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Ccn6/Wisp3 Regulates the IGF2BP2/HMGA2 Signaling Axis in Metaplastic Carcinomas of the Breast

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Abstract

Purpose: Metaplastic breast carcinomas (MBC) are an aggressive subtype of triple negative breast carcinomas (TNBC) in which part or all of the adenocarcinoma transforms into a non-glandular component (e.g. spindled, squamous, or sarcomatous). We discovered that mammary-specific *Ccn6/Wisp3* knockout mice develop MBC with spindle and squamous differentiation that share upregulation of the oncofetal proteins IGF2BP2 (IMP2) and HMGA2 with human MBC. Here, we tested the expression of IGF2BP2 and HMGA2 proteins as biomarkers of MBC, and investigated their contribution to MBC.

Methods: Thirty-one human MBC were arrayed in a tissue microarray (TMA) and immunostained for CCN6, IGF2BP2, and HMGA2. MMTV-cre;*Ccn6*$^{fl/fl}$ tumors and spindle TNBC cell lines were treated with recombinant CCN6 protein or vehicle. HME cells with stable CCN6 shRNA knockdown were treated with IGF2BP2 shRNA knockdown or control, and subjected to invasion and adhesion assays.

Results: CCN6 regulates IGF2BP2 and HMGA2 protein expression in MMTV-cre;*Ccn6*$^{fl/fl}$ MBC, in MDA-MB-231 and −468, and in HME cells. CCN6 recombinant protein reduced IGF2BP2 and HMGA2 proteins, and decreased tumor growth of MMTV-cre;*Ccn6*$^{fl/fl}$ MBC *in vivo*. IGF2BP2 shRNA knockdown was sufficient to reverse the invasive abilities conferred by CCN6 knockdown in HME cells. Analyses of the TCGA Breast Cancer Cohort (n=1,238) showed that IGF2BP2 and HMGA2 are significantly upregulated in MBC compared to other subtypes. In clinical samples, low CCN6 expression is significantly associated with high IGF2BP2/HMGA2 specifically in spindle and squamous MBC.

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Conclusions: These data shed light into the pathogenesis of MBC and demonstrate a novel CCN6/IGF2BP2/HMGA2 oncogenic axis with therapeutic implications.

Keywords

breast cancer; CCN6; WISP3; IGF2BP2; IMP2; HMGA2; metaplastic; spindle; epithelial-mesenchymal transition; EMT

Introduction

Metaplastic breast carcinoma is a rare breast carcinoma that exhibits transformation of part or all of its glandular carcinomatous component into a non-glandular, or metaplastic, component [1,2]. The metaplastic component(s) is most commonly spindle and/or squamous, or heterologous, which include chondroid and osseous elements. These carcinomas account for approximately 1% of breast carcinomas, but up to 14% of breast carcinomas in African and African American women, they are almost invariably triple negative (TNBC) for estrogen and progesterone receptors (ER and PR), and negative for HER2/neu overexpression [3,4]. Compared to other subtypes of TNBC, metaplastic breast carcinomas are the most aggressive and most chemoresistant, with high propensity for metastasis [5,2].

Located on chromosome 6p21-23, *Ccn6/Wisp3* encodes a matricellular protein that is secreted by mammary epithelial cells and mediates epithelial - stromal cross-talk by binding to growth factors (e.g. bone morphogenetic protein 4) and/or cell surface receptors including integrins [6-9]. This protein plays important roles in development, maintenance of an epithelial phenotype, and cell attachment to the matrix. We have shown that normal breast epithelium secretes CCN6 to the extracellular medium, and that low levels of CCN6 characterize biologically aggressive breast cancers, including inflammatory breast carcinomas and metaplastic carcinomas [7,10,11]. Our lab has generated a novel MMTV-cre;*Ccn6*$^{fl/fl}$ knockout mouse using the Cre-lox system to specifically study the effect of *Ccn6* deletion in the mammary epithelium [12]. Using this model, we discovered that Ccn6 is important for normal mammary gland development and tumorigenesis. MMTV-cre;*Ccn6*$^{fl/fl}$ mice mammary glands had fewer terminal buds, decreased breast complexity, and ductal hypoplasia when compared to wild-type mice. Significantly, MMTV-cre;*Ccn6*$^{fl/fl}$ mice developed mammary tumors morphologically and transcriptionally similar to high-grade spindle and squamous human metaplastic carcinomas [12]. Transcriptomic analyses of MMTV-cre;*Ccn6*$^{fl/fl}$ metaplastic tumors and comparison with transcriptional profiles of human metaplastic carcinomas revealed 87 concordantly deregulated genes. Of these, IGF2BP2 and HMGA2 are the top upregulated genes in human and mouse metaplastic carcinomas [12].

IGF2BP2 (insulin growth factor 2 binding protein 2/IMP2) is an oncofetal protein important for embryonic development and downregulated in normal adult tissues, which functions by binding and stabilizing mRNA to extend their half-life [13,14]. Previous studies have shown that IGF2BP2 is elevated in multiple types of carcinomas and that it is associated with cancer growth, migration, adhesion, and energy metabolism [14]. In breast cancer, IGF2BP2

overexpression is associated with decreased cell adhesion and migration [14]. HMGA2 (high mobility group A2), another oncofetal protein, functions as an architectural transcription factor [15]. Similar to IGF2BP2, HMGA2 contributes to embryonic development and tumorigenesis [15]. HMGA2 levels are associated with migration, EMT, and worse outcome in breast cancer [15].

IGF2BP2 and HMGA2 have a reciprocal relationship, as IGF2BP2 prevents Let7-mediated HMGA2 inhibition [16]. Studies have shown that IGF2BP2 gene transcription and protein expression are highly dependent on HMGA2 [17]. IGF2BP2 possesses an AT-rich region in the first intron of its DNA sequence, which allows for HMGA2 mediated regulation. Binding of HMGA2 to this site uncovers a consensus-binding site for NF-kB, inducing expression of IGF2BP2 [17]. However, whether CCN6 regulates the IGF2BP2/HMGA2 axis, and their possible role in metaplastic carcinomas has not been considered previously. Here, we tested the hypothesis that low CCN6 expression coupled with high IGF2BP/HMGA2 may identify a subset of metaplastic carcinomas with spindle/squamous features, and that activation of the IGF2BP2/HMGA2 signaling axis may mediate the invasive abilities of this aggressive form of breast cancer.

Materials and Methods

Cell culture, generation of stable transfectants, and immunoblots.

MDA-MB-231 and MDA-MB-468 and HME cells were purchased from the American Type Culture Collection and grown under recommended conditions. Cell lines were authenticated using STR profiling, and were tested for mycoplasma infection using Sigma LookOut Mycoplasma PCR Detection Kit (Cat MP0035). CCN6 shRNA and scrambled controls were reported previously [18]. The scrambled control vector shRNA-SC and IGF2BP2 knockdown shRNA plasmid (pLKO.1-puro was purchased from Sigma (St. Louis, MO) and packaged at the University of Michigan Vector Core. For stable transductions, the virus-containing supernatant was diluted 1:1 with fresh media and supplemented with 8 μg/ml polybrene to infect HME cells. Selection was initiated in 10 μg/ml puromycin (Sigma-Aldrich Co., St. Louis, MD) 48 hours after infection. Recombinant CCN6 protein (rCCN6) (200 ng/mL) was purchased from PeproTech, and used as in our previous studies [18].

For MDA-MB-231 and −468 breast cancer cells and for HME non tumorigenic breast cells, Western blot analyses were carried out with 100μg of whole cell extract derived as previously reported [19]. Primary antibodies used include: CCN6, IGF2BP2, HMAG2 (Abcam, #ab187666, #ab129071, #ab97276, respectively), E-cadherin, ZEB1, SNAI2/Slug, (Cell Signaling, #3195, #3396, #9585 respectively); β-Actin (Santa Cruz Biotechnology, #SC-47778).

Invasion and Adhesion assays.

In vitro invasion was measured using 24-well matrigel-coated invasion chambers (BD Biosciences, Bedford, MA) according to the manufacturer's instructions, in triplicate. Invasive cells on lower sides of chambers were stained with 0.05% crystal violet, air-dried, photographed, and then counted under a microscope. Cell attachment assays were performed

by trypsinizing 70% confluent cell dishes and seeding 1×10^5 cells in a 12 well plate. After 30 minutes, non-adherent cells were removed by washing wells with PBS three times. Adherent cells were then imaged and entire wells were counted using ImageJ.

In vivo transplantation and CCN6 treatment studies.

MMTV-Cre;*Ccn6*$^{fl/fl}$ mice tumors were collected and implanted into the mammary fat pads of 8-10 week old virgin female syngeneic MMTV-Cre;*Ccn6*$^{fl/fl}$ mice (n=18). Two weeks after implantation, mice were treated with rhCCN6 (1ng/g, i.v., twice a week) or BSA (1ng/g) in the same regimen, and followed by palpation and caliper measurements for 3 weeks. Organoids derived form MMTV-Cre;*Ccn6*$^{fl/fl}$ mice tumors were generated following previous reports [20].

Study population and immunohistochemistry.

Metaplastic breast carcinoma tissues from the Surgical Pathology files at the University of Michigan were retrieved. We identified 31 cases from 1988-2015 with available blocks. All tumor slides were reviewed and tumors classified according to the predominant metaplastic component into spindle, squamous, or chondroid [1,21]. Representative areas were selected to construct a high density tissue microarray (TMA) with duplicate samples. A total of 31 cases (n=77 tissue microarray elements) comprised our patient cohort. Clinical and outcome information on the patients was obtained with institutional review board approval. Patient characteristics including age, race, treatment, and clinical follow up (i.e. recurrence, death) as well as tumor characteristics including, size, nodal involvement, hormone receptor status, and HER-2/neu expression were recorded.

Human tumors arranged in the TMA as well as MMTV-Cre;*Ccn6*$^{fl/fl}$ tumors were subjected to immunohistochemistry to detect CCN6, IGF2PB2, and HMGA2. Five micron-thick paraffin-embedded sections were de-paraffinized in xylene and rehydrated through graded alcohols to water. Heat Induced Epitope Retrieval (HIER) was performed in the Decloaking Chamber (Biocare Medical) with FLEX FTRS Low pH Retrieval buffer, pH6.1 (Dako, North America). Slides were incubated in 3% hydrogen peroxide for 5 minutes to quench endogenous peroxidases. Anti-CCN6 (Abcam # ab224720, dilution 1:400), anti-IGF2BP2 (Abcam # ab129071, dilution 1:100), and anti-HMGA2 (Abcam # ab97276, dilution 1:500) were incubated with the TMAs for 1 hour at room temperature. Antibodies were detected with Envision$^+$ HRP Labeled Polymer (DakoCytomation) for 30 minutes at room temperature. HRP staining was visualized with the DAB$^+$ Kit (DakoCytomation). Negative control slides were run. Slides were counterstained in hematoxylin, blued in running tap water, dehydrated through graded alcohols, cleared in xylene and then mounted with Permount. CCN6, IGF2BP2, and HMGA2 staining was evaluated at least two times for every tissue microarray element and at least four times for each tumor by 2 pathologists (EM and CGK), blinded to tumor stage and clinical information. CCN6 and IGF2BP2 displayed cytoplasmic staining, while HMGA2 was nuclear. For all proteins, expression was scored from 0 to 3 as follows: score 0 no staining, score 1 weak staining, score 2 moderate staining, and score 3 strong staining. Any staining (score ≥1) observed for IGF2BP2 and HMGA2 was considered positive. Expression for CCN6 was evaluated as either low or high

expression. Scores 2 and 3 were considered high and scores 0 and 1 were considered low, respectively.

Results

MMTV-cre;*Ccn6*$^{fl/fl}$ metaplastic carcinomas express high levels of IGF2BP2 and HMGA2

MMTV-cre;*Ccn6*$^{fl/fl}$ mice tumors recapitulate human high grade triple negative spindle/squamous metaplastic carcinomas [12]. However, whether they express IGF2BP2 and HMGA2 proteins is unknown. Here, we found that all high-grade spindled MMTV-cre;*Ccn6*$^{fl/fl}$ tumors showed increased expression of IGF2BP2 and HMGA2 by immunohistochemistry. IGF2BP2 protein was expressed in the cytoplasm of cancer cells, while HMGA2 was exclusively nuclear (Figure 1A). IGF2BP2 and HMGA2 expression was low in adjacent normal mammary epithelium of MMTV-cre;*Ccn6*$^{fl/fl}$ metaplastic carcinomas (Figure 1A).

Our laboratory has previously reported that CCN6 knockdown in non-tumorigenic breast cells induces a spindle and invasive phenotype with up regulation of the EMT transcription factors SNAI1 and ZEB1 [10,22]. Consistent with their spindle morphology, MMTV-cre;*Ccn6*$^{fl/fl}$ tumors also had upregulated expression of EMT regulators, ZEB1 and SNAI2 (Figure 1A). Using a scaffold -free, non-embedded 3D culture method, we generated organoids derived from MMTV-cre;*Ccn6*$^{fl/fl}$ metaplastic carcinomas. Immunohistochemical staining demonstrated high expression of IGF2BP2 and HMGA2 proteins in the spindle cancer cells, similar to the observed expression in whole tumor sections (Figure 1B).

Recombinant human CCN6 protein regulates IGF2BP2 and HMGA2 expression, and is sufficient to reduce growth of MMTV-cre;*Ccn6*$^{fl/fl}$ metaplastic carcinomas in vivo

As CCN6 is a secreted protein, we used recombinant human CCN6 (rhCCN6) to elucidate its role on IGF2BP2 and HMGA2 regulation. We employed MDA-MB-231 and −468 cells, which are TNBC cells with a spindle morphology and gene expression profiles of mesenchymal-like and basal type-A breast carcinomas, respectively [23]. Addition of rhCCN6 was sufficient to reduce IGF2BP2 and HMGA2 protein levels in both breast cancer cell lines (Figure 2A). Furthermore, ectopic overexpression of CCN6 in MDA-MB-231 cells reduced ZEB1 and SNAI2 protein levels compared to controls (Supplementary Fig.1).

To investigate the effect of rhCCN6 in vivo, we established an orthotopic syngeneic model by transplanting MMTV-cre;*Ccn6*$^{fl/fl}$ tumors into the mammary fat pads of mice with the same genetic background (FVB). Mice were treated with rhCCN6 (1ng/g, i.v., twice a week, initiated 2 weeks after transplantation) or BSA in the same regimen. rhCCN6 significantly reduced tumor growth, induced a morphological change from spindled towards epithelial, and reduced the expression of IGF2BP2 and HMGA2, compared to BSA treatment (Figure 2B). Together, these data demonstrate that CCN6 is a novel regulator of the IGF2BP2/HMGA2 signaling axis in spindle metaplastic carcinoma cells and that rhCCN6 is sufficient to reduce tumor growth in vivo.

The effect of CCN6 knockdown on invasion and adhesion requires IGF2BP2

Metaplastic carcinomas in humans and in MMTV-cre;*Ccn6*$^{fl/fl}$ mice are invasive and metastatic with loss of cell adhesions and reduced E-cadherin expression [12]. We have previously reported that CCN6 shRNA knockdown (KD) promotes invasion in HME cells [8]. However, the effect of CCN6 KD on cell adhesion and the mechanistic link with IGF2BP2/HMGA2 axis is unknown. CCN6 KD in HME cells resulted in significant upregulation of IGF2BP2, HMGA2, the EMT transcription factors SNAI2 and ZEB1, and induced downregulation of E-cadherin compared to scrambled controls (Figure 3A). To elucidate whether the effects of CCN6 KD on cell adhesion, invasion, and E-cadherin expression require upregulation of IGF2BP2, we downregulated IGF2BP2 in HME cells with CCN6 KD and controls using two independent shRNAs. IGF2BP2 shRNA rescued the effect of CCN6 KD on HMGA2, ZEB1, SNAI2, and partially rescued E-cadherin expression (Figure 3A).

To ascertain the function of the IGF2BP2 axis on the increased invasion and on the reduced cell adhesion due to CCN6 KD, we subjected these cells to Matrigel invasion and adhesion assays. IGF2BP2 KD reversed the reduced cell adhesion and the increased invasion of CCN6 KD HME cells compared to controls (Figure 3 B-C).

The CCN6 low/ IGF2BP2 high/ HMGA2 high phenotype identifies metaplastic carcinomas with spindle and/or squamous differentiation in clinical samples

Our lab has reported that CCN6 protein is expressed in normal breast lobules and in 67% of invasive carcinomas, while it is low in the majority of metaplastic carcinomas [12]. However, whether CCN6 expression is associated with tumor differentiation is unknown. Using an independent cohort of 31 metaplastic carcinomas arranged in TMAs, we show that low CCN6 is significantly associated with metaplastic carcinomas with spindle and/or squamous differentiation, compared to those with chondroid differentiation. Table 1 summarizes the clinical and pathological features of the patients. Of the 31 metaplastic carcinomas in our cohort, 27 (87%) had low CCN6 and 4 (13%) had high CCN6 ($p<0.002$). Of the 27 metaplastic carcinomas with low CCN6, 25 (92.6%) had spindle and/or squamous metaplastic components, while the 4 (100%) tumors with high CCN6 displayed chondroid differentiation as the predominant metaplastic component ($p=0.003$) (Table 2).

We found that normal breast epithelium adjacent or away from cancer expressed CCN6 and was negative for IGF2BP2 and HMGA2 proteins (Figure 4A). In contrast, 24 of the 27 (88.9%) tumors with low CCN6 and spindle and/or squamous differentiation showed high expression of IGF2BP2 and/or HMGA2 (Figure 4A and Table 3). Supporting the data in human tissues, *in silico* analyses of the TCGA Breast Cancer cohort (n=1,238) using UCSC Xena browser, showed that high mRNA expression of IGF2BP2 and HMGA2 is significantly associated with metaplastic carcinomas compared to other histological subtypes of breast cancer (Figure 4B). Consistent the histological EMT observed in MBC, they exhibit significantly higher SNAI2 and lower CDH1 expression than other breast cancer subtypes (Figure 4B). Together, these data demonstrate that low CCN6 coupled with high IGF2BP2 and/or HMGA2 are novel biomarkers for metaplastic carcinomas with spindle and squamous differentiation, with diagnostic and therapeutic implications.

Discussion

Metaplastic carcinomas are the most aggressive subtype of TNBC with a reported 5-year overall survival was 54% in metaplastic carcinomas compared to 73.3% for TNBC [5], but their pathobiology is still ill defined. This is due in part to the uncommon nature of these tumors, and the limited availability of mouse models that faithfully recapitulate human metaplastic carcinomas with spindle, squamous, or heterologous elements. Our lab has shown that metaplastic carcinomas exhibit mutations in Wnt pathway genes, including point mutations of *Ccn6/Wisp3* [24], and that conditional, mammary-epithelial cell specific *Ccn6/Wisp3* knockout induced development of metaplastic spindle and squamous carcinomas, similar to human disease [12]. In the present study, we utilized this model to discover specific deregulated pathways and tested their significance to human metaplastic carcinomas.

The origin of metaplastic breast carcinomas is currently unknown. There is no pathognomonic precursor, gene mutation(s), or recurrent gene fusions that has been described for metaplastic breast carcinomas, and it is unclear if different mutations contribute to the different subtypes of metaplastic breast carcinomas [25]. Genomic analyses of human tumors showed that metaplastic carcinomas and non-metaplastic TNBCs share TP53 mutations [26-30]. In addition, metaplastic carcinomas harbor more frequent PI3K and Wnt pathway gene mutations than non-metaplastic TNBC [27,24]. However, specific pathways operative in spindle and squamous metaplastic carcinomas have not been reported to date. Transcriptional profiling of MMTV-cre;$Ccn6^{fl/fl}$ tumors [12] and comparison to a previously reported gene expression profile of human metaplastic carcinomas [26] led to the identification of IGF2BP2 and HMGA2 as the two top upregulated genes common to mouse and human tumors. Using cell lines and human tissue samples, we demonstrate that CCN6 loss leads to upregulated IGF2BP2/HMGA2 pathway in metaplastic carcinomas with spindle and squamous components. We validated the expression of these proteins in MMTV-cre;$Ccn6^{fl/fl}$ tumors and in a cohort of 31 human metaplastic carcinomas arranged in a tissue microarray. Our analyses demonstrate that low CCN6 protein is significantly associated with high IGF2BP2 and/or HMGA2 proteins specifically in tumors with spindle and squamous differentiation, compared to those with chondroid elements.

A salient feature of metaplastic carcinomas is the enrichment for markers of stemness and EMT. Using clinical samples, our lab has reported the presence of ALDH1 positive and CD44+/CD24-/low expressing cells in the metaplastic component compared to the glandular component, strongly suggesting that the metaplastic component is less differentiated than the cells forming glands [21]. We also found that in contrast with the E-cadherin positive/ZEB1 negative glandular components, the spindle cells in metaplastic carcinomas showed loss of E-cadherin and ZEB1 upregulation, demonstrating active EMT [21]. Studies by other investigators validated these initial results using gene expression analyses of human tumors showing that metaplastic carcinomas exhibit a mesenchymal stem cell-like gene expression profile with upregulation of stem cell and EMT genes [23]. In the present study, we further demonstrate that MMTV-cre;$Ccn6^{fl/fl}$ tumors have overexpression of ZEB1 and SNAI2/Slug, and that the effect of CCN6 knockdown on the expression of proteins and on breast cancer cell invasion requires IGF2BP2.

The mechanistic link between CCN6, IGF2BP2 and HMGA2 is intriguing in light of their regulation and functions. IGF2BP2 and HMGA2 are oncofetal proteins with central roles during embryonic development, which are reduced in normal adult tissues and are re-expressed in cancer [13-15]. IGF2BP2 and HMGA2 exhibit reciprocal regulation, and their expression is controlled by activation of bone morphogenetic protein (BMP), insulin-like growth factor (IGF), insulin, and Wnt/β-catenin signaling pathways [13,16]. CCN6 was detected during zebrafish development, and was shown to play a role in the development of zebrafish cartilage [31]. We have detected CCN6 expression in the developing murine mammary gland, and CCN6 knockout resulted in developmental defects, demonstrating a role for CCN6 during normal mammary gland development [12]. Adult human breast cells express CCN6 and secrete it into the extracellular medium to regulate the effect of growth factor signaling pathways on the breast epithelia. Our lab and other investigators have demonstrated that conditioned medium containing CCN6 or human recombinant CCN6 protein is sufficient to inhibit BMP by binding to BMP ligand, and to reduce IGF1R activation [7,8]. Although it remains to be shown in the human breast, CCN6 protein derived from zebrafish and human recombinant CCN6 protein antagonized Wnt/β-catenin signaling by interacting with low density lipoprotein receptor 6 (LRP6) and Frizzled 8 in HEK293T human embryonic kidney cells [31].

Available data support that upregulation of IGF2BP2 and HMGA2 leads to enhanced tumor progression. Analysis of publicly available breast cancer expression dataset showed that IGFBP2 is elevated in TNBC where it is associated with worse survival [32,33] and that HMGA2 predicts relapse-free survival and metastasis in patients with TNBC [34]. HMGA2 expression was found significantly associated with the gene expression signature of metaplastic carcinomas [35]. We demonstrate that elevated mRNA expression of IGF2BP2 and HMGA2 is significantly associated with metaplastic carcinoma compared to other breast cancer subtypes, and that low CCN6 protein coupled with high IGF2BP2 and/or HMGA2 proteins marks human metaplastic carcinomas with spindle and squamous differentiation. Taken together, our study delineates a previously unconsidered pathway involved in the development of spindle and squamous metaplastic carcinomas with diagnostic and therapeutic implications.

Supplementary Material

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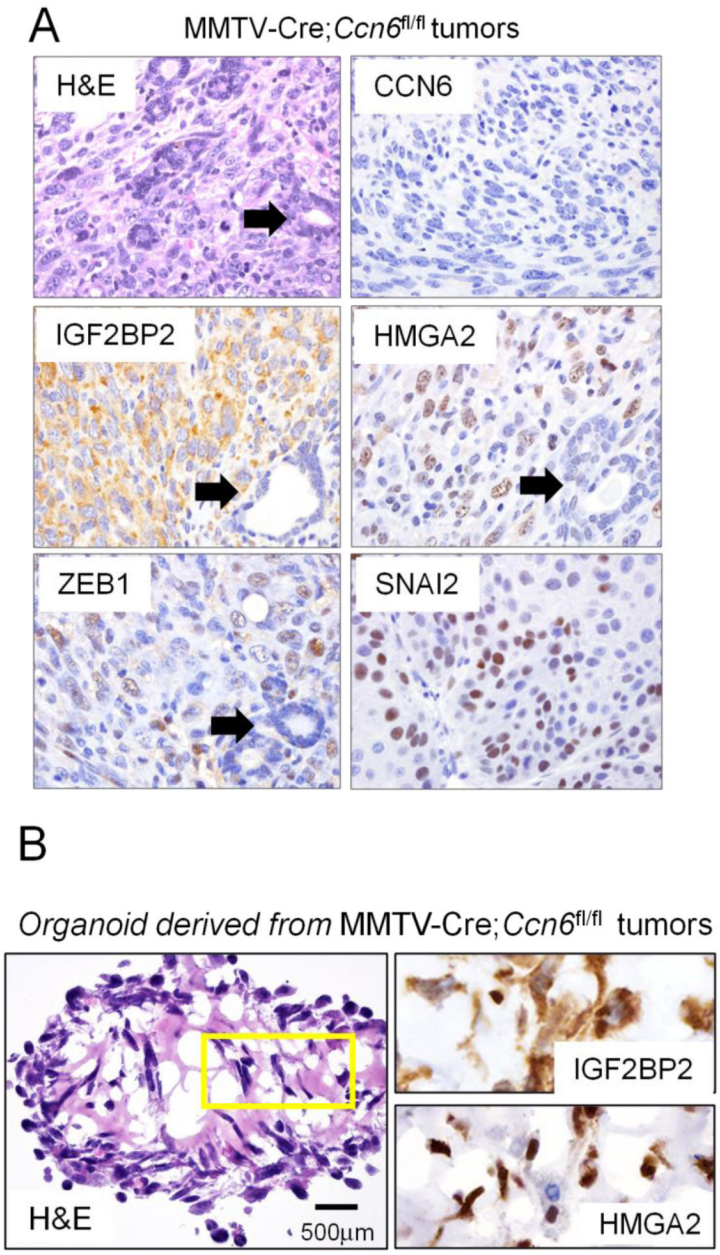

Figure 1. MMTV-cre;*Ccn6*$^{fl/fl}$ metaplastic carcinomas express IGF2BP2 and HMGA2 and EMT markers.

A. Histological sections of MMTV-cre;*Ccn6*$^{fl/fl}$ tumors showing low CCN6, high IGF2BP2 and HMGA2 proteins and upregulated expression of EMT transcription factors ZEB1 and SNAI2. Arrows indicate normal glandular structures in the murine mammary gland. **B.** Organoids developed from MMTV-cre;*Ccn6*$^{fl/fl}$ tumors demonstrating a spindle morphology and high levels of IGF2BP2 and HMGA2 proteins.

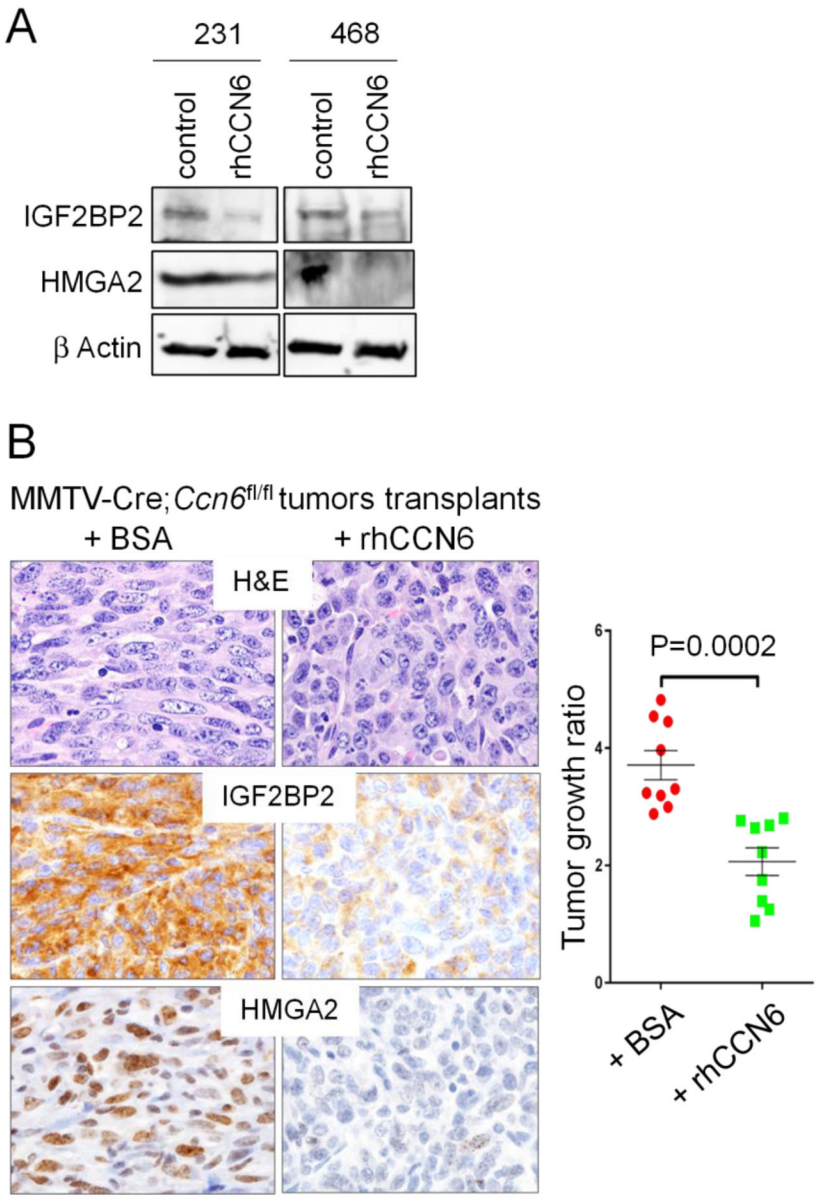

Figure 2. Human recombinant CCN6 protein reduces expression of IGF2BP2 and HMGA2 and regulates metaplastic tumor growth *in vivo*.

A. MDA-MB-231 and −468 breast cancer cells with spindle morphology were treated with rhCCN6 or BSA. **B.** MMTV-cre;*Ccn6*$^{fl/fl}$ tumors were implanted in the mammary fat pads of 8-10 week old female MMTV-cre;*Ccn6*$^{fl/fl}$ mice to avoid the possible contribution of microenvironment-derived CCN6 on tumor growth. Mice were treated with rhCCN6 (1 ng/g) or BSA (1 ng/g) i.v. twice a week starting 2 weeks after tumor implantation. Shown are histological sections of the tumors after 3 weeks of treatment. The graph shows differences in tumor growth at 15 days of treatment with rhCCN6 or BSA.

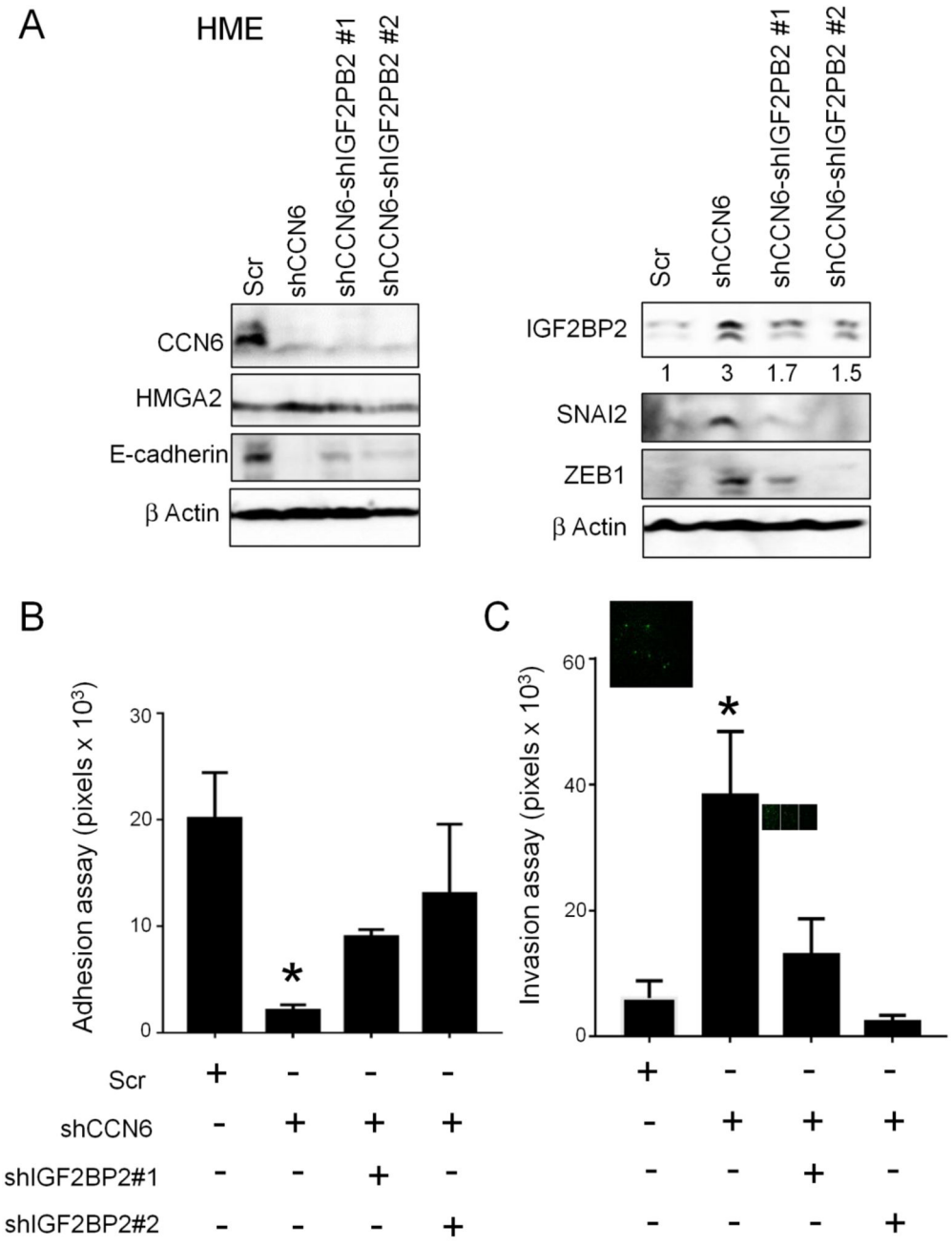

Figure 3. The induction of EMT, increased invasion, and reduced adhesion due to CCN6 knockdown require IGF2BP2.

A. HME cells were transduced with lentivirus containing shRNA CCN6 or scrambled shRNA controls (Scr). shRNA CCN6 HME cells were then transduced with two independent IGF2BP2 shRNAs (#1 and #2) or shRNA controls. Immunoblots were performed using the indicated antibodies. **B-C.** Cells in A were subjected to cell attachment assays and to Matrigel invasion assays. *p<0.05.

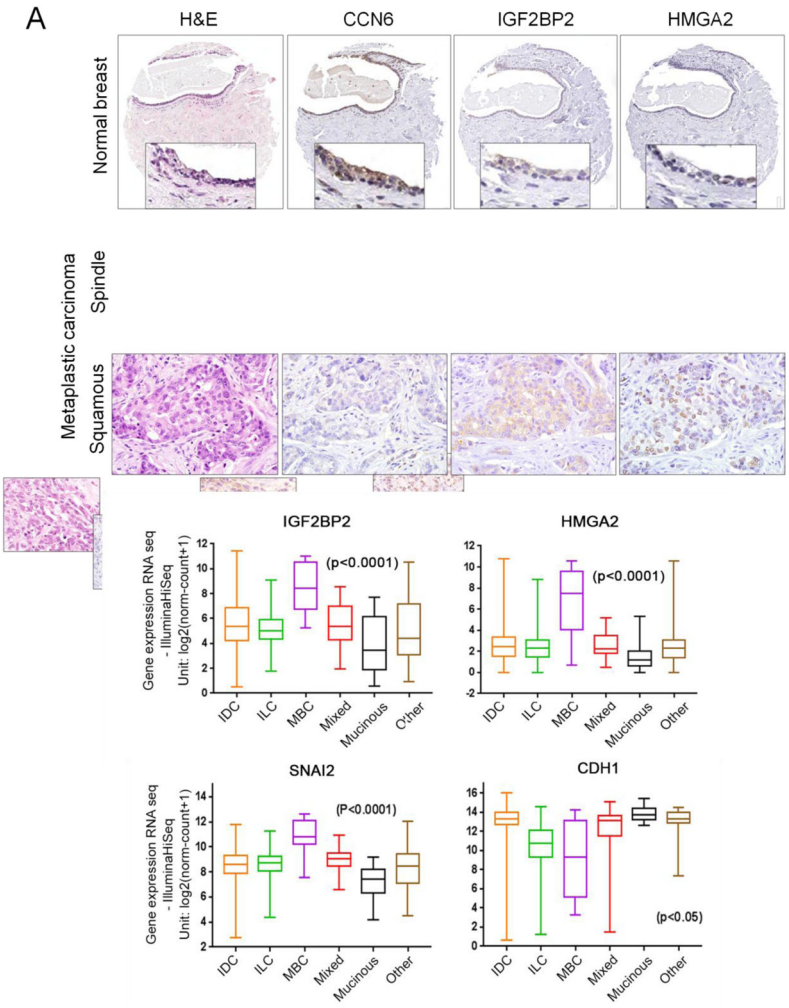

Figure 4. Human spindle and/or squamous metaplastic carcinomas exhibit low CCN6/ high IGF2BP2/ high HMGA2 proteins.

A. Representative images of TMA elements showing normal breast and two metaplastic carcinomas with spindle and with squamous differentiation. 400x magnification. **B.** Analyses of TCGA breast cancer dataset across breast cancer histological subtypes. IDC: invasive ductal, ILC: invasive lobular, MBC: metaplastic, Mixed: mixed invasive ductal and lobular.

Table 1.

Clinical and pathological features of the 31 patients with metaplastic breast carcinoma.

Characteristic	Number (%)
Median age (years)	46.7
Receptor status	
ER negative	28 (90.3)
PR negative	31 (100)
HER2/neu not overexpressed	30 (96.8)
Predominant metaplastic component	
Spindle	9 (29.0)
Squamous	17 (54.8)
Chondroid	6 (19.4)
Stage	
I	4 (12.9)
II	14 (45.2)
III	3 (9.7)
IV	4 (12.9)
Unknown	6 (19.4)
Size	
<2 cm	8 (25.8)
>2 cm, <5 cm	13 (41.9)
>5 cm	5 (16.1)
Unknown	5 (16.1)
Grade	
1	0 (0)
2	6 (19.4)
3	25 (80.6)
Lymph node metastasis	
Yes	11 (35.5)
No	17 (54.8)
Unknown	3 (9.7)
Distant metastasis	
Yes	7 (22.6)
No	20 (64.5)
Unknown	4 (12.9)
CCN6 expression	
Low	27 (87.1)
High	4 (12.9)
HMGA2 expression	
Low	16 (51.6)
High	15 (48.4)
IGF2BP2 expression	

Characteristic	Number (%)
Low	5 (16.1)
High	26 (83.9)

Table 2.

Expression of CCN6 in metaplastic carcinomas according to the predominant metaplastic component in 31 human tumors

Metaplastic component	CCN6			
	Low	High	Total	p-value
Spindle	9 (29%)	0	9	
Squamous	16 (51.6%)	0	16	
Chondroid	2 (6.4%)	4 (12.9%)	6	p=0.003
Total	27	4	31	

Table 3.

Association between CCN6 expression, IGF2BP2, and HMGA2 in metaplastic breast carcinomas.

	CCN6 Low			CCN6 High			
Metaplastic Component	**Both IGF2BP2/ HMGA2 High**	**Either IGF2BP2/ HMGA2 High**	**Both IGF2BP2/ HMGA2 Low**	**Both IGF2BP2/ HMGA2 High**	**Either IGF2BP2/ HMGA2 High**	**Both IGF2BP2/ HMGA2 Low**	**p-value**
Spindle	4	3	2	0	0	0	
Squamous	6	9	1	0	0	0	
Chondroid	0	2	0	3	1	0	
Total	10	14	3	3	1	0	p=0.02