels was provided by its ability to induce a complete reversible blockade of sodium channels in SK-N-SH cells. These results suggest that the secreted CCN3 protein may be acting as a "docking" protein exerting its antiproliferative activity by coordinating the action of ion channels. The truncated nuclear CCN3 isoform reported to be a transcriptional transactivator might be involved in the more direct regulation of gene expression required for cell proliferation and oncogenic transformation.

Dong Xie presented evidence that CCN1 expression is strongly associated with human gliomas, and overexpression of CCN1 in U343 glioma cells induces hallmarks of transformation, such as anchorage independent growth and tumour formation in nude mice. Furthermore, the overexpression of CCN1 leads to increased integrin linked kinase activity, the phosphorylation of glycogen synthase kinase 3β, and the accumulation of nuclear translocated β-catenin. The overexpression of CCN1 also activated the phosphoinositol 3 kinase pathway, resulting in the phosphorylation of both Akt and the antiapoptotic protein Bad. These results indicate that CCN1 can activate multiple signalling pathways in the development of gliomas.

Cell cycle arrest of mesangial cells at the G1 phase, which can be induced by TGF-β, leads to hypertrophy and is one of the earliest abnormalities in diabetic nephrophathy. Nadia Wahab reported that CCN2 can also promote mesangial cell cycle arrest. Similar to the effect of TGF-β, growth arrest is mediated through the upregulation of cyclin dependent kinase inhibitors. Experiments with antisense oligonucleotides indicated that CCN2 mediates TGF-β induced cell cycle arrest and hypertrophy of these cells, suggesting that CCN2 is an effector of TGF-β in mesangial cell function.

INVOLVEMENT OF CCN PROTEINS IN DIFFERENTIATION AND DEVELOPMENT

Targeted gene disruptions for three members of the CCN family have been accomplished, with each showing distinct and interesting phenotypes. Lester Lau showed that CCN1 deficient mice suffer embryonic death, so that CCN1 must be an essential gene. Whereas approximately 30% of CCN1 null mice fail to establish chorioallantoic fusion and die by embryonic day 10.5, most (~ 70%) perish during midgestation as a result of placental insufficiency and loss of vascular integrity in the embryo. CCN1 deficiency results in a specific defect in vessel bifurcation at the chorioallantoic junction, a unique phenotype that is correlated with impaired expression of VEGF-C in the allantoic mesoderm. These findings establish CCN1 as a novel regulator of vascular development, and suggest roles for CCN1 and VEGF-C in vessel bifurcation. Sanja Ivkovic reported that CCN2 knockout mice die perinatally from respiratory failure as a consequence of impaired skeletal development. CCN2 null mice suffer multiple skeletal dysmorphisms as a result of defective chondrocyte proliferation and decreased ECM components. The hypertrophic zones of long bones are expanded because of impaired ECM remodelling, resulting from the loss of matrix metalloproteinases, and growth plate angiogenesis is blocked owing to impaired VEGF expression. These results show that CCN2 is required for skeletal development, and is a key regulator coupling ECM remodelling to angiogenesis at the growth plate. The data from these knockout models show that although different angiogenic mechanisms are perturbed, both CCN1 and CCN2 are intimately involved in VEGF mediated angiogenesis.

In contrast to the embryonic and perinatal lethality caused by CCN1 and CCN2 deficiency, Wendy Kutz showed that targeted disruption of the CCN6 gene results in much more subtle phenotypes. CCN6 null mice are fully viable, surviving in the laboratory for over a year without exhibiting obvious pathologies. Preliminary data indicate that CCN6 null mice have more mature vertebral endplates, consistent with earlier ossification. These results suggest that CCN6 may regulate the timing of angiogenesis and ossification during skeletal maturation in mice. Preliminary experiments also suggest that CCN6 may inhibit angiogenesis in a chick arotic ring assay in culture.

Masaharu Takigawa reported that exogenous CCN2 stimulates the proliferation and differentiation, but not hypertrophy, of articular cartilage cells in culture and the repair of articular cartilage defects in vivo without causing undesired calcification. CCN2 gene expression was induced in osteoblasts, osteocytes, and vascular endothelial cells by various stimuli such as wounding and mechanical stress. Exogenous CCN2 stimulated the repair of the bone defect in vivo via intramembranous ossification, consistent with a role for CCN2 not only in endochondral ossification but also in skeletal growth and bone remodelling. The roles of CCN proteins in chondrocyte differentiation were further explored by Vicki Church, who used the chick homologues CCN4 and CCN6 to examine their expression in the chick limb by in situ hybridisation. In micromass cultures, CCN4 has a small negative effect on chondrogenesis, whereas CCN6 increases chondrogenesis.

The developmental roles of CCN proteins were also examined in *Xenopus laevis*. In both gain of function and loss of function experiments, Branko Latinkic showed that CCN1 is required for gastrulation. This role is mediated in part through the adhesive properties of xenopus CCN1 (xCCN1) and its related ability to modulate the assembly of the ECM. In addition, xCCN1 can either stimulate or inhibit Wnt signalling in a context dependent manner. Sara Mercurio injected synthetic xCCN2 RNA into xenopus embryos and found that most embryos had a short trunk and abnormal heads compared with controls. This phenotype indicates that xCCN2 interferes with the anterior–posterior patterning of the embryo.

Observations by Ken-ichi Katsube indicated that CCN3 can bind to Notch1 via interaction between the extracellular endothelial growth factor domain of Notch1 and the C-terminal cysteine knot domain of CCN3, thereby activating the Notch regulated promoters HES1 and HES5. CCN3 and Notch1 were expressed concomitantly in the presomitic mesoderm and later in the myocytes and chondrocytes, suggesting that they have synergistic effects in mesenchymal cell differentiation. Further studies suggest the possible regulation of Notch signalling by CCN3 through its interaction with the Notch ligand Delta.

CCN PROTEINS IN TUMOUR GROWTH AND OTHER PATHOLOGIES

Tumorigenesis is a complex process that involves a great number of alterations in genes encoding different categories of regulatory factors, such as signal transduction effectors, transcription factors, and receptors. The progressive shift occurring from primary tumours to metastatic nodes also requires changes in cellular adhesion and migration capability, normally controlled by adhesion molecules, both at the cell surface and in the ECM. An increasing number of reports focus on CCN proteins as key regulators of cell growth, the abnormal expression of which is an important feature of cancer cells.

Breast cancer is a major focus in cancer research because it is an important cause of mortality and morbidity in the female population. Previous studies have established the role of CCN1 in breast cancer development. Sushanta Banerjee reported that the CCN5 gene is oestrogen and progesterone responsive and that CCN5 signalling is required for the proliferation of breast cancer derived MCF7 cells. Whereas CCN5 expression is undetected or minimal in normal breast epithelial cells, it is greatly increased in MCF7 and other breast tumour cell lines. Conversely, CCN4 is detected in both normal and tumorous breast cells. No association between CCN5 and grade, size, or age was observed. Oestrogen was proposed to modulate CCN5 expression at both the transcriptionnal and post transcriptional levels. Interestingly, progesterone was reported to antagonise the effects of oestrogen on CCN5 transcription. The inhibition of cancer cell proliferation by antisense oligonucleotides suggests that CCN5 expression is essential for tumorigenesis.

> "An increasing number of reports focus on CCN proteins as key regulators of cell growth, the abnormal expression of which is an important feature of cancer cells"

Previous studies of inflammatory breast cancer (IBC) established that among 17 genes whose expression was altered in tumour samples, CCN6 was specifically lost in IBC. The outcome of these frequently aggressive forms of breast cancer is quite dramatic, with a five year disease free survival of less than 45%. Sofia Merajver reported the effects of CCN6 on the expression of several transformation parameters and

the tumorigenic potential of IBC cells. For these studies, SUM149 IBC cells were transfected with a recombinant CCN6 expression vector. The restoration of CCN6 expression in SUM149 cells resulted in the induction of morphological changes, the upregulation of p27, p21, and cyclin E, a fivefold decrease of growth in soft agar, decreased angiogenesis as measured by the rat aortic ring assay, and a 50% decreased secretion of VEGF, basic fibroblast growth factor (bFGF), and IL-6. Importantly, CCN6 restoration did not suppress tumour growth but was associated with a decrease of tumour cell growth in grafted nude mice. Together, these observations support the idea that CCN6 is a tumour suppressor gene in IBC.

Deciphering the cascade of molecular events leading to the induction of angiogenesis in breast tumours remains a major challenge. In this context, a functional connection between matrix metalloproteinases (MMPs) and CCN2 in MDA231 breast cancer cells was presented by Seiji Kondo. MMPs, which belong to a large family of metalloendopeptidases, have the ability to degrade ECM components such as collagens, fibronectin, laminin, and proteoglycan. They are expressed at the surface of cancer cells. Taking advantage of the correlative increase of active MMP9 and CCN2 concentrations seen a few hours following hypoxic stimulation of MDA231 cells, the authors showed that recombinant CCN2 induced several MMPs and simultaneously decreased the expression of tissue inhibitors of metalloproteinases by vascular endothelial cells. The results suggested a model in which hypoxia stimulates MDA231 cells to release CCN2 as an angiogenic modulator, which in turn promotes invasive cascades by modulating the balance of ECM protein synthesis and degradation in collaboration with MMPs and their inhibitors.

Using a quite different system, Holly Mason reported that overexpression of CCN5 inhibited both the proliferation and motility of myometrial and leiomyoma smooth muscle cells. A similar effect had previously been reported by this group with vascular smooth muscle cells (VSMCs). Leiomyomas are benign tumours of uterine smooth muscle cells. In these tumours, cell hyperproliferation is dependent upon oestrogen and/or progesterone and growth factors. In the rat uterus, oestrogen also induces CCN5 expression. High amounts of CCN5 were detected in quiescent and heparin treated VSMCs, suggesting that CCN5 exerts a potential antiproliferative activity. However, serum stimulated MCF7 cells expressed much higher amounts of CCN5 than serum starved cells and CCN5 expression is maximum at S phase in murine fibroblasts.

Along the same line, Andrew Lake described studies on the effects of overexpressing CCN5 in VSMCs using adenoviral delivery of the gene. Cellular proliferation, motility, and invasiveness were inhibited by CCN5, consistent with the characterisation of the CCN5 gene as a growth arrest gene in VSMCs. These effects of CCN5 may be mediated through changes in the activity or concentrations of MMPs, such as MMP-2, which was shown to be inhibited by CCN5. The expression pattern of CCN5 in balloon injured rat carotid arteries further supported a role for CCN5 in regulating VSMC homeostasis in vivo.

Endometriosis provided another example of an oestrogen dependent disease. It is characterised by a reflux of viable endometrium into the peritoneal cavity and is generally accompanied by infertility. In her communication, Yvonne Janssen reported the use of microarrays to characterise genes whose expression was altered in this disease. Among several others, CCN1 was found to be upregulated in most endometrial samples from women with endometriosis and the increased expression was dependent upon oestrogen. Human endometrial fragments grown in nude mice were shown to express the angiogenic factors VEGF, bFGF, and CCN1, but VEGF expression was not found to be regulated by oestrogen, and VEGF inhibition did not suppress angiogenesis in the nude mouse model used. It was therefore proposed that bFGF and CCN1, whose expression is regulated by oestrogen, represent key angiogenic factors in endometriosis.

CCN PROTEIN EXPRESSION AS A MARKER FOR DISEASE STAGE

The alterations of CCN3 expression that have been reported in many different solid tumours over the past decade set the stage for studies aimed at establishing whether the dosage of CCN3 and other CCN proteins, either in situ or in biological fluids, may be of clinical value.

Lynn Gilmour extended the number and type of cases by reporting for the first time an abnormal degree of CCN3 expression in haematological tumours. By using microarray technology on murine multipotent haemopoietic stem cells transfected with a thermosensitive mutant of the bcr–abl gene fusion expressed in chronic myeloid leukaemia (CML), it was established that CCN3 expression is significantly decreased as a consequence of Bcr–abl activation. The reduction of CCN3 expression was detected both at the RNA and protein levels. Similarly, a significant decrease of CCN3 expression was detected by confocal microscopy in CML tumours. The inducible bcr–abl reduction of CCN3 expression makes these haemopoietic cells a unique system in which to study the molecular mechanisms pertinent to the pathogenesis of CML. The decrease of CCN3 expression in CML also paves the road for future treatment trials based on the ectopic complementation of CCN3 defectiveness in these tumours.

The prognosis value of CCN3 has been previously established in the case of Ewing's sarcoma (EWS). Katia Scotlandi presented results of a study aimed at establishing the role(s) of CCN3 in EWS cells. The isolation of stable transfectants expressing CCN3 under the control of constitutive and inducible promoters permitted the assessment of the functions of CCN3 in EWS cells. In agreement with results obtained in glioblastomas and choriocarcinomas (see below), the expression of CCN3 significantly reduced the growth rate of EWS transfectants ex vivo. Considering the fact that CCN3 expression was associated with a higher risk of developing metastasis, these results were quite unexpected. Interestingly, the expression of CCN3 was shown to decrease the adhesive capacity and increase the motility of the transfected cells, two observations that are fully consistent with CCN3 being associated with a high metastatic potential. For the first time, these experiments have allowed the effects of a CCN protein on cell proliferation, cell adhesion, and motility to be dissociated, and might be of general value and applicability to other proteins inside and outside the CCN family.

For many years, it has been known that gap junction communication plays a crucial role in cell growth control and differentiation. In the continuation of previous studies establishing that an increase of CCN3 expression matched the reduced tumorigenic potential of C6 glioma cells transfected with connexin 43 (Cx43), Christian Naus reported additional evidence suggesting a functional interaction between Cx43 and CCN proteins. In addition to CCN1, CCN4, and CCN3, L19 ribosomal protein, pecanex, and CarG binding protein were also shown to be differentially regulated in transfected cells compared with C6 parental cells. Confocal analysis performed on transfected cells established the colocalisation of CCN3 and Cx43 in these cells, suggesting that they may physically interact. Because aberrant gap junctional intercellular communication (GJIC) is associated with tumorigenesis, CCN3 might be a major player in the GJIC dependent control of cell growth. Although the importance of the colocalisation of CCN3 and Cx43 remains to be determined, a very attractive model was proposed in which CCN3 might interact with zona occludens 1, a tight junction related protein that interacts with the C-terminal tail of Cx43.

In the same vein, Elke Winterhager presented data indicating that the restoration of cell–cell communication via Cx43 protein channels in human choriocarcinoma cells significantly reduced their growth in vitro and their ability to induce tumour growth in vivo. In contrast, Cx40 and trCx43 (a truncated version of Cx43) transfectants demonstrated no obvious changes in cell proliferation in vitro or tumour growth in vivo. Gene microarray analysis of the different Cx transfectants and reverse transcription polymerase chain reaction revealed that CCN3 expression was upregulated in Cx43 transfectants. In agreement with the results reported above, colocalisation of both proteins at the cell membrane was established by double immunolabelling of CCN3 and Cx43 in induced Cx43 transfectants. The association of CCN3 and Cx43 at the cell membrane could be responsible for the growth reduction of the Cx43 transfectants. In contrast, CCN3 could be detected predominantly in the nucleus in both parental cells and Cx40, in addition to trCx43 transfectants. Because colocalisation failed when the Cx43 protein was truncated at the C-terminus end, this part of the Cx43 molecule might be essential for the intracellular interaction. These results reinforced the observations suggesting that cell membrane associated and nuclear CCN3 isoforms have a negative and a positive effect, respectively, on cell growth and that disruption of this balance might be a key element in tumour development (see above).

The measurement of CCN proteins in tumour samples and biological fluids may provide a useful tool for pathology diagnosis or prognosis in cases where a direct correlation between variations of CCN protein concentrations and disease stage is unambiguously established. Reaching this endpoint requires unravelling the processes that control the fate of the protein, including its excretion, transport, maturation, and turnover. Variations in the concentrations of circulating CCN proteins in pathological conditions are to be expected, but may not necessarily be informative in the absence of the above information.

> "The determination of urinary CCN2 concentrations may be a useful tool for identifying those patients with diabetes destined for progressive nephropathy"

With these considerations in mind, Bruce Riser reported data indicating that an increase of urinary CCN2 might be of relevance for the prognosis of progressive sclerosis and end stage disease in the context of diabetes. Although unstimulated mesangial cells (MCs) express low amounts of CCN2, exposure of these cells to recombinant CCN2 increases the deposition of fibronectin and collagen. Both TGF-β, high glucose concentrations, and mechanical stress induce the expression of CCN2 in MCs. Furthermore, a 25 fold induction of glomerular CCN2 is detected early in the glomeruli of diabetic mice, when the expansion of MCs is mild. In the rat, streptozotocin (STZ)-induced hyperglycaemia induces increases in TGF-β, collagen V, fibronectin, kidney weight, and the expansion of mesangial cells. The STZ rat system is a model for human type 1 diabetes. The observation that in this model glomeruli express high amounts of CCN2 before the development of albuminuria suggested that measuring CCN2 concentrations in humans might help in staging this disease. The results that were presented established that a proportion of patients with no clinical nephropathy showed greatly increased expression of CCN2. In patients with diabetes and clinical nephropathy, a direct correlation between urinary concentrations of CCN2 and blood pressure was seen and CCN2 values increased as kidney function decreased. Therefore, the determination of urinary CCN2 concentrations may be a useful tool for identifying those patients destined for progressive nephropathy, and may provide a unique element in the decision to apply early treatment.

Studies of renal and urinary CCN2 protein expression were also reported by Peggy Roestenberg. Using a model of early diabetes in mice, it was shown that urinary CCN2 concentrations increased progressively and correlated with albuminuria. It was also shown that diabetic urinary CCN2 appeared to be derived from local renal production because increases were noted in podocyte production of CCN2 mRNA and protein. Although plasma CCN2 values were not increased in the mouse model, Frans Van Nieuwenhoven showed that CCN2 plasma concentrations were raised in patients with diabetes mellitus type 1, and correlated with albuminuria and creatinine clearance. No correlation was found between CCN2 plasma concentrations and markers of metabolic control, platelet activation, or endothelial dysfunction. Taken together, these data support the possibility that CCN2 is a potentially important pathogenic factor in the development of diabetic nephropathy and that plasma or urine CCN2 values might be a valuable marker of the disease.

Stephen Klaus reported that Fibrogen, Inc (California, USA) has established a large panel (~ 150) of humanised monoclonal antibodies against CCN2. Most of these antibodies recognise various epitopes in domains I, II, and II, with only one antibody recognising the C-terminal domain. These antibodies have been utilised in cell adhesion assays using an antibody sandwich assay. Cells adhered to CCN2 in this manner also spread and show the presence of filopodia and lamellipodia, as seen previously with cells adhered to either CCN1 or CCN2 coated surfaces. These antibodies may provide powerful reagents in the analysis of CCN2 activities and in the development of potential therapeutics.

Noelynn Oliver reported that systemic administration in neonatal mice of microgram amounts of CCN2 together with TGF-β results in the fibrosis of diverse organ systems, in addition to the death of the animal. These effects could be antagonised by a CCN2 monoclonal antibody, which significantly reduced CCN2 mediated collagen deposition. The importance of CCN2 in human fibrotic disease was highlighted by analysis using CCN2 enzyme linked immunosorbent assays, from which it was found that the CCN2 content of numerous fibrotic tissues is raised, the circulating CCN2 concentrations are increased in patients with fibrosis, and plasma and urine CCN2 values correlate with disease severity. The N-terminal fragment was reported to be a good indicator of fibrosis. These data provide an important proof of concept, both regarding the role of CCN2 in fibrotic disease, and the potential usefulness of targeting CCN2 in the disease process.

Authors' affiliations
B Perbal, Laboratoire d'Oncologie Virale et Moléculaire, UFR de Biochimie, Université Paris 7-D. Diderot, 2 Place Jussieu, 75005 Paris, France
D R Brigstock, Department of Surgery, Ohio State University, Colombus Ohio 43205, USA
L F Lau, Department of Molecular Genetics, University of Illinois, Chicago, IL 60607, USA

REFERENCES

1 **Brigstock DR**. The connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) family. *Endocr Rev* 1999;**20**:189–206.
2 **Lau LF**, Lam SC-T. The CCN family of angiogenic regulators: the integrin connection. *Exp Cell Res* 1999;**248**:44–57.
3 **Perbal B**. NOV (nephroblastoma overexpressed) and the CCN family of genes: structural and functional issues. *Mol Pathol* 2001;**54**:57–79.
4 **Mo F-E**, Muntean AG, Chen C-C, *et al*. CYR61 (CCN1) is essential for placental development and vascular integrity. *Mol Cell Biol* 2002;**22**:8709–20.
5 **Bornstein P**. Diversity of function is inherent in matricellular proteins: an appraisal of thrombospondin-1. *J Cell Biol* 1995;**130**:503–6.
6 **Abreu JG**, Ketpura NI, Reversade B, *et al*. Connective-tissue growth factor (CTGF) modulates cell signalling by BMP and TGF-β. *Nat Cell Biol* 2002;**4**:599–604.

7 **Inoki I**, Shiomi T, Hashimoto G, *et al*. Connective tissue growth factor binds vascular endothelial growth factor (VEGF) and inhibits VEGF-induced angiogenesis. *FASEB J* 2002;**16**:219–21.
8 **Sakamoto K**, Yamaguchi S, Ando R, *et al*. The nephroblastoma overexpressed gene (NOV/ccn3) protein associates with Notch1 extracellular domain and inhibits myoblast differentiation via Notch signaling pathway. *J Biol Chem* 2002;**277**:29399–405.
9 **Li CL**, Martinez V, He B, *et al*. A role for CCN3 (NOV) in calcium signalling. *Mol Pathol* 2002;**55**:250–61.
10 **Chen N**, Chen CC, Lau LF. Adhesion of human skin fibroblasts to Cyr61 is mediated through integrin alpha 6beta 1 and cell surface heparan sulfate proteoglycans. *J Biol Chem* 2000;**275**:24953–61.
11 **Desnoyers L**, Arnott D, Pennica D. (2001) WISP-1 binds to decorin and biglycan. *J Biol Chem* 2001;**276**:47599–607.
12 **Segarini PR**, Nesbitt JE, Li D, *et al*. The low density lipoprotein receptor-related protein/α2-macroglobulin receptor is a receptor for connective tissue growth factor. *J Biol Chem* 2001;**276**:40659–67.