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The emerging role of CCN6 in breast cancer invasion

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Abstract

The CCN family of matricellular proteins is essential for cell communication and mediation of epithelial stromal cross-talks with roles in development and cancer. In particular, loss of CCN6 messenger RNA expression has been recognized in highly aggressive breast cancers, especially in inflammatory breast cancer and breast cancers with axillary lymph node metastasis. Recent findings can better explain the relevance of CCN6's reduced expression on human invasive breast carcinomas. CCN6 has been shown to play a role in the process of epithelial to mesenchymal transition (EMT), which converts epithelial cells into migratory mesenchymal-like cells with invasive abilities. Although the mechanism by which CCN6 promotes EMT and invasion has not been fully elucidated, current data suggest that it involves the recruitment of the transcriptional regulators Snai1 and ZEB1 to the E-cadherin promoter.

Introduction

Living cells communicate with the surrounding microenvironment and exchange information through complex signaling pathways in order to carry out most homeostatic biological processes. Matricellular proteins, which include the CCN family of proteins, have the ability to coordinate the extracellular and intracellular signaling pathways and thus modulate the cross-talk between the microenvironment, epithelial and mesenchymal cells. Our laboratory has been devoted to understanding the functions of CCN6 in breast tumorigenesis. Our group has reported that CCN6 has growth and invasion inhibitory functions in invasive breast carcinoma (Huang *et al.*, 2008; Kleer *et al.*, 2004; Kleer *et al.*, 2002; Kleer, 2004; Zhang, 2005). Of special note is that the CCN6 gene is located at chromosome 6q21–22, and loss of one copy of the 6q arm has been shown in 23 to 80% of human breast cancers, making it one of the most frequent sites for allelic loss in human breast cancer (Chappell *et al.*, 1997; Fujii *et al.*, 1996; Rodriguez *et al.*, 2000). CCN6 loss triggers the process of epithelial to mesenchymal transition (EMT) through up-regulation of Snai1 and ZEB-1 and their recruitment and binting to the E-cadherin promoter. These data and their relevance to human breast cancer progression will be discussed herein.

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Structure of CCN6

CCN6 has the modular architecture of the CCN family, which also includes CCN1 (Cyr61), CCN2 (CTGF), CCN3 (Nov), CCN4 (WISP1) and CCN5 (WISP2). The CCN6 gene encodes for a protein of 354 amino acids and 36.9 kDa, with 57% homology to CCN2 (CTGF). CCN proteins share a highly conserved multimodular structure consisting of cysteine-rich motifs. The N-terminal motif, which includes the first 12 cysteine residues, contains a highly conserved IGF binding consensus sequence (GCGCCXXC) which may facilitate the binding to IGF (Byun *et al.*, 2001; Grotendorst and Duncan, 2005; Imai *et al.*, 2000). This motif 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 and 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 domain has been identified in several other signaling peptides (such as TGFβ, platelet derived growth factor, and nerve growth factor) and may participate in dimerization and receptor binding (Perbal *et al.*, 1999). As most CCN proteins, CCN6 contains a cleavable signal peptide which may participate in its secretion into the extracellular matrix (Perbal, 2001; Yang and Lau, 1991). Although integrin receptors and other receptors in the plasma membrane have been shown to be receptors for other CCN proteins (especially CCN1 and CCN3) (Chen *et al.*, 2004; Leu *et al.*, 2003), a receptor for CCN6 has not been identified to date.

CCN6 is secreted and modulates insulin-like growth factor type 1 (IGF-1) signaling

Given the structure of CCN6 protein and its high homology with other CCN proteins, we hypothesized that mammary epithelial cells may secrete CCN6 into the extracellular medium where it may regulate insulin like growth factor (IGF) signaling. The IGFs play a central role in breast cancer development (Pollak, 2004). Compelling epidemiological and clinical data show that high concentrations of IGF-1 in serum are associated with increased mammographic density (one of the strongest predictors of breast cancer risk), and also reliably predict increased breast cancer risk specifically in premenopausal women (Allen *et al.*, 2005; Byrne *et al.*, 2000; Diorio *et al.*, 2005; Eliassen *et al.*, 2007; Schernhammer *et al.*, 2005). In vitro and in vivo studies have shown that IGFs promote the proliferation, survival, and metastatic ability of breast cancer cells (Diorio *et al.*, 2005; Eliassen *et al.*, 2007). The IGF-1 receptor (IGF-1R) promotes breast cancer growth (Surmacz, 2000), metastasis (Carboni *et al.*, 2005; Kim *et al.*, 2007; Sachdev *et al.*, 2004), and its hyperactivation has been linked with increased radioresistance and breast cancer recurrence (Surmacz, 2000; Turner *et al.*, 1997).

Indeed, our laboratory reported that CCN6 protein is secreted from breast epithelial cells and that once in the extracellular medium 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 inflammatory breast cancer cells cells (Kleer, 2004). CCN6 in the conditioned media slowed the growth of SUM149 cells. It was also shown that inhibition of

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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, 2005). This work uncovered a previously undescribed mechanism of CCN6 by demonstrating that it is secreted and that it induces a series of molecular events which result in the modulation of IGF-IR signaling pathways and cellular growth of breast cells. Subsequently, CCN3 (Nov) was found to also modulate the effects on IGF signaling in Ewing's sarcoma cells (Benini *et al.*, 2005). Recently, CCN1 (CTGF) was shown to promote differentiation in rat fibroblasts by enhancing the effects of IGFs (Grotendorst and Duncan, 2005).

CCN6 as an inhibitor of breast cancer invasion

Systematic analysis of CCN6 expression for intensity and frequency showed that CCN6 is expressed in normal breast epithelium but it is lost or reduced in approximately 60% of human invasive carcinomas of the breast (Huang *et al.*, 2008). CCN6 is detected mainly in the cytoplasm of epithelial cells with occasional nuclear expression. In human breast cancer, CCN6 reduction is associated with aggressive inflammatory breast carcinomas and with axillary lymph node metastasis (Huang *et al.*, 2008; van Golen *et al.*, 1999). A body of work has demonstrated a causal role for CCN6 protein down regulation in proliferation, migration and invasion in breast cells (Huang *et al.*, 2008; Zhang, 2005). Utilizing different strategies we have shown that CCN6 down regulation in human mammary epithelial cells caused an increase in anchorage independent growth, motility and invasion (Huang *et al.*, 2008; Zhang, 2005). In aggressive inflammatory breast cancer cell lines with low CCN6 expression, restoration of CCN6 led to a reduction in proliferation, migratory and invasive capacity in vivo and in vitro (Kleer *et al.*, 2004; Kleer *et al.*, 2002; Kleer, 2004).

CCN6 and the process of epithelial to mesenchymal transition

During tumor invasion differentiated epithelial cells lose their cell-cell adhesions and polarity acquiring mesenchymal and migratory properties, events that characterize the epithelial to mesenchymal transition (EMT; Christiansen *et al.*, 2006; Peinado *et al.*, 2007; Polyak and Weinberg, 2009; Thiery and Sleeman, 2006). It is recognized that the acquisition of mesenchymal cell-like features constitutes a central abnormality responsible for progression from non-invasive lesions to invasive carcinoma with the ability to metastasize (Hugo *et al.*, 2007). Although the mechanisms that lead to these changes in cell phenotype are not completely understood, it is becoming clear that one of CCN6's main functions in breast cancer progression is its role in EMT (Huang *et al.*, 2008; Kleer *et al.*, 2007; Kleer *et al.*, 2002).

EMT of breast epithelial cells manifests as the development of a stellate and slender morphology with a characteristic protein expression pattern characterized by decreased expression of epithelial markers (e.g. cytokeratin and E-cadherin), and elevated levels of mesenchymal cell markers (e.g. vimentin; Hugo *et al.*, 2007). E-cadherin repression has been shown to play a major role in EMT in epithelial-derived malignancies (Yook *et al.*, 2005; Yook *et al.*, 2006; Zhou *et al.*, 2004). E-cadherin protein regulates cell adhesion in epithelial cell in a Ca2+-dependent manner via homotypic interactions with E-cadherin molecules on opposing cell surfaces (Nelson and Nusse, 2004). E-cadherin is associated

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with the actin cytoskeleton through peripheral membrane proteins and this association strengthens the cell-cell adhesion of adherens junctions (Nelson and Nusse, 2004). In cancer, down-regulation of E-cadherin is the key step towards invasion, with dominant transcriptional repression being largely responsible for the loss of E-cadherin expression (Fearon, 2003; Nieto, 2002; Thiery, 2002; Yook *et al.*, 2005; Zhou *et al.*, 2004). In breast cancer, low expression of E-cadherin protein is associated with invasion and metastasis (Kowalski *et al.*, 2003; Oka *et al.*, 1993; Palacios *et al.*, 1995). We found decreased or absent E-cadherin expression in 45% of the primary invasive carcinomas of the breast that progressed to develop distant metastases (Kowalski *et al.*, 2003). In patients with locally advanced breast cancer, E-cadherin loss was associated with angiolymphatic invasion and the development of distant metastases (Kleer *et al.*, 2001; Kowalski *et al.*, 2003). Thus, EMT plays a central role in adoption of a mesenchymal-like phenotype, invasion and metastatic spread. Furthermore, EMT has been recently implicated in cancer stem cell-like characteristics, conferring cancer cells the ability to invade adjacent tissues and acquire resistance to chemotherapy treatment (Christiansen and Rajasekaran, 2006; Mani *et al.*, 2008; Polyak and Weinberg, 2009).

Although CCN proteins have been reported to exert a variety of biological functions, it wasn't until recently that CCN6 knockdown was discovered to trigger a phenotypic and gene expression program indicative of EMT. Experiments using stable lentiviral mediated knockdown have shown that downregulation of CCN6 results in EMT of breast epithelial cells, with up regulation of mesenchymal proteins and down regulation of epithelial proteins (Huang *et al.*, 2008; Zhang, 2005). Among these, E-cadherin was strongly suppressed as determined using different methods and cell lines (Huang *et al.*, 2008; Zhang, 2005). The effect of CCN6 on E-cadherin expression was further supported by experiments showing that re-introduction of CCN6 in MDA-MB-231 cells which lack E-cadherin expression, was able to cause an up-regulation of E-cadherin protein evidenced by Western blots and immunofluorescence. The relevance of these intriguing data to human breast cancer is highlighted by the finding that CCN6 expression is positively associated with E-cadherin expression in human breast cancer tissue samples (Huang *et al.*, 2008).

On the role of CCN6 as a regulator of ZEB1 and Snail transcription factors

In recent years several transcription factors have been shown to trigger EMT. Some of these factors are repressors of the E-cadherin gene and are involved in tumor progression. These include zinc-finger proteins of the Snail/Slug family, δEF1/ZEB1, SIP1, and the basic helix-loop-helix E12/E47 factor (reviewed in Peinado *et al.*, 2007). In particular, studies have shown that ZEB1 (δEF1) and Snail (SNAI1) play a role in cancer progression, including breast cancer (Peinado *et al.*, 2007; Weinberg, 2008). ZEB1 was found up-regulated in carcinomas of the breast (Aigner *et al.*, 2007), whereas elevated Snail expression was associated with E-cadherin repression, tumor recurrence and metastasis and poor prognosis in breast cancer (Moody *et al.*, 2005; Peinado *et al.*, 2007).

The transcriptional regulator ZEB1, by binding to the E-boxes of the E-cadherin gene proximal promoter, has been shown to down-regulate E-cadherin, and to induce EMT in breast and other cancers including colorectal and prostatic adenocarcinomas (Aigner *et al.*,

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2007; Eger *et al.*, 2005; Schmalhofer *et al.*, 2009; Spaderna *et al.*, 2008). The molecular events that lead to ZEB1 overexpression have not been fully elucidated, but recent studies implicate extracellular signaling factors including TGFβ, TNFα, IGF-1, as well as COX-2, EGF receptor (EGFR) and estrogen receptor activation (Graham *et al.*, 2008; Schmalhofer *et al.*, 2009). Interestingly, recent work has shown that the miR-200 family of micro-RNAs is associated with down-regulation of ZEB1 and maintenance of the epithelial phenotype in the breast. (Bracken *et al.*, 2008; Burk *et al.*, 2008; Korpal *et al.*, 2008; Park *et al.*, 2008; Shirakihara *et al.*, 2007).

Given this, a major focus of our research is devoted to elucidating the molecular basis of CCN6-mediated EMT in the mammary epithelium. We hypothesize that CCN6 loss may lead to EMT and decreased E-cadherin expression by regulating E-cadherin transcriptional repressors. Our laboratory recently reported that CCN6 regulates EMT and E-cadherin expression in the breast epithelium by increasing the levels of Snai1 and ZEB1 messenger RNA and proteins (Huang *et al.*, 2008). Upon CCN6 knockdown in human mammary epithelial cells, ZEB1 and Snai1 proteins are up-regulated and recruited to the proximal E-cadherin promoter (Huang *et al.*, 2008). Current data suggest that binding to both Snai1 and ZEB1 to the promoter of the E-cadherin gene is required, as inhibition of either gene is able to prevent CCN6's effect on E-cadherin expression (Huang *et al.*, 2008). Whether CCN6 regulates transcription of ZEB1 and Snai1 directly or indirectly through modulation of extracellular growth factor signaling pathways such as IGF-1 are areas of active research in our laboratory. Figure 1 illustrates our working model for CCN6 function in EMT and invasion. We propose that CCN6 regulates Snai1 and ZEB1 and controls epithelial organization by regulating the levels of E-cadherin. Reduced CCN6 expression in breast epithelial cells results in up-regulation of Snai1 and ZEB1 and decreased levels of E-cadherin which contributes to EMT and invasion. Based on our studies showing that CCN6 is an important regulator of IGF-1 signaling, we hypothesize that CCN6 regulates Snai1 and ZEB1 through IGF-1.

Future perspectives

With the identification of CCN6 as a regulator of EMT and invasion in breast cancer a new role for CCN proteins in tumorigenesis is uncovered. Studies aimed at pinpointing the molecular mechanism by which CCN6 regulates ZEB1 and Snai1 expression is an area of important forthcoming research. Restoration of CCN6 expression may not only be a marker of unfavorable outcome in women with breast cancer, but may be a means to prevent or revert the invasion process in breast cancer.

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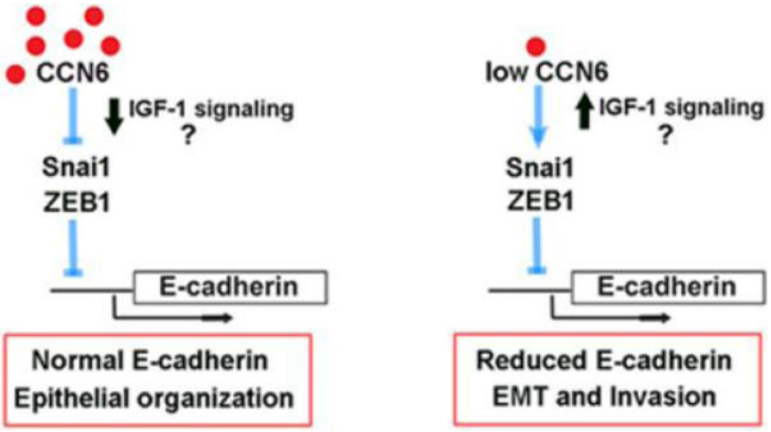

Figure 1. Working model for CCN6 function in EMT and invasion

We propose that CCN6 regulates the levels of Snai1 and ZEB1 in the breast epithelium and controls epithelial organization by regulating the levels of E-cadherin. Reduced CCN6 expression results in up-regulation of Snai1 and ZEB1 and decreased levels of E-cadherin which contributes to EMT and invasion. We hypothesize that CCN6 regulates Snai1 and ZEB1 through IGF-1.