

## CCN6 Knockdown Disrupts Acinar Organization of Breast Cells in Three-dimensional Cultures through Up-regulation of Type III TGF- $\beta$ Receptor<sup>1,2</sup>

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### Abstract

While normal cells in the human breast are organized into acinar structures, disruption of the acinar architecture is a hallmark of cancer. In a three-dimensional model of morphogenesis, we show that down-regulation of the matrix-associated tumor suppressor protein CCN6 (WNT1-inducible-signaling pathway protein 3) disrupts breast epithelial cell polarity and organization into acini through up-regulation of the type III transforming growth factor- $\beta$  receptor (T $\beta$ RIII or betaglycan). Down-regulation of CCN6 in benign breast cells led to loss of tissue polarity and resulted in cellular disorganization with loss of  $\alpha$ 6 integrin-rich basement membrane and the basolateral polarity protein E-cadherin. Silencing of T $\beta$ RIII with shRNA and siRNA rescued the ability of breast epithelial cells to form polarized acinar structures with reduced matrix invasion and restored the correct expression of  $\alpha$ 6 integrin and E-cadherin. Conversely, CCN6 overexpression in aggressive breast cancer cells reduced T $\beta$ RIII *in vitro* and in a xenograft model of CCN6 overexpression. The relevance of our studies to human breast cancer is highlighted by the finding that CCN6 protein levels are inversely associated with T $\beta$ RIII protein in 64% of invasive breast carcinomas. These results reveal a novel function of the matricellular protein CCN6 and establish a mechanistic link between CCN6 and T $\beta$ RIII in maintaining acinar organization in the breast.

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### Introduction

The functional unit of the normal human breast is the terminal duct lobular unit, composed of a small duct that ends in acinar structures [1]. In the acini, normal breast cells are organized with the apical cytoplasm toward a central lumen and the basal portion toward the basement membrane. The normal acinar organization is disrupted in invasive carcinoma. Cancer cells form disorganized clusters and anastomosing structures characterized by loss of cellular polarization and a haphazard invasive pattern [1]. Although the morphologic differences between normal acini and invasive carcinoma are well delineated, the major regulators of this transition need to be elucidated.

The matricellular protein CCN6 (also known as WNT1-inducible-signaling pathway protein 3) was found to have tumor suppressor functions in breast cancer [2–4]. Our laboratory has demonstrated that CCN6 is secreted from epithelial cells in the breast and decreases activation of the insulin-like growth factor-1 (IGF-1) and bone morphogenetic protein (BMP) signaling pathways in breast cancer [5–7]. Recently, it was reported that down-regulation of CCN6 in

benign breast cells induces an epithelial-to-mesenchymal transition and invasion [3]. The function of CCN6 in normal acinar organization is unknown.

The type III transforming growth factor- $\beta$  (TGF- $\beta$ ) receptor (T $\beta$ RIII/betaglycan) is a transmembrane glycoprotein originally identified as a non-signaling co-receptor for TGF- $\beta$ , with a main function of presenting TGF- $\beta$  ligands to the TGF- $\beta$  receptors [8–12]. Subsequent

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studies determined that T $\beta$ RIII regulates TGF- $\beta$  signaling independent of its ligand presentation role [9,12,13]. In cancer, while T $\beta$ RIII has tumor suppressor functions [14–16], it has been reported to promote cancer progression [17]. These data underscore the need to further define the function of T $\beta$ RIII in normal breast and in breast cancer.

In this study, we hypothesized that CCN6 is a key determinant of acinar organization and sought to understand the underlying mechanism. Using benign human mammary epithelial (HME) cells and breast cancer cells, we demonstrate that CCN6 regulates acinar organization through T $\beta$ RIII. In three-dimensional (3D) cultures, CCN6 blockade is sufficient to disrupt acinar organization and cell polarity and to induce T $\beta$ RIII up-regulation. Silencing of T $\beta$ RIII using siRNA and shRNA led to phenotypic reversion with correct apical-basal polarity. Conversely, ectopic expression of CCN6 in breast cancer cells reduced T $\beta$ RIII in 3D and in murine primary tumors and distant metastasis. In clinical specimens from patients with breast cancer, CCN6 expression is inversely associated with T $\beta$ RIII protein levels, providing *in vivo* relevance to the functional results. These data identify a novel axis of cell regulation relevant to breast tumorigenesis by linking a matrix protein with control of acinar organization in the breast.

## Materials and Methods

### Cell Culture

HME cells were obtained from Dr Ethier (Karmanos Cancer Institute). Cell lines were authenticated by morphology and growth characteristics and tested for mycoplasma. HME and their stable cell lines were selected and cultured as previously reported [2,3]. MDA-MB-231 cells were purchased from ATCC (Manassas, VA) and maintained following the manufacturer's instructions.

### Development of Overexpression and Knockdown Cells

The generation of CCN6-Flag overexpressing MDA-MB-231 cells and CCN6 knockdown (CCN6 KD) HME cells have been previously reported [2,3,7]. Briefly, to generate CCN6 and T $\beta$ RIII double stable knockdowns in HME cells, cells were transduced with specific lentivirus and selected by puromycin (5  $\mu$ g/ml; Sigma, St Louis, MO), at 37°C under 5% CO<sub>2</sub>. Lentivirus was packaged at the University of Michigan Vector Core (specific targeting shRNA of T $\beta$ RIII: TRCN0000033429, Open Biosystems (Lafayette, CO); specific targeting shRNA of CCN6: TRCN0000033361, Sigma).

Validated On-Target siRNA were purchased from Dharmacon Thermo Scientific (Chicago, IL; Scrambled siRNA: D-001810-10-05; T $\beta$ RIII siRNA: L-010545-00). Cells were split into complete medium for 24 hours. siRNA oligos were transfected into subconfluent cells with DharmaFECT 1 Transfection reagent (Dharmacon Thermo Scientific) in accordance with the manufacturer's instructions. After 48 hours of growth in complete media, cells were harvested by trypsinization and used for subsequent experiments described below.

### 3D Cultures and Immunocytochemistry

Cells were grown on top of growth factor reduced matrigel (Cat. No. 354230; BD Transduction, Bedford, MA) for 15 days following published protocols [18]. Phase contrast images were taken on a Leica inverted microscope. Immunocytochemical analysis was carried out as reported [18]. Briefly, cells were fixed with freshly prepared 2% para-

formaldehyde for 20 minutes at room temperature followed by permeabilization with phosphate-buffered saline (PBS) containing 0.5% Triton X-100 for 10 minutes at 4°C. The acinar-like structures were rinsed three times with phosphate-buffered saline/glycine for 10 to 15 minutes per wash at room temperature before incubating for primary block with immunofluorescence (IF) buffer (130 mM NaCl, 7 mM Na<sub>2</sub>HPO<sub>4</sub>, 3.5 mM NaH<sub>2</sub>PO<sub>4</sub>, 7.7 mM NaN<sub>3</sub>, 0.1% BSA, 0.2% Triton X-100, and 0.05% Tween-20) + 10% goat serum for 1 to 1.5 hours at room temperature, followed by secondary block [IF buffer + 10% goat serum + 20  $\mu$ g/ml goat anti-mouse Fab fragment (No. 15-006-006; Jackson ImmunoResearch, West Grove, PA)] for 30 to 40 minutes. Primary antibody incubation was done overnight in the secondary block solution at 4°C. The cells were washed three times for 20 minutes with IF buffer at room temperature with gentle rocking. Secondary antibody incubation was done with fluorescent-conjugated secondary antibody in IF buffer + 10% goat serum for 40 to 50 minutes at room temperature. We used Alexa-conjugated antibodies from Molecular Probes (Grand Island, NY) at 1:200. The fluorochromes used were 4',6-diamidino-2-phenylindole (DAPI), Alexa 488, 568, 594, 633, and 680. After washing three times for 15 to 20 minutes each, the cells were mounted with prolong gold anti-fade reagent with DAPI (No. P36931; Molecular Probes, Invitrogen, Grand Island, NY).

### Immunoblot Analyses

Cell lysates were collected using NP-40 lysis buffer containing 50 mM Tris-HCl, (pH 7.4), 1% NP-40, and a mixture of protease inhibitors (Roche, Indianapolis, IN). Samples were boiled in 1 $\times$  sodium dodecyl sulfate loading buffer, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels, and transferred onto a nitrocellulose membrane. For immunoblot analysis, nitrocellulose membranes were blocked with 5% nonfat dry milk and were incubated with corresponding primary antibodies at 4°C overnight. Immunoblot signals were visualized by a chemiluminescence system as described by the manufacturer (Amersham Biosciences, Piscataway, NJ). Blots were reprobated with  $\beta$ -actin to confirm the equal loading of samples.

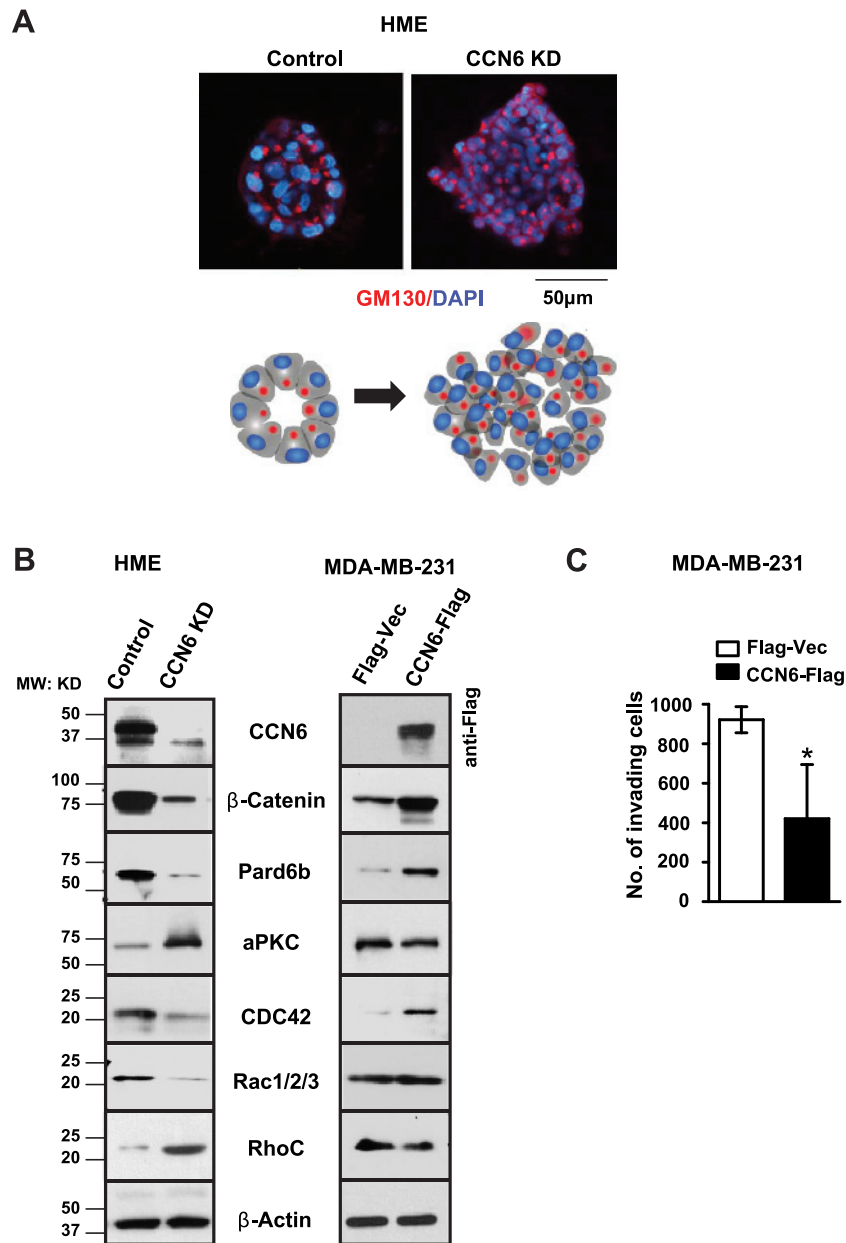
Primary antibodies including anti-CCN6, anti-Pard6b, anti-RhoC-GTPase, anti-CDC42, and anti-Rac1/2/3 (Santa Cruz Biotechnology, Santa Cruz, CA), anti- $\beta$ -catenin, anti-atypical protein kinase C (aPKC; BD Biosciences, San Jose, CA), anti-type I TGF- $\beta$  receptor (T $\beta$ RI), anti-T $\beta$ RIII (Cell Signaling Technology, Danvers, MA), and anti-type II TGF- $\beta$  receptor (T $\beta$ RII; Fischer, Pittsburgh, PA) were used at the manufacturers' recommended dilutions.

### Invasion Assay

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

### Human Breast Tissue Samples, Immunohistochemistry, and Statistical Analyses

A high-density tissue microarray containing 84 primary invasive carcinomas of the breast developed and characterized by our group was employed [19]. A 5- $\mu$ m-thick section was co-immunostained with rabbit polyclonal anti-CCN6 antibody (Orbigen [Atlanta, GA], Cat. No. PAB-11197, 1:300) and rabbit polyclonal anti-T $\beta$ RIII



**Figure 1.** Suppression of CCN6 expression is sufficient to disrupt acinar morphogenesis and polarity. (A) CCN6 KD in nontumorigenic HME cells led to disruption of acinar organization and formation of disorganized cell clusters with mislocalization of the apical polarity protein GM130 compared to controls. (B) Immunoblots of HME and MDA-MB-231 cells showed that CCN6 regulates the expression of cell polarity regulatory proteins. (C) Invasive ability of CCN6-Flag cells and controls (Flag-Vector) in MDA-MB-231 cells was analyzed using the matrigel invasion assay. Note that stable overexpression of CCN6 decreases the invasiveness of MDA-MB-231 cells compared to controls.

(Novus Biologicals [Littleton, CO], Cat. No. 27030002, 1:1500) following standard biotin-avidin complex technique [19]. The TβRIII antibody was incubated for 1.5 hours at room temperature. Antibodies were detected with anti-rabbit Envision<sup>+</sup> HRP Labelled Polymer (DakoCytomation, Carpinteria, CA) for 30 minutes at room temperature. HRP staining was visualized with the DAB<sup>+</sup> Kit (DakoCytomation). A negative control slide with rabbit IgG was run. Slides were counterstained in hematoxylin, blued in running tap water, dehydrated through graded alcohols, cleared in xylene, and mounted with Permount. Expression of CCN6 and TβRIII was evaluated as either low or high on the basis of intensity of staining and percentage of staining cells and following published literature [3,5,20]. Fischer exact test was performed to analyze the association between

TβRIII and CCN6 protein expression. A *P* value < .05 was considered statistically significant.

## Results

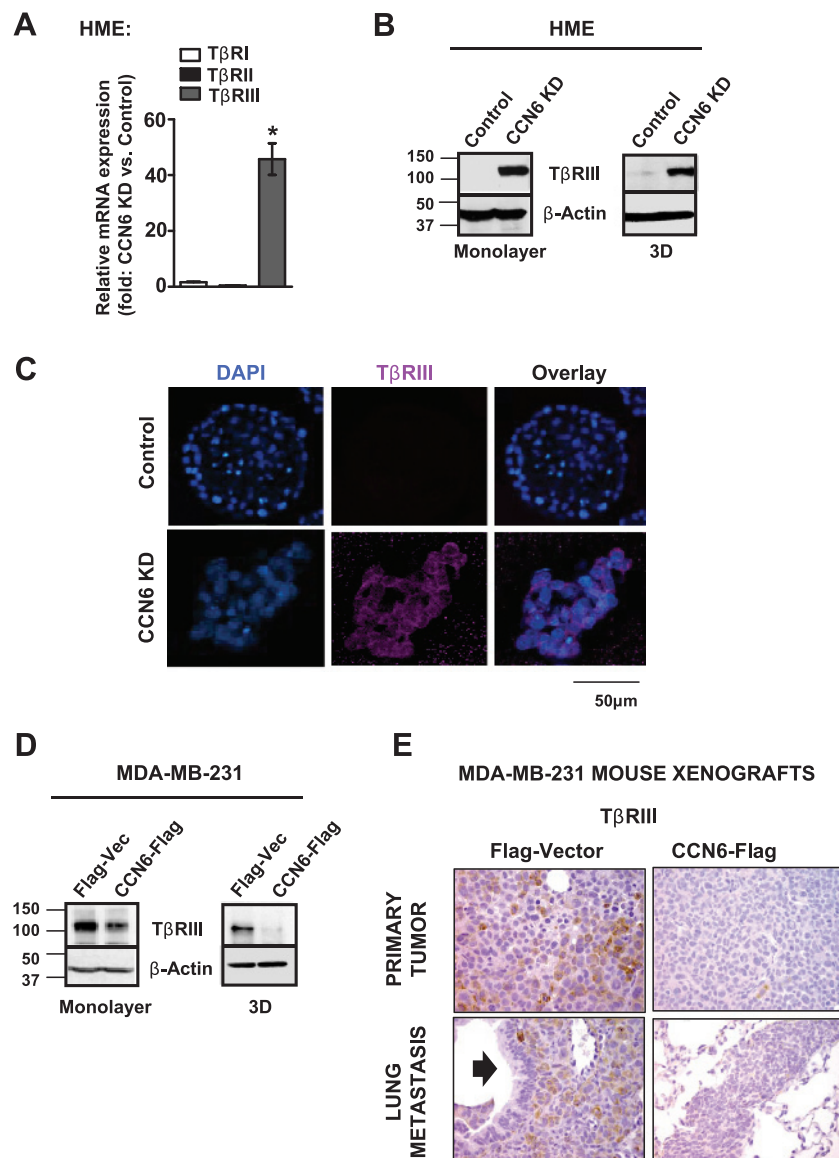
### *CCN6 KD Is a Key Determinant of the Loss of Acinar Organization in 3D Cultures*

Cultured in 3D, nontumorigenic HME cells provide a robust model for studying perturbations of the normal acinar architecture and tissue polarity. Whereas HME cells transduced with the scrambled shRNA controls form polarized round structures resembling normal mammary acini, CCN6 KD HME cells form disorganized colonies with loss of

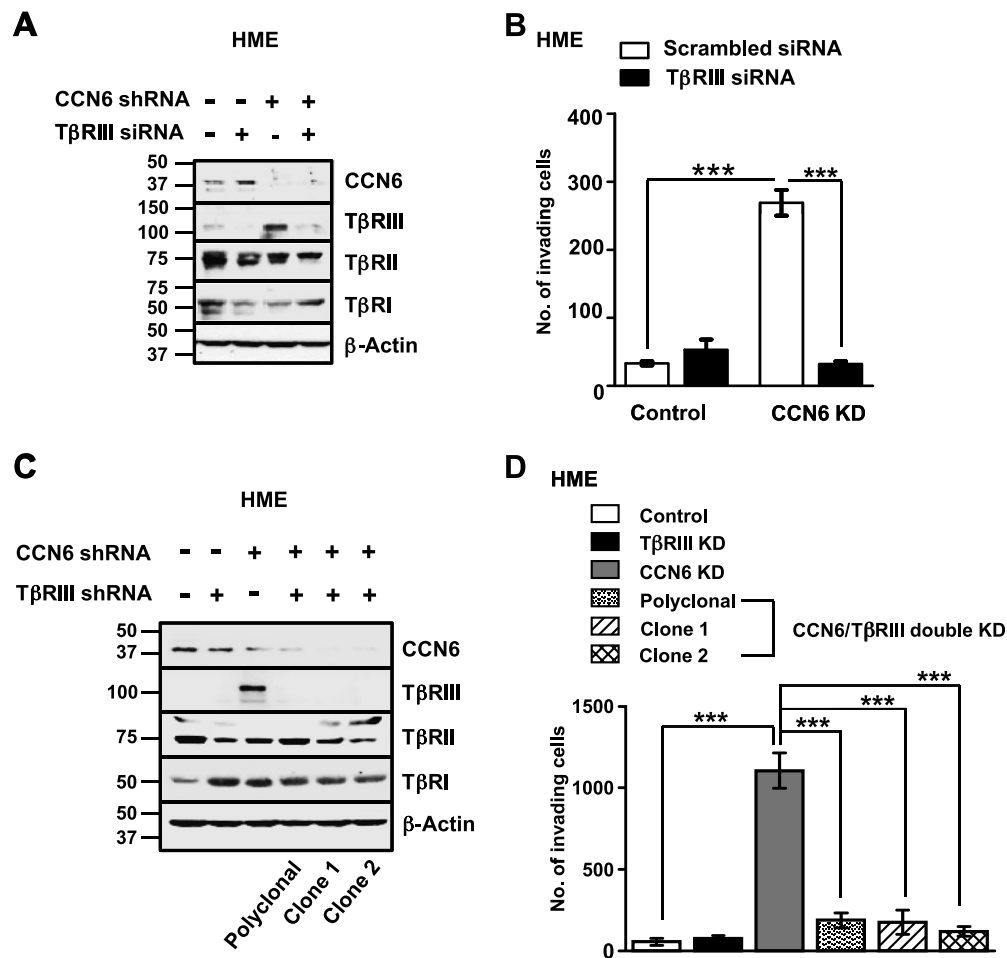
cell organization and polarity. The loss of apical-basal polarity is evidenced by detecting the apical polarity marker GM130 (Figure 1A). Furthermore, CCN6 KD led to altered expression of established regulatory proteins of acinar morphogenesis and cell polarity complex proteins  $\beta$ -catenin, Pard6b, aPKC, CDC42, Rac1/2/3, and RhoC-GTPase (Figures 1B, left, and W1). To validate our results in breast cancer cells, we ectopically overexpressed CCN6 with Flag tag (CCN6-Flag) in MDA-MB-231 cells that have low endogenous CCN6 levels and marked cellular disorganization. CCN6 overexpression led to a protein expression profile toward correct cellular organization and polarity (Figure 1B, right). These protein expression changes induced by ectopic CCN6 were associated with decreased breast cancer cell invasion (Figure 1C).

### CCN6 Regulates the Expression of T $\beta$ RIII Protein in Benign and Breast Cancer Cells

We have recently reported that CCN6 modulates the activity of the TGF- $\beta$  superfamily protein BMP4 in breast cells [7]. However, the possibility that CCN6 may regulate the expression of the TGF- $\beta$  receptors has not been considered previously. Quantitative real-time reverse transcription–polymerase chain reaction revealed that CCN6 KD in HME cells increased the mRNA levels of T $\beta$ RIII but had no significant effects on the levels of T $\beta$ RI and T $\beta$ RII compared to scrambled controls (Figure 2A). Consistently, CCN6 KD led to up-regulation of T $\beta$ RIII protein in HME cells growing in monolayer and in 3D cultures (Figure 2B). No significant



**Figure 2.** CCN6 regulates the expression of T $\beta$ RIII in benign and in breast cancer cells. (A) Quantitative real-time polymerase chain reaction of T $\beta$ RI, T $\beta$ RII, and T $\beta$ RIII revealed that CCN6 KD in HME cells specifically increases the expression of T $\beta$ RIII. No significant effect was observed on the mRNA levels of T $\beta$ RI or T $\beta$ RII between HME CCN6 KD and controls. (B) Immunoblots of HME cells with or without CCN6 KD were grown in monolayers or in 3D. CCN6 KD led to T $\beta$ RIII up-regulation. (C) Representative confocal images of HME CCN6 KD and controls stained with anti-T $\beta$ RIII. DAPI stained the nuclei. (D) Immunoblots of MDA-MB-231 CCN6-Flag cells and controls (Flag-Vec) demonstrated that stable overexpression of CCN6 reduces T $\beta$ RIII protein. (E) Representative pictures of primary tumors and lung metastasis derived from MDA-MB-231 CCN6-Flag and controls, developed previously [7], showed that CCN6 overexpression decreased T $\beta$ RIII protein expression *in vivo*. Black arrow shows normal bronchial epithelium entrapped by the metastatic carcinoma cells.



**Figure 3.** Inhibition of TβRIII is sufficient to rescue the invasive activity induced by CCN6 KD. (A) Immunoblots of HME CCN6 KD cells and controls untreated or treated with TβRIII siRNA. (B) Invasion assay showed that TβRIII siRNA rescued the invasion of CCN6 KD HME cells. (C) Immunoblots of HME CCN6 KD cells and controls stably transduced with TβRIII shRNA or control. Two clones and a polyclonal pool of CCN6/TβRIII double knockdown were tested. (D) Invasion assay showed that down-regulation of TβRIII reversed the invasive ability of CCN6 KD HME cells.

effect of CCN6 KD was seen on the protein levels of TβRI and TβRII (Figure 3, A and C). IF of cells growing in 3D supported these results and revealed that TβRIII protein was increased mainly in the cytoplasm and cellular membranes of CCN6 KD HME cells compared to controls (Figure 2C).

We next investigated whether CCN6 regulates the expression of TβRIII protein in breast cancer cells. Stable CCN6 overexpression in MDA-MB-231 breast cancer cells was sufficient to reduce TβRIII protein expression in cell growing as monolayers and in 3D compared to controls (Figure 2D). The effect of CCN6 overexpression on TβRIII protein was tested in a xenograft model using the same MDA-MB-231 cells (CCN6-Flag and its control) [7]. As shown in Figure 2E, TβRIII protein was reduced in CCN6 overexpressing xenografts and lung metastasis compared to controls.

#### *Inhibition of TβRIII Is Sufficient to Rescue the Invasive, Disorganized Tissue Morphology and Restore Polarization of HME CCN6 KD Cells*

The strong correlation between CCN6 and TβRIII protein expression in nontumorigenic and in breast cancer cells led us to

test whether TβRIII inhibition is sufficient to restore the disrupted tissue architecture and invasive activity induced by CCN6 KD in HME cells. The expression of TβRIII was downregulated in CCN6 KD cells and controls using the following two complementary and independent approaches: transient siRNA inhibition and stable shRNA knockdown.

Western blots show that CCN6 KD induced specific up-regulation of TβRIII protein but had no effect on the levels of TβRI or TβRII (Figure 3, A and C). Furthermore, TβRIII siRNA specifically inhibited TβRIII, with no changes in the protein expression of TβRI or TβRII (Figure 3A). TβRIII siRNA was sufficient to rescue the enhanced invasive capacity of CCN6 KD cells (Figure 3B). For the stable down-regulation of TβRIII and CCN6, we constructed HME cells with CCN6 and TβRIII shRNA double knockdown (CCN6/TβRIII double KD). We generated two stable clones as well as a polyclonal pool. As shown in Figure 3C, the shRNA was effective and specific for TβRIII knockdown. Importantly, TβRIII shRNA rescued the invasiveness of CCN6 KD HME cells compared to controls (Figure 3D).

We next tested the hypothesis that the effects of CCN6 KD on acinar morphogenesis and polarity observed in 3D may be mediated

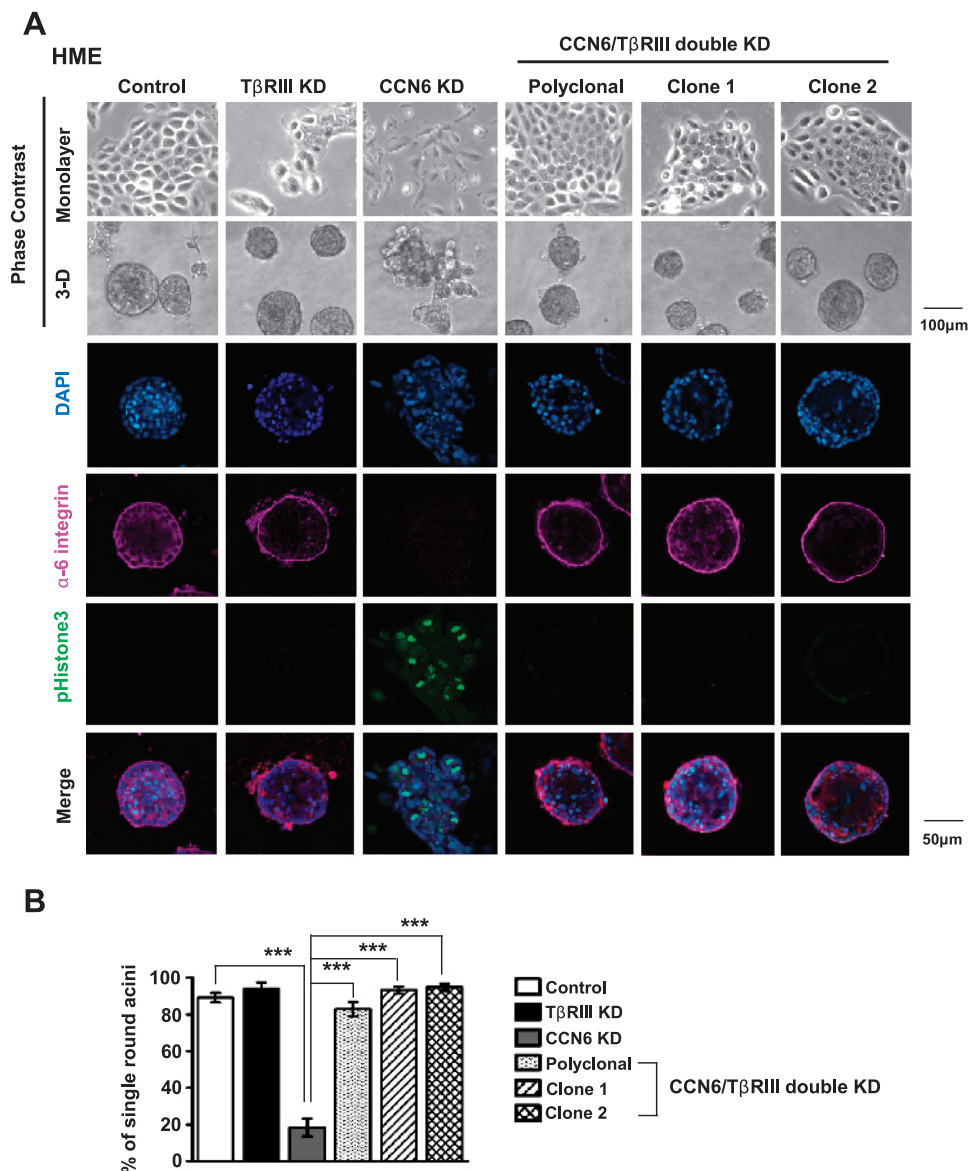
through TβRIII up-regulation. In stark contrast with the polarized and growth-arrested acini formed by HME controls in 3D, CCN6 KD induced the formation of disorganized colonies with loss of the basal polarity marker α6 integrin and increased cell proliferation (pHistone3 staining) (Figure 4A). TβRIII knockdown in HME cells had no effect on acinar polarity and morphogenesis. However, TβRIII knockdown reversed the disorganized morphology and continued proliferation of CCN6 KD HME cells toward the formation of correctly polarized acini in 3D, which recapitulate normal human breast acini. The percentage of acinar structures increased from 18% in CCN6 KD cells to more than 80% in CCN6/TβRIII double KD cells, indicating a rescue of the phenotype (Figure 4B).

Providing further support for the role of CCN6 and TβRIII in acinar organization, TβRIII knockdown in CCN6 KD cells res-

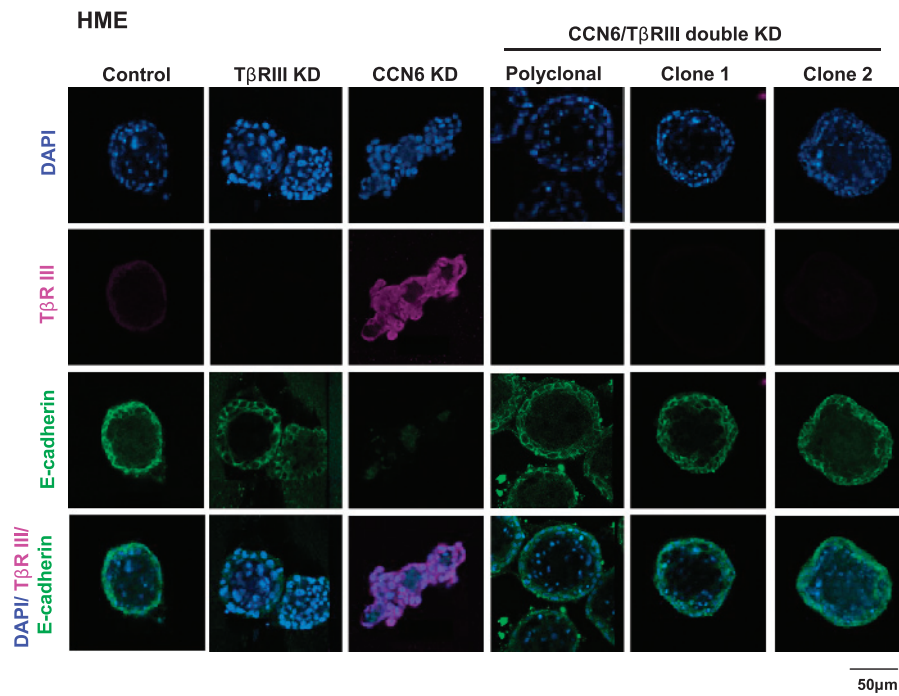
cued the expression of the basolateral polarity protein E-cadherin (Figure 5).

*Low CCN6 Protein Is Associated with High TβRIII in Human Invasive Breast Carcinomas*

The significance of our novel findings to human breast cancer was validated by testing the expression of CCN6 and TβRIII in 84 clinical samples of breast cancer arrayed in a tissue microarray [19]. Consistent with previous observations, CCN6 and TβRIII proteins localized predominantly to the cytoplasm. CCN6 and TβRIII were scored as high when more than 10% of the cancer cells showed moderate or strong staining and as low when staining was present in 10% or less of the tumor cells. We found a significant association between



**Figure 4.** Inhibition of TβRIII expression reverses the disorganized morphology induced by CCN6 KD. (A) Representative phase contrast images of cells grown in monolayer or in 3D showed that TβRIII shRNA reversed the disorganized and proliferative morphology induced by CCN6 KD in HME cells and induced the formation of growth-arrested and polarized acini. Notice the rescue of the basal polarity marker α6 integrin. (B) The bar graph shows the percentage of single-round acinar structures ± SD obtained by counting 100 structures.

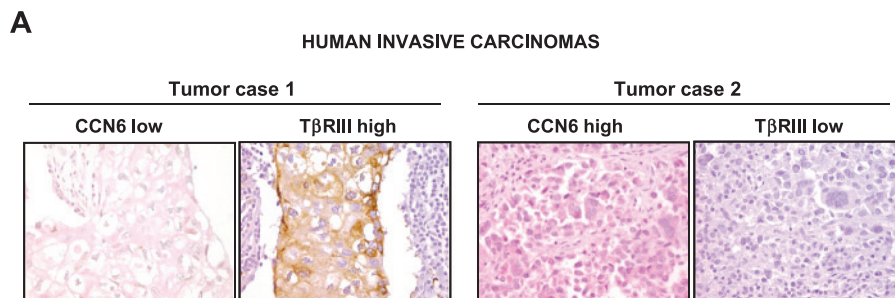


**Figure 5.** TβRIII knockdown rescues E-cadherin expression due to CCN6 KD. CCN6 KD led to TβRIII up-regulation and decreased expression of the lateral polarity marker E-cadherin. TβRIII shRNA inhibition was sufficient to rescue E-cadherin protein expression and to restore the acinar morphology.

CCN6 and TβRIII in invasive breast carcinomas. Of the 84 tumors, 54 (64.3%) exhibited an inverse association between CCN6 and TβRIII proteins. Specifically, 14 (16.7%) had low CCN6 coupled with high TβRIII expression, and 40 (47.6%) had high CCN6 in association with low TβRIII (Fisher exact test,  $P < .041$ ) (Figure 6, A and B).

**Discussion**

In the normal breast, epithelial cells are organized around a central lumen and exhibit apical-basal and lateral polarization [1]. Epithelial cells maintain close contact to each other through epithelial cell-cell adhesion proteins that include E-cadherin and β-catenin. In contrast, invasive breast carcinomas are characterized by disorganized cells



**B**

Association between CCN6 and TβRIII levels in invasive breast carcinoma

	CCN6 low	CCN6 high	Total
TβRIII low	12 (14.3%)	40 (47.6%)	52
TβRIII high	14 (16.7%)	18 (21.4%)	32
Total	26	58	84

Fischer's exact test,  $p=0.041$

**Figure 6.** CCN6 expression is inversely associated with TβRIII expression in human breast tissue samples. (A) Human breast cancer tissues ( $n = 84$ ) immunostained for CCN6 (red) and TβRIII (brown). Tumor case 1 shows an invasive carcinoma with low CCN6 and high TβRIII protein. Tumor case 2 exhibits high CCN6 and low TβRIII protein. (B) The table shows the distribution of CCN6 and TβRIII. We discovered a significant inverse association between CCN6 with TβRIII protein in 64% of invasive carcinomas (Fisher exact test,  $P = .041$ ).

with aberrant or lack of polarization [21]. Despite advances in the characterization of normal and invasive carcinoma phenotypes, the critical regulators of tissue architecture need further investigation.

Using a robust 3D culture model that approximates formation of human breast acini [18,22], we show that CCN6 down-regulation leads to disruption of acinar organization and cell polarity in non-tumorigenic breast epithelial cells. CCN6 KD led to disappearance of basal, apical, and lateral polarity markers  $\alpha 6$  integrin, GM130, and E-cadherin, respectively. Furthermore, CCN6 KD disrupted the expression of cell polarity complex proteins Pard6b, CDC42, and aPKC and of proteins involved in cytoskeletal organization including Rac1/2/3 and RhoC-GTPase. Supporting the novel function of CCN6 in maintaining acinar organization, CCN6 overexpression in breast cancer cells reversed the expression of polarity proteins and inhibited invasion.

From the presented data, CCN6 emerges as a novel regulator of the T $\beta$ RIII. Although substantial data exist on the function of T $\beta$ RI and T $\beta$ RII in tumorigenesis, there are limited data on the function of T $\beta$ RIII. Recent studies have shown that T $\beta$ RIII is an important regulator of cancer cell growth and invasion, with context-dependent roles in cancer progression [9,12]. While T $\beta$ RIII has been demonstrated to be lost or decreased in several cancers [13,14,16] suggesting a tumor suppressor function, other studies have shown that it is upregulated in colon cancer where its increased expression induced cell migration and anchorage-independent capacity [17]. The possibility that CCN6 may regulate the expression and function of the T $\beta$ RIII has not been investigated previously. Our data show that CCN6 down-regulation led to overexpression of T $\beta$ RIII in benign breast cells, whereas CCN6 overexpression in breast cancer cells inhibited T $\beta$ RIII compared to controls. By using independent and complementary strategies including transient siRNA and stable shRNA interference, we provide evidence that the effects of CCN6 KD in coordinating the invasive and disorganized phenotype require up-regulation of T $\beta$ RIII. Furthermore, T $\beta$ RIII knockdown was sufficient to rescue the E-cadherin down-regulation due to CCN6 KD in HME cells.

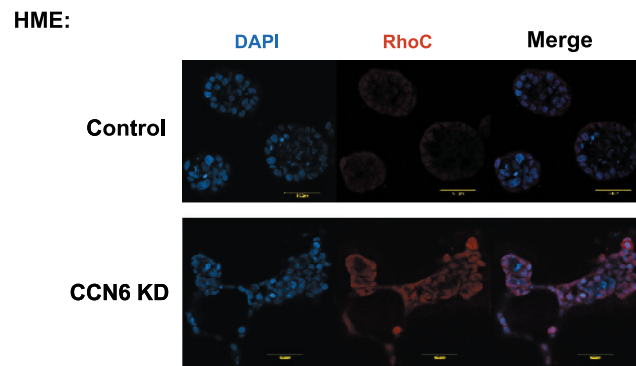
The *in vivo* relevance of the novel mechanistic link between CCN6 and T $\beta$ RIII proteins was determined by testing their expression in xenografts and human tissue samples. In xenograft samples, CCN6 overexpression in MDA-MB-231 cells decreased the levels of T $\beta$ RIII protein in primary and metastatic tumors. In human breast cancer tissue samples, an inverse association between CCN6 and T $\beta$ RIII protein expression was observed in 54 of 84 (64.3%) of invasive carcinomas.

In conclusion, our results demonstrate that CCN6 is a novel regulator of breast tissue architecture. CCN6 down-regulation in nontumorigenic breast cells disrupts cell polarization and acinar formation, leading to a disorganized phenotype with invasive properties. Our results enable us to pinpoint one novel mechanism that involves CCN6-mediated regulation of T $\beta$ RIII. From a clinical perspective, the role of CCN6 in maintaining normal acinar morphology through T $\beta$ RIII is of particular interest, as it may offer new targets to prevent tissue invasion.

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**Figure W1.** CCN6 KD in HME cells leads to increased RhoC protein expression in cells cultured in 3D. Representative confocal images of HME CCN6 KD and controls stained with anti-RhoC are shown. DAPI was used to stain the nuclei.