Cancer Cell International

Review

A structural approach to the role of CCN (CYR61/CTGF/NOV) proteins in tumourigenesis

Nathalie Planque and Bernard Perbal*

Address: Laboratoire d'Oncologie Virale et Moléculaire, UFR de Biochimie, Université Paris 7 – D. Diderot, 2 Place Jussieu- 75 005 PARIS – France

Email: Nathalie Planque - planque@ext.jussieu.fr; Bernard Perbal* - perbal@ccr.jussieu.fr

* Corresponding author

Abstract

The CCN (**C**YR61 [Cystein-rich**61**]/**C**TGF [connective tissue growth factor]/**N**OV [Nephroblastoma overexpressed]) proteins constitute a family of regulatory factors involved in many aspects of cell proliferation and differentiation. An increasing body of evidence indicates that abnormal expression of the CCN proteins is associated to tumourgenesis. The multimodular architecture of the CCN proteins, and the production of truncated isoforms in tumours, raise interesting questions regarding the participation of each individual module to the various biological properties of these proteins. In this article, we review the current data regarding the involvement of CCN proteins in tumourigenesis. We also attempt to provide structural basis for the stimulatory and inhibitory functions of the full length and truncated CCN proteins that are expressed in various tumour tissues.

Introduction

The CCN family of genes presently consists of six members in human also known as: Cyr61 (Cystein rich 61), CTGF (Connective Tissue Growth Factor), Nov (Nephroblastoma Overexpressed gene), WISP-1, 2 and 3 (Wnt-1 Induced Secreted Proteins) (Table 1). The CCN acronym was introduced a decade ago by P. Bork who recognised that the proteins encoded by **C**yr61, **C**TGF, and **N**OV were showing a common multimodular organisation [1] (Figure 1) with structural motifs resembling Insulin-like Growth Factor Binding Proteins (IGFBPs), Von Willebrand factor, thrombospondin, and secreted growth factors. The CCN proteins all contain an N-terminal signal peptide followed by the four structural domains that are largely conserved among the different members of the CCN family (Figures 2 and 3). Their general properties have been extensively reviewed [2–4].

Orthologs of the CCN proteins have been found among vertebrates from *Xenopus* to human. The first gene of the CNN family to be cloned (CCN1 originaly designated 3CH61 and Cyr61), was identified as a growth factor-inducible immediate-early gene in serum-stimulated mouse BALB/c 3T3 fibroblasts. Its chicken ortholog (Cef-10) was cloned as a gene induced following the transformation of chick embryo fibroblasts by the v-src oncogene of the Rous Sarcoma Virus (RSV). Human CCN2, originally designated CTGF, was identified as a mitogen factor present in the conditioned medium of Human Ombilical Vein Endothelial Cells (HUVECs). The mouse ortholog of CCN2 (Fisp12, Fibroblast-inducible secreted protein), was also isolated as a serum-inducible immediate-early gene in NIH3T3 fibroblasts. CCN2 is transcriptionally activated with rapid kinetics in fibroblasts by serum growth factors and is also highly activated by TGFβ.
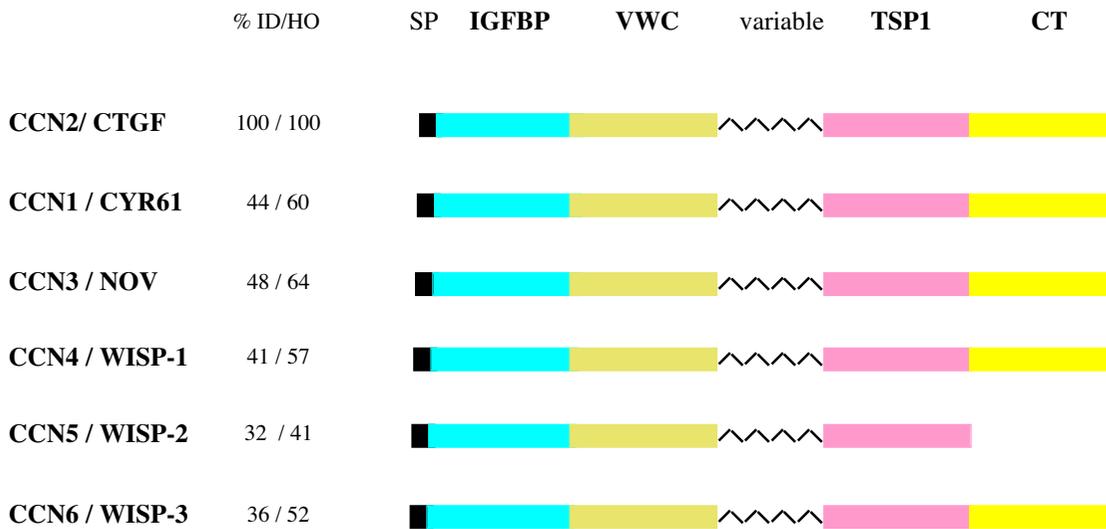
Figure 1
Multimodular structure of the CCN proteins. SP, Signal Peptide ; IGFBP, Insulin-like Growth Factor Binding Protein-like module ; VWC, Von Willebrand Factor-like module ; TSP1, Thrombospondin-like module ; CT, cystein knot containing family of growth regulator-like module. HO : homology ; ID : identity.

Table 1: The CCN family of proteins

	Other Names
CCN1	CYR61, Cef10, IGFBP-rP4, bIG-M1, IGFBP10
CCN2	CTGF, Fisp12 IGFBP-rP2, Hcs24, IGFBP8, HBGF-0.8, ecogenin
CCN3	NOV, IGFBP-rP3, IGFBP9
CCN4	WISP-1, Elm1
CCN5	WISP-2, Cop-1, CTGFL, CTGF-3, HICP
CCN6	WISP-3

CCN3 was cloned as an integration site of the Myeloblastosis Associated Virus (MAV) in chicken nephroblastomas that represent a unique model of the Wilm's Tumour. It was originally designated NOV because it was highly expressed in all MAV-induced nephroblastomas. The NOV protein was the first member of the CCN family to show antiproliferative activity.

Differential hybridisation permitted the isolation the Elm-1 gene (CCN4) which is expressed in low but not high metastatic murine melanoma cells. The rat rCop-1 gene (CCN5) was found to be down-regulated in rat embryo fibroblasts transformed by the cooperation of the H-ras oncogene and oncogenic mutants of the p53 tumor suppressor gene. CCN4 and CCN5 were subsequently shown to be induced by the Wnt oncogene and were therefore designated WISP (Wnt Induced Secreted Proteins). CCN6 was identified as an Expressed Sequence Tag (EST), and was shown to be a Wnt responsive gene.

The CCN genes encode secreted proteins associated with the Extracellular Matrix (ECM) and cell membrane. Results obtained over the past decade have indicated that CCN proteins are matricellular proteins which are involved in the regulation of various cellular functions which include: proliferation, differentiation, survival, adhesion and migration. They are expressed in derivatives of the three embryonic sheets and are implicated in the development of kidney, nervous system, muscle, bone marrow, cartilage and bone. During adulthood, they are
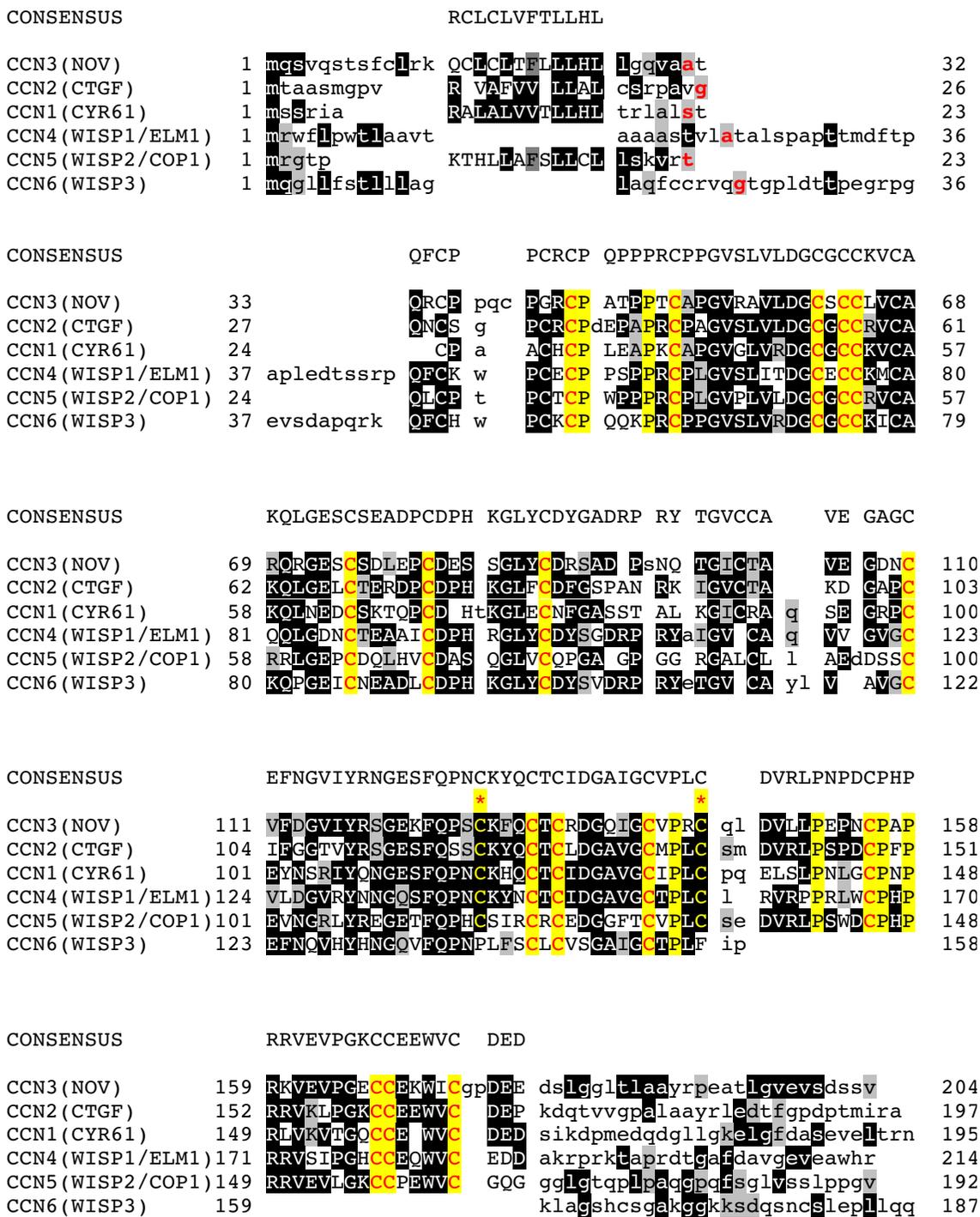
```
CONSENSUS                          RCLCLVFTLLHL

CCN3(NOV)           1  mgsvqstsfclrk QCLCLTFLLLHL lgqvaat                    32
CCN2(CTGF)          1  mtaasmgpv     R VAFVV LLAL csrpavg                    26
CCN1(CYR61)         1  mssria        RALALVVTLLHL trlalst                    23
CCN4(WISP1/ELM1)    1  mrwflpwtlaavt              aaaastvlatalspapttmdftp    36
CCN5(WISP2/COP1)    1  mrgtp         KTHLLAFSLLCL lskvrt                     23
CCN6(WISP3)         1  mgqllfstlllag              laqfccrvqgtgpldttpegrpg    36


CONSENSUS                          QFCP    PCRCP QPPPRCPPGVSLVLDGCGCCKVCA

CCN3(NOV)          33            QRCP pqc PGRCP ATPPTCAPGVRAVLDGCSCCLVCA    68
CCN2(CTGF)         27            QNCS g   PCRCPdEPAPRCPAGVSLVLDGCGCCRVCA    61
CCN1(CYR61)        24            CP a     ACHCP LEAPKCAPGVGLVRDGCGCCKVCA    57
CCN4(WISP1/ELM1)   37  apledtssrp QFCK w  PCECP PSPPRCPLGVSLITDGCECCKMCA    80
CCN5(WISP2/COP1)   24            QLCP t   PCTCP WPPPRCPLGVPLVLDGCGCCRVCA    57
CCN6(WISP3)        37  evsdapqrk  QFCH w  PCKCP QQKPRCPPGVSLVRDGCGCCKICA    79


CONSENSUS                          KQLGESCSEADPCDPH KGLYCDYGADRP RY TGVCCA    VE GAGC

CCN3(NOV)          69  RQRGESCSDLEPCDES SGLYCDRSAD PsNQ TGICTA    VE GDNC   110
CCN2(CTGF)         62  KQLGELCTERDPCDPH KGLFCDFGSPAN RK IGVCTA    KD GAPC   103
CCN1(CYR61)        58  KQLNEDCSKTQPCD HtKGLECNFGASST AL KGICRA q  SE GRPC   100
CCN4(WISP1/ELM1)   81  QQLGDNCTEAAICDPH RGLYCDYSGDRP RYaIGV CA q  VV GVGC   123
CCN5(WISP2/COP1)   58  RRLGEPCDQLHVCDAS QGLVCQPGA GP GG RGALCL l  AEdDSSC   100
CCN6(WISP3)        80  KQPGEICNEADLCDPH KGLYCDYSVDRP RYeTGV CA yl V  AVGC   122


CONSENSUS                          EFNGVIYRNGESFQPNCKYQCTCIDGAIGCVPLC    DVRLPNPDCPHP
                                                       *                 *

CCN3(NOV)         111  VFDGVIYRSGEKFQPSCKFQCTCRDGQIGCVPRC ql DVLLPEPNCPAP   158
CCN2(CTGF)        104  IFGGTVYRSGESFQSSCKYQCTCLDGAVGCMPLC sm DVRLPSPDCPFP   151
CCN1(CYR61)       101  EYNSRIYQNGESFQPNCKHQCTCIDGAVGCIPLC pq ELSLPNLGCPNP   148
CCN4(WISP1/ELM1)  124  VLDGVRYNNGQSFQPNCKYNCTCIDGAVGCTPLC l  RVRPPRLWCPHP   170
CCN5(WISP2/COP1)  101  EVNGRLYREGETFQPHCSIRCRCEDGGFTCVPLC se DVRLPSWDCPHP   148
CCN6(WISP3)       123  EFNQVHYHNGQVFQPNPLFSCLCVSGAIGCTPLF ip               158


CONSENSUS                          RRVEVPGKCCEEWVC  DED

CCN3(NOV)         159  RKVEVPGECCEKWICgpDEE dslgggtlaayrpeatlgvevsdssv      204
CCN2(CTGF)        152  RRVKLPGKCCEEWVC  DEP kdqtvvgpalaayrledtfgpdptmira   197
CCN1(CYR61)       149  RLVKVTGQCCE WVC  DED sikdpmedqdgllgkelgfdasevultrn   195
CCN4(WISP1/ELM1)  171  RRVSIPGHCCEQWVC  EDD akrprktaprdtgafdavgeveawhr      214
CCN5(WISP2/COP1)  149  RRVEVLGKCCPEWVC  GQG gglgtqplpaqgpqfsglvsslppgv     192
CCN6(WISP3)       159                       klagshcsgakggkksdqsncslepllqq   187
```

Figure 2
Alignment of CCN protein sequences. Alignments were performed with the " interalign " program. Conserved residues have been indicated on a black box. Grey boxes indicate positions where more than one amino acid residue is conserved among the CCN proteins. Conserved cysteins are indicated in the yellow boxes.
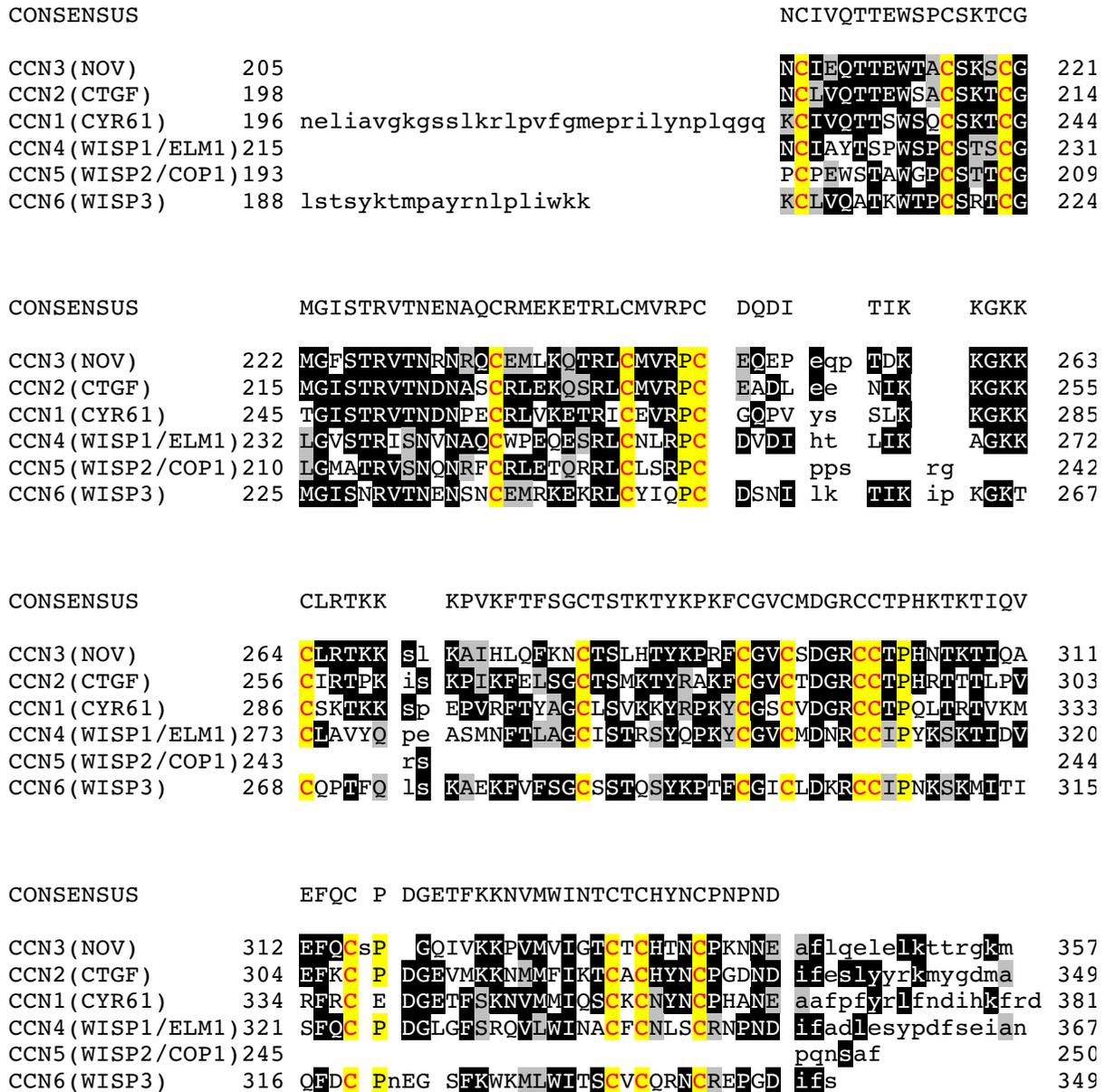
```
CONSENSUS                                            NCIVQTTEWSPCSKTCG

CCN3(NOV)          205                                NCIEQTTEWTACSKSCG   221
CCN2(CTGF)         198                                NCLVQTTEWSACSKTCG   214
CCN1(CYR61)        196 neliavgkgsslkrlpvfgmeprilynplqgq KCIVQTTSWSQCSKTCG 244
CCN4(WISP1/ELM1)   215                                NCIAYTSPWSPCSTSCG   231
CCN5(WISP2/COP1)   193                                PCPEWSTAWGPCSTTCG   209
CCN6(WISP3)        188 lstsyktmpayrnlpliwkk           KCLVQATKWTPCSRTCG   224


CONSENSUS              MGISTRVTNENAQCRMEKETRLCMVRPC DQDI     TIK    KGKK

CCN3(NOV)          222 MGFSTRVTNRNRQCEMLKQTRLCMVRPC EQEP eqp TDK     KGKK   263
CCN2(CTGF)         215 MGISTRVTNDNASCRLEKQSRLCMVRPC EADL ee  NIK     KGKK   255
CCN1(CYR61)        245 TGISTRVTNDNPECRLVKETRICEVRPC GQPV ys  SLK     KGKK   285
CCN4(WISP1/ELM1)   232 LGVSTRISNVNAQCWPEQESRLCNLRPC DVDI ht  LIK     AGKK   272
CCN5(WISP2/COP1)   210 LGMATRVSNQNRFCRLETQRRLCLSRPC      pps     rg          242
CCN6(WISP3)        225 MGISNRVTNENSNCEMRKEKRLCYIQPC DSNI lk  TIK  ip KGKT   267


CONSENSUS              CLRTKK    KPVKFTFSGCTSTKTYKPKFCGVCMDGRCCTPHKTKTIQV

CCN3(NOV)          264 CLRTKK sl KAIHLQFKNCTSLHTYKPRFCGVCSDGRCCTPHNTKTIQA  311
CCN2(CTGF)         256 CIRTPK is KPIKFELSGCTSMKTYRAKFCGVCTDGRCCTPHRTTTLPV  303
CCN1(CYR61)        286 CSKTKK sp EPVRFTYAGCLSVKKYRPKYCGSCVDGRCCTPQLTRTVKM  333
CCN4(WISP1/ELM1)   273 CLAVYQ pe ASMNFTLAGCISTRSYQPKYCGVCMDNRCCIPYKSKTIDV  320
CCN5(WISP2/COP1)   243        rs                                           244
CCN6(WISP3)        268 CQPTFQ ls KAEKFVFSGCSSTQSYKPTFCGICLDKRCCIPNKSKMITI  315


CONSENSUS              EFQC P DGETFKKNVMWINTCTCHYNCPNPND

CCN3(NOV)          312 EFQCsP  GQIVKKPVMVIGTCTCHTNCPKNNE aflqelelkttrgkm   357
CCN2(CTGF)         304 EFKC P DGEVMKKNMMFIKTCACHYNCPGDND ifeslyyrkmygdma   349
CCN1(CYR61)        334 RFRC E DGETFSKNVMMIQSCKCNYNCPHANE aafpfyrlfndihkfrd 381
CCN4(WISP1/ELM1)   321 SFQC P DGLGFSRQVLWINACFCNLSCRNPND ifadlesypdfseian   367
CCN5(WISP2/COP1)   245                                  pqnsaf            250
CCN6(WISP3)        316 QFDC PnEG SFKWKMLWITSCVCQRNCREPGD ifs              349
```

Figure 3

Alignment of CCN protein sequences. Alignments were performed with the " interalign " program. Conserved residues have been indicated on a black box. Grey boxes indicate positions where more than one amino acid residue is conserved among the CCN proteins. Conserved cysteins are indicated in the yellow boxes.

implicated in wound healing, bone fracture repair, and pathologies such as: fibrosis, vascular ailments and tumourigenesis.

The structural similarity observed between CCN and ECM proteins, their localisation in the ECM, and their ability to interact with several types of receptors and regulatory proteins raised the possibility that CCN proteins are adhesive matricellular proteins which represent a new class of signaling molecule playing a critical role in the regulation of cell proliferation. The production of abnormal levels of normal or altered CCN proteins might be associated to or involved in the initiation and establishment of tumours.

Several shorter CCN isoforms have been described in normal conditions. Different truncated CCN2 proteins were detected in biological fluids [[6], Riser, personal communication] and large amounts of a 30 kDa amino-truncated CCN3 isoform was detected in the nervous system of the embryonic rat [7]. In myoblasts, a 30 kDa amino truncated CCN3 isoform was also detected. Upon myotube differentiation the amount of full length CCN3 increased while the amount of truncated CCN3 was found to wane (our unpublished results). Presenly, neither the mechanisms governing the production of these various isoforms, nor the functional relationship that might exist between these different isoforms are elucidated.

The disruption of the CCN3 gene in MAV-induced nephroblastoma [5] provided the first clue that truncated CCN proteins were implicated in tumourigenesis, and suggested that the balance between the negative and positive effects of the full length and truncated CCN3 proteins were critical for normal cell proliferation. These observations raised the possibility that structural post-translational modifications of other CCN proteins might also occur and be critical for their biological properties. In addition, subcellular addressing of the CCN proteins appeared as a key point in these regulatory processes.

In this manuscript we review the current knowledge regarding the implication of CCN proteins in tumourigenesis and attempt to provide structural basis for the stimulatory and inhibitory functions of the various CCN isoforms.

Tumourigenesis and CCN proteins

Original observations which established a role for the CCN proteins in the control of cell proliferation also suggested that CCN1 and 2 were positive regulators of growth – whereas, CCN3-5 were negative [2–4].

An increasing body of evidence now suggests that the biological properties of the CCN proteins is dependent upon the cellular context and their ability to interact with partners involved in complex signaling pathways [8,9]. Recent results a) drew a relationship between abnormal CCN protein expression and tumourigenesis b) indicated that full length secreted CCN proteins can show an antiproliferative activity, whereas truncated isoforms are likely to stimulate proliferation and behave as oncogenes.

Association of CCN protein expression with increased proliferative activity and tumourigenicity

In agreement with the well documented angiogenic properties of CCN1, gastric adenocarcinoma cells transfected with CCN1 gave rise to larger and more vascularised tumours than parental cells when injected into nude mice [10].

In primary human breast carcinomas elevated levels of CCN1, CCN2, and CCN4, but not CCN3 were associated with more advanced features [11]. Increased CCN1 protein expression was observed in a large number of primary breast tumours that were progesterone receptor positive but estrogen receptor negative – suggesting that it might be a novel mediator of progesterone activity in breast cancer [12]. Invasive breast cancer cell lines expressed high levels of CCN1 whereas less tumourigenic breast cancer cells, such as MCF-7, expressed lower; normal breast cells showed almost none. Forced expression of CCN1 in MCF-7 cells was sufficient to induce their growth without anchorage in the absence of estrogen and to form colonies in matrigel in a $\alpha_v\beta_3$ integrin-dependent way. The tumours induced by these cells in ovariectomised athymic nude mice resembled human invasive carcinomas and were highly vascularised [13–15]. These observations suggested that CCN1 was involved in the progression to more advanced stages of breast cancer.

Similarly, serum-stimulated MCF7 cells expressed much higher levels of CCN5 than the serum-starved and CCN5 expression is highest at S-phase in murine fibroblasts. Additionally, the expression of CCN5 was upregulated in Wnt-1 transformed C57MG epithelial cells.

Elevated expression of CCN1 has been detected in pancreatic cancers [16] and within invasive breast carcinomas [13]. The expression of CCN1 was also found to have increased in several types of paediatric tumours including angiofibroma, malignant fibrous histiocytoma, infantile myofibromatosis, and malignant haemangiopericytoma [4].

An elevated expression of CCN2 has also been detected by Northern blotting in human invasive mammary ductal carcinomas [17], dermatofibromas, pyogenic granuloma, endothelial cells of angiolipomas and angioleiomyomas [18], and in pancreatic tumours [19]. A study performed with chondrosarcomas representative of various histolog-

ical grades established that CCN2 expression was closely correlated with increasing levels of malignancy [20].

In agreement with CCN2 playing a role in brain tumour angiogenesis, immunocytochemistry studies indicated that both glioblastoma tumour cells and proliferating endothelial cells stained positive for CCN2 [21]. In astrocytomas, CCN2 expression was particularly elevated in high grade tumours, with a marked effect of CCN2 on cell proliferation. Downregulation of CCN2 expression in these cells was associated with a growth arrest at the G1/S transition while over-expression of CCN2 induced a two-fold increase of the number of cells in the G1 phase. Gene profiling analysis allowed to identify a set of about 50 genes whose expression might account for the proliferative activity of CCN2 in these cells [22]. CCN2 was seen in a higher proportion of mononuclear cells of patients with acute lymphoblastic leukemia [23].

The expression of CCN3 was correlated to increased proliferative index in the case of prostate and renal cell carcinomas [24,25] and higher metastatic potential of the Ewing's carcinoma cells [26]. In addition to the labelling of CCN3 in the cytoplasm of acinar epithelial cells, prostate hyperplasia was showing an intense luminal labeling suggestive of CCN3 secretion in seminal fluid. In prostate tumour cell lines, expression of CCN3 was detected in the cytoplasm of three cell lines derived from metastasis to bone (PC3), brain (DU145) and lymph node (LNCap). As compared to the level of CCN3 in normal prostates, gene profiling and qRT-PCR studies established that the level of CCN3 mRNA was increased around 80 fold in CR2-TAG mice which express the simian virus 40 large T antigen under the control of cryptdin-2 promoter elements [27]; these studies indicated that the expression of CCN3 was not detected in the neuroendocrine cells. No expression was detected in normal adult prostate luminal epithelial PNT1B cells immortalised by SV40-T antigen [24,28], whereas SV40-T immortalised prostate epithelial P69 cells were positive for CCN3 expression. These results suggest that CCN3 expression in these cells might be a marker for epithelial prostatic cell transformation, rather than a feature of increased proliferation and point out a role for CCN3 in the transition from normal to malignant behaviour.

In Renal Cell Carcinomas (RCCs) which represent 85–90% of all kidney tumours, a significantly higher level of secreted CCN3 protein was detected by Western blotting in the conditioned medium of fast growing tumours [25]. An inverse relationship could be drawn between the amount of CCN3 secreted by the tumour cells and the time that was required to establish tumours *in vivo* and for them to reach a given size.

About 60% of the osteosarcoma tumours were found positive for CCN3. Again, an inverse relationship was observed in these tumours between the levels of CCN3 and alkaline phosphatase – an early marker of osteoblastic differentiation. Consequently, as the expression of alkaline phosphatase was associated with loss of agressiveness of osteosarcoma cells, these results indicate that in this system, the expression of CCN3 is likely to be associated with osteoblasts proliferation and therefore represent a marker of bad prognosis. It is worth noting that when injected into chicken embryos, MAV also induces osteopetrosis – a proliferation of osteoblasts. Whether an increase of CCN3 expression is also associated to osteopetrosis remains to be established.

In the case of Ewing's sarcoma, the levels of CCN3 expression varied among the different samples tested and the expression of CCN3 in primary tumours was associated to a significantly higher risk of developing lung and bone metastasis. Patients with primary tumours negative for CCN3 expression did not develop metastasis whereas 50% of the patients with primary tumours staining positive for CCN3 developed metastasis [26]. The expression of CCN3 in Ewing's transfected cells was shown to decrease cellular adhesivity and increase cell motility [29], two observations that are fully consistent with CCN3 being associated with a high metastatic potential.

CCN4 and CCN6 expression was significantly increased in most colon adenocarcinomas [30]. Overexpression of CCN4 induced an accelerated growth and morphological transformation of NRK-49F fibroblastic cells and cell lines established after infection by retrovirus contructs expressing CCN4 was showing increased tumourigenicity. When tested *ex vivo* however, forced expression of CCN4 induced only a slight increase of doubling time and slight decrease of saturation density on the same cells. Inasmuch as these cells did not grow without anchorage, the over expression of CCN4 was not sufficient enough to allievate the need for proper interactions with the ECM and attachment.

Altogether, these observations are in favour of CCN proteins playing a positive role in tumourigenesis by providing the stimulatory effects on cell growth that are required for the increased lifespan of tumour cells. Whether these effects are involving a partial or complete abrogation of apoptotic pathways is an interesting question that deserves attention. The relationship that was drawn between increased expression of CCN proteins and tumourigenicity might also affect the communication of tumour cells with the surrounding medium, as suggested by the effects of CCN3 on Ewing's sarcoma cells. By decreasing the adhesivity of the cells and by providing an increased ability to migrate and invade surrounding tis-

sues, the CCN proteins might be key factors participating to the metastatic potential of tumour cells. The identification of CCN proteins partners should be very helpful in establishing whether abnormal interactions with physiological targets are involved in these processes.

Association of CCN protein expression with reduced proliferative activity and tumourigenicity

The first evidence indicating that the expression of CCN proteins in tumours could be associated to differentiation and growth arrest came from our studies performed with MAV-induced nephroblastomas and Wilm's Tumours. The Myeloblastosis Associated Virus (MAV)-induced avian nephroblastomas constitute a unique model of the Wilm's Tumour [31], a paediatric tumour affecting approximately one in 10,000 children [32]. Large quantities of CCN3 transcripts were detected in all MAV-induced tumours, whereas in normal post-natal kidney the level of CCN3 expression was low [5].

Considering that non-acute retrovirus had been reported to induce tumours by integrating nearby growth regulatory genes in the host genome, we postulated that a) the CCN3 gene was a prefered integration site of MAV within the host genome and b) in these tumours, the expression of CCN3 was stimulated by MAV-LTR enhancer sequences integrated in the vicinity of the CCN3 locus. The use of Bacterial Artificial Chromosomes (BACs) and Fluorescent In Situ Hybridization (FISH) allowed us to recently establish that the CCN3 DNA region is not a preferential site of integration for MAV (C.L. Li, P. Coullin, C. Auffray, A. Bernheim, V. Joliot, R. Zoroob and B. Perbal, manuscript in preparation). Furthermore, the histological analysis of avian nephroblastomas and the expression pattern of CCN3 in the developing avian kidney, established that the MAV target cells express high levels of CCN3 and that elevated levels of CCN3 were detected in the more differentiated tumours (Cherel et al., manuscript in preparation; Chevalier and Perbal, unpublished data).

Analysis of Wilm's Tumours established that blastemal cells committed to abnormal differentiation were positive for CCN3 expression. Tumours with a high degree of stromal components contained the highest levels of CCN3 RNA. In some cases, the quantities of CCN3 RNA were inversely related to the amount of WT1 RNAs detected in the same samples [33]; an observation which, at first glance, was in agreement with the *ex vivo* downregulation of CCN3 promoter activity by WT1 [34]. However, studies performed with a larger panel of samples [35] representative of sporadic, WAGR and DDS histological types of tumours raised the possibility that the variations of CCN3 expression resulted from different relative amounts of WT1 isoforms within these tumours. Similarly, CCN2 –

whose expression is also decreased in Wilm's Tumours, was reported to be a target of WT1 [36].

In the Wilm's Tumours, expression of CCN3 was also shown to match striated muscular differentiation. During the heterotypic differentiation of the blastemal cells that takes place in these tumours, CCN3 expression was detected at an earlier developmental stage than desmin and confocal microscopy also indicated that the CCN3 protein was colocalised with desmin in heterotypic muscular fibres [35]. These observations established the level of CCN3 expression in these tumours as a marker of heterotypic differentiation [35] and suggested for the first time that overexpression of a CCN protein could also be associated with tumour cell growth arrest. A strong association between CCN3 expression and tumour differentiation was also observed in the case of neuroblastomas, chondrosarcomas, rhabdomyosarcomas and other musculoskeletal tumours [4,24,37].

In neuroblastomas with poor pronostic features, CCN3 staining was low moderate within the tumour cells, whereas in tumours with favourable pronostic and no N-myc amplification, the CCN3 staining was strongly detected in the cytoplasm of differentiated ganglion-type cells [4]. Immunohistochemistry, Western blotting analysis, and real-time RT-PCR performed on samples from enchondromas and chondrosarcomas of various grades also the highest levels of CCN3 expression were detected in benign enchondromas, and the lowest levels of CCN3 were detected in the tumours of higher grade. Rhabdomyosarcomas generally expressed significant amounts of CCN3, with the largest quantities being detected in the most differentiated cells [37], an observation in agreement with CCN3 expressed in the developing skeletal muscle and fusing myoloblasts [38].

A reduction of CCN3 expression in tumour cells was also observed in the case of human chronic myeloid leukemia tumours and in murine multipotent haematopoietic stem cells transfected with a thermo sensitive mutant of the bcr-abl gene fusion expressed in chronic myeloid leukaemia (CML) [39]. These results indicated that the tumour-associated reduction of CCN3 expression was not a characteristic specific to solid tumours.

More recently evidence was also obtained for decreased levels of other CCN proteins in tumours. For example, the expression of CCN1 was shown to be downregulated in uterine leiomyomas [40], rhabdomyosarcomas [41], and in 50% of prostate carcinomas [42]. In non-small cell lung cancer (NSCLC), the expression of CCN1 was also decreased markedly, compared to the matched normal samples [43]. In opposition to the stimulatory effect of CCN2 on human embryonal carcinoma cell growth, its

expression was increased when these cells were treated by all-trans retinoic acid and were undergoing neuronal differentiation with concomitant loss of tumourigenicity [44].

Originally reported to be highly expressed in murine cells with a low metastatic potential, CCN4 was detected at similar levels in normal breast epithelial cells and breast tumour cell lines [45]. In primary human colon adenocarcinomas, the expression of CCN5 was significantly decreased and it was not detected in the epithelial tumour cells of mammary carcinoma obtained from Wnt-1 transgenic mice [30]. CCN5 was also downregulated upon transformation of BALB/c3T3 fibroblastic cells. The expression of CCN6 was impaired in most infammatory breast cancer [46] and a relationship was observed between loss of function mutations in CCN6 and progressive pseudorheumatoid dysplasia [47].

CCN proteins may act as antiproliferative agents
Not only the amounts of CCN proteins were low in some tumours, but an increasing body of evidence indicates that overexpression of CCN proteins in tumour cells may result in growth arrest and/or reduced tumourigenicity.

For example, early studies indicated that high levels of CCN3 expression were correlated with reduced tumourigencity and low metastatic potential of glioblastoma cells [48]. By using a series of stable transfectants, we established that CCN3 had a marked antiproliferative effect on glioblastoma cells [49]. Furthermore, the cells expressing CCN3 showed a significant reduction in their ability to induce tumours in nude mice. Inasmuch as the expression of CCN3 was interfering with tumour expansion, but not with early stages of tumour developement, we hypothesised that CCN3 might be cooperating with other proteins at the cell surface or in the ECM to regulate cell proliferation and invasiveness.

In agreement with these observations, confocal analysis performed on the transfected cells established that CCN3 and Connexin-43 (Cx-43) were colocalised [49], an observation raising the possibility that they may physically interact. Similarly, Cx-43 and CCN3 proteins were colocalized at the membrane of choriocarcinoma cells [50]. Inasmuch as an aberrant Gap Junctional Intercellular Communication (GJIC) was associated to tumourigenesis, these results suggested that CCN3 is involved in the control of cell growth at least through its relationship with GJIC. Upon restoration of cell to cell communication via Cx-43 protein channels, human choriocarcinoma cells lost their ability to induce tumour growth *in vitro*, and strongly upregulated the expression of CCN3.

Along the same line, the isolation of stable transfectants expressing CCN3 under the control of constitutive and inducible promoters also permitted to establish that CCN3 was able to significantly reduce the growth rate of EWS transfectants *ex vivo* [29]. Interestingly, transfectants showed reduced adhesivity and increased motility – two features in agreement with CCN3 expression associated with higher metastatic potential (see above).

The expression of CCN1 in NSCLC stable transfectants induced a significant reduction of the proliferation rate that could be partially rescued after addition CCN1 antibodies. The CCN1 transfected cells where arrested in the G1 phase of the cell cycle, showed a decreased activity of CDK2 and an upregulation of p53, p21(WAF1), and pRB2/p130. Interestingly, the transfected cells expressing CCN1 gave rise to smaller tumours than those induced by the parental NSCLC cells [43].

The CCN4 protein was also reported to have marked inhibitory effects on the tumourigenic properties of tumour cells *in vivo*. The absolute number of tumours obtained was dramatically reduced by the expression of CCN4 and the time needed for tumours to reach a given size was considerably higher as a result of the marked inhibitory effect of CCN4 on the growth properties of transfected cells *in vivo*. Furthermore, the transfectants which expressed high levels of CCN4 gave rise to a much smaller number of lung metastasis than those expressing low levels of CCN4. Recently, it was shown that overexpression of CCN4 in lung cancer cells reduced their ability to induce metastasis in nude mice, and impaired their migration within Boyden chambers in response to serum [51].

Early results established the antiproliferative activity of CCN5. Repressed in several transformed derivatives, retroviral-driven ectopic expression of CCN5 induced a negative effect on the growth of rat transformed cells and was accompanied by an enrichment in subG1 DNA content [52]. These observations therefore suggested that overexpression of CCN5 might induce cell death. Furthermore, CCN5 overexpression in stably-transfected rat cells transformed by p53 and H-ras, significantly reduced their tumourigenicity when tested by subcutaneous injection in athymic mice. The expression of CCN5 was detected only after repeated passages of rat and mouse embryo fibroblasts, but not in primary fibroblasts. More recently, overexpression of CCN5 was reported to inhibit both proliferation and motility of myometrial, leiomyoma smooth muscle cells and vascular smooth muscle cells [53]. High levels of CCN5 expression were also detected in quiescent and heparin-treated vascular smooth muscle cells. [53].

In the same light, restoration of CCN6 expression in Inflammatory Breast Cancer (IBC) cells resulted in the induction of morphological changes and a decreased ability to grow in soft agar. Importantly, the restoration of CCN6 expression resulted in tumour growth suppression, but with a decrease of tumour cell growth in grafted nude mice [54], an observation reminiscent to the effects of CCN3 on glioblastoma cells (see above).

Collectively, these results indicated that the expression of CCN proteins was altered in various types of tumours; depending upon the type of cells and tissues, high levels of CCN expression could be associated to either differentiation or increased proliferation and metastasis. They also suggested that CCN proteins may be acting as a tumour suppressor, irrespective of their pattern of expression upon serum stimulation. The differential expression of CCN genes upon serum stimulation previously led to the conclusion that immediate early genes such as CCN1 and CCN2 were encoding positive growth regulators; whereas, genes whose expression was not induced or were repressed by serum were encoding growth suppressors.

Answers lie in the multimodular structure of the CCN proteins

We believe that a better understanding of the structural organisation of the CCN proteins is the key to the understanding of their various biological activities. It is worth pointing that several different signalling proteins have been shown to physically interact with CCN proteins. For example, CCN1,2 and 3 interact with a whole array of Integrins [3,55,56], and CCN3 interacts with Fibulin1C [57], Notch1 [58] and S100A4 proteins [59]. The existence of CCN isoforms which are biologically active have already been proposed to be a key element in regulating the functions of the CCN proteins [4]. Our current working model is based on the hypothesis that post translational modifications of the full length proteins, or alternative splicing of CCN mRNA species, are giving rise to CCN isoforms that may show opposite, or synergistic biological effects, either because their structure dictate different types of protein interaction, and/or because they are adressed to different subcellular compartments. The mosaic structure of the CCN proteins raises interesting questions as to the role and participation of each individual module to the biological function(s) of the full length and truncated CCN proteins.

Signal peptide

All CCN genes encode proteins with a signal peptide (figure 2) that we have recently established to be responsible for secretion (N. Planque, C.L. Li, L. Dassencourt, E. Kerbelec, and B. Perbal unpublished). The signal peptide is encoded by the first exon in the mRNA species encoding full length proteins.

In one of the MAV1-(N) – induced tumours, integration of the proviral genome in the CCN3 gene resulted in the production of a chimeric mRNA species whose translation gave rise to a 28 kDa CCN3 protein deprived of both the signal peptide and module I (Figure 4). Over-expression of this protein by an RSV-based recombinant virus induced morphological transformation of chicken embryo fibroblasts [5]. Interestingly, a 31 kDa aminotruncated CCN3 isoform deprived of module I was also detected in the nucleus of HeLa and 143 osteosarcoma cells [60,61]. It is therefore tempting to draw a relationship between these observations.

Since the absence of signal peptide in the aminotruncated isoform was expected to alter the fate of the CCN3 protein which is otherwise secreted (Figure 5), we proposed that the transforming potential of the truncated CCN3 protein results from its migration to the nucleus in RSV infected cells and in nephroblastoma tumour cells. The interaction of CCN3 with the rpb7 subunit of RNA polymerase II [60] promotes the idea that CCN3 might play a role in the control of transcription. As opposed to the antiproliferative activity of the full length secreted form (see above), we are currently investigating this hypothesis and the possibility that the nuclear isoform of CCN3 is stimulating cell proliferation.

Other CCN3 isoforms have been identified in the cytosol of various cell lines [35]. Whether the intracellular expression of these proteins accounts for the various roles of the CCN3 protein in signalling remains to be established. Along the same line, it is worth noting that, extracellular CCN2 was reported to be transported to the nucleus of endothelial cells [62] where it was proposed to modulate transcription.

In a recent study, the use of transfectants isolated from a human oral squamous cell carcinoma-derived cell line permitted to show that intracellularly expressed full length CCN2 was reducing cell growth and tumourigenicity [63]. It would be interesting to determine whether this observation is specific to CCN2 or whether it also applies to other members of the CCN family.

Module I (IGFBP)

Module I is encoded by the second exon of the CCN genes. According to secondary structure prediction programs, the N-terminus the CCN proteins shows significant differences among which the presence of β-sheets specific to CCN3, 4, 5 and 6 might be related to particular biological function(s).

Module I shares partial identity with the N-terminal part of the Insulin-like Growth Factor Binding Proteins (IGFBPs). However, a careful analysis of nucleotide
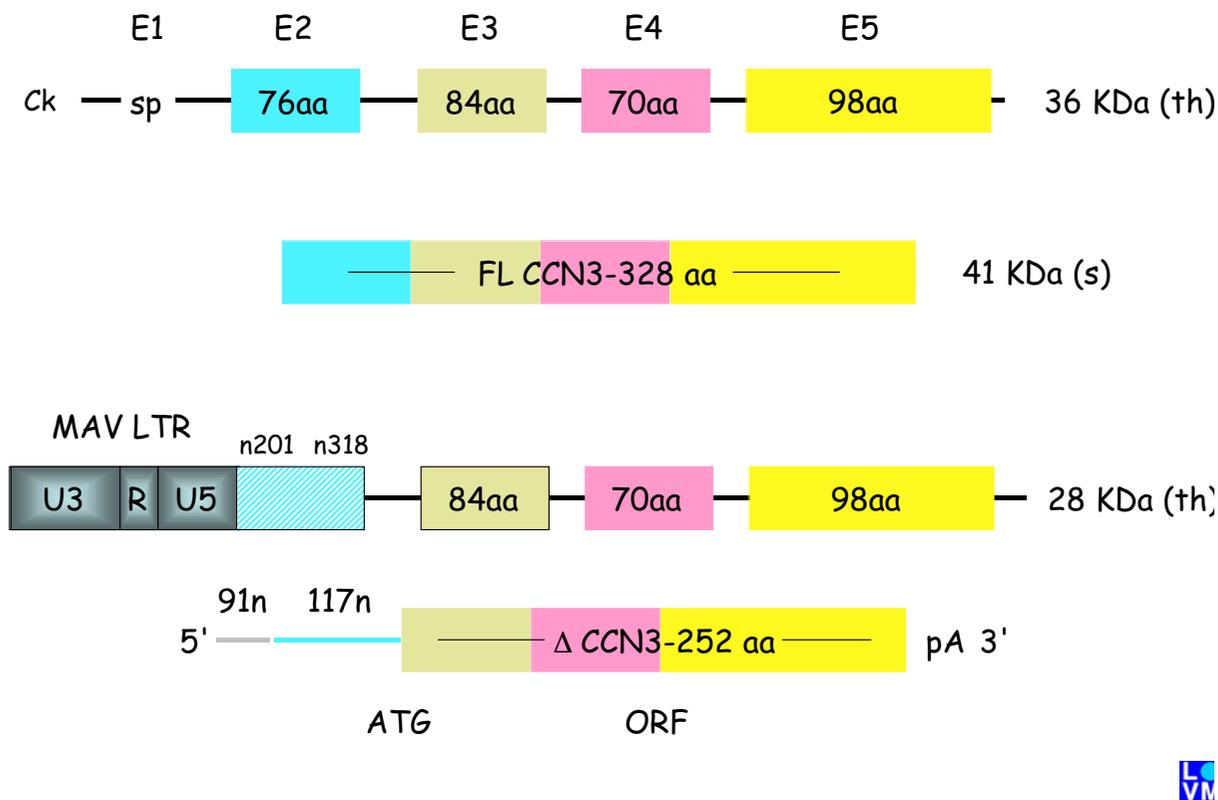
Figure 4
Insertional mutagenesis of MAV. The organisation of the chicken CCN3 gene is represented at the top. The CCN3 chicken protein encode a putative secreted 36 KDa protein. Following post-translational modifications, a full length 41 KDa CCN3 protein is released in the culture medium. In one MAV-induced nephroblastoma, proviral sequences were integrated within exon 2 of the CCN3 gene. As a consequence, MAV LTR was driving the expression of a chimaeric mRNA species encoding an amino truncated CCN3 protein deprived of module I and signal peptide. ORF : open reading frame ; (th) : theoretical ; (s) :secreted. The signal peptide (sp) is encoded by exon 1. E1–E5 : exons encoding the CCN3 protein. n201, n318 indicate the boundaries of the RNA stretch corresponding to the remaining sequences from exon 2. 91 n : 91 nucleotides of viral origin ; 117 n exon 2 sequences which are out of frame in the chimaeric mRNA species.

sequences indicated that the alignment originally proposed for N-terminal cysteine residues of IGFBPs and CCN proteins was not appropriate [64]. Because of this partial sequence identity, it was proposed that CCN proteins bind to IGF. This point remains controversial [65] and the biological significance of the structural relationship between IGFBPs and CCN proteins is still an open topic. *In vitro* crosslinking experiments suggested that full-length CCN2 and CCN3 could bind to IGF with very low affinity [66,67], whereas, ligand blotting did not permit detection of any interaction between CCN3 and IGF [32]. Interestingly, N-terminal truncated forms of CCN2 binds

efficiently to IGF, and the N-terminal region of CCN3 binds to insulin with a much higher affinity than IGFBPs [68].

Proteolysis of the secreted full length CCN proteins that has been reported in the case of CCN2 and CCN3 [6,35,57] might result in the production of CCN-derived peptides with high affinity for ligands that full-length CNN proteins bind only poorly. Amino-truncated CCN2 isoforms were biologically active whereas no specific biological activity has been attributed to the truncated CCN3. Although the molecular processes underlying the produc-
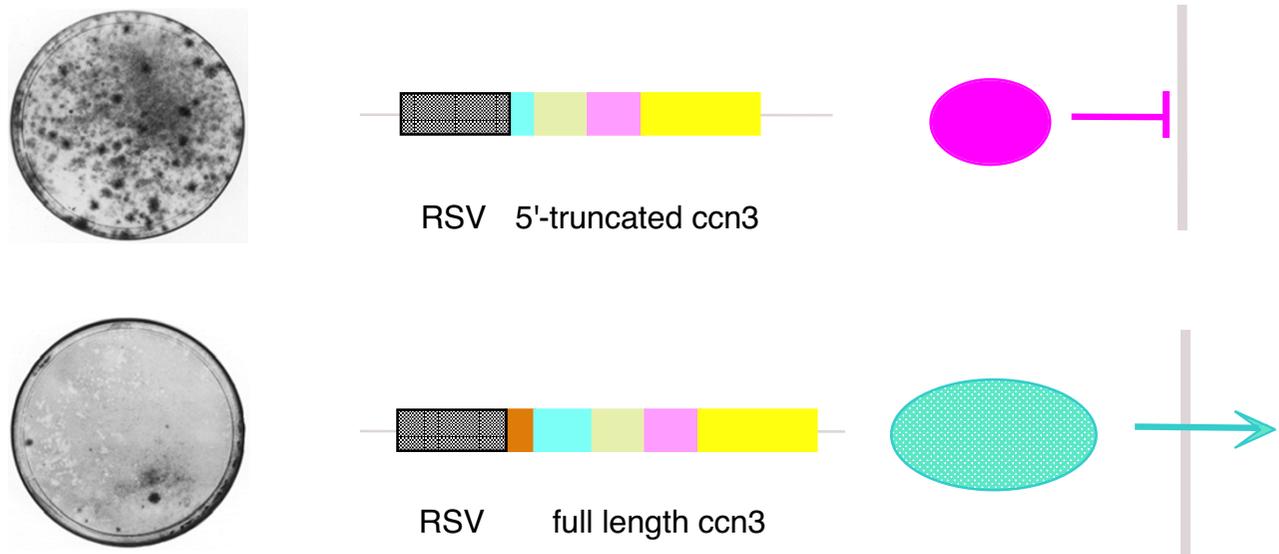
Figure 5
Subcellular localisation of the CCN3 isoforms is critical for biological activity. The cDNA sequences encoding the truncated and the full length CCN3 protein were inserted into RCAS non defective Rous sarcoma virus (RSV)-derived proviral vector. The amino-truncated CCN3 protein which is deprived of signal peptide (top) is not secreted and induces morphological transformation of chicken embryo fibroblasts, whereas the secreted full length CCN3 protein induces cell growth arrest.

tion of these secreted isoforms is presently unknown, it is important to note that proteolysis occur at the same amino acid residues in both CCN2 and CCN3 [57].

Module II (VWC)

Module II includes a stretch of 70amino acid residues – which shares sequence identity with the Von Willebrand Factor Type C repeat (VWC). It is encoded by exon 3 and is well conserved among all members of the CCN family with the exception of CCN6, which lacks four of the 10 cysteine residues contained in this module. The VWC motif is also present in other secretory proteins such as α-collagen, thrombospondin 1 and 2, mucins, and Drosophila SOG (Short Gastrulation) protein. In addition to the VWC motif, exon 3 also encode a region devoid of cysteines that may serve as a hinge connecting the N- and C-terminal halves of the proteins. This region is highly charged and is longer in CCN1 than in the other CCN

members (48aa's versus 10 –15aa's). Secondary structure predictions shows great structural differences in this region among the different members of the CCN family. The variable region of CCN6 only contains α-helix whereas in the other CCN members it contains β-sheets and β-turns.

Module II contains sequences that were proposed to drive protein oligomerisation. Inasmuch as CCN4 protein lacking module II are produced in scirrhous gastric carcinoma by alternative splicing [69], it would be interesting to determine whether the deletion of this module alters the capacity of CCN4 to oligomerise and confers on this protein new biological properties. Should it be the case, alternative splicing of VWC sequences would represent an additional mean to produce oncogenic variant forms of CCN proteins.

Module III (TSPI)

Module III contains sequences sharing identity with the Thrombospondin type 1 repeat (TSP1) (WSXCSXXCG), which is thought to be implicated in the binding of sulfated glycoconjugates [70] and to be important for cell adhesion. The position of five of the six cysteines contained in this domain is conserved in other proteins (e.g. properdine, F-spondine, circumsporozoid protein) which contain a TSP1 repeat and are implicated in the interactions of the cells with the ECM.

The presence of a TSP module in the CCN proteins suggested that they might be involved in functional interactions with components of the ECM or play a role in cell adhesion. To elaborate, CCN3 physically interacts with fibulin1C, a matricellular protein involved in attachment [48]. CCN1 promotes adhesion, migration and mitogenesis of human skin fibroblasts, and adhesion of vascular smooth muscle cells [71–74]. Additionally, CCN2 promotes primary human skin fibroblast adhesion [72]. In both case, these functions involve binding to cell surface receptors – among which integrins and HSPG. CCN1 up-regulates expression of matrix metalloproteinases 1 and 3 [73] which promote matrix remodelling. The binding of CCN4 to sulfate proteoglycans decorin and biglycan mediates and modulates the interaction of CCN4 with the surface of fibroblasts [75].

However, there is no indication as yet to assign a direct role for the TSP1 module in the interaction of CCN proteins with their substrate. The construction of recombinant CCN proteins lacking module 3 (our unpublished results, and L. Lau personal communication) should be informative. It will be particularly interesting to establish whether the TSP1 module plays biological functions by itself or in cooperation with other modules and whether its intrinsic biological properties are modulated by its association with other modules in the CCN proteins. Recently, a variant CCN1 protein lacking the TSP1 module, has been detected in serum-induced normal human fibroblasts [76].

It is also of high interest that in the (A)9 repeat located at the junction of the second and third module in CCN6, is the target for frameshift mutations [77]. The resulting truncated CCN6(A)8 protein is deprived of both module III and IV. CCN6(A)8 was detected in 31% of colorectal carcinomas with microsatellite instability (MSI) and in 21% of MSI-positive gastric carcinomas [78]. These observations reinforced the idea that the different CCN modules can function individually and that alterations of the CCN protein structure is associated with tumourigenesis [4].

Module IV (CT)

Module IV, also designated CT, is encoded by exon5. It is the less conserved of the four domains at the level of nucleotide sequence, but it appears to be critical for several of the biological functions attributed to the CCN proteins. Module IV resembles the CT domain of several extracellular protein including, Von Willebrand's factor and mucins. Sequences similarities to Heparin-binding motifs are also found within this domain [6].

Within the CT module, six cysteines form a structure known as "cystin knot", which was implicated in the dimerisation of growth factors such as TGF-β, PDGF, and Nerve Growth Factor (NGF), [79]. The presence of a cystin knot in the CCN proteins might allow both homotypic and heterotypic interactions with proteins containing a similar structure. Dimerisation has not been conclusively demonstrated for any of the CCN proteins to date, but it has been shown that module IV is sufficient to permit the interaction of CCN3 with CCN2 in the two hybrid system and by GST pull down assay [57].

The CT domain is sufficient to allow interaction of CCN3 with Fibulin 1C, and Notch1 [57,58]. In CCN2, it is sufficient to mediate cell adhesion in a divalent cation- and heparin-dependent way [80]. Similarly, deletion of the CT module in CCN1 abrogates its capacity to stimulate integrin-dependent adhesion of fibroblasts [73]. Therefore, it seems that the CT module may be critical for the control of cell adhesion. An isoform of CCN2 lacking the CT domain (CTGF-L) is also expressed in human primary osteoblasts, fibroblasts, ovary, testes, and heart [81]; however, not much is known about its biological activities.

In the CCN proteins, module IV is thought to act synergistically with module II in the formation of the high molecular species which have been detected with all CCN proteins. Although there is no experimental data as yet indicating that any of their biological properties are dependent upon their ability to oligomerise, it is highly probable that the absence of four cysteines in module II of CCN6, and the absence of domainIV in CCN5 might alter their oligomerisation capacity and confer on these two proteins new biological properties.

Conclusion and perspectives

The bulk of data obtained thus far regarding the involvement of the CCN protein in tumourigenensis reinforces the idea that their biological properties might be dependent upon the cellular context; thereby suggesting that they may show opposite functions in different cell types. The variety of proteins interacting with members of the CCN family pointed out the complexity of the regulatory circuitry in which these proteins are acting.

It has been proposed by Lau and co-workers that the interaction of CCN proteins with integrins accounts for most of their biological properties both in normal and pathological conditions. In as much as the CCN3 protein was shown a) to interact with several proteins playing key roles in various signaling pathways, b) modulate cytosolic ion concentrations, and c) regulate transcription; we would like to broaden this view. Our current working hypothesis is based on the assumption that the CCN proteins are "docking" proteins permitting a coordinated interaction of the various receptors and co-factors. The multimodular structure of the CCN proteins provides the basis for multiple interactions and for combinatorial events; they are expected to vary greatly among tissues and cell types from cellular generation to death. The adaptive response of cells to complex environmental changes requires a precise coordination of signalling pathways. Our current knowledge put the CCN family of cell growth regulators at a central place in the control of these fundamental biological processes.

Note
We follow in this manuscript the recommendations for a unified nomenclature of the CCN proteins [82]

References
1. Bork P: **The modular architecture of a new family of growth regulators related to connective tissue growth factor.** *FEBS Lett* 1993, **327**:125-130.
2. Brigstock DR: **The connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) family.** *Endocr Rev* 1999, **20**:189-206.
3. Lau LF and Lam SC: **The CCN family of angiogenic regulators: the integrin connection.** *Exp Cell Res* 1999, **248**:44-57.
4. Perbal B: **NOV (nephroblastoma overexpressed) and the CCN family of genes: structural and functional issues.** *Mol Pathol* 2001, **54**:57-79.
5. Joliot V, Martinerie C, Dambrine G, Plassiart G, Brisac M, Crochet J and Perbal B: **Proviral rearrangements and overexpression of a new cellular gene (nov) in myeloblastosis-associated virus type 1-induced nephroblastomas.** *Mol Cell Biol* 1992, **12**:10-21.
6. Brigstock DR, Steffen CL, Kim GY, Vegunta RK, Diehl JR and Harding PA: **Purification and characterization of novel heparin-binding growth factors in uterine secretory fluids. Identification as heparin-regulated Mr 10,000 forms of connective tissue growth factor.** *J Biol Chem* 1997, **272**:20275-20282.
7. Su BY, Cai WQ, Zhang CG, Martinez V, Lombet A and Perbal B: **The expression of ccn3 (nov) RNA and protein in the rat central nervous system is developmentally regulated.** *Mol Pathol* 2001, **54**:184-191.
8. Planque N and Perbal B: **The CCN proteins: a new family of cell growth regulators.** In *Recent Research Developments in Human Pathology* Edited by: *Pandalai SG. Research Signpost* .
9. Perbal B, Brigstock DR and Lau L: **Report of the second international workshop on the CCN family of genes.** *Mol Pathol* 2003, **56**:80-85.
10. Babic A, Kireeva M, Kolesnikova T and Lau L: **CYR61, a product of a growth factor-inducible immediate early gene, promotes angiogenesis and tumour growth.** *Proc Natl Acad Sci* 1998, **95**:6355-6360.
11. Xie D, Nakachi K, Wang H, Elashoff R and Koeffler HP: **Elevated levels of ccn1, ccn2 and ccn4 in primary breast cancers associated with more advanced features.** *Cancer Res* 2001, **61**:8917-8923.
12. Sampath D, Winneker RC and Zhang Z: **The angiogenic factor Cyr61 is induced by the progestin R5020 and is necessary for mammary adenocarcinoma cell growth.** *Endocrine* 2002, **18**:147-159.
13. Tsai MS, Hornby AE, Lakins J and Lupu R: **Expression and function of CYR61, an angiogenic factor, in breast cancer cell lines and tumour biopsies.** *Cancer Res* 2000, **60**:5603-5607.
14. Tsai MS, Bogart DF, Castaneda JM, Li P and Lupu R: **Cyr61 promotes breast tumourigenesis and cancer progression.** *Oncogene* 2002, **21**:8178-8185.
15. Xie D, Miller CW, O'Kelly J, Nakachi K, Sakashita A, Said JW, Gornbein J and Koeffler HP: **Breast cancer. Cyr61 is overexpressed, estrogen-inducible, and associated with more advanced disease.** *J Biol Chem* 2001, **276**:14187-14194.
16. Wenger C and Gress TM: **CCN-family members in pancreatic diseases [abstract].** *Mol Pathol* 2001, **54**:119.
17. Frazier K and Grotendorst G: **Expression of Connective Tissue Growth Factor mRNA in the Fibrous Stroma of Mammary Tumours.** *Int J Biochem Cell Biol* 1997, **29**:153-161.
18. Igarashi A, Hayashi N, Nashiro K and Takehara K: **Differential expression of connective tissue growth factor gene in cutuneous fibrohistiocytic and vascular tumour.** *J Cutan Pathol* 1998, **25**:143-148.
19. Wenger C, Ellenrieder V, Alber B, Lacher U, Menke A, Hameister H, Wilda M, Iwamura T, Beger HG, Adler G and Gress TM: **Expression and differential regulation of connective tissue growth factor in pancreatic cancer cells.** *Oncogene* 1999, **18**:1073-1080.
20. Shakunaga T, Ozaki T, Ohara N, Asaumi K, Doi T, Nishida K, Kawai A, Nakanishi T, Takigawa M and Inoue H: **Expression of connective tissue growth factor in cartilaginous tumours.** *Cancer* 2000, **89**:1466-1473.
21. Pan LH, Beppu T, Kurose A, Yamauchi K, Sugawara A, Suzuki M, Ogawa A and Sawai T: **Neoplastic cells and proliferating endothelial cells express connective tissue growth factor (CTGF) in glioblastoma.** *Neurol Res* 2002, **24**:677-683.
22. Rubenstein J, Shen A, Haqq C, Ginziger D, Hyun W, Brigstock D and Shuman M: **Connective tissue growth factor is expressed in malignant astrocytic tumors and is involved in cell-cycle regulation [abstract].** *Mol Pathol* 2003, **56**:72.
23. Vorwerk P, Wex H, Hohmann B, Mohnike K, Schmidt U and Mittler U: **Expression of components of the IGF signalling system in childhood acute lymphoblastic leukaemia.** *Mol Pathol* 2002, **55**:40-45.
24. Maillard M, Cadot B, Ball RY, Sethia K, Edwards D, Perbal B and Tatoud R: **Differential expression of novH proto-oncogene in human prostate cell lines and tissues.** *Mol Pathol* 2001, **54**:275-280.
25. Glukhova L, Angevin E, Lavialle C, Cadot B, Terrier M, Lacombe J, Perbal B, Bernheim A and Goguel AF: **Specific Genomic Alterations Associated With Poor Prognosis In High Grade Renal Cell Carcinomas.** *Cancer Genet Cytogenet* 2001, **130**:105-110.
26. Manara MC, Perbal B, Benini S, Strammiello R, Cerisano V, Perdichizzi S, Serra M, Astolfi A, Bertoni F, Alami J, Yeger H, Picci P and Scotlandi K: **The expression of ccn3(nov) gene in musculoskeletal tumours.** *Am J Pathol* 2002, **160**:849-859.
27. Hu Y, Ippolito JE, Garabedian EM, Humphrey PA and Gordon JI: **Molecular characterization of a metastatic neuroendocrine cell cancer arising in the prostates of transgenic mice.** *J Biol Chem* 2002, **277**:44462-44474.
28. Lopez-Bermejo A, Buckway CK, Devi GR, Hwa V, Plymate SR, Oh Y and Rosenfeld RG: **Characterization of insulin-like growth factor-binding protein-related proteins (IGFBP-rPs) 1, 2, and 3 in human prostate epithelial cells: potential roles for IGFBP-rP1 and 2 in senescence of the prostatic epithelium.** *Endocrinology* 2000, **141**:4072-4080.
29. Scotlandi K, Benini S, Manara MC, Cerisano V, Perdichizzi S, Strammiello R and Perbal B: **Biological role of NOVH in Ewing's sarcomac cells [abstract].** *Mol Pathol* 2003, **56**:73.
30. Pennica D, Swanson TA, Welsh JW, Roy M, Lawrence D, Lee J, Brush J, Taneyhill L, Deuel B, Lew M, Watanabe C, Cohen R, Melhem M,

Finley G, Quirke P, Goddard A, Hillan K, Gurney A, Botstein D and Levine A: **WISP genes are members of the connective tissue growth factor family that are up-regulated in Wnt-1-transformed cells and aberrantly expressed in human colon tumours.** *Proc Natl Acad Sci USA* 1998, **95:**14717-14722.

31. Perbal B: **Contribution of MAV-1-induced nephroblastoma to the study of genes involved in human Wilms' tumour development.** *Critical Reviews in Oncogenesis* 1994, **5:**589-613.

32. Beckwith JB, Kiviat NB and Bonadio JF: **Nephrogenic rests, nephroblastomatosis, and the pathogenesis of Wilms' tumour.** *Pediatric Pathology* 1990, **10:**1-36.

33. Martinerie C, Huff V, Joubert I, Badzioch M, Saunders G, Strong L and Perbal B: **Structural analysis of the human nov proto-oncogene and expression in Wilms tumour.** *Oncogene* 1994, **9:**2729-2732.

34. Martinerie C, Chevalier G, Rauscher FJ 3rd and Perbal B: **Regulation of nov by WT1: a potential role for nov in nephrogenesis.** *Oncogene* 1996, **12:**1479-1492.

35. Chevalier G, Yeger H, Martinerie C, Laurent M, Alami J, Schofield P and Perbal B: **NovH: Differential Expression in Developing Kidney and Wilms' Tumours.** *Am J Pathol* 1998, **152:**1563-1575.

36. Stanhope-Baker P and Williams BR: **Identification of connective tissue growth factor as a target of WT1 transcriptional regulation.** *J Biol Chem* 2000, **275:**38139-38150.

37. Le AT, Yu C, Yeger H, Perbal B and Alman BA: **NOV (CCN3) regulation in the growth plate and CCN family member expression in cartilage neoplasia.** *Journal of Pathology* .

38. Kocialkowski S, Yeger H, Kingdom J, Perbal B and Schofield P: **Expression of the human nov gene in first trimester fetal tissues.** *Anat Embryol* 2001, **203:**417-427.

39. Gilmour L, Price S, Whetton AD, Perbal B and Irvine AE: **Deregulation of nov expression in chronic myeloid leukaemia [abstract].** *Mol Pathol* 2003, **56:**73.

40. Sampath D, Zhu Y, Winneker RC and Zhang Z: **Aberrant expression of Cyr61, a member of the CCN (CTGF/Cyr61/Cef10/NOVH) family, and dysregulation by 17 beta-estradiol and basic fibroblast growth factor in human uterine leiomyomas.** *J Clin Endocrinol Metab* 2001, **86:**1707-1715.

41. Genini M, Schwalbe P, Scholl FA and Schufer BW: **Isolation of genes differentially expressed in human primary myoblasts and embryonal rhabdomyosarcoma.** *Int J Cancer* 1996, **66:**571-577.

42. Pilarsky CP, Schmidt U, Eissrich C, Stade J, Froschermaier SE, Haase M, Faller G, Kirchner TW and Wirth MP: **Expression of the extracellular matrix signaling molecule Cyr61 is downregulated in prostate cancer.** *Prostate* 1998, **36:**85-91.

43. Tong X, Xie D, O'Kelly J, Miller CW, Muller-Tidow C and Koeffler HP: **Cyr61, a member of CCN family, is a tumour suppressor in non-small cell lung cancer.** *J Biol Chem* 2001, **276:**47709-47714.

44. Freemantle SJ, Kerley JS, Olsen SL, Gross RH and Spinella MJ: **Developmentally-related candidate retinoic acid target genes regulated early during neuronal differentiation of human embryonal carcinoma.** *Oncogene* 2002, **21:**2880-2889.

45. Banerjee S, Saxena N, Sengupta K, Tawfik O, Mayo MS and Banerjee SK: **WISP-2 gene in human breast cancer: role of estrogen and progesterone [abstract].** *Mol Pathol* 2003, **56:**71.

46. Kleer CG, Zhang Y, Pan Q, van Golen KL, Wu Z-F and Merajver SD: **WISP3 is a novel tumor suppressor gene for inflammatory breast cancer [abstract].** *Mol Pathol* 2003, **56:**72.

47. Hurvitz JR, Suwairi WM, Van Hul W, El-Shanti H, Superti-Furga A, Roudier J, Holderbaum D, Pauli RM, Herd JK, Van Hul EV, Rezai-Delui H, Legius E, Le Merrer M, Al-Alami J, Bahabri SA and Warman ML: **Mutations in the CCN gene family member WISP3 cause progressive pseudorheumatoid dysplasia.** *Nat Genet* 1999, **23:**94-98.

48. Li WX, Martinerie C, Zumkeller W, Westphal M and Perbal B: **Differential expression of nov H and CTGF in human glioma cell lines.** *Mol Pathol* 1996, **49:**91-97.

49. Gupta N, Wang H, McLeod TL, Naus CC, Kyurkchiev S, Advani S, Yu J, Perbal B and Weichselbaum RR: **Inhibition of glioma cell growth and tumourigenic potential by CCN3 (NOV).** *Mol Pathol* 2001, **54:**293-99.

50. Gellhaus A, Propson S, Gabriel H-D, Gruemmer R, Duerig J, Klein-Hitpass BL, Maass K, Perbal B and Winterhager E: **NOV is associated with CX43 but not with CX40 and TRCX43 in human choriocarcinoma transfectants: evidence for potential growth-regulatory functions [abstract].** *Mol Pathol* 2003, **56:**75.

51. Soon LL, Yie TA, Shvarts A, Levine AJ, Su F and Tchou-Wong KM: **Overexpression of WISP-1 downregulated motility and invasion of lung cancer cells through inhibition of Rac activation.** *J Biol Chem* 2003, **278:**11465-11470.

52. Zhang R, Averboukh L, Zhu W, Zhang H, Jo H, Dempsey PJ, Coffey RJ, Pardee AB and Liang P: **Identification of rCop-1, a New Member of the CCN Protein Family, as a Negative Regulator for Cell Transformation.** *Mol Cell Biol* 1998, **18:**6131-6141.

53. Mason HR, Nowak RA and Castellot JJ Jr: **CCN5 inhibits the proliferation and motility of human myometrial and leiomyoma smooth muscle cells [abstract].** *Mol Pathol* 2003, **56:**73.

54. Kleer CG, Zhang Y, Pan Q, van Golen KL, Wu ZF, Livant D and Merajver SD: **WISP3 is a novel tumour suppressor gene of inflammatory breast cancer.** *Oncogene* 2002, **21:**3172-3180.

55. Brigstock DR: **Angiogenesis: the shared role of CTGF and CYR61.** *CCN Newsletter* 2002, **1:**5-8.

56. Lin C, Leu SJ, Chen N, Tebeau C, Lin SX, Yeung CY and Lau L: **CCN3 (NOV) is a novel angiogenic regulator of the CCN protein family.** *J Biol Chem* . Apr 13 2003

57. Perbal B, Martinerie C, Sainson R, Werner M, He B and Roizman B: **The C-terminal domain of the regulatory proteinNOVH is sufficient to promote interaction with fibulin 1C: a clue for a role of NOVH in cell-adhesion signaling.** *Proc Natl Acad Sci U S A* 1999, **96:**869-874.

58. Sakamoto K, Yamaguchi S, Ando R, Miyawaki A, Kabasawa Y, Takagi M, Li CL, Perbal B and Katsube K: **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:**29399-29405.

59. Li CL, Martinez V, He B, Lombet A and Perbal B: **A role for CCN3 (NOV) in calcium signalling.** *Mol Pathol* 2002, **55:**250-261.

60. Perbal B: **Nuclear localisation of NOV protein : a potential role for nov in the regulation of gene expression.** *Mol Pathol* 1999, **52:**84-91.

61. Thomopoulos GN, Kyurkchiev S and Perbal B: **Immunocytochemical localisation of NOVH protein and ultrastructural characteristics of NCI-H295R cells.** *J Submicrosc Cytol Pathol* 2001, **33:**251-260.

62. Wahab NA, Brinkman H and Mason RM: **Uptake and intracellular transport of the connective tissue growth factor.** *Biochem J* 2001, **359:**89-97.

63. Moritani NH, Kubota S, Nishida T, Kawaki H, Kondo S, Sugahara T and Takigawa M: **Suppressive effect of overexpressed connective tissue growth factor on tumor cell growth in a human oral squamous cellcarcinoma-derived cell line.** *Cancer Lett* 2003, **192:**205-214.

64. Perbal B: **Les protéines CCN: Quand multimodulaire rime avec multifonctionnel.** *Médecine/Sciences* 2002, **18:**745-756.

65. Grotendorst GR, Lau LF and Perbal B: **CCN proteins are distinct from and should not be considered members of the insulin-like growth factor-binding protein superfamily.** *Endocrinology* 2000, **141:**2254-2256.

66. Kim HS, Nagalla SR, Oh Y, Wilson E, Roberts CT jr and Rosenfeld RG: **Identification of a family of low-affinity insulin-like growth factor binding proteins (IGFBPs):characterisation of connective tissue growth factor as a member of the IGFBP super family.** *Proc Natl Acad Sci USA* 1997, **94:**12981-12986.

67. Burren C, Wilson E, Hwa V, Oh Y and Rosenfeld R: **Binding Properties and Distribution of Insulin-Like Growth Factor Binding Protein-Related Protein 3 (IGFBP-rP3/NovH), an Additional Member of the IGFBP Superfamily.** *J Clin Endocri and Metab* 1999, **84:**1096-1103.

68. Hwa V, Oh Y and Rosenfeld RG: **The insulin-like growth factor-binding protein (IGFBP) superfamily.** *Endocr Rev* 1999, **20:**761-787.

69. Tanaka S, Sugimachi K, Saeki H, Kinoshita J, Ohga T, Shimada M, Maehara Y and Sugimachi K: **A novel variant of WISP1 lacking a Von Willebrand type C module overexpressed in scirrhous gastric carcinoma.** *Oncogene* 2001, **20:**5525-5532.

70. Holt GD, Pangburn MK and Ginsburg V: **Properdin binds to sulfatide [Gal(3-SO4)beta 1–1 Cer] and has a sequence homology with other proteins that bind sulfated glycoconjugates.** *J Biol Chem* 1990, **265:**2852-2855.

71. Chen CC, Chen N and Lau LF: **The angiogenic factors Cyr61 and connective tissue growth factor induce adhesive signaling in**

primary human skin fibroblasts. *J Biol Chem* 2001, **276:**10443-10452.

72. Chen CC, Mo FE and Lau LF: **The angiogenic factor Cyr61 activates a genetic program for wound healing in human skin fibroblasts.** *J Biol Chem* 2001, **276:**47329-47337.

73. Grzeszkiewicz TM, Kirschling DJ, Chen N and Lau LF: **CYR61 stimulates human skin fibroblast migration through Integrin alpha vbeta 5 and enhances mitogenesis through integrin alpha vbeta 3, independent of its carboxyl-terminal domain.** *J Biol Chem* 2001, **276:**21943-21950.

74. Grzeszkiewicz TM, Lindner V, Chen N, Lam SC and Lau LF: **The angiogenic factor cysteine-rich 61 (CYR61, CCN1) supports vascular smooth muscle cell adhesion and stimulates chemotaxis through integrin alpha(6)beta(1) and cell surface heparan sulfate proteoglycans.** *Endocrinology* 2002, **143:**1441-1450.

75. Desnoyers L, Arnott D and Pennica D: **WISP-1 binds to decorin and biglycan.** *J Biol Chem* 2001, **276:**47599-47607.

76. Leng E, Malcom T, Gai G, Estable M and Saowski I: **Organization and expression of the Cyr61 gene in normal human fibroblasts.** *J Biomed Sci* 2002, **9:**59-67.

77. Thorstensen L, Diep CB, Meling GI, Aagesen TH, Ahrens CH, Rognum TO and Lothe RA: **WNT1 inducible signaling pathway protein 3, WISP-3, a novel target gene in colorectal carcinomas with microsatelliteinstability.** *Gastroenterology* 2001, **121:**1275-1280.

78. Tanaka S, Sugimachi K, Shimada M, Maehara Y and Sugimachi K: **Variant WISPs as targets for gastrointestinal carcinomas.** *Gastroenterology* 2002, **123:**392-393.

79. Schlunegger MP and Grutter MG: **Refined crystal structure of human transforming growth factor beta 2 at 1.95 A resolution.** *J Mol Biol* 1993, **231:**445-458.

80. Ball DK, Rachfal AW, Kemper SA and Brigstock DR: **The heparin-binding 10 kDa fragment of connective tissue growth factor (CTGF) containing module 4 alone stimulates cell adhesion.** *J Endocrinol* 2003, **176:**R1-R7.

81. Kumar S, Hand AT, Connor JR, Dodds RA, Ryan PJ, Trill JJ, Fisher SM, Nuttall ME, Lipshutz DB, Zou C, Hwang SM, Votta BJ, James IE, Rieman DJ, Gowen M and Lee JC: **Identification and cloning of a connective tissue growth factor-like cDNA from human osteoblasts encoding a novel regulator of osteoblast functions.** *J Biol Chem* 1999, **274:**17123-17131.

82. Brigstock DR, Goldschmeding R, Katsube KI, Lam SCT, Lau LF, Lyons K, Naus C, Perbal B, Riser B, Takigawa M and Yeger H: **Proposal for a unified CCN nomenclature.** *Mol Pathol* 2003, **56:**127-128.