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REVIEW

CCN family of proteins: critical modulators of the tumor cell microenvironment

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Abstract The CCN family of proteins consisting of CCN1 (Cyr61), CCN2 (CTGF), CCN3 (NOV), CCN4 (WISP-1), CCN5 (WISP-2) and CCN6 (WISP-3) are considered matricellular proteins operating essentially in the extracellular microenvironment between cells. Evidence has also been gradually building since their first discovery of additional intracellular roles although the major activity is triggered at the cell membrane. The proteins consist of 4 motifs, a signal peptide (for secretion} followed consecutively by the IGFBP, VWC, TSP1 and CT (C-terminal cysteine knot domain) motifs, which signify their potential binding partners and functional connections to a variety of key regulators of physiological processes. With respect to cancer it is now clear that, whereas certain members can facilitate tumor behavior and progression, others can competitively counter the process. It is therefore clear that the net outcome of biological interactions in the matrix and what gets signaled or inhibited can be a function of the interplay of these CCN 1–6 proteins. Because the CCN proteins further interact with other key proteins, like growth factors in the matrix, the balance is not only important but can vary dynamically with the physiological states of tumor cells and the surrounding normal cells. The tumor niche with its many cell players has surfaced as a critical determinant of tumor behavior, invasiveness, and metastasis. It is in this context that CCN proteins should be investigated with the potential of being recognized and validated for future therapeutic approaches.

Keywords CCN proteins · Cancer

Introduction

Since their first discovery in the early 90s' a great deal has been learned about the CCN family (the first three named after Cyr61, CTGF, NOV) and later joined by Wnt related WISP1/CCN4, WISP2/CCN5, WISP3/CCN6 proteins in mammals (see A. Perbal and B. Perbal in this issue). We first presented a perspective on the CCN family of proteins in 2007 (Yeger and Perbal 2007). Discussions on the structure-functional relationships of the CCN family of proteins (Perbal 2001; Holbourn et al. 2008; Krupska et al. 2015) and subsequently, following a number of reviews in recent years, we learned more about their biological roles and in particular about cancer (Gurbuza and Chiquet-Ehrismann 2015;Wells et al., 2015a b; Li et al. 2015). As part of a larger family of matricellular proteins the CCN family has been implicated in both health and disease with impacts on cell proliferation, angiogenesis, tumorigenesis, tumor progression, wound healing, and immune functions (Murphy-Ullrich and Sage 2014). One member of the CCN family, CCN2, has received a great deal of attention because of its implication in the severe condition of fibrosis in a number of diseases (Kubota and Takigawa 2015). With strong evidence of direct implication in the process of fibrosis, tightly associated with the functions of TGFβ1, efforts are underway to demonstrate clinical utility in blocking the actions of CCN2 (Liu et al. 2015; Aguiar et al. 2014; Wallace et al. 2013; Morales et al. 2013]. TGFβ1, called the master regulator of fibrosis that signals through the SMAD pathway (Meng et al. 2016) is complexly regulated and is

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not considered (or shown) to be a good therapeutic target. However, CCN2 appears to be a better candidate as a therapeutic target, but the role of CCN2 in cancer has yet to be adequately studied or implicated. Other members, in particular CCN1, CCN3 and CCN6 have, in contrast, grown considerably in attention as being implicated in tumor progression and metastasis in a number of prominent adult cancers [see below]. In fact, their intimate involvement is starting to single them out as potential therapeutic targets.

When considering the molecular aspects of the functional domains of CCN proteins, although the primary structures appear rather similar, subtle differences in their sequences dictate ultimate protein interactions, binding partners, and obvious non-redundant biological functions. In fact the divergence in the cysteine-knot domain and the lack of this domain in CCN5 along with significant sequence differences in the variable hinge region imbues these different CCN proteins with quite a difference in overall 3D structure. Thus it would be easy to imagine that CCN proteins could be competitive for binding partners but with different outcomes and modulation of the malignant phenotype and process.

CCN proteins and cancer

Li et al. (2015) recently reviewed the roles of the CCN1–6 expression levels in tumorigenesis and cancer metastasis. They compiled data on the relative expression levels of the CCN1–6 proteins in developing tissues and different tissues. From a transcripts perspective it is interesting to note that all are expressed in the fetal state but then the pattern becomes more restricted during childhood and re-expands during adulthood. For example and noteworthy, CCN5 expression during fetal life disappears during childhood and reappears at fetal levels during adulthood. This might suggest that it has a highly specific role to play relative to other CCN proteins in mature and possibly aging tissues. Along this same line, whereas CCN1 and CCN2 are highly expressed in many, but not all cancers, CCN5 appears a lot more restricted to particular cancers, notably renal, uterine, head and neck and chondrosarcoma. A similar restricted pattern is seen for CCN6, somewhat broader for CCN4 and more so for CCN3. So then do relative transcripts levels in particular cancers match expression in the normal tissue counterpart? For CCN6 both normal cervix and cervical tumors correspond, and GI tumors match at the site of stomach. Of note both normal mammary tissue and breast cancers correspond, although at low levels. However, the CCN6 protein has been recently shown to play a major role in breast cancer development and progression in studies from the Kleer group who originally made this discovery (Huang et al. 2016a, b and refs therein). Thus it is obvious that much work is required to dissect the functions of the proteins in context of the initiation site and subsequent metastases.

On the other hand, Jia et al. (2016) have presented a more complex picture in reviewing the role of CCN proteins in hepatocellular carcinoma. In clinical samples whereas CCN2 and CCN3 are positively correlated with HCC development, CCN1 is both negatively and positively correlated with HCC development while no differences were noted for CCN5 (Jia et al. 2016). They did not have data for CCN6 in HCC. In their assessment special emphasis was placed on the role of CCN proteins as multitasking signal integrators in the inflammatory microenvironment. Since CCN proteins can be involved in binding of inflammatory factors, and inflammation is a core component of the cancer process, CCN proteins may be modulators of the immune reaction to cancer cells.

Chang et al. (2014) reviewed the role of proteins CCN1, CCN2, CCN3, CCN4, CCN5 and CCN6 in colon carcinoma. CCN1 correlated positively with the early stage in tumor development and with migration of tumor cells in culture. CCN4 and CCN6 correlated positively with tumor grade. In line with this, CCN4 promotes cell cycle checkpoint progression, accelerating growth and inhibiting apoptosis. Oppositely, CCN5 correlated negatively with tumor grade. It should be noted that indeed the building consensus on CCN5 is that of a competitive inhibitor (note it lacks the CT domain). CCN2 presents a mixed picture. Positively correlated with early stage in tumor development but negatively correlated with metastasis and patient survival and found to inhibit invasion and metastasis. Also it is negatively correlated with prevalence of peritoneal carcinomatosis. CCN3 data was not available but it is interesting to note that while CCN3 may have an anti-tumor role in myeloma, in several solid cancers CCN3 inhibits proliferation but stimulates migration and invasion (Perbal 2013; see more on this below). A propos, Kees and colleagues reviewed the roles of CCN proteins in hematological malignancies (Wells et al. 2015a, b). Again a rather complex picture emerges with respect to expression levels of CCN proteins in the tumor cells and associated stroma. Overexpression of CCN1 in AML proved beneficial while overexpression in the mesenchymal stem cells in multiple myeloma proved inhibitory.

Along this line, CCN4 appears to be an important factor for survival of human mesenchymal stromal cells (Schlegelmilch et al. 2014) protecting against TRAIL-induced apoptosis, reflecting on activities at the cell membrane where CCN proteins operate (see below). Other outcomes of CCN1 overexpression in peripheral T-cell lymphoma and angioimmunoblastic lymphoma are as yet unknown. An interesting observation is that CCN2 expression is reduced in multiple myeloma and an N-terminal fragment of CCN2 is increased in serum suggesting degradative processing of secreted CCN. Internalization of cleaved fragments (intact domains) proteolytically generated through the linkage peptides

has been investigated from the earliest work on CCN proteins (Perbal 2001, 2013). More recent studies build credibility for autogenerated functional cleaved peptides (Gonzalez-Guerrico et al. 2016). Thus it is conceivable that CCN fragments could be found in the circulation with a high probability of detection only limited by the methods used. It would behoove the CCN field to employ LC-MS to search for such material as it might lead to validating CCN peptides as key biomarkers.

The above discussion very much suggests that the roles of CCN proteins would be exquisitely cell and environment context dependent and perhaps integrated with the inflammatory state of the cancer under analysis. This then also raises the question about the CCN protein binding partners (receptors) on cells, tumor and normal, that could interact with CCN proteins. The current knowledge on CCN receptors has been recently reviewed by Lau (2016) who brings out some relevant connections to be considered. Receptors include the primary binding integrins (interacting with multiple sites on CCN proteins) but also HSPGs, LRPs joint with Wnt binding frizzled, Notch, TrkA, and M6P/IGF2R, with signaling pathways leading to activation of AKT, ROS, FAK, Ca2+ release, p53, MAPKs and NFkB. Figure 1 shows the multimodular architecture and some of the binding partners for the individual domains. In other words, a potential panoply of coordinating signaling pathways that can be triggered where almost all have roles in tumorigenesis and progression, as well as being actionable in normal cells.

In the following sections we present cancer relevant studies and nuances on the individual CCN proteins. The intention is to scrutinize for new insights and potential for future investigations, basic and translational.

CCN1 (Cyr61)

Long et al. (2013) studying a number of renal carcinoma cell lines showed upregulation of multidrug resistance in vinblastine resistant cell lines correlated with increased expression of CCN1 by ELISA. This was mediated by αVβ3 integrin blocked by an antibody with CCN1 treatment specifically triggering the PI3K/Akt pathway. They concluded that CCN1 and the αVβ3 integrin are associated with drug resistance. Saglam et al. (2014) showed that higher CCN1 expression is associated with increased in situ (intraepithelial) malignant grade in a series of breast carcinoma cases studied by immunohistochemistry and correlated with increasing cell cycle progression (cyclin D1). Bartel et al. (2012) demonstrated by immunohistochemistry and westerns that CCN1 and CCN2 were inversely expressed in borderline ovarian tumors, and loss of CCN2 was associated with poor prognosis of invasive ovarian carcinomas assessed with Kaplan-Meier survival plots. Ishida et al. (2015) carried out Kaplan-Meier survival analysis in a series of patients with high CCN1 expression in gliomas. There was no significant difference in progression free survival (PFS) or overall survival (OS), but in patients with totally resected tumors high CCN1 predicted a significantly shorter PFS and OS. The

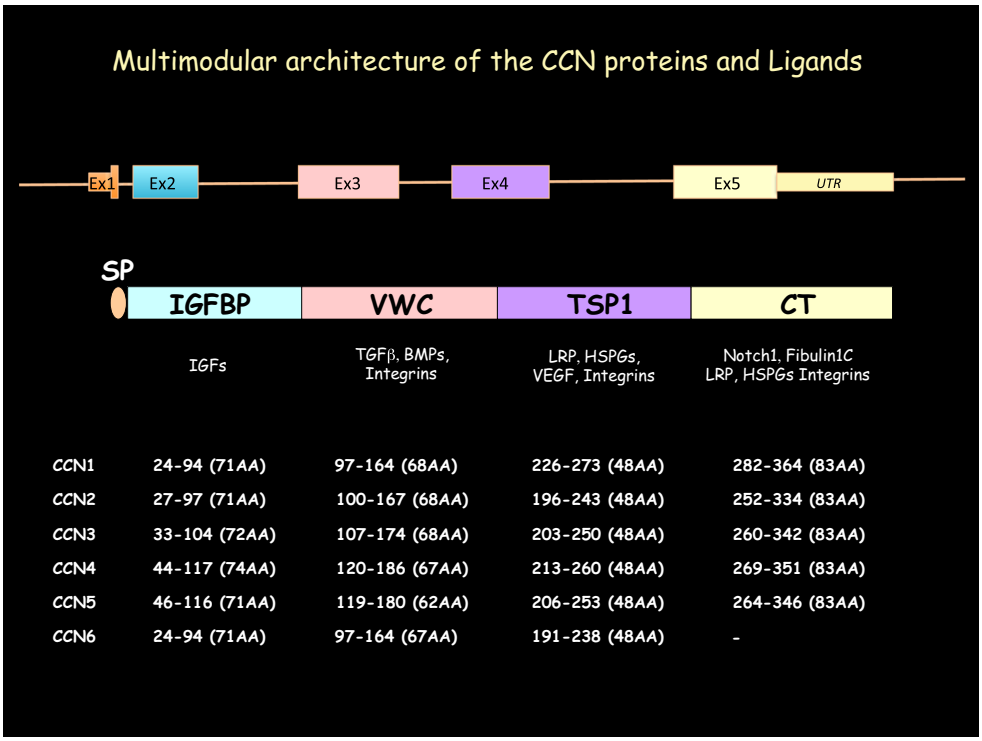

Fig. 1 Multimodular architecture of the CNN proteins and Ligands

authors explained this on the observation that CCN1 may be important in maintaining tumor stemness. Since CCN1 can be detected in cerebrospinal fluid it might serve as prognostic marker. Jeong et al. (2014) examined CCN1 expression in six colorectal cell lines and 20 sets of paired normal and tumor tissues by western blot. 4/6 lines were higher in CCN1 and 16/20 tumor tissues. Higher CCN1 was associated with significant cancer-specific mortality and a lesser duration of survival. Thus CCN1 may serve as a prognostic marker for CRC. Li et al. (2012b) showed that higher CCN1 expression was found in advanced, poorly differentiated, hepatocellular carcinomas and that this malignant progression was mediated by β-catenin which upregulates CCN expression via the β-catenin/TCF4 transcription complex. Forcing expression of CCN1 in HepG2 cell line promoted xenograft tumor growth with signs of an induced inflammatory state. Emre and Imhof (2014) discussed the emerging role of CCN1 as an important factor in controlling leucocyte migration and production of cytokines. Coupled with the ability of CCN1 and CCN2 to mediate proangiogenesis in cancer (Chintalapudi et al. 2008), and CCN1 for αVβ5 mediated cancer cell migration (Jandova et al. 2012), we have here a scenario of facilitated tumor cell invasion.

Haque et al. (2012) reported that CCN1 is a critical regulator of sonic hedgehog in pancreatic carcinogenesis. Working with pancreatic cancer cell lines they showed that CCN1 silencing interfered with Shh downstream signaling and inhibition of Notch1 degradation thus sustaining the proliferative drive. Again CCN1 activity was mediated through αVβ3 integrin binding. Interestingly, Huang et al. (2016a, b) recently showed that transgenic expression of CCN1 in Rip1CYR mice caused irregular islet morphology. When Rip1CYR mice were crossed with RipTag2CYR mice (model of beta cell carcinogenesis) the tumors were more invasive and vascularized. CCN1 modulated α6β1-dependent invasion and adhesion. Overall CCN1 appeared to act as a tumor promoting gene in pancreatic neuroendocrine tumors.

The picture that emerges is one of a CCN protein that promotes a more aggressive cancer phenotype while facilitating the invasive microenvironment. Importantly, assays that can detect CCN1 in blood and other fluids could lead to development of a predictive biomarker for many cancers. A review by Lau (2011) had already discussed in detail this biomarker aspect and several others relative for normal tissue development and pathology while Chong et al. (2012) presented a detailed schema depicting the role and integrating partners of CCN matricellular proteins in cancer progression. There is no doubt that the CCN proteins are intimately involved in the intricate biological processes underlying tumor stromal interactions in the tumor niche.

CCN2 (CTGF)

CCN2 or Connective Tissue Growth Factor (CTGF), as the name implies, came into prominence in the earliest days of CCN investigations when it was convincingly connected with the process of fibrosis and mediation by TGFβ (reviewed in Kubota and Takigawa 2015, Cicha and Goppelt-Struebe 2009. Fibrosis as connected with disease or other roles in normal tissue development are not discussed here. Older studies associated CCN2 expression with the aggressive and metastatic phenotype in breast, glioblastoma, esophageal, gastric and hepatocellular carcinomas (ref in Chang et al. 2013). A metastasis suppressing effect of CCN2 in lung cancer had been previously reported. In the current study Chang et al. (2013) used a series of lung adenocarcinoma cell lines, transfected CCN2, and determined the effect on cell survival. They demonstrated that CCN2 bound to the EGF receptor (also recently shown by Rayego-Mateos et al. 2013) and induced EGFR degradation. Apparently the CCN2 binding suppressed phosphorylation of c-Src leading to expression of the death-associated protein kinase (DAPK) and resulting anoikis. The thinking is that for lung cancer CCN2 could synergize with anti-EGFR antibody for an enhanced therapeutic outcome. Supporting xenograft studies in SCID mice indicated that CCN2 increased DAPK expression and an enhanced suppression of metastasis. Not surprising, the C-terminal module of CCN2 activates EGFR (Rodrigues-Diez et al. 2015) and downstream inflammatory pathway signaling, another factor mitigating the cancer process.

Wells et al. (2015a) summarized studies describing the topography of dysregulated CCN2 expression in a variety of neoplasms (27 currently) in humans. Those with higher CCN2 expression and resulting poor incomes for most, except gallbladder cancer and lymphoma, generally showed increased proliferation, drug resistance, angiogenesis migration and metastasis. Cancers (e.g. lung) with lower CCN2 expression) showed opposite effects on the same parameters. Elevated CCN2 expression in stromal cells is associated with the desmoplastic reaction and general stromal effects on tumor cells, likely reflecting the known association with hypoxic microenvironments, cancer associated fibroblasts that modulate angiogenesis, tumor cell migration and metastatic behavior (Rachfal et al. 2004; Gilkes et al. 2014; Shiga et al. 2015; Kim et al. 2014). The fact that these can be suppressed in some cancers and stimulated in others suggests a complex scenario of associated interacting factors. Finally, Lacle et al. (2015) performed a clinicopathological prognostic study on CCN2 expression in 109 male breast cancers and 75 female breast cancers. The major source of CCN2 was found in the stroma which here correlated with grade and proliferation while that in tumor cells did not. However no correlation with prognosis was found. This raises a cautionary note about the actual prognostic value of CCN2 or its role in cancer. Nevertheless

more studies need to be done with respect to the role CCN2 might have in tumor-stromal interactions. One such example comes from the studies by Hutchenreuther et al. (2015) who showed that in CCN2-deficient mice type 1 collagen producing stromal cells lacking CCN2 were unable to impede the spontaneous lung metastasis of B16(F10) mouse melanoma tumor cells apparently due to lack of the matricellular protein periostin. Loss of CCN2 in the tumor cells reduced their ability for invasion through collagen with reduced expression of periostin. Interestingly, due to non-correlation with BRAF mutant melanoma in humans, CCN2 might constitute a therapeutic target in BRAF-inhibitor resistant melanoma.

On another note, Patel et al. (2014) studied the effects of hypoxia (commonly found in most rapidly growing cancers) and CCN2 on chordoma U-CH1 cells. By RT-PCR analysis U-CH1 express multiple CCN proteins basally (CCN1, CCN2, CCN3, CCN5). While hypoxia upregulates progenitor-like markers and increased cell growth and tumorsphere formation CCN2 treatment had a more pronounced effect under normoxia and promoted increased expression of CCN1–3 and 5 but also stimulated tumor-sphere formation. Interestingly, this highlights how multiple CCN proteins might contribute to the net outcome suggesting an increased degree of stemness and progenitor like behavior. Tsai et al. (2014) studying osteosarcoma implicated CCN2 in resistance to apoptosis produced by cisplatin, a drug commonly used in treatment. Interestingly, CCN2 promoted FAK, MEK, ERK mediated survival signaling and knockdown by shRNA facilitated cisplatin treatment in a mouse model. Finally, from a therapy point of view, Chang et al. (2016) studied extracts of the medicine plant *Nelumbo nucifera* Gaertn (Nymphaeaceae) (NLE) as potential therapy for breast cancer. Using the MDA-MB-231 metastatic cell line and the chicken chorioallantoic membrane and Matrigel in nude mice angiogenesis models showed that NLE blocked HUVEC capillary formation and siRNA knockdown of CCN2 in MDA-MB231 reduced MMP2 and VEGF and attenuated PI3K-AKT-ERK activation making a connection between this signaling pathway, CCN2 function, and a potential therapeutic for triple negative breast cancer. Taken together, therapeutic targeting of CCN2 might reflect an ability to actual target the tumor CSC niche sequestering the metastatic phenotype.

CCN3 (NOV)

As hinted at in the last section on CCN2 multiple CCN proteins can contribute to the ultimate phenotype and physiological status of biological processes. Perbal (2001); Bleau et al. (2005); Kawaki et al. (2008) and Riser et al. (2009) presented the view of co-regulation by CCN proteins where CCN3 may act as a counter-regulator interfering with a pathogenic process mitigated via another CCN isoform. Much attention has been paid to the role of CCN2 in driving fibrosis in different organs and Riser's focus on diabetic nephropathy is emphasized. However, there is a block of studies that support CCN3 as highly pro-tumorigenic. Chen et al. (2012); Chen et al. (2014a); Cui et al. (2014), and Ueda et al. (2015) make the case for prostate, bladder cancer, pancreatic cancer, and colorectal cancer, respectively. Liu et al. (2015), make a case for CCN3 as enhancing renal cell carcinoma cell migration, by upregulation of ICAM and COX-2 expression via the Akt pathway (note it is commonly triggered by CCN proteins). However, no correlation based on staining was seen with grade or tumor stage. Similarly, Wagener et al. (2013) showed a similar migratory effect of CCN3 on Jeg3, a choriocarcinoma cell line. On the other hand, Yao et al. (2015) reported that CCN3 inhibits proliferation of osteosarcoma, here using ADNOV infection to turn on and ADsiNOV to downregulate CCN3. In this situation, the p38 and JNK activation of MAPKs were triggered leading to apoptosis. CCN3 did promote migration of the osteosarcoma cells [wound assay]. Whether this suggests differences in regulatory mechanisms in carcinomas versus sarcomas, and furthermore on specific cell behaviors, still has to be investigated. The CCN3 link with prognosis in osteosarcoma and Ewing's tumors in patients has been previously reported (Perbal et al. 2008; Manara et al. 2002). In Ewing's sarcoma, CCN3 plays a dual role, inhibiting proliferation while promoting migration and invasion of the same cells (Benini et al. 2005). In comparison, melanomas overexpressing CCN3 show a higher metastatic potential studied as xenografts (Vallacchi et al. 2008), while overexpression of CCN3 in human prostate cell lines (Maillard et al. 2001) and high expression in high grade renal cell carcinomas (Glukhova et al. 2001) favor an active role in tumorigenesis and progression.

Again, and from another type of cancer perspective, studies from the Irvine lab (McCallum et al. 2006, 2009; Suresh et al. 2013) investigating the role of CCN3 in myelopoiesis and leukemia, provided evidence that exogenous CCN3 decreases NOTCH1 signaling and BCR-ABL causes downregulation of CCN3 allowing the NOTCH1 drive to continue suggesting a form of combinatorial therapy including CCN3. Whether future studies will identify similar therapeutic permutations involving CCN proteins have yet to be realized. At least for CCN3, tumor type, stage in development, and malignant context are key factors to consider.

CCN4 (Wisp-1)

The next series of CCN proteins CCN4, CCN5 and CCN6 were discovered subsequently to CCN1–3 and present similarities and differences with respect to roles in cancer. In fact, more recent studies, as will be discussed, highlight significant contributions to the cancer phenotype and new insights into how novel therapeutic approaches may be envisioned. WISP-1, a Wnt-1 induced secreted protein, was identified and found

to be structurally related to CCN proteins (Pennica et al. 1998). It is a β-catenin responsive oncogene (as are other CCN genes- a common feature), and was renamed CCN4, in order. Klinke (2014) investigated the immunological side of breast cancer progression and immunsurveillance. Gene signatures of type1 cell-mediated cytotoxic immunity were considered and IL-12 focused on as an important cytokine. Both in silico hierarchical clustering analysis and in vitro determinations using reverse phase protein array, tissue microarrays, and flow cytometric profiling of breast cancer cell lines provided evidence of CCN4 as a paracrine inhibitor of the type 1cell-mediated immunity via IL-12 signaling and promoting type-2 immunity. Essentially the high expression of CCN4 in breast cancer associates with a distinct immune signature. Chiang et al. (2015) further showed that CCN4 is higher in breast cancer than in normal breast, and that ectopic expression of CCN4 in MCF-7 significantly increased growth in vitro and in xenografts, and induced EMT in the typical manner with coordinate upregulation of β-catenin. Forced expression blocked expression of a tumor suppressor, NDRG1, identifying CCN4 as an oncogene in the process. CCN4 appears to be able to promote angiogenesis in oral squamous carcinoma via upregulating VEGF-A (Chuang et al. 2015) and VEGF-C dependent lymphangiogenesis by inhibiting miR-300 (3′-UTR binding repressor of VEGF-C) in these cells (Lin et al. 2016). CCN4 is re-expressed in 67 % of esophageal squamous cell carcinoma patients and can predict patient prognosis after surgery, after radiotherapy and radioresistance (Li et al. 2014; Zhang et al. 2015a). In a similar vein, osteoblast-derived CCN4 increases VCAM-1 expression and enhances prostate cancer metastasis (in the context of bone metastasis) by downregulating a suppressor, miR-126 (Tai et al. 2014). Ono et al. (2013) had reported on using anti-CCN antibodies to interfere with bone metastasis in a mouse prostate cancer model (TRAMP model). They revealed CCN 4 expression at the bone-tumor interface and in the stroma suggesting an earlier role. This CCN mediated upregulation of adhesion molecules and the EMT process, intimately associated with the migratory and invasive phenotype in metastasizing cancer, appears to be a recurring theme and altogether conceivable given the motif structures of the CCN proteins. Obviously, CCN protein expression responds to the dynamics of the tumor cell microenvironment as one might predict. So how else can CCN proteins function in a predictive capacity? Chen et al. (2014b, 2015) analyzed CCN4 polymorphisms (SNPs) with respect to platinum-based chemotherapy toxicity in lung cancer patients. 28 SNPs were identified, however not statistically significantly related to increased risk of overall severe toxicity. Of these, 5 SNPs were related in subgroup analysis differentiating at the 55 yr. old mark and predictive of gastrointestinal toxicity. Finally, it is worth noting that CCN4 has been reported to be an important survival factor for human mesenchymal stromal cells (Schlegelmilch et al. 2014). Using shRNA to knock down CCN4 and DNA microarray analysis they showed that CCN deficiency caused TRAIL-induced apoptosis of primary hMSCs. In context of cancer this might suggest that CCN4 helps to maintain the tumor-stromal niche where conceivably, as a secreted protein, it is a factor supporting the stromal cells associated with tumor cells.

CCN5 (Wisp-2)

The CCN5 protein is structurally unique in that it is missing the C-terminal domain shown to interact with a large number of matrix molecules, integrins and receptors, such as Notch 1 and LRP6 (reviewed in Russo and Castellot 2010). As mentioned above it has now been found to interact with the EGFR. Like CCN2, CCN5 is expressed broadly in the different tissues and organs of the body. What highlights this CCN protein is the evidence that it can actually interfere with the activities of other CCN isoforms, and thus a competitive type of dominant negative CCN protein. Banerjee and Banerjee (2012) termed CCN5 as a micromanager of breast cancer progression capable of preventing the EMT process in breast and pancreatic cancer cells. However, in cancers undergoing malignant progression loss of CCN5 activity may permit cancer progression. In effect, here CCN5 along with CCN3 as another example, functions as the brakes for the CCN family where the other CCN proteins may act as drivers. In fact, a similar relationship had been gleaned from previous studies on other CCN proteins, like CCN3 (note CCN6 later on), which showed growth inhibitory properties ex vivo and anti tumor effects in vivo in glioblastoma (Gupta et al. 2001), choriocarcinomas (Gellhaus et al. 2004) and Ewing's sarcoma (Benini et al. 2005), with interaction by other CCN members. The scenario is very much like other signaling intermediates, e.g. kinases versus dephosphorylation enzymes, MMPs versus TIMPS, etc. Ji et al. (2014) recently reviewed the role of CCN5 in a variety of human cancers and the nuances regarding its activity in proliferation, motility, invasiveness and EMT. Because CCN5, as other CCN proteins, may bind to multiple receptors, here being the IGFBP module, it may be induced by growth factors and still contribute positively to one behavioral trait, e.g. proliferation, but then interfere with subsequent behaviors, e.g. EMT of tumor cells. Thus the picture is becomes one of a context dependent and spatiotemporal modulated mechanism with interplay of combinatorial partners. It is therefore worth considering some more recent evidence as well. In terms of fibrosis, CCN2 and CCN5 have opposing effects on fibroblast proliferation and transdifferentiation induced by TGFβ (Xu et al. 2015). Ferrand et al. (2014) knocked down CCN5 by shRNA transfection of breast cancer cell lines and showed that this led to emergence of a more stem cell like character with increased tumorsphere formation and tumor formation in immunodeficient mice. Gene expression

analysis confirmed the increase in stemness, connection with TGFβ inducible SMAD3 transcription, and promotion of EMT, essentially unblocking the tumorigenic drive. Interestingly glucocorticoids that induce CCN5 lead to attenuation of invasiveness of oestrogen receptor- negative MDA-MB 231 breast cancer cells. Glucocorticoids only downregulated the estrogen receptor, and not CCN5, in the receptor positive cells thereby attenuating the signaling. This implicates the steroid nuclear receptors in CCN regulation and raise the question about the role of CCNs when localized into the nucleus, a long time observation (Perbal 1999, 2006), yet to be fully explored.

Moreover, it appears that CCN5 activates the CTL-induced killing but loss of CCN5 function promotes evasion of immunosurveillance and tumor progression in breast cancer (Akalay et al. 2015). Again here the increase in stemness shown in vitro with knockdown of CCN5 and correlation of stemness marker KLF4 with EMT in tumor specimens highlights the potential of CCN5 as a therapeutic modality. Loss of CCN5 was correlated with the metastatic, invasive phenotype of gall bladder cancer and resulting poor prognosis (Yang et al. 2014). CCN5 knockout in Caco-2 colorectal cancer cells increased invasiveness and motility and upregulation of MMPs that facilitate tumor invasion (Frewer et al. 2013). Finally, Ji et al. (2015) studied expression of CCN5 in a large cohort of 324 cases of gastric cancer and found that increased expression of CCN5 correlated with favorable clinical features and disease-free survival. When CCN5 was knocked down in gastric cancer cell lines, proliferation, migration and invasion were promoted. Overexpression of CCN5 suppressed metastasis with reversal of EMT, loss of MMPs, via JNK and ERK signaling. Gastric cancer motility was shown to be attenuated by PLC-γ and JNK inhibitors. Thus, although, one might view CCN5 as a tumor suppressor, it could also be viewed in terms of epigenetics, where epigenetic modulation of CCN proteins contributes to the ultimate interplay of CCN proteins.

CCN6 (Wisp-3)

CCN6 (WISP-3) has grabbed the limelight in the last few years stemming from a significant body of work from the Kleer lab working on breast cancer (see C. Kleer article, this issue). Lorenzatti et al. (2011) had essentially showed that exogenous CCN6 blunted the IGF1 signaling required to upregulate EMT and invasiveness of MDA-MB-231, suggesting CCN6 as a therapeutic target. Pal et al. (2012a) followed up by identifying the signaling pathway partners BMP4-TAK1 using p38 kinases to regulate acinar morphogenesis and invasion of breast cancer cells. In clinical specimens CCN6 expression was inversely associated with BMP4 and phospho-p38 kinase levels in 69 % of invasive breast carcinomas using a 71-sample microarray. This data supported a tumor suppressor role for CCN6 in breast cancer in terms of limiting invasion and metastasis. Pal et al. (2012b) had already shown that CCN6 knockdown disrupts acinar organization in 3D culture of normal mammary epithelium by upregulation of the typeIII TGF-β receptor, a co-receptor for TGF-β family members, suggesting that CCN6 may be required for normalizing differentiation through proper formation of junctions and extracellular matrix. In further recent studies, Huang et al. (2016a, b) showed that ectopic overexpression of CCN6, in MDA-MB-231 cells and cells derived from patient tumors, induced EMT sand reduced the tumor initiating cell fraction and activity. Interestingly, these effects were mediated through a CCN6/SLUG signaling axis through Notch1 and required the TSP1(thrombospondin 1) motif of CCN6. CCN6 protein levels were inversely correlated with the transcriptionally active fragment of Notch1 (NIC1) in ~70 % of invasive breast carcinomas. This links the BMP4/p38 observation with the Notch1 pathway. However, how CCN6 is turned down has yet to be determined, but does beg the question about the interaction with other CCN isoforms. In addition one might ask how CCN proteins inter-modulate with metabolic programming in tumor cells. Gonzalez-Guerrico et al. (2016), using the normal breast cell line MCF10 (normal) and modified oncogenic variants, reported that breast cancer cells are upregulated in fatty enzyme synthase (FASN) and turning FASN signaling off reverses proliferation, EMT, and the angiogenic switch. In fact, correction of exacerbated lipogenesis appeared to be able to reprogram cancer cells back to a normal tissue architecture, representing a type of differentiation therapy that again suggests epigenetic modulation of an aggressive malignant phenotype. Along this line of thinking, it is interesting to note that CCN6 localizes to mitochondria and when depleted there is increased ROS and oxidative phosphorylation. In addition, the anti-oxidant detoxification pathway mediated by Nrf2 represses CCN6 (Patraa et al. 2016), and many advanced cancers overexpress Nrf2, so is this one way in which CCN6 expression could be suppressed?

From a different perspective, CCN6 is associated with malignant progression in other cancer types. In gastric cancer (Fang et al. 2014), in bladder cancer (Zeng et al. 2015), is associated with a more invasive and metastatic phenotype. In contrast, with respect to the homeostatic balance of different CCN proteins, in hepatocellular carcinoma (Zhang et al. 2015a, 2015b), where CCN3 is overexpressed while CCN1, CCN4 and CCN2 are reduced as compared to normal controls. This study data suggested that CCN4 and CCN1 might counter the progression enhanced by CCN3. However, as this is a RT-PCR base study one cannot draw solid conclusions since knowledge of the CCN protein expression and levels is important. Finally, information is now being gathered on mutations in CCN genes and relevance to disease causation. Bhavani et al. (2015) performed a molecular analysis on 114 families with progressive pseudorheumatoid dysplasia. The current paper analyzed 54 families and identified 16 causative

mutations in CCN6. 49/54 had homozygous and 5/54 had heterozygous mutations. Eight mutations were considered pathogenic and included 4 splice mutations, 2 deletions, 1 nonsense mutation and 1 insertion. Note that the hallmark pathological feature is deformation of the skeleton, generally upper torso. It would be interesting to know whether mutations in CCN genes associate with specific cancers.

Summary and perspective

In this review studies are presented on the CCN family of proteins in cancers with emphasis mainly on the most recent publications given that substantial progress is being made for the individual CCN proteins. It is evident that the role of a particular CCN protein as potentiating tumor progression or inhibiting progression is related to the tumor type. In some instances evidence for competition between CCN proteins is starting to be revealed and in a higher order evidence for contributions from several of the different CCN proteins. On the other hand, co-operation amongst CCN proteins is seen for normal tissue development (Hara et al. 2016) and in theory may happen in cancer as well. A common emerging theme gleaned here is the effect of CCN proteins on the EMT process, upregulation in expression of MMPs, and the resulting invasion and metastasis of cancer cells. Note that in a recent comprehensive review on EMT, the CCN proteins have yet to be factored in (Nieto et al. 2016). Given that CCN proteins can bind to and interact with a growing number of recognized receptors (Fig. 1), which then activate different signaling pathways, the complexity of the system is appreciable. Oppositely, it would appear that CCN proteins or their degradation products can localize to intracellular compartments where they can elicit other functions, e.g. metabolic perturbation and possibly transcription (Fig. 2).

Thus far only a select number of cancers have been studied and mainly adult cancers of central concern like breast, prostate and pancreas. Little attention has been being paid to pediatric cancers (Leal et al. 2011). Molecular genetic investigations are also few in number and the high- powered molecular profiling methodologies now available have yet to be applied in a concerted manner.

Also it is worth noting few studies have yet explored the epigenetic regulation of CCN proteins aside from a number of miRNA studies. Wu et al. (2014) performed meta-analysis of microarray data and identified CCN3 as a top androgen-repressed gene. Methylation analysis around the CCN3 promoter established epigenetic regulation. In inhibition of the androgen receptor and EZH2 restored CCN3 expression and androgen deprivation in mice and prostate cancer patients. CCN3 inhibits prostate cancer cell growth in vitro and in vivo. Kikuchi et al. (2007) had shown that CCN2 expression was reduced in ovarian cancer cell lines but was restored after treatment with 5-aza-2′-deoxycytidine, affecting DNA methylation. Along with the other studies discussed above and Chen et al. (2014c) (exosomal delivery of miRNAs by stellate cells in liver) demonstrating miRNA regulation of CCN expression, the emerging evidence supports the epigenetic modulation of cancer growth by CCN proteins. From a therapeutic point of view, and considering the biological activities of the different motifs, it would be reasonable to envision that peptides could be used as therapeutics although these might still be a lot more complicated to administer than epigenetic modulators. Thus the emerging idea is that one may capitalize on the different motifs of CCN proteins to produce the desired therapeutic effect and outcome whether stimulatory or inhibitory.

Taking this thinking further, it may be feasible to exploit the concerted and sequential action of action of CCN proteins which do not play redundant functions. This is based on the evidence supporting 1) combinatorial events could be provided by their unique tetramodular structure, 2) CCN proteins can interact with themselves and with other regulatory proteins (homotypic and heterotypic interactions) sharing homologous modules (such as the CT domain which can build dimers with, for example, HCG, TGFβ, PDGF, and NGF).

If we were to summarize the current knowledge on CCN proteins the following could be said. 1) All the CCN proteins have the ability to bind at their four sites (except for CCN5) a series of common ligands. Does this strengthen resulting signals or cause some sort of steric interference to modulate the outcome? 2) The CCN proteins and their potential ligands are differentially expressed in a temporal manner and in the various cellular and also tissue compartments. Hence different combinations are subject to a spatiotemporal regulation that can account for the wide array of biological functions in which CCN proteins participate. 3) From the more recent studies it is becoming obvious that spatiotemporal regulation and expression open up opportunities to modulate their biological effects in a tissue specific and cancer developmental way. This has been viewed as a centralized communication network (Perbal 2013).

The modular structure of the CCN proteins offers a wide array of combinations (see Fig. 1) that may help in the understanding of their many functions and how they are involved in coordinating signaling. In fact, at the fine biochemical and molecular levels CCN proteins

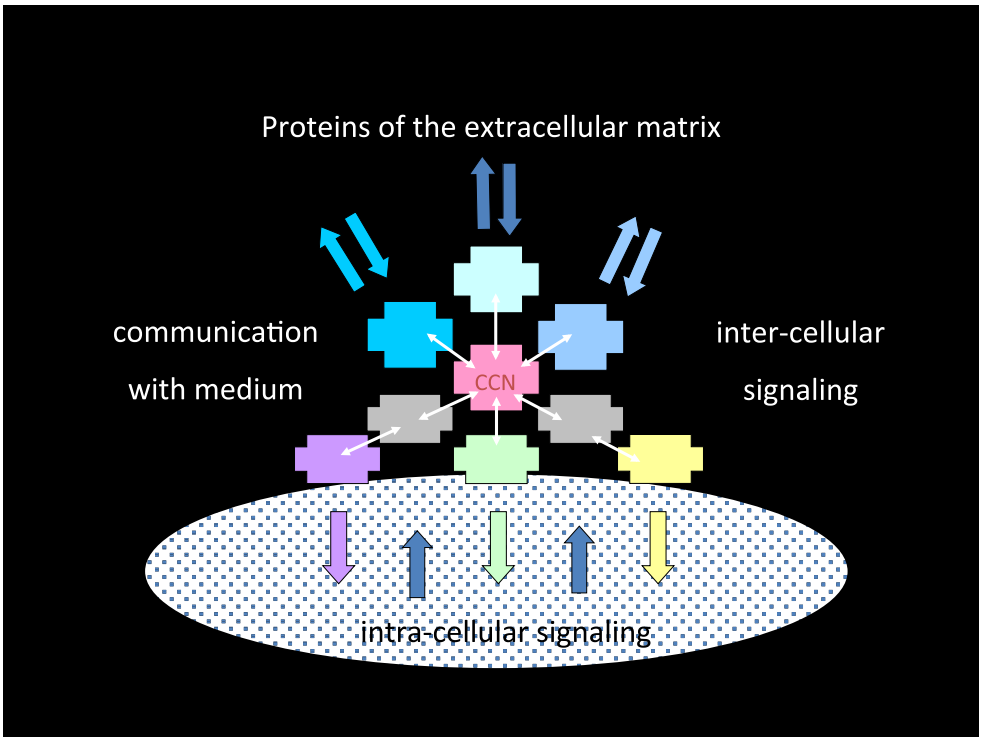

Fig. 2 Proteins of the extracelluar matrix

like CCN5 and CCN3 may be capable of physically restricting some interactions to interfere with potential formation of homodimers and heterodimers that govern net CCN protein functioning. One could draw similarities with other regulatory pathways where checkpoints serve as molecular switches.

What then else could be entertained as approaches to help with understanding the role of CCN proteins in cancer? The ability to systematically explore combinations of microenvironmental stimuli that includes CCN 1–6 proteins on tumor cell behavior could be considered with an approach reported by Malta et al. (2016). They constructed an array with 741 distinct combinations of 38 different ECM components to study embryonic stem cells and the derived endoderm progenitors. The study revealed modulatory effects on endoderm differentiation, including effects on lineage choice, cell adhesion and survival during the differentiation process. In terms of molecular approaches, following the wave of CRISPR technology that is washing over just about every area of scientific investigation, CRISPR mediated approaches will likely want to be incorporated by CCN investigators to finely manipulate CCNs in vitro and in vivo. It is obvious, with compelling evidence, that the family of CCN family of proteins have an impact on the process of cancer as matricellular proteins that not only bridge the highway between tumor cells and the surrounding normal cells but also direct the macromolecular traffic. It behooves more CCN investigators to discover new vistas using such technology.

There is an undisputed relationship between cancer and aging. At the recent 8th ICCNS meeting, Dr. Judith Campisi, the 2016 ICCNS Springer Awardee, presented work on this topic (Perbal et al. 2016). Although senescent cells, increasingly found in aging tissue, might be thought of a default route for removal of cancer cells, these cells in fact, are potent producers of proinflammatory cytokines, growth factors, and chemokines- the signature of the senescence-associated secretory phenotype (SASP) (Lecot et al. 2016). Adding fuel to the fire, SASP cells can indeed fuel cancer progression. Considering that the factors just mentioned would operate at the interface between cells in the matricellular compartment, we have little knowledge of how CCN proteins participate in the SASP response. This certainly constitutes an area open for intensive investigation and of relevance both to cancer and aging.

Finally, the ICCNS workshops have in general taken place in a sunny clime. It is therefore both intriguing and relevant that Vitamin D may play an important role in the regulation of CCN genes with implications for general health and in pathological conditions such as fibrosis and cancer (reviewed in Piszczatowski and Lents 2016). We predict that continuing research on the CCN family of proteins will shed a bright light on their biological roles.

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