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REVIEW

Dual roles of CCN proteins in breast cancer progression

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Abstract The tumor microenvironment has a powerful effect on the development and progression of human breast cancer, which may be used therapeutically. Despite efforts to understand the complex role of the tumor microenvironment in breast cancer development, the specific players and their contributions to tumorigenesis need further investigation. The CCN family of matricellular proteins comprises six members (CCN1–6; CYR61, CTGF, NOV, WISP1–3) with central roles in development, inflammation, and tissue repair. CCN proteins also exert functions during pathological processes including fibrosis and cancer by regulating extracellular signals in the cellular environment. Studies have demonstrated that all six CCN proteins exert functions in breast tumorigenesis. Although CCN proteins share a multimodular structure in which most cysteine residues are conserved within structural motifs, they may have opposing functions in breast cancer progression. A better understanding of the functions of each CCN member will assist in the development of specific therapeutic approaches for breast cancer.

Keywords Breast cancer · Stem cells · Differentiation · CCN · CYR61 · CTGF · NOV · WISP1 · WISP2 · WISP3 · Matricellular · Metastasis · CCN6

Introduction

Invasive breast carcinomas are a heterogeneous group of malignant epithelial tumors that arise in the breast parenchyma, invade adjacent tissue and exhibit metastatic ability. Invasive breast carcinomas have various histological morphologies under the light microscope and may exhibit different clinical behaviors and treatment responses. Pathologists recognize various morphological subtypes of invasive carcinoma. Most invasive tumors in the breast are invasive ductal carcinomas, accounting for approximately 80 % of invasive breast cancer. Invasive lobular carcinomas account for 10–15 % of all breast cancers, and the remainder constitute special histological types including mucinous, tubular, micropapillary, and others. The morphologic features, degree of differentiation and mitotic activity are robust surrogates for biological behavior. Hormonal receptor status (estrogen and progesterone receptor, ER and PR), and human epidermal growth factor receptor 2 (HER-2/neu) status have prognostic value and predict treatment response (Elledge et al. 1998; Osborne 1998; Slamon and Pegram 2001). It has become increasingly evident that breast cancer heterogeneity extends beyond morphology and ER, PR, and HER-2/neu expression. More recently, invasive carcinomas have been classified based on their gene expression profiles into at least four groups including luminal A, luminal B, HER2, and triple negative subtypes. Of note, these subtypes are also heterogeneous and maybe further refined (Burstein et al. 2015).

In addition to the intrinsic characteristics of breast cancer cells, the interactions between cancer cells and the cellular and components of the microenvironment, including matricellular proteins, are important for breast cancer progression (Pein and Oskarsson 2015). Pathologists have noticed the presence of a desmoplastic stroma associated with invasive carcinomas decades ago, which results from increased deposition of extracellular matrix (ECM) proteins (Rosen 2009). It is now recognized that stromal desmoplasia alters the chemical composition and the mechanical properties of the ECM (Paszek et al. 2005; Provenzano et al. 2009). Breast cancer cells respond to

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these changes in the ECM through deregulated signaling pathways, which promote neoplastic functions and result in loss of normal architecture, invasion, and increased proliferation (Paszek et al. 2005; Provenzano et al. 2009). While CCN proteins have been studied mainly to regulate angiogenesis, development, and mesenchymal tissue homeostasis, there have been compelling reports demonstrating their roles in cancer, including breast cancer.

The CCN family: members and nomenclature

The CCN protein family includes CCN1 (Cyr61), CCN2 (CTGF) CCN3 (Nov), CCN4 (WISP1), CCN5 (WISP2), and CCN6 (WISP3). Encoded by different genes, CCN proteins share a highly conserved multimodular structure consisting of four cysteine-rich motifs. An N-terminal signal peptide is followed by the N-terminal motif, which includes the first 12 cysteine residues and contains the highly conserved IGF binding consensus sequence (GCGCCXXC) (Byun et al. 2001; Grotendorst and Duncan 2005; Imai et al. 2000; Perbal 2001; Yang and Lau 1991). This domain is followed by a von Willebrand factor-like motif (VWC), and the thrombospondin type 1 motif (TSP-1) involved in cell-cell interactions and possibly inhibition of angiogenesis. The carboxy-terminal motif (CT) is present in all CCN proteins forms a "cysteine knot", since the protein is folded into two highly twisted antiparallel pairs of beta-strands and contains three disulfide bonds. The CT module has been identified in several other signaling peptides (such as transforming growth factorβ, platelet derived growth factor, and nerve growth factor) and may participate in dimerization and receptor binding (Perbal et al. 1999). Importantly, CCN5 lacks the CT module (Zhang et al. 1998).

The multimodular organization of the CCN proteins suggests that they may interact with other proteins to exert specific functions, and perhaps that the biological functions of the CCN proteins depend on the availability of interacting proteins and substrates. This may explain the cell and context-specific biological effects of the CCN family members, and their wide range or physiological and pathological functions (Leask and Abraham 2006; Perbal 2001). They are mediators of epithelial-stromal cross-talk, and have been reported to interact with key signaling molecules such as cell surface integrins, and Notch1 (Leask and Abraham 2006). Although CCN proteins are best known for their roles in the extracellular matrix, a number of studies show that they may function intracellularly and that certain CCN proteins may have nuclear functions (Planque et al. 2006). Available data suggest that CCN proteins may exert tumor promoting and/or tumor suppressing functions in breast cancer. In this review, I have attempted to segregate CCN proteins into those with primarily pro-tumorigenic functions and those with primarily tumor inhibitory functions, based on the most compelling available published literature to date, summarized in Fig. 1.

CCN proteins as breast cancer promoters

The CCN proteins with oncogenic functions in breast cancer include mainly CCN1 and CCN2. Early work by Lupu and colleagues identified CCN1 as a mediator of Heregulin signaling in breast cancer cells (Tsai et al. 2000). Indeed, CCN1 was associated with Heregulin-induced breast cancer migration and metastasis, as well as increased tumor angiogenesis likely through interactions with the $\alpha_v\beta_3$ integrin receptor (Espinoza et al. 2014; Tsai et al. 2000).

Initial immunohistochemical studies showed that CCN1 protein was expressed in approximately 30 % of breast carcinomas, especially estrogen receptor (ER) positive-HER-2/neu negative tumors, compared to normal breast tissues (Tsai et al.

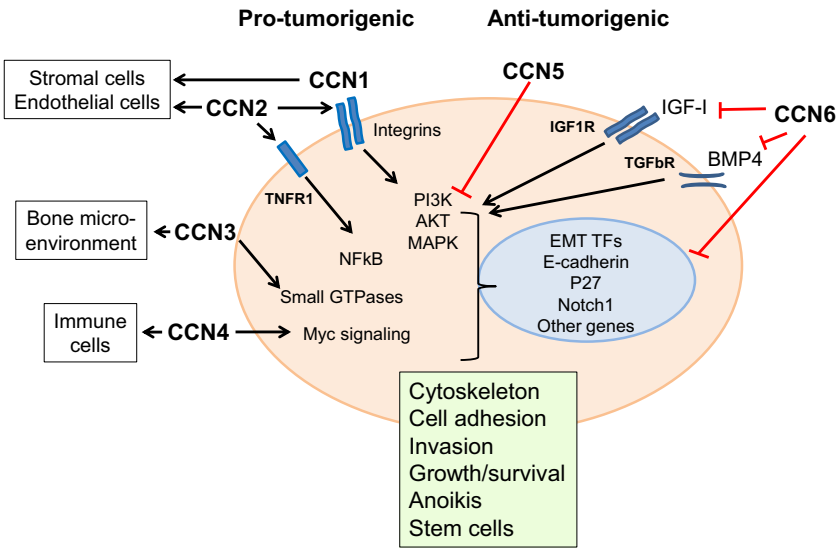

Fig. 1 Functions of CCN proteins in breast cancer. Based on published literature CCN1/Cyr61, CCN2/CTGF, CCN3/Nov, and CCN4/WISP1 have tumor promoting effects, while CCN5/WISP2 and CCN6/WISP3 exert tumor suppressor functions. Further work is necessary to identify binding partners, other receptors, and signaling pathways regulated by CCNs in breast cancer cells

2000). Importantly, high expression of CCN1 was associated with the presence of lymph node metastasis and worse prognosis in breast cancer patients (Jiang et al. 2004). CCN1 overexpression in ER positive MCF7 breast cancer cells led to increased tumor size and vascularization in human breast cancer xenografts in nude mice (Tsai et al. 2002). In vitro, CCN1 mRNA and protein were induced by estradiol in MCF7 cells, and CCN1 was sufficient to induce estradiol-independence and antiestrogen resistance (Tsai et al. 2002).

Further supporting the tumor promoting functions of CCN1, Sanchez-Bailon et al. found that CCN1 suppression in triple negative breast cancer cells reduced invasion and transendothelial migration compared to controls (Sanchez-Bailon et al. 2015). A neutralizing anti-CCN1 antibody inhibited breast cancer cell migration. Earlier this year, Huber and coworkers identified CCN1 as a novel urokinase-type plasminogen activator receptor (uPAR) interacting protein in triple negative breast cancer cells (Huber et al. 2016). Detection of interacting CCN1-uPAR proteins was associated with expression of tumor biomarkers, as well as increased tumor grade. CCN1 was also found to enhance breast tumor vascularization and to promote spontaneous metastasis downstream of Hedgehog (Hh) signaling (Harris et al. 2012). Breast cancer-derived CCN1 was also reported to regulate fibroblast production of MMP1 to increase breast cancer cell migration and invasion (Nguyen et al. 2006). These representative studies provide strong evidence for a tumor promoting effect of CCN1 in breast cancer and show promise for the use of CCN1 and its downstream signaling proteins as biomarkers of cancer progression and therapeutic targets.

Similar to CCN1, high levels of CCN2 mRNA expression were detected in 55 % of human invasive carcinomas and associated with features of aggressive breast cancer (Xie et al. 2001). CCN2 overexpression in breast cancer cells increased resistance toward doxorubicin and paclitaxel (Wang et al. 2009). Koeffler and colleagues (Chien et al. 2011) dissected the role of CCN2 in breast tumorigenic properties by transfecting MCF-7 cells with full-length CCN2, and with four mutant constructs in which one of the motifs had been deleted. Forced expression of CCN2 in breast cancer cells was associated with increased migration and angiogenesis. The migratory activity was dependent on the CT motif of CCN2. Furthermore, they showed that recombinant CT-CCN2 recapitulates the activity of the full-length protein. Interestingly, the CT motif contains a binding site for integrin $\alpha_v\beta_3$ (Gao and Brigstock 2004). These data demonstrate that the CT motif behaves as a critical regulator of CCN2 function, and suggest a therapeutic possibility.

The role of CCN3 in breast tumorigenesis needs further investigation. The expression of CCN3 and its relationship to outcome of breast cancer patients remains unclear. A study found that CCN3 overexpression was associated with better patient prognosis (Jiang et al. 2004). However, CCN3 overexpression was also reported to predict resistance to endocrine therapy (Ghayad et al. 2009). It is interesting to note that CCN3 may play a role in breast cancer cell morphology, migration, and metastasis. Sin et al. reported that CCN3 reorganizes the actin cytoskeleton of the breast cancer cells MDA-MB-231 with the formation of multiple cell protrusions and increases migration, possibly by activating the small GTPase Rac1 (Sin et al. 2009). Ouellet et al. reported that CCN3 is expressed in human samples of metastatic breast cancer to the bone, and using the 4 T1 mouse breast cancer model, they showed that CCN3 enhances breast cancer bone metastasis (Ouellet et al. 2011). This important effect of CCN3 in metastasis may depend on the ability of CCN3 to impair osteoblast differentiation and promoting osteoclastogenesis, providing a favorable environment for osteolytic breast cancer bone metastasis (Ouellet et al. 2011).

Limited available literature on CCN4 in breast cancer suggests that this protein exerts pro-tumorigenic functions. CCN4 mRNA expression was elevated in breast cancer samples compared to normal breast. High and low CCN4 mRNA levels have been associated with poor outcome in breast cancer patients (Davies et al. 2007; Xie et al. 2001). A recent study shed light into the function of CCN4 in breast tumorigenesis by demonstrating that recombinant CCN4 protein promoted the growth of MCF7 and MDA-MB-231 cells, induced EMT, and enhanced migration and invasion. In xenograft studies, CCN4 overexpression increased tumor formation of MCF7 cells (Chiang et al. 2015). Further studies are needed to understand how CCN4 regulates breast tumorigenesis.

CCN proteins as breast cancer suppressors

While CCN1–4 exert primarily tumor promoting functions in breast cancer, CCN5 and CCN6 have been shown to reduce breast turmorigenesis. It is important to recognize that CCN5 is the only CCN protein that contains only three structural motifs and lacks the CT module. Thus, the different protein structure suggests that CCN5 may function differently than other CCN family members. Early studies showed that CCN5 mRNA expression exhibited a biphasic pattern, being expressed in non-invasive breast lesions and reduced during the transition between ductal carcinoma in situ (non-invasive) and invasive carcinoma, suggesting that CCN5 may indeed suppress invasion (Banerjee et al. 2008). In invasive carcinomas, CCN5 levels were associated with the degree of tumor differentiation, which is a surrogate marker for prognosis. Thus, low CCN5 levels were detected in poorly differentiated breast carcinomas compared to well-differentiated tumors (Banerjee et al. 2008). Functionally, overexpression of CCN5 reduced breast cancer cell growth, migration, and

invasion (Dhar et al. 2008). The same group recently reported that CCN5 overexpression resulted in growth arrest of triple negative breast cancer by enhancing p27Kip1 expression and nuclear translocation (Haque et al. 2015).

An important aspect of CCN5 tumor suppressor function may be its role in cell differentiation. It has been reported that CCN5 knockdown in MCF7 cells induced an epithelial to mesenchymal transition and estradiol-independent growth. In contrast, CCN5 overexpression in MCF7 cells promoted epithelial differentiation. CCN5 overexpression in the poorly differentiated MDA-MB-231 cells led to reduced proliferation and invasion (Fritah et al. 2008). This group most recently extended their observation on the tumor suppressor role of CCN5 by demonstrating that CCN5 downregulation in MCF7 breast cancer cells not only induced an epithelial to mesenchymal transition but also increased the number of stem cells (Ferrand et al. 2014). Taken together these investigations support a tumor suppressor function for CCN5.

The matricellular protein CCN6 was originally identified in 1999 as a gene lost in 80 % of the most lethal form of locally advanced breast cancer termed inflammatory breast cancer (IBC) compared to non-inflammatory breast cancers (van Golen et al. 1999). This was achieved using a modified version of the differential display technique and in situ hybridization analysis of human breast tissues (van Golen et al. 1999). Later studies revealed that CCN6 is also lost or reduced in nearly 50 % of non-IBC tumors and is significantly associated with worse survival (Huang et al. 2008; Pal et al. 2012a). Studies in my laboratory spanning over a decade have shown that CCN6 exerts inhibitory functions in breast cancer growth, invasion, and metastasis (Huang et al. 2008; Huang et al. 2016; Kleer et al. 2002; Pal et al. 2012a; Pal et al. 2012b). The main mechanisms mediating CCN6 tumor inhibitory functions are: 1. by regulating the process of epithelial to mesenchymal transition (Huang et al. 2008; Pal et al. 2012a), 2. by interacting and modulating the tumorigenic effects of growth factor signaling proteins in breast cancer (e.g. insulin-like growth factor I (IGF-1), bone morphogenetic protein 4 (BMP4) and transforming growth factor β (TGFβ)) (Pal et al. 2012a; Pal et al. 2012b; Zhang et al. 2005), and 3. by regulating the number of tumor initiating/stem cells (Huang et al. 2016).

Both IGF-1 signaling and BMP4/TGFβ signaling pathways are key promoters of breast cancer progression (Barcellos-Hoff and Akhurst 2009; Pollak 2004; Surmacz 2000). Our laboratory reported that CCN6 protein is secreted from breast epithelial cells and that CCN6 is able to decrease the IGF-1-induced activation of the IGF receptor (IGF-1R) and two of its main downstream signaling proteins, IRS-1 and ERK-1/2, in SUM149 IBC cells (Kleer et al. 2004). Importantly, CCN6 protein in the conditioned media slowed the growth of SUM149 cells. It was also shown that inhibition of CCN6 in HME cells results in the loss of a growth regulatory function that protects HME cells from the tumorigenic effects of growth factors, particularly IGF-1 (Zhang et al. 2005).The recent connection between CCN6 and BMP4 signaling is intriguing. Pal et al. reported that CCN6 suppresses breast cancer metastasis by binding to BMP4 and blocking BMP4-mediated activation of TAK1 kinase and p38 MAPK in breast cancer (Pal et al. 2012a). Detailed studies dissecting the functional domains of CCN6 showed that the TSP-1 domain is required for binding to BMP4 and for the invasion inhibitory effect of CCN6 in breast cancer cells (Pal et al. 2012a).

It is interesting to note that CCN5 and CCN6 are important in epithelial cell differentiation and induce a mesenchymal to epithelial transition to reduce breast cancer migration and invasion. Similarly, CCN5 and CCN6 reduce the number of breast cancer initiating/stem cells in vivo and in vitro. Huang et al. showed that the effect of CCN6 on epithelial differentiation and stem cells is exerted through a novel CCN6/Slug/Notch1 and requires an intact TSP-1 domain of CCN6 (Huang et al. 2016). These data suggest that modulation of CCN6 levels may be a potential therapeutic strategy to halt breast cancer progression.

Conclusion and perspectives

As initially defined, matricellular proteins are dynamically expressed secreted proteins found in the extracellular matrix that exert regulatory rather than structural functions, and are deregulated in physiological and pathological processes (Bornstein 1995; Bornstein and Sage 2002). CCN proteins have been mainly studied in development, angiogenesis, and fibrosis, but there is accumulating compelling evidence that they exert important roles in tumorigenesis, including breast cancer. The structural motifs of CCN proteins, which are homologous to ECM-associated proteins, may explain the numerous reported binding partners and multiple signaling pathways modulated by CCNs. In addition, the interactions with other extracellular proteins and growth factors may dictate the pro-and/or anti-tumorigenic functions of CCNs. A specific CCN receptor has not been identified to date. Rather, CCN proteins bind to different multi-ligand receptors, especially to integrins (Lau 2016). Despite remaining gaps in knowledge, published data show that CCNs play important roles in breast cancer progression, that they are promising prognosticators and, as secreted proteins, may have therapeutic potential.

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