Blockade of CCN6 (WISP3) Activates Growth Factor–Independent Survival and Resistance to Anoikis in Human Mammary Epithelial Cells

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Abstract

CCN6 is a secreted cysteine-rich matricellular protein (36.9 kDa) that exerts growth-inhibitory functions in breast cancer. Reduction or loss of CCN6 protein has been reported in invasive carcinomas of the breast with lymph node metastasis and in inflammatory breast cancer. However, the mechanism by which CCN6 loss promotes breast cancer growth remains to be defined. In the present study, we developed lentiviral-mediated short hairpin RNA CCN6 knockdown (KD) in nontumorigenic mammary epithelial cells MCF10A and HME. We discovered that CCN6 KD protects mammary epithelial cells from apoptosis and activates growth factor–independent survival. In the absence of exogenous growth factors, CCN6 KD was able to promote growth under anchorage-independent conditions and triggered resistance to detachment-induced cell death (anoikis). On serum starvation, CCN6 KD was sufficient for activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. Growth factor–independent cell survival was stunted in CCN6 KD cells when treated with either human recombinant CCN6 protein or the PI3K inhibitor LY294002. Targeted inhibition of Akt isoforms revealed that the survival advantage rendered by CCN6 KD requires specific activation of Akt-1. The relevance of our studies to human breast cancer is highlighted by the finding that low CCN6 protein levels are associated with upregulated expression of phospho-Akt-1 (Ser473) in 21% of invasive breast carcinomas. These results enable us to pinpoint one mechanism by which CCN6 controls survival of breast cells mediated by the PI3K/Akt-1 pathway. Cancer Res; 70(8); 3340–50. ©2010 AACR.

Introduction

CCN6 is a cysteine-rich protein that belongs to the CCN (Cyr61, CTGF, Nov) family of matricellular proteins, with developmental functions (1–3). Recent studies have shown that the CCN protein family members also play important roles in tumorigenesis, including cancer cell proliferation, survival, adhesion, and invasion (4–9). CCN proteins are mostly secreted and extracellular matrix associated and have been proposed to connect signaling pathways and facilitate cross talks between epithelium and stroma (1, 10).

CCN6 is a tumor suppressor gene found to be downregulated in the most aggressive form of locally advanced breast cancer, inflammatory breast cancer, as well as noninflammatory breast cancers with lymph node metastasis (5, 11). Immunohistochemical studies of human breast tissue samples have shown that whereas normal epithelium expresses CCN6 protein, CCN6 is reduced or lost in 60% of invasive carcinomas (5, 6). The high frequency of reduction or loss of CCN6 in breast cancer suggests a potential role in initiation and/or progression of human breast cancer.

Activation of phosphatidylinositol 3-kinase (PI3K) and protein kinase B (Akt) is a frequent alteration in human cancer, including carcinomas of the breast (12–15). Although PI3K/Akt activation is a common pathway for several extracellular growth factors, PI3K/Akt signaling can be activated by growth factor–independent mechanisms. A major function of the PI3K/Akt signaling pathway is the promotion of cell survival and resistance to apoptosis and anoikis (12–14, 16–19). Anoikis, or detachment-induced apoptosis, is a protective mechanism by which epithelial cells die on losing contact with the extracellular matrix. The acquisition of anoikis resistance has been shown to promote breast cancer progression and metastasis by enabling cancer cells to survive in the vascular or lymphatic channels (20, 21).

CCN6 has been proposed to participate in cell survival, and it has been studied to modulate insulin-like growth factor–mediated growth and proliferation (6, 22). However, no studies have been carried out to investigate the growth factor–independent functions of CCN6 and the link between CCN6 and survival signaling pathways. In the present study, we show that CCN6 blockade confers a distinct survival advantage to nontumorigenic breast epithelial cells MCF10A.
and HME. CCN6 downregulation leads to growth factor–independent survival and proliferation and is sufficient to trigger anchorage-independent growth of mammary epithelial cells. We show that CCN6 knockdown (KD) protects cells from apoptosis and anoikis. Mechanistically, our data show that growth factor–independent survival and anoikis resistance conferred by CCN6 KD require activation of the PI3K/Akt-1 signaling pathway.

Materials and Methods

Cell culture. HME cell line was developed and provided by S.P. Ethier (Karmanos Cancer Institute, Detroit, MI); spontaneously immortalized human mammary epithelial cells, MCF10A, were obtained from the American Type Culture Collection and maintained as described previously (5). Serum deprivation medium consisted of DMEM/F-12 (MCF10A cells) or F-12 (HME cells) supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin. This medium was deprived of exogenous factors and serum. Cells were maintained in a 37°C incubator with 5% CO₂.

Transfections and transductions. Short hairpin RNA (shRNA) sequence (5′-CGGCCATTAGTACAACACT-TGAACTGAGTTCAGGTGTTGTA-3′) targeting human CCN6 (NM_003880; Sigma) and shRNA sequence (5′-CCGGCGATCGCTTCTTTGGCCTGATCTCGAGATACCGG-CAAAGAGCGATGTTTGG-3′) targeting human Akt-1 (NM_00104031; Open Biosystems) were cloned into pLKO.1-puro vectors. Both shRNA-containing plasmids were packaged into lentiviral particles at the Vector Core (University of Michigan, Ann Arbor, MI). Human Akt-1 small interfering RNA (siRNA) oligonucleotides (sense, 5′-CGGCGGATCGCTTCTTTGGCCTGATCTCGAGATACCGG-CAAAGAGCGATGTTTGG-3′; antisense, 5′-AUGGUCGCGGCGGCUUGG-3′), human Akt-2 siRNA oligonucleotides (sense, 5′-CAGAAGUCG-CAGCUGAAGA-3′; antisense, 5′-UUCUCACUGCGCAUUCUG-3′), and human siRNA negative control oligonucleotides were purchased from Sigma.

To generate stable MCF10A and HME cell lines with CCN6 or Akt-1 KD, 1 × 10⁶ cells per 100-mm plate were transduced with the corresponding lentivirus-containing supernatant diluted 1:1 with fresh serum-free medium for 48 h. Stable clones were selected for antibiotic resistance with 10 μg/mL puromycin (Sigma) for 3 wk. For siRNA oligonucleotide transfections, cells were split into complete medium for 24 h before subconfluence. siRNA oligos were transfected into subconfluent cells with Oligofectamine (Invitrogen) in accordance with the manufacturer’s instructions. After 48 h of growth in serum deprivation medium, cells were harvested by trypsinization and used for the experiments described below.

Western blot analysis. Cell lysates were collected using NP40 lysis buffer containing 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% NP40, and a mixture of protease inhibitors (Roche). Samples were boiled in 1× SDS loading buffer, separated by SDS-PAGE gels, and transferred onto a nitrocellulose membrane. For immunoblot analysis, nitrocellulose membranes were blocked with 5% nonfat dry milk and incubated with corresponding primary antibodies at 4°C overnight. Immunoblot signals were visualized by a chemiluminescence system as described by the manufacturer (Amersham Bioscience). Blots were reprobed with β-actin to confirm the equal loading of samples. Primary antibodies, including anti-CCN6, anti–Akt-2, anti-phospho-Akt-2 (Ser⁷²²), anti-Cdc25A, and anti-Cdc25C (Santa Cruz Biotechnology); anti-Akt, anti–Akt-1, anti–Akt-3, anti-phospho-Akt (Ser⁷²³), anti-glycogen synthase kinase-3β (GSK-3β), anti-phospho-GSK-3β (Ser³⁷), anti-Cdc2, and anti–cyclin D1 (Cell Signaling); anti-phospho-Akt-1 (Ser⁷²²); Upstate Biotechnology); anti-phospho-AKT-3 (Ser⁷²²) (Abgent); and anti–β-actin (Sigma), were used at the manufacturers’ recommended dilutions.

Proliferation and viability assays. For trypsin blue staining (Life Technologies), 2 × 10⁵ cells were seeded into each well of six-well plates in complete culture media. For the WST-1 assay (Roche), 5 × 10³ cells were seeded into 96-well microplates in complete culture media. For both assays, the next day, culture media were replaced with serum deprivation medium for 18 h. Subsequently, cells were treated with recombinant CCN6 (200 ng/mL; PeproTech) or with the PI3K/Akt inhibitor LY294002 (20 μmol/L; Invitrogen). Each treatment was performed in triplicate. Cell viability was determined by counting cells at 0, 24, 48, 72, and 96 h after treatment. Proliferation using the WST-1 assay was determined at the same time points following the manufacturers’ protocols. Data points represent the mean ± SD.

Cell synchronization and flow cytometry. MCF10A and HME CCN6 KD and control cells were maintained in complete medium until they reached 50% confluency. At this time, cells were washed, switched to serum deprivation medium, and synchronized at the G1-S boundary with double thymidine block. Synchronized cells were released to progress through the cell cycle over the next 12 h. Cell cycle was analyzed at indicated time points by flow cytometry after staining with propidium iodide (PI; 50 μg/mL). The percentage of cells in G₁, S, or G₂ phase is presented as the mean of three independent experiments. Whole-cell lysates were collected at the same time points and analyzed by immunoblot with indicated antibodies. β-Actin served as the loading control.

Apoptosis assay. Cells (2 × 10⁵ or 5 × 10⁵ per well) were seeded into each 100-mm plate or each well of 96-well microplates in complete culture media, respectively. The next day, culture media were switched to serum deprivation media for 18 h before treatment with recombinant CCN6 (200 ng/mL) or LY294002 (20 μmol/L) for 24 h. Caspase activity was quantified using Apo-ONE Homogeneous Caspase-3/7 Assay kit (Promega) following the manufacturer’s instruction. Annexin V/PI staining (Invitrogen) of cells in 100-mm plates was performed and analyzed by flow cytometry at the Flow Cytometry Core of the University of Michigan.

Anoikis assay. Anoikis was induced by culturing cells in poly-HEMA–coated plates according to published procedures (23). Briefly, a 12% solution of poly-HEMA in 95% ethanol was mixed overnight, clarified by centrifugation at 2,500 rpm, and diluted 1:10 in 95% ethanol. Dishes were coated with the diluted poly-HEMA solution (4 mL per 100-mm plate or 100 μL per well of 96-well microplate). Cells (1 × 10⁵) in 100 μL serum-deprived medium were seeded into
phospho-Akt-1 (Ser473) was evaluated as either low or high visualized in red with Vulcan Red (Biocare Medical), and stain non-biotin-avidin complex technique. Staining for CCN6 was proliferation (26). Growth factor CCN6 protein. A well-known characteristic of MCF10A and they are human, mostly diploid, nontumorigenic, and express cell lines were chosen for CCN6 downregulation because scrambled shRNA sequences (Fig. 1A). MCF10A and HME Control cells were transduced with a lentivirus containing MCF10A and HME cells using shRNAs in lentiviral vectors. breast epithelial cells, we generated stable CCN6 KDs in notype of reduced or absent CCN6 expression in human deprivation, CCN6 downregulation caused a 5-fold reduction with the Annexin V assay and flow cytometry. Under serum This result was further strengthened by analysis of apoptosis CCN6 KD cells survived under serum deprivation (Fig. 1B). able to grow at similar levels in complete medium, only phenotype and aberrantly activated signaling (26). We increased phosphorylation at Ser473 of Akt, which is required for the number of colonies in soft agar (Fig. 3A), indicating that besides enhancing survival, CCN6 loss promotes anchorage-independent growth in the absence of exogenous growth factors in the media. We reasoned that the ability to grow under anchorage-independent conditions may be a direct consequence of anoikis resistance, critical to breast cancer progression and metastasis (21). CCN6 KD and controls were placed on poly-HEMA–coated plates to prevent cell attachment, and analyzed for cell proliferation and apoptosis using WST-1 and caspase-3/7 assays. Figure 3B shows that CCN6 KD induced anchorage-independent growth under serum deprivation. The significance of the PI3K/Akt pathway for cell survival was performed using 24-well plate Biocoat Growth Factor Reduced Matrigel Invasion Chambers (BD Biosciences) according to the manufacturer’s procedures, in triplicate.

**Human breast tissue samples, immunohistochemistry, and statistical analyses.** A high-density tissue microarray containing 118 primary invasive carcinomas of the breast developed and characterized by our group was used (24). A 5-μm-thick section was comminuted with rabbit polyclonal anti-CCN6 antibody (1:300; Orbigen) and rabbit polyclonal anti–phospho-Akt-1 (Ser⁴⁷³; 1:150; Millipore) following a non-biotin-avidin complex technique. Staining for CCN6 was visualized in red with Vulcan Red (Biocare Medical), and staining for phospho-Akt-1 (Ser⁴⁷³) was visualized in brown with the DAB⁺ kit (DakoCytomation). Expression of CCN6 and phospho-Akt-1 (Ser⁴⁷³) was evaluated as either low or high based on intensity of staining and percentage of staining cells (5, 6, 25). χ² Test was performed to analyze the association between phospho-Akt-1 (Ser⁴⁷³) and CCN6 protein expression. A P value of <0.05 was considered statistically significant.

**Results**

**CCN6 KD confers growth factor–independent survival to mammary epithelial cells.** To determine the oncogenic phenotype of reduced or absent CCN6 expression in human breast epithelial cells, we generated stable CCN6 KDs in MCF10A and HME cells using shRNAs in lentiviral vectors. Control cells were transduced with a lentivirus containing scrambled shRNA sequences (Fig. 1A). MCF10A and HME cell lines were chosen for CCN6 downregulation because they are human, mostly diploid, nontumorigenic, and express CCN6 protein. A well-known characteristic of MCF10A and HME cells is their growth factor requirement for cellular proliferation (26). Growth factor–independent proliferation is a common hallmark in cancer cells containing oncogenic phenotypes and aberrantly activated signaling (26). We analyzed cell survival in MCF10A CCN6 KD and control cells at 0, 24, 36, 72, and 96 hours using a trypan blue assay. Although CCN6 KD and control mammary epithelial cells were able to grow at similar levels in complete medium, only CCN6 KD cells survived under serum deprivation (Fig. 1B). This result was further strengthened by analysis of apoptosis with the Annexin V assay and flow cytometry. Under serum deprivation, CCN6 downregulation caused a 5-fold reduction in the percentage of apoptotic cells compared with controls (Fig. 1C).

To determine if the improved cell growth of CCN6 KD cells under serum deprivation was associated with differences in cell cycle, we performed DNA content analysis by flow cytometry. As expected, synchronized, serum-starved control MCF10A cells exhibited a block at the G₁–S boundary. In contrast, CCN6 KD cells failed to arrest and progressed through the cell cycle overcoming the growth arrest conditions (Fig. 2A). Treatment with recombinant CCN6 protein blocked the cell cycle progression induced by CCN6 KD. The growth factor–independent cell cycle progression of CCN6 KD cells was associated with increased levels of G₁–S transition regulatory proteins (Fig. 2B). Similar results were obtained with HME cells (Supplementary Fig. S1). Collectively, these data show that the increased viability and proliferation of CCN6 KD mammary epithelial cells under serum deprivation result from both resistance to apoptosis and cell cycle progression.

**CCN6 KD protects cells from anoikis and promotes anchorage-independent growth under serum deprivation.** We next investigated the effect of CCN6 downregulation on anchorage-independent growth, a characteristic of neoplastic transformation. MCF10A and HME CCN6 KD cells and controls were plated in soft agar and incubated for 21 days in serum deprivation medium. CCN6 KD significantly increased the number of colonies in soft agar (Fig. 3A), indicating that besides enhancing survival, CCN6 loss promotes anchorage-independent growth in the absence of exogenous growth factors in the media.

The invasion assay. In vitro invasion was performed using 24-well plate Biocoat Growth Factor Reduced Matrigel Invasion Chambers (BD Biosciences) according to the manufacturer’s procedures, in triplicate.

In the absence of the PI3K inhibitor or CCN6 recombinant protein, immunoblots were carried out. To determine the oncogenic phenotype of reduced or absent CCN6 expression in human breast epithelial cells, we generated stable CCN6 KDs in MCF10A and HME cells using shRNAs in lentiviral vectors. Control cells were transduced with a lentivirus containing scrambled shRNA sequences (Fig. 1A). MCF10A and HME cell lines were chosen for CCN6 downregulation because they are human, mostly diploid, nontumorigenic, and express CCN6 protein. A well-known characteristic of MCF10A and HME cells is their growth factor requirement for cellular proliferation (26). Growth factor–independent proliferation is a common hallmark in cancer cells containing oncogenic phenotypes and aberrantly activated signaling (26). We analyzed cell survival in MCF10A CCN6 KD and control cells at 0, 24, 36, 72, and 96 hours using a trypan blue assay. Although CCN6 KD and control mammary epithelial cells were able to grow at similar levels in complete medium, only CCN6 KD cells survived under serum deprivation (Fig. 1B). This result was further strengthened by analysis of apoptosis with the Annexin V assay and flow cytometry. Under serum deprivation, CCN6 downregulation caused a 5-fold reduction in the percentage of apoptotic cells compared with controls (Fig. 1C).

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Ser473 (Fig. 4C, lane 4). Following LY294002 treatment, phospho-Akt expression dropped to almost undetectable levels (Fig. 4C, lane 6). After treatment with recombinant CCN6 protein, CCN6 KD cells showed a significant decrease in phospho-Akt levels (Fig. 4C, lane 5).

Survival curves for MCF10A CCN6 KD and controls in the presence of LY294002 (20 μmol/L) and/or recombinant CCN6 protein (200 ng/mL) show that treatment with LY294002 abrogated the CCN6 KD-induced cell survival effects. Likewise, addition of recombinant CCN6 protein compromised the survival advantage conferred by CCN6 KD on mammary epithelial cells (Fig. 4C).

We next tested the hypothesis that PI3K/Akt is also required for the resistance to apoptosis induced by CCN6 KD. We performed two apoptosis assays: Annexin V and caspase-3/7 cleavage in the presence of LY294002 and/or recombinant CCN6 protein. Given the multiple functions of PI3K/Akt in cell survival, it is not surprising that treatment with LY294002 increased apoptosis of MCF10A control cells. However, LY294002 treatment of CCN6 KD cells rescued the effects of CCN6 KD on apoptosis to levels similar to controls and to cells treated with recombinant CCN6 protein (Fig. 4D). Altogether, these results show that CCN6 protein can rescue the survival and apoptotic effects of CCN6 KD in mammary epithelial cells and that the activation of the PI3K/Akt pathway is essential in the cell survival mechanism of CCN6.

**Akt-1 is the main Akt isofrom mediating CCN6 effect on survival.** Three Akt isoforms have been characterized to date in mammalian cells: Akt-1 (PKBα), Akt-2 (PKBβ), and Akt-3 (PKBγ; refs. 27, 28), all of which are serine-threonine kinases. Studies have shown that the physiologic relevance and function of the individual isoforms are distinct (27, 28). To further define which Akt isofrom transduced the survival signal of CCN6 downregulation, we investigated the effect of CCN6 KD on the levels and phosphorylation of Akt-1.
Akt-2, and Akt-3. CCN6 KD in mammary epithelial cells increased the levels of Akt-1 and Akt-2 but did not influence Akt-3 expression or phosphorylation compared with controls (Fig. 5A).

Studies in cultured cells and murine models have identified potentially specific actions of each Akt isoform in breast cancer (29–32). It has been recently shown that whereas Akt-1 plays an important role in mammary tumor growth, Akt-2

**Figure 2.** Mammary epithelial cells with CCN6 KD fail to arrest in G1/S under serum deprivation. A, fluorescence-activated cell sorting analysis of serum-starved MCF10A CCN6 KD cells and controls, with or without CCN6 recombinant (re-CCN6) protein (200 ng/mL) based on PI staining at increasing collection times (0–12 h). DNA histograms for each specified time point are representative of three independent cell cycle analyses. Corresponding values for each cell population (G1, S, and G2) are expressed as percentages of cells found within each phase of the cell cycle. Note the failure of CCN6 KD MCF10A cells to arrest under serum deprivation, which was reverted by treatment with CCN6 protein. B, lysates of G1–S-synchronized MCF10A CCN6 KD and controls were analyzed by immunoblot with antibodies against the G1–S transition regulators. β-Actin serves as the loading control.
is mainly involved in breast cancer invasion and metastasis (30–32). We hypothesized that CCN6 KD may promote survival of mammary epithelial cells through specific activation of Akt-1. We further hypothesized that Akt-2 may mediate the previously reported invasion effects of CCN6 (5, 7, 22, 33). To test these hypotheses, we used siRNAs to transiently downregulate Akt-1 or Akt-2 on CCN6 KD mammary epithelial cells and controls. Specific blockade of Akt-1 completely reverted the increased growth factor–independent proliferation of CCN6 KD cells compared with siRNA control cells (Fig. 5B and C; Supplementary Fig. S3). This contrasts with the modest decrease in proliferation observed on Akt-2 inhibition on CCN6 KD cells compared with siRNA control cells (Fig. 5B and C; Supplementary Fig. S3). These data show that the survival mechanism induced by CCN6 KD requires principally Akt-1. We also noted that inhibition of Akt-1 and Akt-2 decreased at least in part CCN6 KD-mediated invasion in mammary epithelial cells (Supplementary Fig. S4).

To provide further evidence for a role for Akt-1 in the increased cell survival conferred by CCN6 KD, we developed stable short hairpin shRNAs targeting Akt-1 in lentivirus and stably transduced them into CCN6 KD cells and controls. Effective shRNA downregulation of Akt-1 was confirmed by immunoblots (Fig. 6A). Akt-1 shRNA significantly reduced the proliferation of CCN6 KD cells either attached or when grown in poly-HEMA–coated plates (Fig. 6A). Akt-1 shRNA also reverted the reduced levels of cleaved caspase-3 and caspase-7 resulting from CCN6 KD (Fig. 6B). Taken together, these results indicate that Akt-1 is the principal isoform mediating the growth advantage of CCN6 KD in mammary epithelial cells. Our data show that the ability of CCN6 KD cells to resist anoikis and survive in suspension necessitates Akt-1.

**CCN6 downregulation is associated with increased Akt-1 phosphorylation in human invasive breast carcinomas.** To assess the relevance of our *in vitro* studies to human breast cancer, we simultaneously investigated the expression of CCN6 and phospho-Akt-1 proteins in 118 primary invasive cancers. The results showed a significant correlation between CCN6 downregulation and Akt-1 phosphorylation in invasive breast carcinomas (Fig. 7A).

**Figure 3.** CCN6 reduction promotes anchorage-independent growth and protects mammary epithelial cells from anoikis under serum deprivation.

- **A.** MCF10A and HME cells were tested for their ability to grow in soft agar under serum deprivation. Colonies ≥100 μm in diameter were counted using ImageJ software. Columns, mean number of colonies; bars, SD. Each assay was performed in triplicate. Representative images from soft agar assay plates are shown.
- **B.** Above cell lines were grown in serum deprivation medium using poly-HEMA–coated plates for 24 h. Left, cell survival was analyzed by WST-1 assay; right, cell apoptosis was determined by caspase-3/7 activity assay. Columns, mean; bars, SD. Experiments were performed in triplicate.

* *, P < 0.001 (CCN6 KD versus controls).
Figure 4. CCN6 KD requires activation of PI3K/Akt pathway to increase cell survival and inhibit apoptosis. A, immunoblot analyses of MCF10A cells with CCN6 KD and controls reveals increased phosphorylation of Akt at Ser473 and one of its downstream molecules. B, immunoblot of HME cells with CCN6 KD shows increased phospho-Akt Ser473, similar to MCF10A cells. Specific inhibition of PI3K/Akt pathway with LY294002 (20 μmol/L) can reverse the upregulation of phospho-Akt Ser473 induced by CCN6 KD. C, immunoblots of MCF10A CCN6 KD cells and controls under serum deprivation for 48 h in the presence or absence of recombinant CCN6 (200 ng/mL) or LY294002 (20 μmol/L). Lane 4, CCN6 KD upregulates phospho-Akt Ser473. Treatment with CCN6 protein (lane 5) reduces phospho-Akt Ser473 in CCN6 KD cells. Addition of LY294002 (lane 6) reduces phospho-Akt Ser473 to nearly undetectable levels. Cells were tested for their ability to survive by the trypan blue assay in serum deprivation at the specified time points. Treatment with recombinant CCN6 protein or LY294002 rescues the survival advantage of CCN6 KD. D, cells were subjected to caspase-3/7 and Annexin V assays. Treatment with recombinant CCN6 protein or LY294002 rescues the apoptosis resistance of CCN6 KD. Columns, mean of three independent experiments performed in triplicate; bars, SD. *, P < 0.001 (CCN6 KD versus all of other experimental groups).
carcinoma tissue samples arrayed in a tissue microarray (24). Double immunohistochemical analysis showed that when present, CCN6 protein was predominantly cytoplasmic and that phospho-Akt-1 protein was localized to the nuclei of breast cancer cells (Fig. 6C; refs. 5, 6, 25). CCN6 and phospho-Akt-1 were scored as high when >10% of the cancer cells showed moderate or strong staining and were scored as low when staining was present in <10% of tumor cells (5, 6, 25). We found a novel significant association between CCN6 and phospho-Akt-1 protein expression in invasive carcinomas of the breast. Of the 118 tumors, 69 (58.5%) exhibited reciprocal expression of CCN6 and phospho-Akt-1 proteins (37.3% had high CCN6 and low phospho-Akt-1, and 21.2% had low CCN6 and high phospho-Akt-1; \( P = 0.027, \chi^2 \) test). Expression of both proteins was seen in 37 of 118 (31.4%) tumors, and absent CCN6 and phospho-Akt-1 expression occurred in 12 of 118 (10.1%) tumors (Fig. 6C and D).

**Discussion**

This study reveals that CCN6 downregulation provides survival advantage to mammary epithelial cells and points to a new mechanism by which CCN6 downregulation triggers growth factor–independent survival and protects mammary epithelial cells from detachment-induced apoptosis (anoikis). This mechanism implicates the activation of PI3K/Akt-1 signaling pathway.

A fundamental difference between normal and cancer cells is their requirement for extracellular signals (26). Whereas normal cells die on deprivation of extracellular growth factors,
cancer cells have the ability to survive and proliferate in their absence (26). We observed that CCN6 KD was sufficient to increase survival of mammary epithelial cells in serum-free conditions. To elaborate these conclusions, we investigated the effect of CCN6 on survival under serum deprivation using two complementary approaches: downregulation of CCN6 by shRNAs and treatment with human recombinant CCN6 protein. By measuring cell viability at different time points, it was concluded that mammary epithelial cells transduced with the scrambled shRNA were unable to proliferate under serum deprivation; rather, they underwent apoptosis. In contrast, CCN6 KD cells not only survived under serum deprivation but also continued to grow after 4 days while exhibiting significantly decreased apoptosis. Treatment with recombinant CCN6 protein completely reverted the growth and survival effects of CCN6 KD.

CCN6 KD had a striking effect on cell cycle progression. CCN6 downregulation resulted in failure to cell cycle block under growth arrest conditions. Treatment of CCN6 KD cells with recombinant CCN6 protein rescued this effect and restored the G1-S boundary block in serum-deprived mammary epithelial cells. These data not only reveal a novel cell cycle function of CCN6 but also strongly suggest that cell cycle delay may be important for the reported tumor-suppressive role of CCN6 in breast cancer (7).

Our group has previously reported that CCN6 loss is associated with a highly metastatic form of invasive breast carcinoma, termed inflammatory breast cancer, as well as with

Figure 6. Inhibition of Akt-1 rescues anoikis and apoptosis resistance in CCN6 KD mammary epithelial cells. A, MCF10A cells with CCN6 KD, Akt-1 KD, CCN6/Akt-1 double KD, and corresponding control cells were grown in serum deprivation medium for 24 h. Western blot analyses of phospho-Akt-1 and total Akt-1 levels are shown. Cell lines were grown in serum deprivation medium, with or without poly-HEMA–coated treatment, for 24 h. Cell survival was assayed by WST-1 assay. Akt-1 KD rescues the increased proliferation conferred by CCN6 KD in attached or detached mammary epithelial cells. B, cell apoptosis was determined by caspase-3/7 activity assay. Akt-1 KD rescues the decrease in apoptosis induced by CCN6 KD in mammary epithelial cells to levels similar to controls. Columns, mean; bars, SD. Data are representative of at least three independent experiments. *, P < 0.001 (CCN6 KD cells versus control or CCN6 KD/Akt-1 double KD cells).

C, human breast cancer tissue samples (n = 118) coimmunostained for CCN6 (red) and phospho-Akt-1 Ser473 (brown). Representative invasive ductal carcinoma with high CCN6 expression and low phospho-Akt1 (left), and another tumor with low CCN6 protein and high phospho-Akt1 (right).

D, distribution of CCN6 and phospho-Akt1 protein expression in our cohort of 118 consecutive primary invasive carcinomas of the breast. We discovered a significant association between CCN6 and phospho-Akt-1 proteins (P = 0.027, χ2 test).
noninflammatory invasive breast cancers with lymph node metastasis (5, 11). Data presented here show that CCN6 regulates a crucial step in metastatic dissemination: the acquisition of resistance to detachment-induced apoptosis (anoikis). Resistance to anoikis allows cancer cells to survive after detachment from the matrix and facilitates survival in lymphatic and blood vessels (21, 34). CCN6 KD in MCF10A and HME cells is sufficient to prevent anoikis and promote growth under anchorage-independent conditions in the absence of exogenous growth factors. The induction of anoikis resistance may contribute to the enhanced metastases observed in invasive carcinomas with low CCN6 protein expression.

The serine/threonine kinase Akt has been identified as a major effector of PI3K in the promotion and maintenance of cell survival, being able to act on cell proliferation and on the suppression of apoptosis and anoikis pathways (14, 16–19). By using independent and complementary strategies, including pharmacologic, transient, and stable RNA interference, we provide direct evidence that the survival advantage and resistance to apoptosis and anoikis induced by CCN6 KD necessitate the activation of PI3K/Akt signaling. To understand the specific contribution of each Akt isoform on the survival mechanisms triggered by CCN6 downregulation, we undertook two approaches: transient and stable downregulation of Akt-1 and Akt-2 in CCN6 KD cells and controls. Our data show that the growth factor–independent survival triggered by CCN6 KD requires principally phosphorylation of Akt-1. Both transient and stable Akt-1 inhibition reverted the prosurvival and antiapoptotic and anti-anoikis effects of CCN6 KD in mammary epithelial cells. The relevance of the novel mechanistic association between CCN6 and phospho-Akt-1 proteins to human breast cancer is supported by the finding that 37.3% of primary invasive carcinomas exhibited high CCN6 and low phospho-Akt-1, and that 21.2% exhibited low CCN6 and high phospho-Akt-1 proteins. We found that 31.4% had high levels and 10.1% had low levels of both proteins.

Despite the observation that CCN6 KD induced upregulation of Akt-2 phosphorylation at Ser373, our experiments show that Akt-2 seems to have a dispensable role in mediating the survival effects of CCN6. Although more work is needed to further understand the contribution of Akt-2 to CCN6 function, our preliminary studies suggest that Akt-2 may play a role in mediating the invasive phenotype of CCN6 KD cells.

In conclusion, our results show a previously undescribed function of CCN6 during breast tumorigenesis. We have identified that CCN6 KD promotes growth factor–independent survival of benign breast cells and is sufficient to induce anchorage-independent growth and anoikis resistance under serum deprivation. Our results enable us to pinpoint one mechanism by which CCN6 controls survival of breast cells, implicating the PI3K/Akt-1 pathway. We show that Akt-1 activation is indispensable for driving CCN6-mediated growth factor–independent survival of mammary epithelial cells. In view of our results and based on the profound effects of exogenously added recombinant CCN6 protein, we propose that modulation of CCN6 levels may be a valid strategy to prevent or halt neoplastic progression in the breast.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Blockade of CCN6 (WISP3) Activates Growth Factor–Independent Survival and Resistance to Anoikis in Human Mammary Epithelial Cells

Wei Huang, Maria E. Gonzalez, Kathy A. Toy, et al.

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