AD_____

Award Number: DAMD17-02-1-0490

TITLE: Detection of Metastatic Potential in Breast Cancer by RhoC-GTPase and WISP3 Proteins

PRINCIPAL INVESTIGATOR: Celina G. Kleer, M.D.

CONTRACTING ORGANIZATION: University of Michigan
Ann Arbor, MI 48109-1274

REPORT DATE: May 2007

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-05-2007	Final	17 Apr 2002 – 16 Apr 2007

4. TITLE AND SUBTITLE

Detection of Metastatic Potential in Breast Cancer by RhoC-GTPase and WISP3 Proteins

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-02-1-0490

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Celina G. Kleer, M.D.

E-Mail: kleer@med.umich.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Michigan
Ann Arbor, MI 48109-1274

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Breast cancer is the most common type of life-threatening cancer, and the second most common cause of cancer related deaths of women in the United States. Even though the larger the primary tumor, the greater the likelihood of metastases, this is not always the case. There are many small breast cancers with a highly aggressive and metastatic behavior and discouraging outcome that remain under treated because there is no marker capable of identifying them. In this proposal we will study the utility of detecting RhoC GTPase and WISP3 proteins by immunohistochemistry as biological prognostic markers capable of identifying breast cancers with high propensity to metastasize, independently of tumor size. The impact of this study is that we will develop a clinically useful test to detect which invasive cancers will metastasize, and that will allow clinicians to institute early treatment before the development of metastases. This will impact on patient outcome. We will also study the predictive power of RhoC GTPase and WISP3 expression in the response of breast cancer to farnesyl transferase inhibitors, a new gene-targeted treatment modality for advanced cancers.

15. SUBJECT TERMS
WISP3, inflammatory breast cancer, RhoC-GTPase, prognostic factor

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	58	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

Introduction

The goals of the Career Development Award were to: 1. assist me in developing my laboratory and establish myself as an independent investigator in the field of breast cancer research at the University of Michigan; and 2 understanding the clinical utility of RhoC-GTPase and WISP3 proteins in breast cancer patients.

As discussed in detail in the following report, we have achieved both goals successfully. The Award enabled me to establish my research laboratory in the Medical Sciences Research Building 1 at the University of Michigan Medical School, and get promoted to the rank of Associate Professor with tenure. The Award allowed me to make substantial and seminal contributions on the role of RhoC GTPase and WISP3 genes in the development and progression of breast cancer and also discover the role of these and other potentially relevant proteins as breast cancer biomarkers. This award resulted in over 20 peer reviewed publications that advanced the field of breast cancer research.

Body

We have made significant contributions on the proposed tasks. Below are brief descriptions of key accomplishments according to the approved statement of work (SOW):

Task 1. To determine whether the concordant alterations of RhoC-GTPase over-expression and WISP3 loss are prognostic indicators and predictors of survival in breast cancer patients. Months 1-24.

a. Identify and retrieve the breast cancer tissue blocks and slides (489 cases total). Months 1-6.
b. Histopathologic study of the cases and selection of adequate tumor areas to construct the tissue microarrays. Categorize the breast cancers according to stage. Months 6-9.
c. Construction of the tissue microarrays, one containing 400 breast cancers of all anatomic stages and the other containing 89 cases of locally advanced breast cancer. Months 9-15.
d. Immunohistochemical analysis for RhoC-GTPase and WISP3 proteins, and other markers (ER, PR, HER2/neu, Ki-67, microvessel density and apoptosis). Months 16-19.
e. Interpretation and grading of the immunohistochemical studies and statistical analyses. Months 20-24.

Task 1

Identify and retrieve the breast cancer tissue blocks and slides.
By performing a computerized search of the breast cancer database at the Department of Pathology, University of Michigan, using the words “breast” and “cancer” and “breast” and “carcinoma” from years 1987-1991. We identified 385 consecutive invasive breast cancer patients. Of the 385 cases, 236 cases were available for study. The reasons for this were: 1. unavailability of tissue slides or blocks, and 2. primary resection performed at a referring institution.

In addition, 60 cases of locally advanced breast cancer, of which 30 are inflammatory breast cancers, and 30 are stage matched, non-inflammatory breast cancer were identified from the pathology files.

Histopathologic study of the cases and selection of adequate tumor areas to construct the tissue microarrays. Categorize the breast cancers according to stage.

The P.I. reviewed all the hematoxylin and eosin stained sections from all these cases and annotated the pathologic characteristics of each tumor using the following template:

Summary for Invasive Carcinomas.

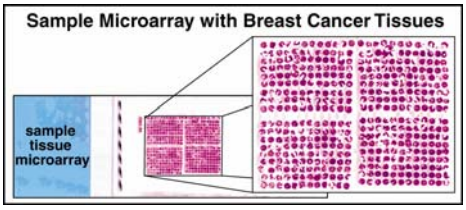
Greatest dimension of invasive carcinoma (microscopic):						**cm**
Involvement of surgical margin:	**Positive (at ink)**				**Close (<= 0.2 cm)**	
	Negative (>0.2 cm)					
If margin positive:		**Single focus**			**Multiple foci**	
If margin close:		**Single focus**			**Multiple foci**	
Histopathological grade (Elton and Ellis):		**1**	**2**	**3**		
Positive lymph nodes /total lymph nodes:			**/**			
Highest axillary node positive:		**Yes**		**No**		**N/A**
Extranodal extension:		**Yes**		**No**		**N/A**
Extensive DCIS:		**Yes**		**No**		**N/A**
DCIS > 25% of tumor:		**Yes**		**No**		
Extratumoral DCIS:		**Yes**		**No**		
Microcalcifications:	**None**		**within inv/DCIS**		**within benign ducts**	
Hormonal receptors:	ER:	**POS**	**NEG**	PR:	**POS**	**NEG**
Her2neu overexpression:		**POS (2+**	**3+)**	**NEG**		
T	***N***	***M***				

Development of a breast cancer database

We developed a relational database in Microsoft Access to store the pathological and clinical information. The idea behind this decision was to be able to link the results of the TMA scoring with the patient pathological and clinical information. Clinical and treatment information was extracted by chart review, performed with IRB approval. The P.I. was involved in all steps of the database design and development, and learned how to perform database queries.

Construction of the tissue microarrays

We have constructed four high density tissue microarrays (TMAs) that will enable us to characterize WISP3 and RhoC expression in a wide range of normal breast and breast disease, and to study associations between expression of these proteins and patient outcome. The figure below is a schematic representation of a TMA.



In order to construct the tissue arrays, the P.I. reviewed all cases histologically and selected the areas to array. At least three different areas of the tumors were selected and at least three tissue cores (0.6 mm in diameter) were sampled from each donor block. TMAs are assembled using the manual tissue puncher/array

(Beecher Instruments). This instrument consists of thin-walled stainless steel needles with an inner diameter of approximately 600 μm and stylet used to transfer and empty the needle contents. The assembly is held in an X-Y position guide that is manually adjusted by digital micrometers. Small biopsies are retrieved from selected regions of donor tissue and are precisely arrayed in a new paraffin block. Cores are inserted into a 45 x 20 x 12 mm recipient block and spaced at a distance of 0.8 mm apart.

Immunohistochemical analysis for RhoC-GTPase and WISP3 proteins, and other markers (ER, PR, HER2/neu, Ki-67, microvessel density and apoptosis).

We have optimized the conditions for immunohistochemistry for the anti-RhoC antibody and applied it to the constructed TMAs successfully. We used 1:400 dilution of antibody incubated overnight, and microwave antigen retrieval. Below are examples of tissues stained using RhoC antibody:

We have worked closely with Covance in developing two antigenic peptides and immunizing rabbits to obtain polyclonal antibodies against WISP3. The following peptides were synthesized and polyclonal antibodies were obtained:

Ac-CSGAKGGKKDSDQSN-CONH2
Ac-CPEGRPGEVSDAPQRKQ-CONH2.

After evaluating 4 different anti-WISP3 antibodies, we selected the one that worked better for Western blot and gave a specific band (shown below).

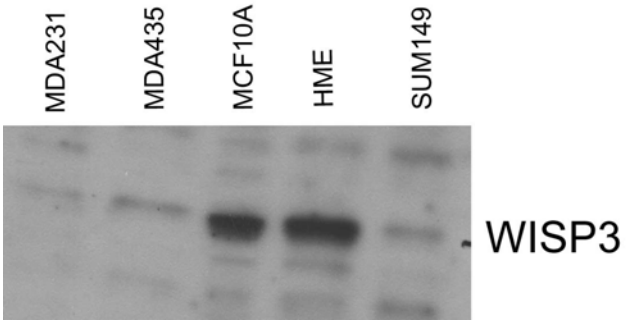


Figure 2. Western immunoblot of cell lysates of five different breast cancer cell lines (MDA231, MDA435, SUM149), and HPV immortalized human mammary epithelial cells (HME), and spontaneously immortalized human mammary epithelial cells (MCF10A). WISP3 protein is expressed in normal cells, and its expression decreases in breast cancer cells.

We have optimized the conditions for the anti-WISP3 antibody for immunohistochemistry and we have applied it to the TMAs successfully. We use the antibody at 1:100 dilution, with 60 minutes incubation and microwave antigen retrieval. Below are examples of the tissues microarray samples stained with anti-WISP3 antibody. We have also stained the TMAs for estrogen receptor, progesterone receptor and HER-2/neu.

Interpretation and grading of the immunohistochemical studies and statistical analyses.

I evaluated the immunohistochemistry for RhoC, ER, PR and HER-2/neu in all the TMAs, and with the assistance of Kent Griffith, the biostatistician, have analyzed the results which are shown below. I am in the process of evaluating the immunohistochemistry for WISP3, to explore its clinical relevance.

Below is the summary of our RhoC analyses.

We found that RhoC expression increases with breast cancer progression. All samples of normal breast epithelium had negative to weak staining, whereas staining intensity increased in hyperplasia, DCIS, invasive carcinoma, and metastases (Kruskal-Wallis $p<0.001$).
RhoC expression was associated with negative ER expression and worse histologic grade. In patients with invasive carcinoma, high RhoC expression was an independent predictor of death from breast cancer, and of local-recurrence free survival. The hazard ratio for local recurrence for patients with high RhoC levels as compared with those with low RhoC levels was 2.37, with a 95% confidence interval of 1.18-4.77 ($p=0.015$), Figure 3.

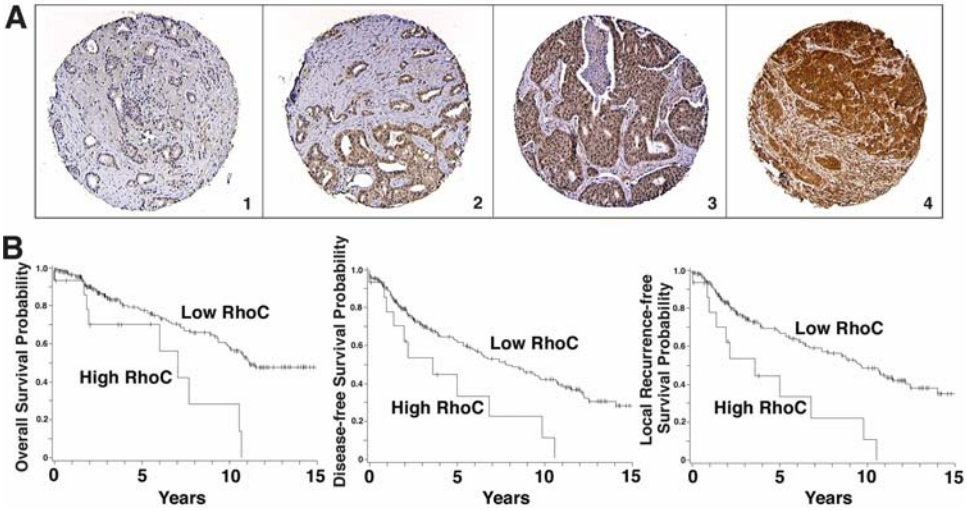


Figure 3. **RhoC protein expression is associated with survival in patients with breast cancer. A.** Tissue microarray elements containing representative invasive carcinomas with negative (1), weak (2), moderate (3), and strong (4) RhoC staining intensities. Original magnification 40x. **B.** High RhoC expression in invasive carcinomas is associated with worse overall, disease-free, and local recurrence-free survival.

These studies show that RhoC expression increases with breast cancer progression and RhoC protein levels in tumor tissue, as measured by immunohistochemistry, are strongly associated with survival and local recurrence in patients with breast cancer. This not only extends our initial observations (Kleer et al, *Am J Pathol* 2002 Feb;160(2):579-84), but also suggests that RhoC may have a role in the local invasiveness and progression of breast carcinoma. Our studies suggest that RhoC protein levels may be first altered in carcinoma *in situ*, the precursor of invasive carcinoma. Clinically, our retrospective study suggests that RhoC levels may prove useful after further validation, to identify patients with breast cancer that are likely to recur locally. This work was published in *Breast Cancer Research and Treatment* 93(2):101-10, 2005.

Task 1. To define the role of Rho-GTPase and WISP3 in the clinical setting as independent predictors of survival in patients with locally advanced breast cancer. Months 24-36.

a. Histopathologic study of 89 cases of locally advanced breast cancer that were previously retrieved
from the pathology files (first 6 months). Selection of adequate areas to construct the tissue microarray. Months 24-27.

b. Development of the tissue microarray, and immunohistochemical analysis of RhoC-GTPase, WISP3 and other biomarkers (ER, PR, HER2/neu, Ki-67, microvessel density and apoptosis). Months 28-33.

c. Interpretation and grading of the immunohistochemical stains and statistical analyses. Months 33- 36.

Task 2.

a. Histopathologic study of 89 cases of locally advanced breast cancer that were previously Retrieved from the pathology files (first 6 months). Selection of adequate areas to construct the tissue microarray. Months 24-27.
So far, we have identified 60 cases of locally advanced breast cancer, of which 30 are inflammatory breast cancers, and 30 are stage matched, non-inflammatory breast cancer were identified from the pathology files. We evaluated them histologically and chose the areas to construct a TMA

In addition to the IBC cases from the UM, we have obtained 30 IBC samples from Egypt, through a collaboration with Dr. Amr Soliman (School of Public Health, UM). Interestingly, we found epidemiologic and histopathological differences in US IBC cases and Egyptian IBC cases. Specifically, age at diagnosis was significantly younger in Egyptian cases [mean 46.9±standard deviation (SD) 10.8] than U.S. cases (58.8±13.5) (P= 0.01). Peau d'orange was more common (P=0.02) and number of tumor emboli was remarkably higher (P=0.0003) in IBC from Egypt when compared to the U.S.

d. Development of the tissue microarray, and immunohistochemical analysis of RhoC-GTPase, WISP3 and other biomarkers (ER, PR, HER2/neu, Ki-67, microvessel density and apoptosis). Months 28-33.
We have constructed a TMA with these tissues, and stained them for RhoC, ER, PR and HER-2/neu. We have stained the TMA for WISP3 as well and are in the process of evaluating the immunohistochemical results.

We have stained the 30 IBC cases from Egypt using the anti-RhoC antibody and also using a marker for lymphatic vessels (LYVE-1).

e. Interpretation and grading of the immunohistochemical stains and statistical analyses. Months 33- 36.
RhoC, ER, PR and Her-2/neu stains have been evaluated and analyzed in conjunction with Task 1. We are in the process of interpreting the immunohistochemical results for WISP3 staining in this group as well. Once this is performed, we will analyze the value of RhoC and WISP3 expression in predicting response to therapy in this group of tumors.

In addition, in the IBC cases from Egypt, we found that RhoC is highly expressed. LYVE-1-positive lymphatic invasion was observed in 46% and 50% of the IBC tumors from Egypt and the U.S., respectively. A high level of RhoC overexpression was observed in 84% of IBC tumors from Egypt and 13% from the U.S. (P=0.0001). In summary, these exciting new data suggest that IBC patients from Egypt present with graver clinical signs and distinct molecular characteristics when compared to the U.S. The higher number of tumor emboli and the further elevated level of RhoC expression might contribute to the dismal phenomenon of IBC commonly seen in Egypt. This work was presented the AACR meeting on 2006, and is the subject of a manuscript in preparation.

Task 3. To study in detail the *in vivo* effect of WISP3 loss in modulating the response of invasive breast carcinomas with RhoC-GTPase over-expression to farnesyl transferase inhibitors. Months 24-36.

a. Prepare a panel of cell lines (SUM149 wt, SUM149/WISP3, HME/RhoC, SUM185 wt and MCF10AT wt). Since all these cell lines have been prepared in our preliminary work, getting them ready for injection with take approximately 3 weeks. Month 25-26.
b. In vivo mice experiment (injection of cell lines, tumor development and treatment with farnesyl transferase inhibitor). Months 27-30.
c. Histological and immunohistochemical study of the excised tumors stained with anti-RhoC and anti-WISP3 antibodies. Months 30-32.
d. Analysis of the immunostains. Months 32-34.
e. Statistical analyses. Months 34-36.

We have not yet initiated the experiments in Task 3. We have been very concerned with the role of RhoC and WISP3 as markers of prognosis and response to therapy in breast cancer given the urgent need for novel biomarkers in the clinic. We have also devoted our attention to elucidating the mechanism of action of these genes. The experiments proposed in Task 3 will commence this year.

<u>In addition to the Tasks we have contributed to the following projects:</u>

1. We have performed seminal work in understanding WISP3 function, and how WISP3 and RhoC may cooperate to determine a highly aggressive inflammatory breast cancer phenotype, which we have published (Kleer et al, WISP3 and RhoC guanosine triphosphatase cooperate in the development of inflammatory breast cancer. *Breast Cancer Res*. 2004;6(1):R110-5). In this work, we found that antisense inhibition of WISP3 in HME cells increased RhoC mRNA and resulted in an increase in cellular proliferation, anchorage independent growth and VEGF levels in the conditioned media. Conversely, restoration of WISP3 expression in the highly malignant IBC cell line, SUM149, was able to decrease the expression of RhoC protein. This provides further evidence in support that these two genes act in concert to give rise to the highly aggressive IBC phenotype. We propose a model of this interaction as a starting point for further investigations. This manuscript is included in the appendix.

2. We have made an important contribution by elucidating that WISP3 is a secreted protein and that it modulates IGF signaling. This work is seminal, as no other tumor suppressor gene has ever been defined specifically for Inflammatory Breast Cancer. Previously, we have demonstrated that WISP3

has tumor suppressor functions in IBC (Kleer et al, *Oncogene*, 21, 3172-3180, 2002), and we have gained insight into WISP3 as a modulator of IGF signaling. This work was presented at the AACR meeting in Washington DC, July, 2003 as an oral presentation, and has been recently published and is included in the appendix. (Kleer et al, *Neoplasia* 2004 Mar-Apr;6(2):179-85).

Briefly, in this work we found that WISP3 is secreted into the conditioned media and into the lumens of normal breast ducts. Once secreted, WISP3 was able to decrease, directly or through induction of other molecule(s), the IGF-1-induced activation of the IGF-IR, and two of its main downstream signaling molecules, IRS1 and ERK-1/2 in SUM149 IBC cells. Furthermore, WISP3 containing conditioned media decreased the growth rate of SUM149 cells. This work sheds light into the mechanism of WISP3 function by demonstrating that it is secreted, and that once in the extracellular media it induces a series of molecular events that lead to modulation of IGF-IR signaling pathways and cellular growth in IBC cells.

3. We have established for the first time a stable siRNA and shRNA inhibition of WISP3 expression in human mammary epithelial cells (HME) and characterized its functions. Surprsingly, we found that WISP3 inhibition resulted in epithelial to mesenchymal transition (EMT) of HME cells and induced motility and invasion. Moreover, these cells were more sensitive to the growth and proliferative effects of IGF-1 in the medium. These experiments suggest that WISP3 may be a key a regulator of IGF-1 effects in HME cells. We have published this work (Zhang et al, *Breast Cancer Research* 7(6):R1080-9, 2005).

We have recently found that lentiviral silencing of WISP3 in HME cells decreases E-cadherin through transcriptional repression, mainly by regulating the levels of Snail, a key repressor of E-cadherin. These results have been presented at the Epithelial to Mesenchymal transition meeting in Vancouver, 2005, and are part of a manuscript in preparation in our laboratory. A review article published this year in a special Epithelial to Mesenchymal Transition Issue of *Cells, Tissues and Organs* is included in the appendix (Kleer et al, *CTO* Vol. 185, No. 1-3, pp 95 - 99 , 2007).

5. We have identified that EZH2 is a marker of aggressive breast cancer and that it promotes the neoplastic transformation of human mammary epithelial cells (Kleer CG, *Proceedings of the National Academy of Sciences*, 100(20): 11606-11, 2003). For this study, we used the tissue microarrays constructed and stained them using a polyclonal antibody for EZH2, a transcriptional repressor. We found that EZH2 expression was an independent factor that predicts death from breast cancer. We have included a copy reprint of this paper in the appendix section.

We have also investigated the mechanism of action of EZH2 (Zeidler et al, *Neoplasia* 7(11):1011-9, 2005. Featured article and Cover), and discovered that is a promising biomarker of preneoplastic progression in the breast (Ding et al, *Cancer Research* 66(8):4095-9, 2006. Selected as a Cancer Research Highlight).

6. I have embarked on several fruitful collaborations with other members of the Breast Oncology Program at UM, and at other institutions.

In summary, this Award helped us complete several manuscripts dealing with key aspects of WISP3 and RhoC expression in breast cancer. We have developed key reagents and resources that will enable

us to move forward in testing their clinical usefulness. We have also completed a major effort in understanding the function of WISP3 gene as it contributes to the inflammatory breast cancer phenotype.

Key Research Accomplishments

- Constructed four high density tissue microarrays
- Developed a relational database with the patient information
- Generated and tested a polyclonal antibody against WISP3
- Validation of RhoC as a novel tissue biomarker that predicts local recurrence and survival in patients with breast cancer.
- Investigated the mechanisms of cooperation between RhoC and WISP3 in determining the inflammatory breast cancer phenotype.
- Elucidated that WISP3 is a secreted protein and that it modulates IGF-I signaling cascade in inflammatory breast cancer
- Discovered that WISP3 inhibition in HME cells leads to epithelial to mesenchymal transition, and triggers invasion and motility.
- Discovered that EZH2 is a marker of aggressive breast cancer and that it promotes neoplastic transformation of mammary epithelial cells.

Training component of the Award

During the period of the Career Development Award, I have obtained protected time from my clinical responsibilities and established my research laboratory at the University of Michigan. I became an appreciated and active member of the Breast Oncology Program. I have also become the Director of the Breast Pathology Fellowship Program at UM, a member of the Execute Committee of the Molecular and Cellular Pathology Graduate Program at UM, and have been invited as an ad hoc reviewer to the DOD BCRP and the tumor progression and metastasis study section of the NCI. I have also been promoted to the rank of Associate Professor of Pathology with tenure at UM. All of these accomplishments are a direct result of the DOD Career Development Award.

Reportable Outcomes

We are in a position to report that RhoC over expression is an early marker of aggressive breast cancers, even when they are small, and that it is a promising marker of prognosis and local recurrence in patients with breast cancer.

We can state that WISP3 is able to ameliorate the highly malignant features of inflammatory breast cancer. Specifically, WISP3 has growth and angiogenic inhibitory functions, at least in part though modulating IGF-receptor signaling pathways.

We can state that EZH2 is a marker of aggressive breast cancer, and that it can predict prognosis.

Research Manuscripts published for the period of the Career Development Award (2002-2007):

Kleer CG, van Golen KL, Zhang Y, Wu Z-F, Rubin MA, Merajver SD.Characterization of RhoC Expression in Benign and Malignant Breast Disease: A Potential New Marker for Small Breast Carcinomas with Metastatic Potential. *Am J of Pathol*. 160(2), 579-584, 2002.

Kleer CG, Zhang Y, Pan Q, van Golen KL, Wu Z-F, and Merajver SD. WISP3 Isa Novel Tumor Suppressor Gene of Inflammatory Breast Cancer. *Oncogene* 21, 3172-3180, 2002.

Zhou M, Chinnaiyan AM, Kleer CG, Lucas PC and Rubin MA. Alpha-Methylacyl-CoA racemase: a novel tumor marker overexpressed in several human cancers and their precursor lesions. *Am J Surg Pathol*. 26(7): 926-931, 2002.

Kleer CG, Tseng MD, Gutsch DE, Rochford AR, Wu Z-F, Joynt LK, Helvie MA, Chang T, van Golen KL, Merajver SD. Epstein-Barr virus is implicated in the pathogenesis of breast fibroadenomas in immunocompromised hosts. *Modern Pathology* 15(7):759-764, 2002.

Pan Q, Kleer CG, van Golen KL, Irani J, Bottema KM, Bais C, De Carvalho M, Mesri EA, Robins DM, Dick R, Brewer GJ and Merajver SD. Copper deficiency induced by tetrathiomolybdate suppresses tumor growth and angiogenesis. *Cancer Research* 62(17):4854-9, 2002.

Valdez R, Thorson J, Finn WG, Schnitzer B, and Kleer CG. Lymphocytic Mastitis/Diabetic Mastopathy: A Molecular, Immunophenotypic, and Clinicopathologic Evaluation of Eleven Cases. *Modern Pathology* 16: 223-228, 2003.

Rao DS, Bradley SV, Kumar PD, Hyun TS, Saint-Dic D, Oravecz-Wilson K, Kleer CG, and Ross TS. Altered receptor trafficking in Huntingtin Interacting Protein 1-transformed cells. *Cancer Cell* 3: 471-482, 2003.

Sabel MS, Schott AF, Kleer CG, Merajver SD, Cimmino V, Diehl KM, Hayes DF, Chang A, Pierce LJ. Sentinel Node Biopsy Prior to Neoadjuvant Chemotherapy. *American Journal of Surgery* 186:102-105, 2003.

Pan Q, Bao LW, Kleer CG, Brewer GJ, and Merajver SD. Antiangiogenic tetrathiomolybdate enhances doxorubicin therapy against breast carcinoma. *Molecular Cancer Therapeutics* 2:617-622, 2003.

Collisson EA, Kleer CG, Wu M, De A, Gambhir SV, Merajver SD, Kolodney MS. Atorvastatin Prevents RhoC Isoprenylation, Invasion and Metastasis in Human Melanoma Cells. *Molecular Cancer Therapeutics*, 2:941-948, 2003.

Kleer CG, Cao Q, Varambally S, Shen R, Ota I, Tomlins SA, Ghosh D, Sewalt RG, Otte AP, Hayes DF, Sabel MS, Livant D, Weiss SJ, Rubin MA and Chinnaiyan AM. EZH2 is a Marker of Aggressive Breast Cancer and Promotes Neoplastic Transformation of Breast Epithelial Cells. *Proceedings of the National Academy of Sciences,* 100(20):11606-11, 2003.

Kowalski PJ, Rubin MA and Kleer CG. E-Cadherin Expression in Primary Carcinomas of the Breast and its Distant Metastases. *Breast Cancer Research*, 5:R217-R222, 2003.

Kleer CG, Zhang Y, Pan Q, Wolf J, Wu M, Wu Z-F, Merajver SD. WISP3 and RhoC-GTPase Cooperate in the Development of Inflammatory Breast Cancer. *Breast Cancer Research* 6(1): R110-5, 2004.

Ray ME, Yang ZQ, Albertson D, Kleer CG, Washburn JG, Macoska JA, Ethier SP. Genomic and Expression Analysis of the 8p11-12 Amplicon in Human Breast Cancer Cell Lines. *Cancer Research* 64(1):40-47, 2004.

Van Den Eynden GG, Van Der Auwera I, Van Laere S, Colpaert CG, Van Dam P, Merajver S, Kleer CG, Harris AL, Van Marck EA, Dirix LY, Vermeulen PB. Validation of a tissue microarray to study differential protein expression in inflammatory and non-inflammatory breast cancer. *Breast Cancer Res Treat.* 85(1):13-22, 2004.

Kleer CG, Zhang Y, Pan Q, Merajver SD. WISP3 is a Secreted Tumor Suppressor Protein that Modulates IGF Signaling in Inflammatory Breast Cancer. *Neoplasia,* 6(2):179-85, 2004.

Koker M and Kleer CG. P63 Expression in Breast Cancer: A Highly Sensitive and Specific Marker of Metaplastic Carcinoma. *Am J Surg Pathol,* 28(11):1506-12, 2004.

Marcus B., Arenberg D., Kleer C.G., Chepeha D., Schmalbach C., Pan Q., Hanash S, Kuick R., Lee J., Merajver SD, Teknos TN. Prognostic Factors in Oral Cavity and Oropharyngeal Squamous Cell Carcinoma: The Impact of Tumor-Associated Macrophages. *Cancer*, 101(12):2779-87, 2004.

Roubidoux MA, Sabel MS, Bailey JE, Kleer CG, Klein KA, Helvie MA. Small (<2.0-cm) Breast Cancers: Mammographic and US Findings at US-guided Cryoablation – Initial Experience. *Radiology*, 233(3):857-67, 2004.

Stearns V, Gallagher MA, Kleer CG, Singh B, Freedman M, Haddad B, Isaacs C, Warren R, Brown M, Trock B and Hayes DF. A Pilot Study to Establish a Clinical Model to Perform Phase II Studies of Breast Cancer Chemopreventive Agents in Women at High Risk Using Biomarkers as Surrogate Endpoints for Activity. *Clin Cancer Res*, 10(24):8332-40, 2004.

Pu RT, Schott AF, Sturtz DE, Griffith KA, and Kleer CG. Pathologic Features of Breast Cancer Associated With Complete Response to Neoadjuvant Chemotherapy: Importance of Tumor Necrosis. *Am J Surg Pathol*, 29(3):354-58, 2005.

Witniewicz A, Shen R, Lnu S, Mehra, R, Chinnaiyan AM, Sabel MS, Rubin MA, and Kleer CG. Alpha Methylacyl-CoA Racemase (AMACR) Protein Expression is Associated with the Degree of Differentiation in Breast Cancer Using Quantitative Image Analysis. *Breast Cancer Biomarkers, Epidemiology and Prevention*, 14(6):1418-23, 2005. Cover Article.

Khan, A., Sabel, M.S., Nees, A., Diehl, K.M., Cimmino, V.M., Kleer, C.G., Schott, A.F., Hayes, D.F., Chang, A.E., and Newman, L.A. Comprehensive Axillary Evaluation in Neoadjuvant Chemotherapy Patients with Ultrasonography and Sentinel Lymph Node Biopsy. *Annuals of Surgical Oncology* 12(9):1-8, 2005.

Schott, A.F., Roubidoux, M.A., Helvie, M.A., Hayes, D.F., Kleer, C.G., Newman, L.A., Pierce, L.J., Griffith, K.A., Murray, S., Hunt, K.A., Paramagul, C., and Baker, L.H. Clinical and Radiologic Assessments to Predict Breast Cancer Pathologic Complete Response to Neoadjuvant Chemotherapy. *Breast Cancer* 92(3):231-8, 2005.

Kleer, C.G., Griffith, K., Sabel, M.S., Van Golen, K.L., Gallagher, G., Wu, Z.F., and Merajver, S.D. RhoC-GTPase is a Novel Tissue Biomarker Associated with Biologically Aggressive Carcinomas of the Breast. *Breast Cancer Research and Treatment* 93(2):101-10, 2005.

Pan, Q., Bao, L.W., Kleer, C.G., Sabel, M., and Merajver, S.D. Protein Kinase C is Elevated in High Grade Breast Cancer and a Novel Target for RNA interference Anticancer Therapy. *Cancer Research* 65(18):8366-71, 2005.

Zeidler, M., Varambally, S., Cao, Q., Chinnaiyan, A.M., Ferguson, D.O., Merajver, S.D., and Kleer, C.G. The Polycomb Group Protein EZH2 Impairs DNA Repair in Human Mammary Epithelial Cells. *Neoplasia* 7(11):1011-9, 2005. Featured article and Cover.

Zhang, Y., Pan, Q., Zhong, H., Merajver, S.D., and Kleer, C.G. Inhibition of CCN6 (WISP3) Expression Promotes Neoplastic Transformation and Enhances the Effects of IGF-1 on Breast Epithelial Cells. *Breast Cancer Research* 7(6):R1080-9, 2005.

Mehra, R., Varambally, S., Shen, R., Ding, L., Sabel, M.S., Ghosh, D., Chinnaiyan, A.M.*, and Kleer, C.G*. Identification of GATA3 as a Breast Cancer Prognostic Marker by Global Gene Expression Meta-Analysis. *Cancer Research* 65(24):11259-64, 2005.

Krop, I., März, A., Carlsson, H., Li, X., Bloushtain-Qimron, N., Hu, M., Gelman, R., Sabel, M.S., Schnitt, S., Ramaswamy, S., Kleer, C.G., Enerbäck, C., and Polyak, K. A Putative Role for Psoriasin in Breast Tumor Progression. *Cancer Research* 65(24):11326-34, 2005.

Paramagul CP, Helvie MA, Robidoux MA, Kleer CG, Newman LA. Lobular carcinoma in situ (LCIS): Clinical and mammography findings of subsequent breast cancers. *Journal of Women's Imaging* 7(4):181-189, 2005.

Ben-David, M.A., Kleer, C.G., Paramagul, C., Griffith, K.A., and Pierce, L.J. Is LCIS a Component of Breast Cancer a Risk Factor for Local Failure Following Breast- Conserving Therapy? Results of a Matched Pair Analysis. *Cancer* 106(1):28-34, 2006.

O'Malley, F.P., Mohsin, S.K., Badve, S., Bose, S., Collins, L.C., Ennis, M., Kleer, C.G., Pinder, S.E., and Schnitt, S.J. interobserver Reproducibility in the Diagnosis of Flat Epithelial Atypia of the Breast. *Modern Pathology* 19(2):172-9, 2006.

Newman, E.L., Kahn, A., Diehl, K.M., Cimmino, V.M., Kleer, C.G., Chang, A.E., Newman, L.A., and Sabel, M.S. Does the Method of Biopsy Affect the Incidence of Sentinel Lymph Node Metastases? *The Breast Journal* 12(1):53-57, 2006.

Ding, L., Erdmann, C., Chinnaiyan, A.M., Merajver, S.D., and Kleer, C.G. Identification of EZH2 as a Molecular Marker for a Precancerous State in Morphologically Normal Breast Tissues. *Cancer Research* 66(8):4095-9, 2006. Selected as a Cancer Research Highlight.

Hird, R.B., Chang, A., Cimmino, V., Diehl, K., Sabel, M., Kleer, C.G., Helvie, M., Schott, A., Young, J., Hayes, D., Newman, L. Impact of estrogen receptor expression and other clinico pathologic features on tamoxifen use in ductal carcinoma in situ. *Cancer* 15;106:2113-8, 2006.

Kuefer, R., Day, K.C., Kleer, C.G., Sabel, M.S., Hofer, M.D., Varambally, S., Zorn, C.S., Chinnaiyan, A.M., Rubin, M.A., Day, M.L. The ADAM15 disintegrin is associated with aggressive prostate and breast cancer disease. *Neoplasia* 8(4): 319-329, 2006.

Newman, EA, Guest AB, Helvie MA, Roubidoux MA, Chang AE, Kleer CG, Diehl KM, Cimmino VM, Pierce L, Hayes D, Newman LA, Sabel MS. Changes in surgical management resulting from case review at a breast cancer multidisciplinary tumor board. *Cancer*, 22;107(10):2346-2351, 2006.

Kleer, C.G., Teknos, T.N., Islam, M., Marcus, B., Lee, J.S.J., Pan, Q., and Merajver, S.D. RhoC-GTPase Expression as a Potential Marker of Lymph Node Metastasis in Squamous Cell Carcinomas of the Head and Neck. *Clinical Cancer Res*, 12(15):4485-90, 2006.

Ding L, and Kleer CG. Enhancer of zeste 2 as a Marker of Preneoplastic Progression in the Breast. *Cancer Res*. 66(19)9352-5, 2006.

Zeidler M, and Kleer CG. The Polycomb Group Protein Enhancer of Zeste 2: Its Links to DNA Repair and Breast Cancer. *J Mol Histol*. 37(5-7):219-223, 2006

Kunju L, and Kleer CG. Significance of Flat Epithelial Atypia on Mammotome Core Needle Biopsy: Should it be Excised? *Hum Pathol.* 38(1):35-41, 2007

Maturen, K.E., Paramagul, C.P., Roubidoux, M.A., Kleer, C.G., Weadock W.J., Abate, S.A. Interactive Computer Teaching Module for Radiologic-Pathologic Correlations in Breast Imaging. *MedEdPORTAL*, In press.

Ostrander J.H., Daniel A.R., Lofgren K, Kleer CG and Lange CA. Breast Tumor Kinase (Brk/PTK6) Regulates Heregulin-Induced Activation of Erk5 and p38 MAP Kinases in Breast Cancer Cells. *Cancer Research* 67(9):4199-209, 2007.

Kleer CG, Zhang Y, Merajver SD. CCN6 (WISP3) as a New Regulator of the Epithelial Phenotype in Breast Cancer. *Cells Tissues Organs.* Vol. 185, No. 1-3, pp 95 - 99 , 2007

Privette LM, Gonzalez ME, Ding L, Kleer CG and Petty EM. Altered Expression of the Early Mitotic Checkpoint Gene, *CHFR*, in Breast Cancers: Implications for Tumor Suppression. *Cancer Research*, In press.

McHugh JB, Fullen DR, Ma L, Kleer CG, Su LD. Expression of polycomb group protein EZH2 in nevi and melanoma. *Journal of Cutaneous Pathology*. In press.

Abstracts presented at scientific meetings during the period of the Award (2002-2007):

Kleer, C.G. and Rubin, M.A. Increased Expression of α-methyl acyl-CoA racemase (AMACR) is Associated with Tumor Size and Lymph Node Metastases in Breast Cancer. Presented at the IAP meeting, Chicago, IL, Feb. 2002.

Valdez, R., Thorson, J., Finn, W.G., Schnitzer, B., and Kleer, C.G. Lymphocytic Mastitis/Diabetic Mastopathy: A Molecular, Immunophenotypic and Clinicopathologic Evaluation of Eleven Cases. Presented at the IAP meeting, Chicago, IL, Feb. 2002.

Kleer, C.G. and Afify, A.M. HER-2/neu Over-Expression: Comparison between Three Immunohistochemical Assays. Presented at the IAP meeting, Chicago, IL, Feb. 2002.

Kleer, C.G., Van Golen, K.L., Zhang, Y., Wu, Z-F, Rubin, M.A., and Merajver, S.D. RhoC Expression in Benign and Malignant Breast Disease: A Promising Marker of Metastatic Potential in Small Tumors. Presented at the AACR meeting, San Francisco, CA, Apr. 2002.

Pan, Q., Kleer, C.G., Van Golen, K.L., Irani, J., Bottema, K.M., Bais, C., De Carvalho, M., Mesri, E.A., Robins, D.M., Dick, R., Brewer, G.J., and Merajver, S.D. Copper Deficiency Induced by Tetrathiomolybdate Suppresses Tumor Growth and Angiogenesis. Presented at the AACR meeting, San Francisco, CA, Apr. 2002.

Kleer, C.G., Van Golen, K.L., Zhang, Y., Wu, Z-F, Rubin, M.A., and Merajver, S.D. RhoC Expression in Benign and Malignant Breast Disease: A Promising marker of Metastatic Potential in Small Tumors. ASCO meeting, Orlando, FL, May 18, 2002.

Kleer, C.G., Chang, T., Pan, Q., Rubin, M.A., Merajver, S.D. Characterization of VEGF-C Expression in Breast Tissues Using High Density Tissue Microarrays. *Histopathology* 41(1): 1-63. Oral presentation, International Academy of Pathology, Amsterdam, 2002.

Sturtz, D.E., Schott, A.F., and Kleer, C.G. Pathologic Features of Breast Cancer Associated with Complete Response to Neoadjuvant Chemotherapy: Importance of Histologic Type and Grade. Platform presentation, USCAP meeting, Washington DC, 2003.

Kleer, C.G., Zhang, Y., Pan, Q., Merajver, S.D. Characterization of WISP3, A Novel Tumor Suppressor Gene for Inflammatory Breast Cancer, in Normal Breast and Breast Disease. Platform presentation, USCAP meeting, Washington DC, 2003.

Kleer, C.G., Shen, R., Wolf, J., and Rubin, M.A. Characterization of Platelet Derived Growth Factor Receptor Expression in Breast Cancer Identifies Inflammatory Breast Cancer as a Potential Target for Treatment with PDGF-b Inhibitors. Poster presentation, USCAP meeting, Washington DC, 2003.

Kleer, C.G., Shen, R., Chinnaiyan, A.M., and Rubin, M.A. EZH2 is Overexpressed During Breast Cancer Progression and is a Predictor of Poor Outcome in Patients with Breast Cancer. Poster presentation, USCAP meeting, Washington DC, 2003.

Kleer, C.G., Zhang, Y., Pan, Q., and Merajver, S.D. WISP3 is a Secreted Protein and Modulates IGF Signaling in Inflammatory Breast Cancer. Mini symposium AACR Meeting, Washington DC, July 11-14, 2003.

Koker, M. and Kleer, C.G. Metaplastic Carcinoma of the Breast: P63 is a Highly Sensitive and Specific Marker. Platform presentation, USCAP Meeting, Vancouver, BC, 2004.

Koker M., and Kleer, C.G. Smooth Muscle Actin and p63 in the Diagnosis of Difficult Lesions of the Breast. Poster presentation, USCAP Meeting, Vancouver, BC, 2004.

Koker, M., Griffith K., Newman, L., Sabel, M., Rubin, M.A., and Kleer, C.G. Pathologic Factors Predictive of Sentinel Lymph Node Metastasis in Patients with Breast Cancer. Poster presentation, USCAP Meeting, Vancouver, BC, 2004.

Witkiewicz, A., Rubin, M.A., and Kleer, C.G. AMACR Expression in Breast Adenocarcinomas. Poster presentation, USCAP Meeting, Vancouver, BC, 2004.

Kleer, C.G., Cao, Q., Varambally, S., Shen, R., Ota, I., Tomlins, S.A., Ghosh, D., Sewalt, R.G., Otte, A.P., Hayes, D.F., Sabel, M.S., Livant, D., Weiss, S.J., Rubin, M.A., and Chinnaiyan, A.M. EZH2 is a Marker of Aggressive Breast Cancer and Promotes Neoplastic Transformation of Breast Epithelial Cells. Platform presentation, USCAP Meeting, Vancouver, BC, 2004.

Kleer, C.G., Griffith, K., Sabel, S., Gallagher, G., Merajver, S.D. RhoC GTPase is a New Tissue Biomarker Predictive of Local Recurrence in Patients with Breast Cancer. Poster presentation, AACR meeting, Orlando, FL, 2004.

Zhang, Y., Monroe, S., Merajver, S.D., and Kleer, C.G. Inhibition of WISP3 (CCN6) Promotes the Neoplastic Transformation and Enhances the Effects of IGF-1 on Breast Epithelial Cells. Pathology Research Symposium, University of Michigan Pathology Graduate Program, Nov. 2004.

Kleer, C.G., Griffith, K., Sabel, M.S., Van Golen, K.L., Gallagher, G., Wu, Z.F., and Merajver, S.D. RhoC-GTPase is a Novel Tissue Biomarker Associated with Biologically Aggressive Carcinomas of the Breast. Poster Discussion Session, Breast Cancer Symposium, San Antonio, TX, Dec. 8-11, 2004.

Mehra, R., Chinnaiyan, A.M., and Kleer, C.G. GATA3 as a Novel Prognostic Marker for Breast Cancer. Platform presentation, USCAP meeting, San Antonio, TX, Feb 28- Mar 3, 2005.

Mohsin, S.K., Badve, S., Bose, S., Kleer, C.G., Pinder, S.E., and O'Malley, F. Assessment of Variability in Diagnosing 'Atypia' in Columnar Cell Lesions (CCL) of the Breast. Poster Session, USCAP meeting, San Antonio, TX, Feb 28-Mar 3, 2005.

Mehra, R., Varambally, S., Poisson, L.M., Rhodes, D.R., Ghosh, D., Chinnaiyan, A.M., and Kleer, C.G. Overexpression of Minichromosome Maintenance Protein 2 is Associated with Tumor Aggressiveness and Outcome in Breast Cancer. Platform presentation, USCAP meeting, San Antonio, TX, Feb 28- Mar 3, 2005.

Zhang, Y., Monroe, S., Merajver, S.D., and Kleer, C.G. Inhibition of WISP3 (CCN6) Promotes the Neoplastic Transformation and Enhances the Effects of IGF-1 on Breast Epithelial Cells. Era of Hope Meeting, Department of Defense Breast Cancer Research Program, Poster presentation, Philadelphia, PA, June 8-11, 2005.

Zhang, Y., Pan, Q., Zhong H., Merajver, S.D., and Kleer, C.G. Inhibition of WISP3 (CCN6) Promotes a Mesenchymal Phenotype and Enhances the Effects of IGF-1 on Breast Epithelial Cells. Epithelial Mesenchymal Transition (EMT) Conference. Vancouver, BC, Canada, October 1-3, 2005. Poster Presentation.

Wei, I.*, Pu, R.*, Zhang, Y., Merajver, S.D., and Kleer, C.G. Analysis of WISP3 Promoter Methylation in Inflammatory Breast Cancer. Student Biomedical Research Forum, University of Michigan Medical School, Ann Arbor, MI, Nov. 2005.

Hayes, M.J. and Kleer, C.G. Expression of the Undifferentiated Cell Marker P63 in Primary invasive Carcinomas of the Breast and Their Nodal Metastases. Laboratory Investigation 86(1): 29A. Presented at USCAP meeting, Atlanta, GA, Feb. 2006.

Kunju, L.P. and Kleer, C.G. Significance of Flat Epithelial Atypia (FEA) on Mammotome Core Needle Biopsy: Should It Be Excised? Laboratory Investigation 86(1): 32A. Presented at USCAP meeting, Atlanta, GA, Feb. 2006.

Zeidler, M., Varambally, S., Cao, Q., Chinnaiyan, A.M., Ferguson, D.O., Merajver, S.D., and Kleer, C.G. The Polycomb Group Protein EZH2 Impairs DNA Repair in Human Mammary Epithelial Cells. AACR meeting, April 1-5, 2006, Washington DC. Poster Presentation.

Wei, I.*, Pu, R.*, Zhang, Y., Merajver, S.D., and Kleer, C.G. Analysis of WISP3 Promoter Methylation in Inflammatory Breast Cancer. XXVI Congress of the International Academy of Pathology, Montreal, Sept. 16-21, 2006.

Hayes, MJ, Witkiewicz, A, Kleer, C.G. Aberrant b-Catenin Expression is Associated with Morphology and Prognosis in Metaplastic Mammary Carcinomas. United States and Canadian Academy of Pathology, San Diego, CA, March 24-30, 2007.

Kunju, LP, Kleer, C.G. Carcinoma and Grade 1 (Well Differentiated) Invasive Ductal Carcinoma: Comparision of Associated Flat Epithelial Atypia and Other Intra-Epithlial Lesions. United States and Canadian Academy of Pathology, San Diego, CA, March 24-30, 2007.

Lo, AC., Soliman, AS., Kleer, C.G, Alford, SH., Eissa, S., Hablas, A., Khaled, HM., Omar, S., Merajver, S. Molecular Epidemiologic Features of Inflamitory Breast Cancer: A Comparison Between Egyptian and U.S. Patients. AACR meetingApril 14-18, 2007, Los Angeles, CA.

Georgopolis, A., Soliman, A., Kleer, CG., Lo, AC., Eissa, S., Khaled, H., Omar, S., Merajver, SD. Molecular Differences Between Inflammatory Breast Cancers in Egyptian Patients. AACR meeting, April 14-18, 2007, Los Angeles, CA.

Conclusion:
We are encouraged by our progress in all areas. We want to move forward and test the clinical utility of WISP3 and in combination with RhoC and other markers, in detecting aggressive breast cancer phenotypes before they develop metastases. We also wish to explore the relationship between WISP3 and the IGF-receptor pathway in more depth. These are the directions we are moving on for this year.

References:
Manley, S., Mucci, N.R., De Marzo, A.M. & Rubin, M.A. Relational database structure to manage high-density tissue microarray data and images for pathology studies focusing on clinical outcome: the prostate specialized program of research excellence model. Am J Pathol 159, 837-43, 2001

Kleer CG, Zhang Y, Pan Q, van Golen KL, Wu Z-F, and Merajver SD. WISP3 Is a Novel Tumor Suppressor Gene of Inflammatory Breast Cancer. Oncogene 21, 3172-3180, 2002.

Kleer CG, van Golen KL, Zhang Y, Wu Z-F, Rubin MA, Merajver SD.
Characterization of RhoC Expression in Benign and Malignant Breast Disease: A Potential New Marker for Small Breast Carcinomas with Metastatic Potential. Am J of Pathol. 160(2), 579-584, 2002.

Valdez R, Thorson J, Finn WG, Schnitzer B, and Kleer CG. Lymphocytic Mastitis/ Diabetic Mastopathy: A Molecular, Immunophenotypic, and Clinicopathologic Evaluation of Eleven Cases. Modern Pathology 16: 223-228, 2003.

Kleer CG, Cao Q, Varambally S, Shen R, Ota I, Tomlins SA, Ghosh D, Sewalt RG, Otte AP, Hayes DF, Sabel MS, Livant D, Weiss SJ, Rubin MA and Chinnaiyan AM. EZH2 is a Marker of Aggressive Breast Cancer and Promotes Neoplastic Transformation of Breast Epithelial Cells. Proceedings of the National Academy of Sciences, 100(20):11606-11, 2003.

Kowalski PJ, Rubin MA and Kleer CG. E-Cadherin Expression in Primary Carcinomas of the Breast and its Distant Metastases. Breast Cancer Research, 5:R217-R222, 2003.

Kleer CG, Zhang Y, Pan Q, Wolf J, Wu M, Wu Z-F, Merajver SD. WISP3 and RhoC-GTPase Cooperate in the Development of Inflammatory Breast Cancer. Breast Cancer Research 6(1): R110-5, 2004.

Ray ME, Yang ZQ, Albertson D, Kleer CG, Washburn JG, Macoska JA, Ethier SP. Genomic and Expression Analysis of the 8p11-12 Amplicon in Human Breast Cancer Cell Lines. Cancer Research 64(1):40-47, 2004.

Van Den Eynden GG, Van Der Auwera I, Van Laere S, Colpaert CG, Van Dam P, Merajver S, Kleer CG, Harris AL, Van Marck EA, Dirix LY, Vermeulen PB. Validation of a tissue microarray to study differential protein expression in inflammatory and non-inflammatory breast cancer. Breast Cancer Res Treat. 85(1):13-22, 2004.

Kleer CG, Zhang Y, Pan Q, Merajver SD. WISP3 is a Secreted Tumor Suppressor Protein that Modulates IGF Signaling in Inflammatory Breast Cancer. Neoplasia, 2004 Mar-Apr;6(2):179-85.

APPENDIX- Selected Publications resulting directly from the Award

Kleer CG, Zhang Y, Pan Q, Merajver SD. WISP3 is a Secreted Tumor Suppressor Protein that Modulates IGF Signaling in Inflammatory Breast Cancer. Neoplasia, 6(2):179-85, 2004.

Kleer, C.G., Griffith, K., Sabel, M.S., Van Golen, K.L., Gallagher, G., Wu, Z.F., and Merajver, S.D. RhoC-GTPase is a Novel Tissue Biomarker Associated with Biologically Aggressive Carcinomas of the Breast. Breast Cancer Research and Treatment 93(2):101-10, 2005.

Zhang, Y., Pan, Q., Zhong, H., Merajver, S.D., and Kleer, C.G. Inhibition of CCN6 (WISP3) Expression Promotes Neoplastic Transformation and Enhances the Effects of IGF-1 on Breast Epithelial Cells. Breast Cancer Research 7(6):R1080-9, 2005.

Kleer, C.G., Teknos, T.N., Islam, M., Marcus, B., Lee, J.S.J., Pan, Q., and Merajver, S.D. RhoC-GTPase Expression as a Potential Marker of Lymph Node Metastasis in Squamous Cell Carcinomas of the Head and Neck. Clinical Cancer Res, 12(15):4485-90, 2006.

Kleer CG, Zhang Y, Merajver SD. CCN6 (WISP3) as a New Regulator of the Epithelial Phenotype in Breast Cancer. Cells Tissues Organs. Vol. 185, No. 1-3, pp 95 - 99 , 2007

Neoplasia • Vol. 6, No. 2, March/April 2004, pp. 179–185 179

www.neoplasia.com

WISP3 (CCN6) Is a Secreted Tumor-Suppressor Protein that Modulates IGF Signaling in Inflammatory Breast Cancer[1]

Celina G. Kleer, Yanhong Zhang*, Quintin Pan† and Sofia D. Merajver†*

Departments of *Pathology and †Internal Medicine, Division of Hematology and Oncology, University of Michigan Medical Center, Ann Arbor, MI, USA

Abstract

Inflammatory breast cancer (IBC) is the most lethal form of locally advanced breast cancer. We have found that WISP3 is lost in 80% of human IBC tumors and that it has growth- and angiogenesis-inhibitory functions in breast cancer *in vitro* and *in vivo*. WISP3 is a cysteine-rich, putatively secreted protein that belongs to the CCN family. It contains a signal peptide at the N-terminus and four highly conserved motifs. Here, for the first time, we investigate the function of WISP3 protein in relationship to its structural features. We found that WISP3 is secreted into the conditioned media and into the lumens of normal breast ducts. Once secreted, WISP3 was able to decrease, directly or through induction of other molecule(s), the IGF-1–induced activation of the IGF-IR, and two of its main downstream signaling molecules, IRS1 and ERK-1/2, in SUM149 IBC cells. Furthermore, WISP3 containing conditioned media decreased the growth rate of SUM149 cells. This work sheds light into the mechanism of WISP3 function by demonstrating that it is secreted and that, once in the extracellular media, it induces a series of molecular events that leads to modulation of IGF-IR signaling pathways and cellular growth in IBC cells.

Neoplasia (2004) **6**, 179–185

Keywords: IGF-binding proteins, MAPK signaling, cell proliferation, cell cycle control, ERK-1/2 phosphorylation.

Introduction

Inflammatory breast cancer (IBC) is the most lethal form of locally advanced breast cancer [1,2]. It is also a very distinct clinical and pathological type of carcinoma. Clinically, patients present with what has been classically termed ''peau d'orange,'' characterized by skin thickening and dimpling, also with nodularity, erythema, and, often, nipple retraction [1–4]. IBC is highly angiogenic and angioinvasive. Clusters of malignant cells invade the dermal lymphatics, forming tumor emboli that likely cause the clinical symptoms, and disseminate to distant sites [1].

In our previous work, we found that WISP3 is lost in 80% of human IBC tumors and is a key genetic determinant of the IBC phenotype [5]. WISP3 has growth-, invasion-, and angiogenesis-inhibitory functions in IBC *in vitro* and *in vivo* [6]. WISP3 is a member of the CCN family of proteins, which also includes connective tissue growth factor (CTGF), Cyr61, Nov, WISP1, and WISP2 [7,8]. A putatively secreted protein with a secretory signal peptide at the NH_2 terminus, WISP3 contains 36 conserved cysteine residues that are organized into four highly conserved modules: 1) a motif associated with insulin-like growth factor binding protein (IGFBP) (GCGCCXXC); 2) a von Willebrand type C–like motif; 3) a thrombospondin 1 module; and 4) a carboxyl-terminal domain putatively involved in dimerization [8,9]. The role of each of these conserved domains in the function of the CCN proteins, in general, and of WISP3, in particular, remains to be elucidated.

IGF-I and its major receptor, IGF-IR, play an important role in normal breast biology and in the development of breast cancer [10–13]. A large body of work implicates the IGF family in breast cancer progression. High concentrations of IGF-I 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 [14]. *In vitro*, IGF-I is a strong mitogen for human breast cancer cells and has been found in the epithelial and stromal component of breast cancers [13]. High expression of IGF-IR has been demonstrated in most primary human breast cancers when compared to normal or benign breast tissues, and hyperactivation of IGF-IR in breast cancer has been linked to increased radioresistance and cancer recurrence at the primary site [13,15,16]. High levels of IRS-1, a major signaling molecule downstream of the IGF-IR, correlate with tumor size and shorter disease-free survival in ER^+ breast cancer patients [17,18]. Based on the protein sequence of WISP3 and the important role of IGF signaling in breast cancer, we hypothesized that WISP3 is secreted into the extracellular medium and that the growth-inhibitory effect of

Address all correspondence to: Sofia D. Merajver, MD, PhD, and Celina G. Kleer, MD, Department of Internal Medicine, 7217 CCGC, 1500 East Medical Center Drive, Ann Arbor, MI 48108-0948, USA. E-mail: smerajve@umich.edu

[1]This work was supported, in part, by Army grants DAMD17-02-1-0490 (C.G.K.), DAMD17-02-1-491 (C.G.K.), and DAMD-17-00-1-0345 (S.D.M.); National Institutes of Health grants K08CAO9O876 (C.G.K.), RO1CA77612 (S.D.M.) and 1 P50-CADE97258 (S.D.M.); and a grant from the John and Suzanne Munn Endowed Research Fund of the University of Michigan Comprehensive Cancer Center (C.G.K.).

Received 5 September 2003; Revised 2 December 2003; Accepted 3 December 2003.

DOI 10.1593/neo.03316

WISP3 in IBC may be dependent, at least in part, on modulation of IGF-I signaling. To test this hypothesis, we investigated the downstream effects of WISP3 starting at the IGF-IR receptor and signaling pathway. Here, we demonstrate that WISP3 is a secreted protein and that, once in the conditioned media, it can effectively modulate IGF-IR activation and its signaling cascade and the cellular growth of IBC cells.

Materials and Methods

Cell Culture and Transfections

SUM149 cells derive from a primary IBC that has lost WISP3 expression [6,19]. SUM149 cells and their transfectants were cultured in Ham's F-12 media supplemented with 5% fetal bovine serum (FBS), hydrocortisone (1 μg/ml), insulin (5 μg/ml), fungizone (2.5 μg/ml), gentamycin (5 μg/ml), and penicillin/streptomycin (100 μ/ml each), at 37°C under 10% CO_2. HEK-293 cells derived from human embryonic kidney epithelial cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. SUM149 and HEK-293 cells were transfected with HIS^- tagged (pcDNA 3.1/V5-HIS TOPO TA expression vector; Invitrogen, Carlsbad, CA) and $Flag^-$ tagged (pFlag-CMV vector; Sigma, St. Louis, MO) full-length WISP3 cDNA, and clonal cell lines were established as described previously [6]. Control cell lines were generated by transfecting the SUM149 and HEK-293 cell lines with the empty vectors. The inserts were confirmed by sequencing. Cells were incubated in serum-free medium. WISP3 and control conditioned media were collected 3 days later. The conditioned media were cleared of cell debris by centrifugation, and concentrated 10-fold through a Centriplus YM-10 column (Millipore, Bedford, MA) before use.

IGF-I Stimulation

Seventy percent confluent SUM149 cells were shifted to serum-free medium. After 24 hours of starvation, the cells were cultured in WISP3 and control conditioned media for 24 hours. Subsequently, SUM149 cells were stimulated with 20 ng/ml human recombinant IGF-I (Upstate Biotechnology Inc., Lake Placid, NY) for 15 minutes.

Immunoprecipitation and Western Blotting

Cells were lysed in lysis buffer composed of 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM PMSF, and 1 μg/ml aprotinin. The lysates were clarified by centrifugation at 14,000*g* for 10 minutes. A total of 500 μg of cell lysates was incubated with 1 μg/ml anti–IGF-IR mAb (Calbiochem, San Diego, CA) overnight at 4°C. Immune complexes were precipitated by adding 50 μl of protein A/G plus agarose bead slurry for 2 hours. The agarose beads were collected and washed three times with ice-cold lysis buffer, and resuspended in 25 μl of 2 × Laemmli sample buffer for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Fifty micrograms of protein extract was separated by SDS-PAGE and transferred onto a PVDF membrane (Amersham Pharmacia Biotech). The precipitated IGF-IR was detected with anti–IGF-IR β subunit polyclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA). Tyrosine phosphorylation of immunoprecipitated IGF-IR was assessed with anti–phosphotyrosine mAB PY20 (Transduction Laboratories, Lexington, KY). Total IGF-IR, phosphorylated and total IRS1, and ERK-1/2 were measured with appropriate antibodies (Transduction Laboratories; Upstate Biotechnology Inc.). WISP3 expression was confirmed by Western blot using a polyclonal anti–WISP3 antibody (gift from Dr. Warman) and an antibody against the HIS tag (Invitrogen). The protein bands were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). All experiments were repeated at least three times and the optical density of the bands was quantified by densitometry (Scio Image software for Win 95/98, version 0.4). Statistical analysis was performed using 95% confidence intervals for the estimates of the means. A P value of $< .05$ was considered statistically significant.

Effect of WISP3 in the Proliferation of SUM149 cells

SUM149 cells were plated in 96-well tissue culture plates at a density of 5×10^4 cells/ml in Ham's F-12 media with 5% FBS. One hundred microliters of serum-free medium was added for 24 hours. Ten-fold concentrated WISP3 and control conditioned media were added in the presence and absence of IGF-I simulation as described above. MTT reagents were added 24 hours later according to the manufacturer's protocol (Sigma), and the plate was read at a wavelength of 595 nm. The experiment was performed in triplicate.

Human Breast Tissues and Immunohistochemistry

WISP3 protein expression was studied by immunohistochemistry in normal human breast tissues obtained from 10 reduction mammoplasty procedures. Immunohistochemical analysis was performed by using a polyclonal anti–WISP3 antibody at 1:500 dilution with overnight incubation and microwave antigen retrieval [20]. The detection reaction followed the Dako Envision$^+$ System Peroxidase kit protocol (Dako, Carpinteria, CA). Diaminobenzidine was used as chromogen and hematoxylin was used as counterstain. Positive and negative controls were tumor xenografts derived from cell lines shown to express high levels of WISP3 (SUM149 cell line stably transfected with WISP3) and from a cell line that does not express WISP3 (SUM149 wild type), respectively.

Results

WISP3 Protein Is Secreted by Human Breast Epithelial Cells

WISP3 protein contains a multimodular structure with a secretory signal peptide at the N-terminus. To investigate whether WISP3 is secreted by breast epithelial cells, SUM149 IBC cells previously characterized with a loss in WISP3 expression were stably transfected to express full-length WISP3. Conditioned media from SUM149/WISP3–overexpressing clones were collected and

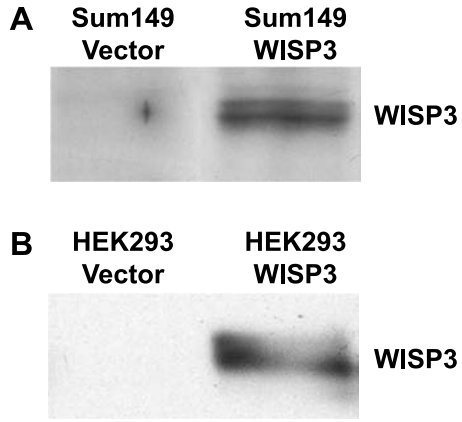


Figure 1. *WISP3 protein is secreted and detected in the conditioned media. (A) Western immunoblot using anti–WISP3 polyclonal antibody detects WISP3 protein in the conditioned media of SUM149 cells transfected with WISP3 full-length cDNA. WISP3 protein is not detected in the conditioned media of SUM149 cells transfected with the empty vector. (B) Western immunoblot of the conditioned media of HEK-293 cells transfected with WISP3, detected using an anti–HIS antibody. WISP3 is not detected in the conditioned media of the control cells.*

detected for WISP3 by Western blot analysis using a polyclonal anti–WISP3 antibody. WISP3 protein was detected in the media of SUM149-expressing WISP3 (SUM149/WISP3), and not in the media of empty vector–transfected SUM149 cells (SUM149/Flag) (Figure 1*A*). In order to explore these results from a different perspective, we performed transient transfections of WISP3 in SUM149 cells using a HIS^- tagged full-length WISP3 expression vector. In this case, WISP3 protein was detected in the conditioned media using both anti–WISP3 antibody and anti–HIS antibody (data not shown). To specifically address whether WISP3 would be detected in the conditioned media of a nonmammary cell, we repeated these experiments with the HEK293 cell line, derived from human embryonic kidney epithelial cells. WISP3 was detected in the conditioned media of these cells and not in the conditioned media of the empty vector controls (Figure 1*B*).

In situ expression of WISP3 protein was determined by immunohistochemical analysis of normal breast tissues derived from reduction mammoplasty procedures. In all 10 tissues examined, WISP3 protein was expressed at low levels in the cytoplasm of normal epithelial cells from ducts and acini and, interestingly, was present in the luminal secretions of ducts and lobules (Figure 2*A*). Xenografts derived from wild-type SUM149 cells ($WISP3^-$) and from SUM149/$WISP3^+$ cells were used as negative and positive controls, respectively (Figure 2, *B* and *C*).

WISP3 Containing Conditioned Media Reduces IGF-I–Induced IGF-IR Activation and Signaling Pathways

The effect of WISP3 on the activation of the IGF-I signaling pathway was studied in SUM149 cells derived from a primary IBC [5,6,19,21]. The activation of the IGF signaling

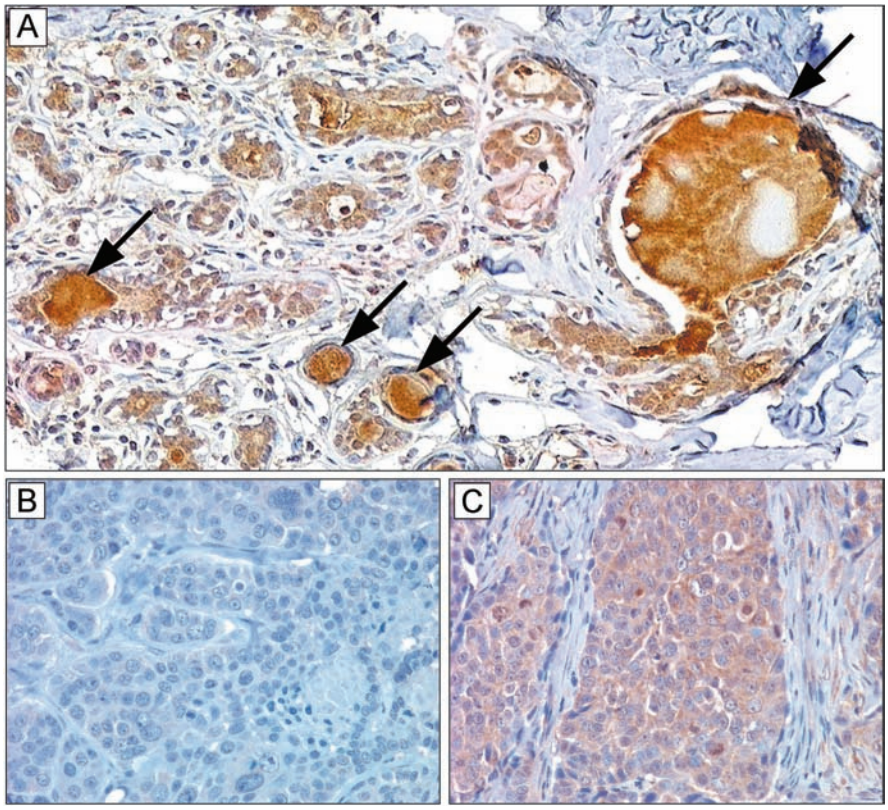


Figure 2. *In situ expression of WISP3 protein in normal breast tissues. (A) Immunohistochemical analysis of normal breast tissues using a polyclonal anti–WISP3 antibody shows that WISP3 protein is expressed at low levels in the cytoplasm of normal epithelial cells and in the luminal secretions of ducts and acini (arrows) (× 200). (B and C) Xenografts derived from wild-type SUM149 and SUM149/WISP3 cells were used as negative and positive controls, respectively (× 400).*

cascade plays a central role in breast cancer development and progression. To investigate whether the presence of WISP3 in the conditioned media has an effect on IGF-IR signaling pathways in IBC cells, phosphorylation of IGF-IR, IRS1, and ERK-1/2 was determined in wild-type SUM149 cells in the presence or absence of WISP3 in the conditioned media. Experiments were carried out under baseline conditions without addition of IGF-I and after stimulation with IGF-I. In the presence of WISP3 in the conditioned media, SUM149 cells exhibited decreased IGF-IR phosphorylation (Figure 3). The effect of WISP3 in the phosphorylation of the IGF-IR was evident in the presence of IGF-I stimulation because WISP3 was able to ameliorate the effect of IGF-I stimulation on the activation of the IGF-IR (Figure 3). Similarly, WISP3 conditioned media decreased the IGF-I–induced IRS1 and ERK-1/2 phosphorylation (Figures 4 and 5).

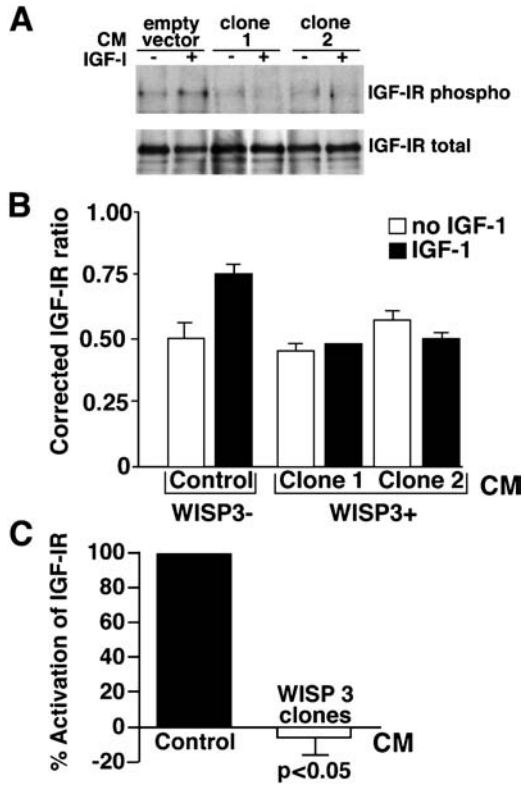


Figure 3. *WISP3 decreases IGF-I–induced phosphorylation of the IGF-IR. (A) Western blot analysis of SUM149 cell lines bathed in WISP3⁺ and control (WISP3⁻) conditioned media. The experiment was carried out under baseline conditions (without IGF-I) and after stimulation with 20 ng/ml IGF-I. The IGF-IR was precipitated from 500 μg of protein lysate with an anti–IGF-IR mAb and subsequently detected by immunoblot with an anti–IGF-IR β subunit polyclonal Ab. Tyrosine phosphorylation of immunoprecipitated IGF-IR was assessed with an anti–phosphotyrosine mAb PY20. (B) Relative protein levels of IGF-IR phosphorylation normalized for total IGF-IR. Blots were scanned and the pixel intensity measured using Scn Image program. Results are expressed as mean ± SEM of three independent experiments. (C) Quantitation of the differences in the percent activation of the IGF-IR. The difference in normalized IGF-IR phosphorylation under baseline conditions and after IGF-I stimulation was calculated for each cell line. Results were corrected using the difference in IGF-IR phosphorylation in the absence of IGF-I stimulation as reference. WISP3 was able to decrease the IGF-I–induced activation of the IGF-IR (t test, P < .05).*

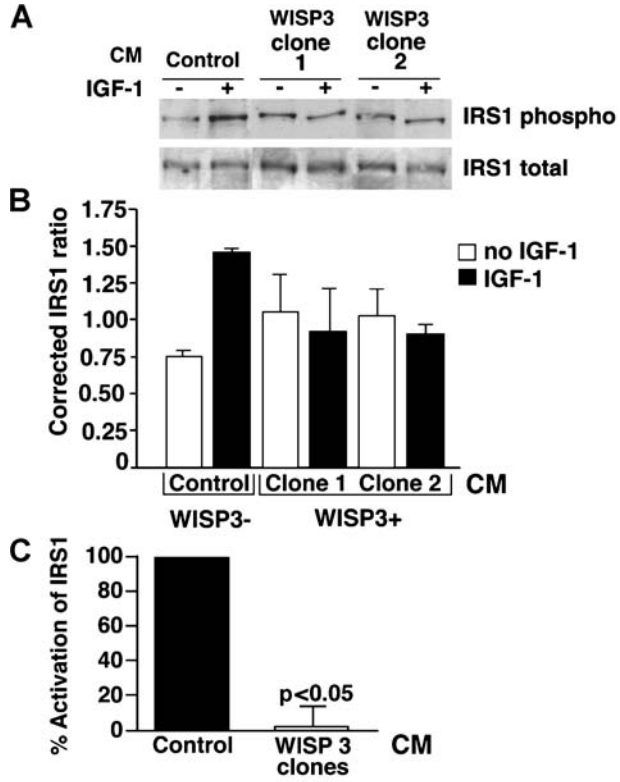


Figure 4. *WISP3 decreases IGF-I–induced phosphorylation of IRS1. (A) Western blot analysis of SUM149 cell lines bathed in WISP3⁺ and control WISP3-conditioned media. The experiment was carried out under baseline conditions (without IGF-I) and after stimulation with IGF-I. The expression of phosphorylated IRS1 was detected by Western blot using a polyclonal antibody against phosphorylated IRS1. The Western blot was stripped and probed with an antibody against total IRS1. (B) Relative protein levels of IRS1 phosphorylation normalized for total IRS1. Blots were scanned and the pixel intensity measured. Results are expressed as mean ± SEM of three independent experiments. (C) The differences in the percent activation of IRS-1 were quantitated. The difference in the corrected IRS1 phosphorylation under baseline conditions and after IGF-I stimulation was calculated for each cell line and results were normalized using the difference in IRS1 phosphorylation in the absence of IGF-I stimulation as reference. WISP3-rich conditioned media was able to decrease the phosphorylation of IRS-1 triggered by IGF-I (t test, P < .05).*

WISP3 Containing Conditioned Media Reduces the Growth of IBC Cell Lines

After establishing that the presence of WISP3 in the conditioned media was able to modulate IGF-I signaling pathways, its effect on cellular proliferation was determined in the presence and absence of IGF-I stimulation. SUM149 cells bathed in the WISP3 conditioned media had significantly lower growth rates than the control SUM149 cells bathed in control (WISP3-deficient) conditioned media, both in the presence and absence of IGF-I stimulation (*t* test, $P < .05$; Figure 6).

Discussion

We have previously demonstrated that WISP3 is lost in 80% of IBC tumors and that it has tumor-suppressor functions in IBC [5,6]. Studies on the SUM149 IBC cell line showed that restoration of WISP3 expression has potent growth- and

angiogenesis-inhibitory functions *in vitro* and *in vivo* [6]. Restoration of WISP3 resulted in a significant decrease in anchorage-independent growth in soft agar and cellular proliferation, as well as a drastic decrease in the invasive capabilities of the SUM149 cells, which are highly invasive in their wild-type state. Furthermore, restoration of WISP3 expression in SUM149 cells resulted in a biologically relevant decrease in the level of angiogenic factors (VEGF, bFGF, and IL-6) in the conditioned media of the cells. *In vivo*, restoration of WISP3 expression in SUM149 cells caused a drastic decrease in tumor volume and rate of tumor growth when injected in nude mice [6]. Taken together, this body of work had strongly supported a tumor-suppressor role for WISP3 in mammary tumor progression. In the present study, we sought to discover the molecular mechanisms underlying the tumor-suppressor function of WISP3.

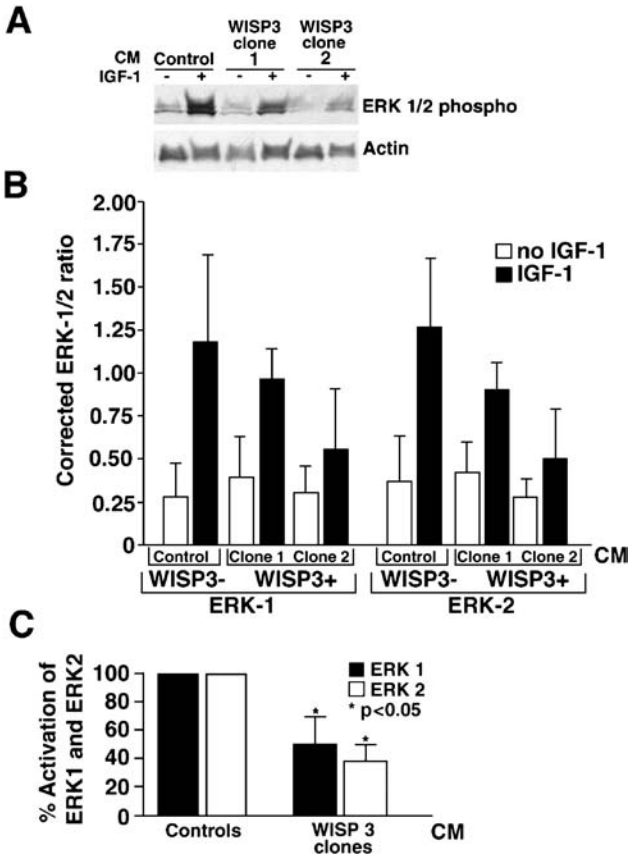


Figure 5. *WISP3 decreases IGF-I–induced phosphorylation of ERK-1/2. (A) Western blot analysis of SUM149 cell lines bathed in WISP3⁺ and control (WISP3⁻) conditioned media, under baseline conditions (without IGF-I) and after stimulation with IGF-I. The expression of ERK-1/2 was detected by Western blot using a polyclonal antibody against phosphorylated ERK-1/2. The blot was stripped and probed with an antibody against β-actin. (B) Relative protein levels of ERK-1/2 phosphorylation normalized using actin. Blots were scanned and the pixel intensity measured. Results are expressed as mean ± SEM of three independent experiments. (C) Quantitation of the differences in the percent activation of ERK-1/2. The difference in the corrected ERK-1/2 phosphorylation under baseline conditions and after IGF-I stimulation was calculated for each cell line. Results were normalized using the difference in ERK-1/2 phosphorylation in the absence of IGF-I stimulation as reference. WISP3-rich conditioned media ameliorated the phosphorylation of ERK 1 and ERK 2 induced by IGF-I stimulation (t test, P < .05).*

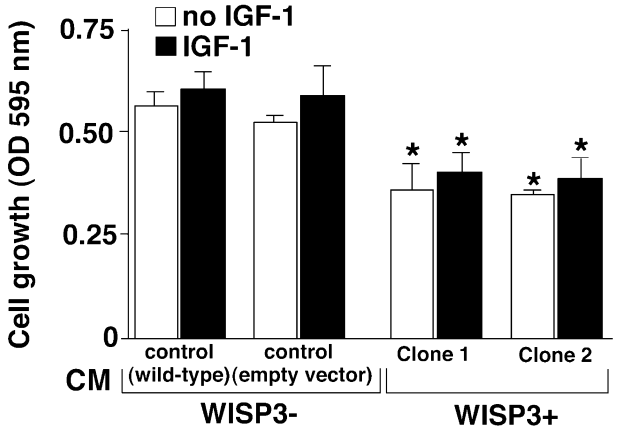


Figure 6. *WISP3 decreases the growth of IBC cells. The growth of SUM149 cells was measured in the absence (WISP3⁻) and presence of WISP3 (WISP3⁺) in the conditioned media under baseline conditions (no IGF-I) and after IGF-I stimulation by an MTT assay. SUM149 cells were grown in 96-well plates at a density of 5 × 10⁴ cells/ml. Serum-starved cells were incubated for 24 hours in the WISP3 and control conditioned media, with or without IGF-I stimulation. Results are expressed as mean ± SEM of three independent experiments. WISP3⁺ conditioned media decreases significantly the growth of SUM149 cells (t test, P < .05 for both clones).*

WISP3 belongs to the CCN family of proteins, which are highly conserved, putatively secreted proteins with important roles in development during chondrogenesis and skeletogenesis [7]. The CCN proteins have been recently also implicated in carcinogenesis [7,22–26]. It is not well understood, however, how the functions of the CCN proteins in development relate to their role in cancer. Moreover, their expression during tumorigenesis cannot be generalized across different tissue types. This may be due to tissue-specific functions of the CCN proteins, perhaps mediated by their multimodular structure and the presence of different affinities for binding partners and ligands in different tissues [7]. The presence of different receptors and differential processing of the CCN proteins (e.g., cleavage by proteases) may account also for their diverse functions in different tissues. In this paper, we focused on determining whether WISP3 is secreted into the conditioned media and its relationship to IGF signaling pathways.

Analysis of the protein sequence of WISP3 revealed that it contains a signal peptide at the N-terminal region that may participate in the secretion of the protein into the extracellular media [7–9]. Indeed, by Western blot, using two different specific antibodies, we were able to detect WISP3 protein in the conditioned media of SUM149 and HEK293 cells transfected with WISP3. Furthermore, consistent with these results, by immunohistochemical analysis, WISP3 protein was detected in the secretions accumulated in the lumens of ducts and lobules in normal breast tissues. The fact that WISP3 is secreted and present in the conditioned media (thereby alluding to its stability in solution) led us to the hypothesis that it may directly or indirectly regulate IGF signaling.

Although the signaling pathways that are required for the effects of IGF-I in breast cancer have not been completely elucidated, the contribution of IGF-I–induced IGF-IR

activation appears to be critical in hormone-dependent and -independent breast cancer [27–30]. IGF-I is locally released by breast cancer cells and stromal fibroblasts, and it is involved in autocrine and paracrine stimulation of the mammary epithelium [31]. In breast cancer cells, when IGF-I binds IGF-IR, signaling occurs mainly through activation of IRS-1 and RAS-dependent phosphorylation of MAP kinase with subsequent activation of nuclear transcription factors [32,33]. IGF-I signaling promotes cell growth, survival, and motility of breast cancer cells, as well as resistance to therapeutic interventions [10–12,14–18]. We hypothesized that expression of WISP3 could result in a series of molecular events that leads to the modulation of IGF-IR activation and downstream signaling. Contributing to this hypothesis is the fact that we have shown that WISP3 is secreted into the media where it has the opportunity to directly or indirectly modulate the strength of IGF signaling. Indeed, in the presence of IGF-I, WISP3 containing conditioned media decreased IGF-IR phosphorylation and the phosphorylation of two main downstream IGF-IR signaling molecules, IRS1 and ERK-1/2. This inhibition was not evident under baseline conditions, without stimulation with IGF-I. Our experiments thus show that even relatively small concentrations of WISP3 secreted by WISP3-transfected cells are able to modulate, directly or indirectly, IGF-I signaling in the setting of IGF-I stimulation.

A major growth-regulatory IGF-IR downstream pathway that regulates breast cancer growth and survival converges on ERK-1/2 cascade [34]. We observed a decrease in ERK-1/2 phosphorylation by addition of WISP3 containing conditioned media in the presence of IGF-I stimulation. ERK-1/2 influence chromatin remodeling and activation of gene expression, leading to enhanced cellular proliferation and decreased apoptosis [35–37]. Specifically, ERK-1/2 have been shown to activate the transcription of key genes involved in cell cycle progression including cyclin D1 and cyclin E. We have shown previously that restoration of WISP3 expression in the highly malignant SUM149 IBC cell line markedly decreased the levels of cyclin E and PCNA, a reliable marker of cellular proliferation [6].

The mechanism whereby WISP3 may modulate IGF-IR activation in the presence of IGF-I remains to be elucidated. WISP3 contains a highly conserved motif (GCGCCXXC) characteristic of IGFBPs, which may provide the proper protein folding to interact with IGF-like ligands, thereby enabling interference with IGF signaling. Although initial studies reported that two other CCN proteins, CTGF (CTGF) and Nov, specifically bind to IGF-I [38,39], these results have not been subsequently built on and they remain to be duplicated by other investigators. Whether WISP3 physically binds to IGF-I warrants further investigation, in light of out data.

Another mechanism that may explain the modulation of IGF-IR phosphorylation by WISP3-containing conditioned media is the formation of a WISP3/IGF-I complex that may bind to the IGF-IR and occupy IGF binding sites, but the complex may be either inhibitory or may be only a weak agonist of the receptor. In another system [40], this hypothesis is supported by recent data showing that IGF-I can still freely bind to the receptor even when complexed to a truncated N-terminal fragment of IGF-binding protein 5 (mini-IGFBP5); interestingly, the N-terminal portion of IGFBP5 has high homology to the N-terminal portion of WISP3. Mini-IGFBP5 binding to IGF-I resulted in incomplete inhibition of receptor binding [40]. In a similar manner, a WISP3/IGF-I complex might still bind to the IGF-IR but exert only a weak agonist effect, effectively resulting in physiologic antagonism of IGF-I action under conditions of high IGF-I stimulation. WISP3-rich conditioned media was able to significantly decrease the proliferation rate of IBC cells. The fact that this effect was seen both in the presence and absence of IGF-I simulation suggests that, in addition to interfering with IGF-I signaling pathways, WISP3 may have IGF-independent functions in IBC. In sum, we show that WISP3 is a secreted protein that modulates IGF-I signaling pathways, leading to a decrease in the growth of IBC cells.

Acknowledgements

We thank Robin Kunkel and Elizabeth Horn for artwork; S. Ethier for the SUM149 and HME cell lines; Wendy Kutz and Matthew Warman from Case Western Reserve University for providing anti–WISP3 antibody; and Michelle LeBlanc and the Immunohistochemistry Core of the University of Michigan Cancer Center for tissue processing and immunohistochemistry.

References

[1] Lee BJ (1924). Inflammatory carcinoma of the breast: a report of twenty-eight cases from the breast clinic of Memorial Hospital. *Surg Gynecol Obstet* **39,** 580–595.

[2] Jaiyesimi IA, Buzdar AU, and Hortobagyi G (1992). Inflammatory breast cancer: a review. *J Clin Oncol* **10,** 1014–1024.

[3] Merajver SD, Weber BL, Cody R, Zhang D, Strawderman M, Calzone KA, LeClaire V, Levin A, Irani J, Helvie M, August D, Wicha M, Lichter A, and Pierce LJ (1997). Breast conservation and prolonged chemotherapy for locally advanced breast cancer: the University of Michigan experience. *J Clin Oncol* **15,** 2873–2881.

[4] Swain SM, Sorace RA, Bagley CS, Danforth Jr, DN, Bader J, Wesley MN, Steinberg SM, and Lippman ME (1987). Neoadjuvant chemotherapy in the combined modality approach of locally advanced nonmetastatic breast cancer. *Cancer Res* **47,** 3889–3894.

[5] van Golen KL, Davies S, Wu ZF, Wang Y, Bucana CD, Root H, Chandrasekharappa S, Strawderman M, Ethier SP, and Merajver SD (1999). A novel putative low-affinity insulin-like growth factor–binding protein, LIBC (lost in inflammatory breast cancer), and RhoC GTPase correlate with the inflammatory breast cancer phenotype. *Clin Cancer Res* **5,** 2511–2519.

[6] Kleer CG, Zhang Y, Pan Q, van Golen KL, Wu ZF, Livant D, and Merajver SD (2002). *WISP3* is a novel tumor suppressor gene of inflammatory breast cancer. *Oncogene* **21,** 3172–3180.

[7] Perbal B (2001). *NOV* (nephroblastoma overexpressed) and the *CCN* family of genes: structural and functional issues. *Mol Pathol* **54,** 57–79.

[8] Pennica D, Swanson TA, Welsh JW, Roy MA, Lawrence DA, Lee J, Brush J, Taneyhill LA, Deuel B, Lew M, Watanabe C, Cohen RL, Melhem MF, Finley GG, Quirke P, Goddard AD, Hillan KJ, Gurney AL, Botstein D, and Levine AJ (1998). *WISP* genes are members of the connective tissue growth factor family that are up-regulated in wnt-1–transformed cells and aberrantly expressed in human colon tumors. *Proc Natl Acad Sci USA* **95,** 14717–14722.

[9] Hurvitz JR, Suwairi WM, Van Hul W, El-Shanti H, Superti-Furga A, Roudier J, Holderbaum D, Pauli RM, Herd JK, Van Hul EV, Rezai-Delui

H, Legius E, Le Merrer M, Al-Alami J, Bahabri SA, and Warman ML (1999). Mutations in the *CCN* gene family member *WISP3* cause progressive pseudorheumatoid dysplasia. *Nat Genet* **23,** 94–98.

[10] Bartucci M, Morelli C, Mauro L, Ando S, and Surmacz E (2001). Differential insulin-like growth factor I receptor signaling and function in estrogen receptor (ER)–positive MCF-7 and ER-negative MDA-MB-231 breast cancer cells. *Cancer Res* **61,** 6747–6754.

[11] Furstenberger G, and Senn HJ (2002). Insulin-like growth factors and cancer. *Lancet Oncol* **3,** 298–302.

[12] Kleinberg DL, Feldman M, and Ruan W (2000). IGF-I: an essential factor in terminal end bud formation and ductal morphogenesis. *J Mammary Gland Biol Neoplasia* **5,** 7–17.

[13] Surmacz E (2000). Function of the IGF-I receptor in breast cancer. *J Mammary Gland Biol Neoplasia* **5,** 95–105.

[14] Byrne C, Colditz GA, Willett WC, Speizer FE, Pollak M, and Hankinson SE (2000). Plasma insulin–like growth factor (IGF) I, IGF-binding protein 3, and mammographic density. *Cancer Res* **60,** 3744–3748.

[15] Turner BC, Haffty BG, Narayanan L, Yuan J, Havre PA, Gumbs AA, Kaplan L, Burgaud JL, Carter D, Baserga R, and Glazer PM (1997). Insulin-like growth factor-I receptor overexpression mediates cellular radioresistance and local breast cancer recurrence after lumpectomy and radiation. *Cancer Res* **57,** 3079–3083.

[16] Resnik JL, Reichart DB, Huey K, Webster NJ, and Seely BL (1998). Elevated insulin-like growth factor I receptor autophosphorylation and kinase activity in human breast cancer. *Cancer Res* **58,** 1159–1164.

[17] Rocha RL, Hilsenbeck SG, Jackson JG, VanDenBerg CL, Weng C, Lee AV, and Yee D (1997). Insulin-like growth factor binding protein-3 and insulin receptor substrate-1 in breast cancer: correlation with clinical parameters and disease-free survival. *Clin Cancer Res* **3,** 103–109.

[18] Lee AV, Jackson JG, Gooch JL, Hilsenbeck SG, Coronado-Heinsohn E, Osborne CK, and Yee D (1999). Enhancement of insulin-like growth factor signaling in human breast cancer: estrogen regulation of insulin receptor substrate-1 expression *in vitro* and *in vivo*. *Mol Endocrinol* **13,** 787–796.

[19] Ethier SP, Mahacek ML, Gullick WJ, Frank TS, and Weber BL (1993). Differential isolation of normal luminal mammary epithelial cells and breast cancer cells from primary and metastatic sites using selective media. *Cancer Res* **53,** 627–635.

[20] Shi SR, Key ME, and Kalra KL (1991). Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J Histochem Cytochem* **39,** 741–748.

[21] van Golen KL, Wu ZF, Qiao XT, Bao LW, and Merajver SD (2000). RhoC GTPase, a novel transforming oncogene for human mammary epithelial cells that partially recapitulates the inflammatory breast cancer phenotype. *Cancer Res* **60,** 5832–5838.

[22] Soon LL, Yie TA, Shvarts A, Levine AJ, Su F, and Tchou-Wong KM (2003). Overexpression of WISP-1 down-regulated motility and invasion of lung cancer cells through inhibition of Rac activation. *J Biol Chem* **278,** 11465–11470.

[23] Vilmos P, Gaudenz K, Hegedus Z, and Marsh JL (2001). The Twisted gastrulation family of proteins, together with the IGFBP and CCN families, comprise the TIC superfamily of cysteine rich secreted factors. *Mol Pathol* **54,** 317–323.

[24] Kireeva ML, Mo FE, Yang GP, and Lau LF (1996). Cyr61, a product of a growth factor–inducible immediate-early gene, promotes cell proliferation, migration, and adhesion. *Mol Cell Biol* **16,** 1326–1334.

[25] Tong X, Xie D, O'Kelly J, Miller CW, Muller-Tidow C, and Koeffler HP (2001). Cyr61, a member of CCN family, is a tumor suppressor in non-small cell lung cancer. *J Biol Chem* **276,** 47709–47714.

[26] Xie D, Nakachi K, Wang H, Elashoff R, and Koeffler HP (2001). Elevated levels of connective tissue growth factor, WISP-1, and CYR61 in primary breast cancers associated with more advanced features. *Cancer Res* **61,** 8917–8923.

[27] Godden J, Leake R, and Kerr DJ (1992). The response of breast cancer cells to steroid and peptide growth factors. *Anticancer Res* **12,** 1683–1688.

[28] Jackson JG, White MF, and Yee D (1998). Insulin receptor substrate-1 is the predominant signaling molecule activated by insulin-like growth factor-I, insulin, and interleukin-4 in estrogen receptor–positive human breast cancer cells. *J Biol Chem* **273,** 9994–10003.

[29] Jackson JG, and Yee D (1999). IRS-1 expression and activation are not sufficient to activate downstream pathways and enable IGF-I growth response in estrogen receptor negative breast cancer cells. *Growth Horm IGF Res* **9,** 280–289.

[30] Peyrat JP, Bonneterre J, Dusanter-Fourt I, Leroy-Martin B, Djiane J, and Demaille A (1989). Characterization of insulin-like growth factor 1 receptors (IGF1-R) in human breast cancer cell lines. *Bull Cancer* **76,** 311–319.

[31] Satyamoorthy K, Li G, Vaidya B, Patel D, and Herlyn M (2001). Insulin-like growth factor-1 induces survival and growth of biologically early melanoma cells through both the mitogen-activated protein kinase and beta-catenin pathways. *Cancer Res* **61,** 7318–7324.

[32] Resnicoff M, and Baserga R (1998). The role of the insulin-like growth factor I receptor in transformation and apoptosis. *Ann NY Acad Sci* **842,** 76–81.

[33] Baserga R (1999). The IGF-I receptor in cancer research. *Exp Cell Res* **253,** 1–6.

[34] Santen RJ, Song RX, McPherson R, Kumar R, Adam L, Jeng MH, and Yue W (2002). The role of mitogen-activated protein (MAP) kinase in breast cancer. *J Steroid Biochem Mol Biol* **80,** 239–256.

[35] Murphy LO, Smith S, Chen RH, Fingar DC, and Blenis J (2002). Molecular interpretation of ERK signal duration by immediate early gene products. *Nat Cell Biol* **4,** 556–564.

[36] Volmat V, Camps M, Arkinstall S, Pouyssegur J, and Lenormand P (2001). The nucleus, a site for signal termination by sequestration and inactivation of p42/p44 MAP kinases. *J Cell Sci* **114,** 3433–3443.

[37] Volmat V, and Pouyssegur J (2001). Spatiotemporal regulation of the p42/p44 MAPK pathway. *Biol Cell* **93,** 71–79.

[38] Kim HS, Nagalla SR, Oh Y, Wilson E, Roberts CT Jr, and Rosenfeld RG (1997). Identification of a family of low-affinity insulin-like growth factor binding proteins (IGFBPs): characterization of connective tissue growth factor as a member of the IGFBP superfamily. *Proc Natl Acad Sci USA* **94,** 12981–12986.

[39] Burren CP, Wilson EM, Hwa V, Oh Y, and Rosenfeld RG (1999). Binding properties and distribution of insulin-like growth factor binding protein–related protein 3 (IGFBP-rP3/NovH), an additional member of the IGFBP superfamily. *J Clin Endocrinol Metab* **84,** 1096–1103.

[40] Zeslawski W, Beisel HG, Kamionka M, Kalus W, Engh RA, Huber R, Lang K, and Holak TA (2001). The interaction of insulin-like growth factor-I with the N-terminal domain of IGFBP-5. *EMBO J* **20,** 3638–3644.

Breast Cancer Research and Treatment (2005) 93: 101–110
DOI 10.1007/s10549-005-4170-6

Report

RhoC-GTPase is a novel tissue biomarker associated with biologically aggressive carcinomas of the breast

Celina G. Kleer[1,5], Kent A. Griffith[2,5], Michael S. Sabel[3,5], Gary Gallagher[1], Kenneth L. van Golen[4,5], Zhi-Fen Wu[4,5], and Sofia D. Merajver[4,5]
[1]*Department of Pathology and Internal Medicine;* [2]*Department of Biostatistics and Internal Medicine;* [3]*Department of Surgery and Internal Medicine;* [4]*Division of Hematology/Oncology;* [5]*Comprehensive Cancer Center, University of Michigan, Ann Arbour, MI, USA*

Key words: Rhoc, breast cancer, biomarkers, tissue microarrays, inflammatory breast cancer, pathology

Summary

Background. There is a need for reliable predictors of breast cancer aggressiveness that will further refine the staging classification and help guide the implementation of novel therapies. We have identified RhoC as being nearly always overexpressed in the most aggressive form of breast cancer, inflammatory breast cancer (IBC); in subsequent work we identified RhoC to be a promising marker of aggressive behavior in breast cancers less than 1 cm in diameter. We hypothesized that RhoC expression would identify aggressive, non-IBC tumors breast cancer patients at any stage with worse outcomes defined as recurrence and/or metastasis.

Methods. We constructed four high-density tissue microarrays (TMAs) using 801 tissue cores from 280 patients. These tissues represent a wide range of normal breast and breast disease, including intraductal hyperplasia, ductal carcinoma *in situ* (DCIS), invasive carcinomas, and distant metastases. The TMAs were immunostained using a polyclonal anti-RhoC antibody developed in our laboratory. Cytoplasmic RhoC expression was scored as negative, weak, moderate, or strong by a previously validated scoring schema.

Results. RhoC expression increases with breast cancer progression. All samples of normal breast epithelium had negative to weak staining, whereas staining intensity increased in hyperplasia, DCIS, invasive carcinoma, and metastases (Kruskal–Wallis $p < 0.001$). In patients with invasive carcinoma, high RhoC expression was associated with features of aggressive behavior including high histologic grade, positive lymph nodes, and negative hormonal receptor status. High RhoC expression was a predictor of overall survival in patients with breast cancer (log rank test, $p = 0.002$) and was associated with 100% increase in the risk of death as compared to patients with low RhoC expression. Importantly, high RhoC was an independent predictor of poor response to doxorubicin-based chemotherapy with a hazard ratio of 3.1 and a 95% CI of 1.2–7.7 ($p = 0.02$).

Conclusion. RhoC expression increases with breast cancer progression and RhoC protein level in tumor tissue is strongly associated with biologically aggressive invasive carcinomas of the breast. RhoC expression, if validated, may identify patients who are less likely benefit from doxorubicin therapy and suggests RhoC overexpression as a new target for intervention.

Introduction

Breast cancer remains the second most common cause of cancer related deaths for women in the United States [1]. With the most advanced current treatment options, it is a fact that once patients develop distant metastases, they succumb to the disease [2]. The most important prognostic indicators in breast cancer that are in current use in the clinic are components of the staging system, such as primary tumor size and the presence of lymph node metastases [3]. Although these parameters are the most powerful prognostic factors available, they are not as precise as desired in predicting which tumors will recur locally and/or metastasize distally [4]. There are small invasive carcinomas that follow an aggressive clinical course and large tumors that do not recur or metastasize. Approximately one-third of women with node-negative breast cancer experience recurrences, whereas approximately one-third of patients with positive lymph nodes are free of disease 10 years after the primary tumor diagnosis. In addition to size and lymph nodes, other morphologic features, such as histological grade, vascular invasion, and molecular markers have been investigated for their potential to predict outcome, but in general, they have had limited value so far [4–6]. These data highlight the need for more sensitive and specific markers of aggressive behavior.

Through a modified version of the differential display technique and *in situ* hybridization of breast tissues, we previously identified RhoC, a gene involved in cell

polarity and motility, as being overexpressed in the most lethal form of locally advanced breast cancers, inflammatory breast cancer (IBC) [7]. We demonstrated that RhoC functions as a transforming oncogene for human mammary epithelial cells giving rise to a highly motile and invasive phenotype [8,9]. Invasive breast carcinomas that developed metastases exhibited higher levels of RhoC protein than invasive carcinomas that did not metastasize [10]. This body of work led us to hypothesize that RhoC overexpression may occur early in breast cancer progression and that it may identify a group of invasive, non-IBC tumors with a highly aggressive phenotype.

Methods

Selection of patients and tissue microarray development

Breast tissues were obtained from the Surgical Pathology files at the University of Michigan with Institutional Review Board approval. A total of 280 cases (n = 801 tissue microarray elements) were reviewed by the study pathologist (CGK) and arrayed in four high-density tissue microarrays (TMAs), as previously described [11,12]. At least three tissue cores (0.6 mm diameter) were sampled from each block to account for tumor heterogeneity. The TMAs contained the whole spectrum of breast pathology, with samples of normal breast (n = 76), intraductal hyperplasia (n = 26), ductal carcinoma *in situ* (n = 22), invasive carcinoma (n = 639), and breast cancer metastases (n = 38). The invasive carcinomas were obtained from 233 largely consecutive patients (n = 639 tissue microarray elements) with follow-up information at the University of Michigan between 1987 and 1991. Clinical and outcome information on the 233 patients was obtained by chart review performed by the surgeon on the study (MSS) with IRB approval. In our cohort of 233 breast cancer patients, 211 had follow-up information. The median duration of follow-up was 3.6 years (range 15 days–17 years). Clinical and pathological variables were determined following well-established criteria. The histologic grade was assessed according to the method described by Elston and Ellis [13]; angiolymphatic invasion was classified as either present or absent.

Immunohistochemical studies

Immunohistochemistry was performed on the TMAs by using a standard biotin–avidin complex technique and a polyclonal antibody against RhoC that was previously validated by immunoblot and immunohistochemistry [10]. RhoC expression was evaluated at least three times for every tissue microarray element and at least nine times for each tumor, using an internet based tool (TMA Profiler, University of Michigan, Ann Arbor, MI) [11,14]. Using this method, the pathologist is blinded to tumor stage and clinical information. The median value of all measurements from a single individual was used for subsequent analyses. As observed previously [10], RhoC protein was strongly expressed in the cytoplasm of myoepithelial cells and vascular smooth muscle cells, which served as consistent internal positive controls. Cytoplasmic RhoC expression was scored from 1 to 4 by comparison to the positive internal controls [10,11,15]. Strong, diffuse staining was considered score = 4, whereas moderate and low diffuse staining was scored as 3 and 2, respectively. Negative staining was scored as 1. Based on our previous work dealing with the biological characterization of RhoC as an oncogene, we defined high RhoC expression when there was strong staining (score = 4) and low RhoC expression, when staining was negative, weak, or moderate (scores = 1–3).

Statistical analysis

The association between RhoC protein expression and the pathologic diagnoses of the tissue microarray element was assessed using the general estimating equation. The ordinal expression categories for RhoC were modeled using the multinomial distribution with the cumulative logit link. Tissue microarray elements were clustered by patient. The model calculates the odds of a higher expression score versus a lower score, with the odds ratio and 95% confidence intervals reported.

The median RhoC expression score by patient was calculated for the subset of invasive carcinoma microarray elements. In instances where the calculated median was the midpoint between expression categories, the median was rounded to the higher category. Possible associations between the median RhoC expression score and clinical and pathologic features of the patient were assessed using the cumulative-logit multinomial model. Also called the proportional-odds model, the model calculates the odds of a higher expression score compared to a lower score across the ordinal categories of expression. The appropriateness of the proportional-odds assumption across categories was tested using the χ^2 score test. The odds ratio and 95% confidence intervals are reported.

Overall survival time, time to breast cancer specific mortality, and time to treatment failure were calculated from the date of surgery until the subjects' date of death, date of death due to breast cancer, or the date of diagnosed treatment failure, respectively. Patients experiencing competing events were censored at the date of the competing event. For example, for calculations of breast cancer specific mortality, patients dying from other causes were censored on that date. Treatment failures included the diagnosis of local recurrence and the development of regional and distant metastases. Patients not experiencing any failure events were censored on their last date of follow-up or date of death. The analyzable sample included those patients with primary invasive tumor specimens arrayed for whom clinical follow-up data were available (N = 211).

Univariate associations between time-to-event endpoints and the clinical and pathologic characteristics,

which included median RhoC expression, were assessed using the log-rank test statistic. The probability of events was estimated using the product-limit method of Kaplan and Meier. Multivariate associations were modeled using Cox proportional hazards regression. Clinical and pathologic characteristics with univariate log-rank test statistics with *p*-values less than 10% were included in multivariate models. The most parsimonious multivariate models were constructed using backward, stepwise elimination, with a *p*-value less than or equal to 5% necessary for a covariate to be retained. Hazard ratios and 95% confidence intervals are reported.

Results

RhoC protein expression is elevated in breast cancer

On the basis of our previous work characterizing RhoC as an oncogene in IBC and its protein expression in breast tissues, we sought to determine whether RhoC is upregulated as breast cancer develops. To this end, we evaluated the expression of RhoC protein in a wide range of breast tissues (280 cases, $n = 801$ tissue microarray elements) by immunohistochemistry, to characterize its expression *in situ*. RhoC expression was observed mainly in the cytoplasm (Figure 1(a)), consistent with our previous observations [10]. Invasive breast carcinomas that expressed high levels of RhoC and those that expressed low levels of RhoC were readily apparent. RhoC protein levels were elevated in invasive carcinoma when compared to normal, intraductal hyperplasia, and DCIS (Table 1 and Figure 1). The odds of a higher RhoC expression levels were 2 times, 8 times, 12 times, and 8 times higher than normal epithelium, for intraductal hyperplasia, DCIS, invasive carcinoma, and metastatic deposits, respectively (Table 2).

Elevated RhoC expression is associated with aggressive breast cancer and poor prognosis

By using our breast cancer tissue microarray data, we evaluated the clinical pathological associations of RhoC

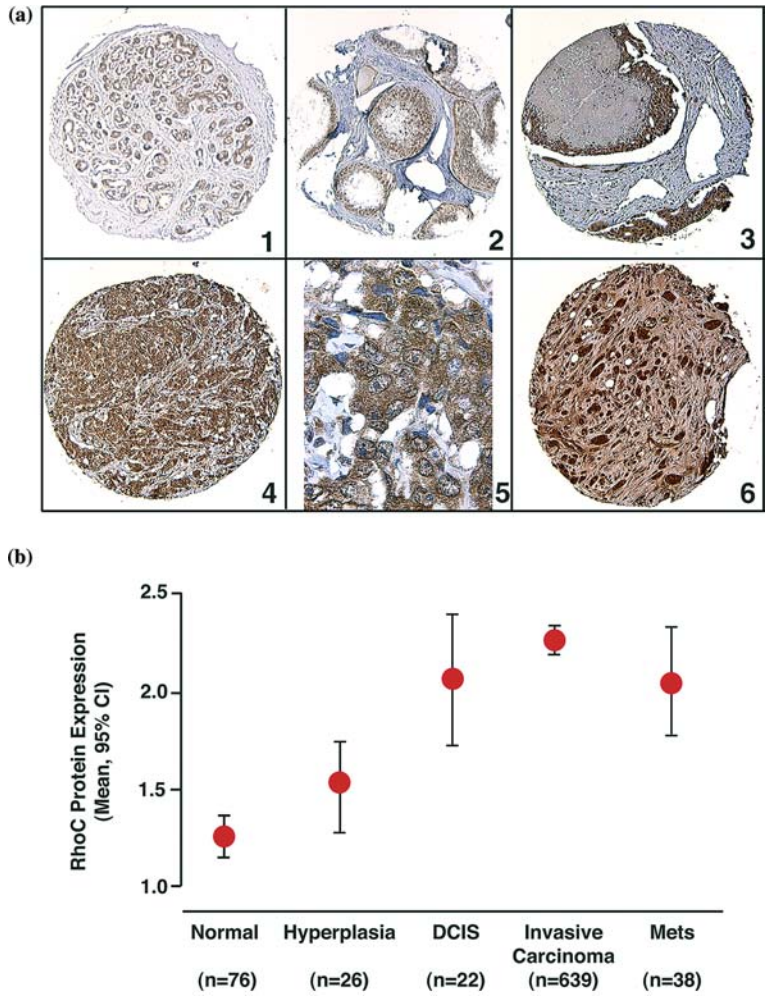


Figure 1. RhoC protein expression increases with breast cancer progression. (a) Tissue microarray samples of a normal breast lobule (1) and intraductal hyperplasia (2) with negative and weak RhoC expression. Ductal carcinoma *in situ* with comedo-necrosis (3) and invasive ductal carcinoma (4) with moderate and high RhoC expression, respectively. High power magnification of an invasive ductal carcinoma showing cytoplasmic accumulation of RhoC protein (5) Metastatic breast carcinoma in bone (6) with high expression of RhoC. (b) Mean RhoC expression increases with the severity of the diagnosis. Original magnification 40× and 100×.

Table 1. Frequency of RhoC protein expression in breast tissue samples as determined by immunohistochemistry

Breast tissue	Cores	Staining intensity, *n (%)*				Mean intensity
		1	2	3	4	
Normal epithelium	76	58 (76)	16 (21)	2 (3)	0	1.26
Intraductal hyperplasia	26	14 (54)	11 (42)	1 (4)	0	1.50
Ductal carcinoma *in situ*	22	6 (27)	9 (41)	7 (32)	0	2.05
Invasive carcinoma	639	140 (22)	249 (39)	203 (32)	47 (7)	2.25
Metastasis	38	12 (32)	13 (34)	13 (34)	0	2.03
Total	801					2.11

Table 2. Odds of higher RhoC expression according to the tissue diagnosis

Diagnosis	Odds ratio	95% CI	*p*-Value
Normal epithelium	1.00		
Intraductal hyperplasia	2.46	1.01–6.00	0.0487
Ductal carcinoma *in situ*	8.41	3.35–21.14	<0.0001
Invasive carcinoma	12.16	7.00–21.14	<0.0001
Metastasis	8.03	3.64–17.70	<0.0001

protein levels in breast cancer. In our cohort of 233 breast cancer patients ($n = 801$ samples), 211 had follow-up information. The median age of the study population was 58 years (range 28–99 years). The clinical and pathological characteristics of the patients are summarized in Table 3. The breakdown of treatment modalities in this group of patients is summarized in Table 4. Ninety-three patients (44.1%) received chemotherapy following surgery. In 90 of 93 patients (97%) the treatment consisted of a doxorubicin and cyclophosphamide combination regimen, with the remaining three patients receiving taxol alone.

After a median follow-up of 3.6 years (range: 15 days–17 years), 42 of the 226 patients (18.6%) died of breast cancer. The 5- and 10-year disease specific survival rates for the entire cohort of patients were 60% and 38%, respectively.

High RhoC expression was present in a subset of invasive carcinomas (13 of 211, 6.2%). The association between RhoC protein levels and clinical characteristics is shown on Table 5. RhoC expression was strongly associated with the presence of positive axillary lymph nodes (Fisher's exact test, $p = 0.0026$), one of the strongest known predictors of survival. High RhoC expression was also associated with increasing histologic tumor grade (Fisher's exact test, $p = 0.016$), a measure of the degree of tumor differentiation and poor prognostic indicator. Grade II and III tumors were three and six times more likely to have a high RhoC expression when compared to grade I tumors, respectively. High RhoC expression was associated with negative estrogen receptor status (Fisher's exact test, $p = 0.033$) and negative progesterone receptor status (Fisher's exact test, $p = 0.004$). Notably, despite the small number of tumors, RhoC overexpression was strongly associated with features of poor outcome in patients with breast cancer.

We next investigated the prognostic value of RhoC protein expression by interrogating the dataset about its prediction of aspects of the outcome in patients with newly diagnosed breast cancer. As expected, at the univariate level, the stage of disease, lymph node status, and histological tumor grade were associated with overall and disease-specific survival (Tables 6 and 7). Hormone receptor status was inversely associated with outcome. We found a strong and consistent association between RhoC protein levels and overall patient outcome. Higher RhoC protein levels were associated with all the important clinical outcomes that comprise 'poor prognosis': shorter disease-free interval after initial surgical treatment, lower overall survival, and a high probability of breast cancer-specific death (Figure 2). The 10-year overall survival for patients with tumors expressing high RhoC levels was 23% and by contrast to 53% for low levels of RhoC (log rank, $p = 0.002$, Figure 2b).

The best multivariable model predictive of overall survival included tumor stage, negative PR, the presence of vascular invasion, and treatment with radiotherapy, chemotherapy and tamoxifen (Table 8). High RhoC expression was a marginally significant independent predictor of outcome. Patients with high RhoC levels had a 100% higher risk of death when compared to patients with low RhoC expression (hazard ratio 2, 95% CI 1.0–4.1, $p = 0.067$).

RhoC is a promising predictive factor of response to doxorubicin-based chemotherapy

In our cohort of 211 breast cancer patients, 93 (44.1%) received adjuvant chemotherapy consisting in 90 of the 93 patients of a doxorubicin and cyclophosphamide combination regimen (Table 4). We sought to determine whether RhoC expression could predict survival in chemotherapy treated patients. Tumor stage, positive lymph node status, estrogen and progesterone receptor status, lymphovascular invasion, tamoxifen use, and median RhoC expression all had significant univariate associations with survival for chemotherapy treated patients. The multivariate model indicates that median RhoC expression was found to be independently

Table 3. Clinico-pathologic characteristics of the 211 patients with invasive carcinomas

Characteristics	*N* (%)†
Race	
White	172 (81.5)
Black	26 (12.3)
Other/Unknown	13 (6.2)
Menopause status	
Pre	43 (20.4)
Peri	19 (9.0)
Post	129 (61.1)
Unknown	20 (9.5)
Breast cancer type	
Ductal	149 (70.6)
Lobular	19 (9.0)
Ductal and Lobular	9 (4.3)
Other/Unknown	34 (16.1)
Tumor stage	
I	65 (30.8)
II	72 (34.1)
III	47 (22.3)
IV	5 (2.4)
Unknown	22 (10.4)
Tumor size (cm)	
≤ 2	109 (51.7)
>2	85 (40.3)
Unknown	17 (8.0)
Tumor grade	
I	24 (11.4)
II	92 (43.6)
III	77 (36.5)
Unknown	18 (8.5)
Estrogen receptor	
Positive	137 (64.9)
Negative	68 (32.2)
Unknown	6 (2.8)
Progesterone receptor	
Positive	113 (53.6)
Negative	92 (43.6)
Unknown	6 (2.8)
Her2/Neu status	
Positive over expressed	36 (17.7)
Negative not over expressed	165 (77.6)
Unknown	10 (4.7)
Lymphovascular invasion	
Present	61 (28.9)
Absent	147 (69.7)
Unknown	3 (1.4)
Lymph nodes	
Negative	92 (43.6)
1–3 positive nodes	46 (21.8)
>4 positive nodes	39 (18.5)
Unknown	34 (16.1)
Median RhoC expression	
1	33 (15.6)
2	94 (44.6)
3	71 (33.7)
4	13 (6.2)

Table 4. Treatment characteristics of the patients with invasive carcinomas ($N = 211$)

Characteristics	*N* (%)†
Neoadjuvant chemotherapy	
Yes	17 (8.1)
No	192 (91.0)
Unknown	2 (0.9)
Surgery type	
Mastectomy	132 (62.6)
Lumpectomy	74 (35.1)
None/Unknown	5 (2.4)
Adjuvant chemotherapy	
Yes	93 (44.1)
No	108 (51.2)
Unknown	10 (4.7)
Adjuvant radiotherapy	
Yes	95 (45.0)
No	104 (49.3)
Unknown	12 (5.7)
Tamoxifen therapy	
Yes	96 (45.5)
No	99 (46.9)
Unknown	16 (7.6)

associated with overall survival following chemotherapy, with a hazard ratio of 3.1 and a 95% CI of 1.2–7.7 ($p = 0.0176$) (Table 9).

Discussion

In this study based on unselected patients with primary invasive carcinomas of the breast treated by standard of care at our institution between 1987 and 1991, we tested the hypothesis that RhoC protein levels are associated with highly aggressive breast cancer. Furthermore, we examined the expression of RhoC in the whole spectrum of breast tissues, ranging from normal breast, intraductal hyperplasia, ductal carcinoma *in situ*, invasive carcinomas, and breast cancer metastases. We found that a high level (4+) of RhoC protein is present only in invasive carcinomas and not present in normal breast epithelium, hyperplasia, or ductal carcinoma *in situ*. RhoC protein expression increased steadily from normal breast, to fibrocystic changes, to DCIS, and invasive carcinomas. The strongest RhoC expression was observed in locally advanced breast cancer and in

Table 5. Association of RhoC expression with other clinical and pathologic features

Characteristic:	Fisher's exact *p*-value	Median RhoC Staining Intensity, *N* (%)			
		1	2	3	4
Tumor stage:	0.8520				
1		11 (33.3)	33 (35.1)	17 (23.9)	4 (30.8)
2		10 (30.3)	29 (30.9)	29 (40.9)	4 (30.8)
3		9 (27.3)	17 (18.1)	17 (23.9)	4 (30.8)
4		0	3 (3.2)	2 (2.8)	0
Tumor size (cm):	0.5792				
≤ 2		11 (33.3)	38 (40.4)	32 (45.1)	4 (30.8)
>2		19 (57.6)	51 (54.3)	32 (45.1)	7 (53.9)
Tumor grade:	0.0166				
I		8 (24.2)	13 (13.8)	3 (4.2)	0
II		14 (42.4)	43 (45.7)	31 (43.7)	4 (30.8)
III		9 (27.3)	29 (30.9)	30 (42.3)	9 (69.2)
Positive lymph nodes:	0.0026				
Zero		16 (48.5)	45 (47.9)	26 (36.6)	5 (38.5)
1–3		4 (12.1)	19 (20.2)	21 (29.6)	2 (15.4)
4+		9 (27.3)	10 (10.6)	16 (22.5)	4 (30.8)
Lymphovascular invasion:	0.6962				
Present		10 (30.3)	23 (24.5)	22 (31.0)	6 (46.2)
Absent		23 (69.7)	69 (73.4)	48 (67.6)	7 (53.9)
Estrogen receptor:	0.0336				
Positive		23 (69.7)	65 (69.2)	46 (64.8)	3 (23.1)
Negative		10 (30.3)	26 (27.7)	22 (31.0)	10 (76.9)
Progesterone receptor:	0.0043				
Positive		22 (66.7)	55 (58.5)	35 (49.3)	1 (7.7)
Negative		11 (33.3)	35 (37.2)	34 (47.9)	12 (92.3)
Her2/Neu expression:	0.6965				
Positive		4 (12.1)	15 (15.9)	15 (21.1)	2 (15.4)
Negative		29 (87.9)	73 (77.6)	52 (73.2)	11 (84.6)

metastatic breast cancer. These findings suggest that accumulation of RhoC protein is an early and progressive event in the development of breast cancer, thereby justifying efforts aimed at developing novel therapeutic interventions that may prevent the increase in RhoC protein expression.

In the group of patients with invasive carcinomas, very high RhoC expression occurred in a small subset (13 of 211, 6.2%). However, those patients with high levels of RhoC protein in the tumor cells had uniformly a worse outcome than patients with low RhoC expression, despite of aggressive multimodality treatment. Consistently, high RhoC expression was associated with positive lymph nodes, higher histologic grade, and with negative ER and PR protein expression, all known markers of more aggressive disease. Patients with high RhoC expression had a 5- and 10-year overall survival of 57.5% and 23%, respectively, in contrast to patients with low RhoC expression, who had a 5- and 10-year overall survival of 70.5% and 53%, respectively (log rank test, $p = 0.002$). In the multivariable Cox regression analysis, patients with high RhoC levels had 100% increase in the risk of death as compared to patients with low RhoC levels (hazard ratio of 2, 95% CI of 1–4.1, $p = 0.067$). This suggests that RhoC overexpression is a specific alteration that occurs infrequently in early breast ancer, but when present, it signals a biologically aggressive tumor phenotype with high likelihood of recurrence and poor survival despite different treatment interventions. We suggest that this finding is clinically highly relevant and, if further validated, it may be the basis of a new clinically applicable test.

Notably, when we analyzed the predictive value of RhoC in a group of breast cancer patients treated uniformly with a combination regimen of doxorubicin and cyclophosphamide, high RhoC levels were independently associated with overall survival after chemotherapy. Although the number of patients with high RhoC expression is low overall, our data suggest that RhoC may identify a small group of patients who have a poor survival despite doxorubicin-based chemotherapy. This is clinically relevant because, if further validated in a larger cohort of uniformly treated patients, it may identify patients who might benefit from other chemotherapeutic agents or alternative molecular

Table 6. Univariate analysis of overall survival

Characteristic	5-year		10-year		Log-rank *p*-value
	Estimate	95% CI	Estimate	95% CI	
Tumor stage					<0.0001
1	83.5	73.5–93.5	71.8	59.0–84.6	
2	78.2	68.0–88.4	53.2	40.0–66.4	
3 or 4	47.5	33.3–61.5	33.3	19.0–47.8	
Estrogen receptor					0.0078
Positive	75.0	66.9–83.0	55.8	45.9–65.7	
Negative	57.0	44.7–62.3	39.4	26.6–52.2	
Progesterone					0.0001
Positive	80.1	71.9–88.3	62.4	51.7–73.0	
Negative	57.0	46.3–67.7	37.4	26.2–48.5	
Lymphovascular invasion					0.0080
Absent	74.1	66.2–82.0	59.4	50.1–68.8	
Present	59.9	47.4–72.3	34.7	21.6–47.9	
Tumor grade					0.0245
I/II	78.1	69.9–86.4	58.8	48.2–69.3	
III	60.2	48.9–71.5	42.0	30.1–54.0	
Positive lymph nodes					0.0010
Zero	82.4	73.9–90.9	66.5	55.3–77.7	
1–3	78.1	65.3–90.8	56.3	39.7–72.9	
4+	53.7	37.3–70.0	37.4	19.8–54.9	
Tamoxifen use					0.0447
Yes	83.4	75.4–91.5	61.1	49.4–72.7	
No	62.0	52.1–71.9	45.1	34.5–55.8	
Median RhoC expression					
1	80.8	65.1–96.5	58.0	35.9–80.1	0.0209
2	69.3	59.6–79.1	48.5	37.4–59.7	
3	67.6	55.8–79.5	56.9	43.7–70.1	
Low (1, 2, or 3)	70.5	63.7–77.3	52.9	44.9–60.9	
High (4)	57.5	28.9–86.1	23.0	0.0–50.2	

therapies. More research is needed in this direction to further define the prognostic utility of RhoC.

The clinical significance of elevated RhoC protein in breast cancer is linked to and completely consistent with its biological functions. RhoC is a ras homology gene, with highly conserved motifs and shares a high degree of homology to RhoA, another member of the family [16–18]. Rho proteins in general, and RhoC and RhoA in particular, are involved in cytoskeletal reorganization, specifically in the formation of actin stress fibers and focal adhesion contacts [16–18]. When immortalized human mammary epithelial cells are transfected with RhoC, they undergo a striking change in the cytoplasmic shape and they become motile and invasive [9]. In our laboratory, we discovered the strong link between RhoC overexpression and inflammatory breast cancer, the most aggressive form of locally advanced breast cancer known [7–9,19]. Thus, it is not surprising that RhoC overexpression occurs in a small group of biologically aggressive non-IBC tumors with high propensity to recur and metastasize and which respond poorly to doxorubicin-based adjuvant treatment.

Recently, Rho proteins have been implicated in breast tubulogenesis and differentiation, probably through regulation of cell contractility [20]. Our descriptive observations support this notion since RhoC protein levels increased with decreasing differentiation of the invasive carcinomas. For example, well-differentiated invasive carcinomas with prominent tubule formation, monotonous appearing cells, and rare mitoses expressed little or no RhoC protein whereas poorly differentiated carcinomas that grew in disorganized sheets of pleomorphic malignant cells and exhibited a brisk mitotic activity expressed high levels of RhoC protein.

Since our initial reports of RhoC overexpression in breast cancer our findings have been supported by other investigations. RhoC overexpression has been found in malignancies derived from different cell lineages including non-small cell lung carcinoma, hepatocellular carcinoma, ovarian carcinoma, melanoma, pancreatic carcinoma, and gastric carcinoma [7,10,21–29]. In these malignancies, RhoC has been implicated in neoplastic transformation, progression, invasion, and metastases. Taken together, these data suggest that RhoC may be involved in a global, rather than a tissue type specific mechanism of tumor progression.

Rho proteins are prenylated in order to exert their functions and to localize appropriately to the sub

Table 7. Univariate analysis of disease free survival

Characteristic	5-year		10-year		Log-rank *p*-value
	Estimate	95% CI	Estimate	95% CI	
Tumor stage					< 0.0001
1	96.1	90.8–100	90.6	81.8–100	
2	85.7	77.0–94.4	67.3	54.0–80.5	
3 or 4	59.4	44.3–74.4	48.2	31.5–64.9	
Tumor size (cm)					
≤ 2	90.0	83.2–96.7	81.1	71.5–90.7	0.0500
> 2	74.3	64.3–84.3	66.5	55.0–78.1	
Tumor grade					0.0500
I/II	90.6	84.7–96.6	77.8	68.1–87.5	
III	69.3	58.3–80.3	59.5	47.2–71.9	
Positive lymph nodes					< 0.0001
Zero	93.5	87.9–99.0	86.0	77.3–94.7	
1–3	82.3	70.4–94.3	67.8	51.5–84.2	
4+	62.0	45.4–78.6	46.5	26.9–66.1	
Estrogen receptor					0.0041
Positive	86.6	80.1–93.0	74.5	65.4–83.7	
Negative	64.9	52.4–77.3	54.4	40.4–68.6	
Progesterone					0.0003
Positive	92.4	86.9–97.9	79.4	69.8–89.1	
Negative	65.1	54.3–75.8	55.2	43.1–67.4	
Lymphovascular invasion					< 0.0001
Absent	87.0	80.8–93.3	79.3	71.0–87.5	
Present	64.4	51.8–76.9	47.3	32.8–61.8	
Tamoxifen use					0.0121
Yes	87.8	80.7–95.0	80.9	71.6–90.2	
No	74.9	65.8–84.1	60.2	48.9–71.6	
Median RhoC expression					0.0736
1	92.1	83.1–100	72.7	51.4–94.0	
2	80.2	71.4–89.0	72.8	62.2–83.4	
3	78.7	68.3–89.1	68.6	55.7–81.6	
Low (1, 2, or 3)	81.5	75.5–87.4	71.6	63.9–79.2	
High (4)	64.7	36.2–93.2	32.4	0.0–67.1	

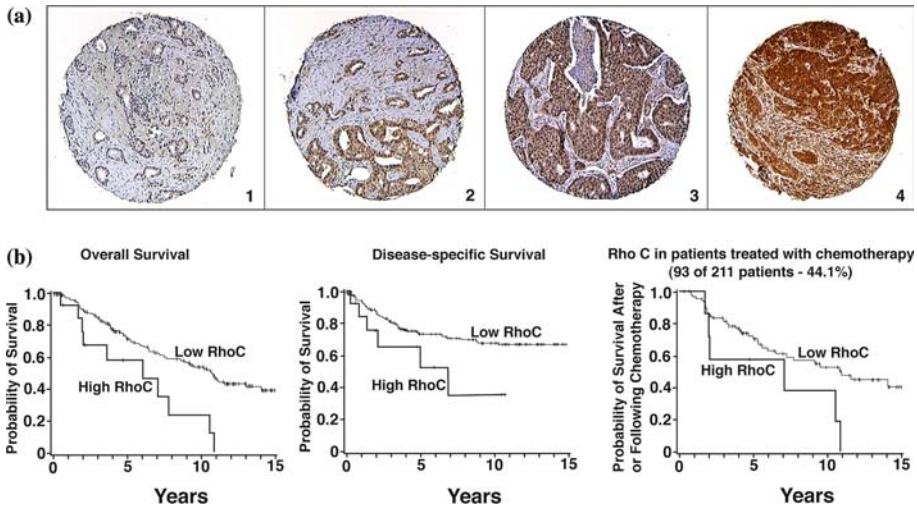


Figure 2. RhoC protein expression is associated with survival in patients with breast cancer. (a) Tissue microarray elements containing representative invasive carcinomas with negative (1), weak (2), moderate (3), and strong (4) RhoC staining intensities. Original magnification 40×. (b) High RhoC expression in invasive carcinomas is associated with worse overall, disease-free, and survival following doxorubicin and cyclophosphamide treatment.

Table 8. Best multivariate model predicting overall survival

Patient/tumor characteristic	HR	95% CI	*p*-Value
Tumor stage			
1	1.0		
2	2.2	1.2–4.0	0.0119
3 or 4	5.6	2.7–11.5	< 0.0001
Lymphovascular invasion			
Absent	1.0		
Present	1.7	0.1–2.7	0.0274
Progesterone receptor			
Positive	1.0		
Negative	1.9	1.2–3.1	0.0059
Median RhoC			
Low expression	1.0		
High expression	2.0	1.0–4.1	0.0670
Radiotherapy			
No	1.0		
Yes	0.6	0.4–1.0	0.0543
Chemotherapy			
No	1.0		
Yes	0.3	0.2–0.6	0.0001
Tamoxifen			
No	1.0		
Yes	0.5	0.3–0.8	0.0030

Table 9. Best multivariate model predicting overall survival for patients receiving chemotherapy

Patient/tumor characteristic	HR	95% CI	*p*-value
Tumor stage			
1	1.0		
2	1.2	0.3–4.3	0.8114
3 or 4	4.2	1.3–13.9	0.0194
Median RhoC			
Low expression	1.0		
High expression	3.1	1.2–7.7	0.0176
Tamoxifen			
No	1.0		
Yes	0.4	0.2–0.9	0.0374

cytoplasmic membrane space [30–34]. Prenylation can be inhibited by farnesyl transferase inhibitors (FTIs) and FTIs are effective in modulating tumor growth in ras-transformed tumor cells [35–39]. Our group has previously found that FTIs were able to reverse of the RhoC-induced phenotype (even though RhoC is not itself farnesylated), manifested by a significant decrease in anchorage-independent growth, motility, and invasion [39]. Thus, we suggested that FTIs may be useful therapeutic compounds in RhoC overexpressing tumors. Another potentially useful strategy against RhoC phenotypes is represented by the HMGCoA (3-hydroxy-3-methylglutaryl-CoA) reductase inhibitors (statins). In particular, atorvastatin has been clearly shown to inhibit RhoC driven phenotypes in melanoma cells [40].

In summary, we discovered that RhoC expression increases with breast cancer progression and that it is associated with markers of aggressive disease and poor survival. Importantly, we found that RhoC overexpression is a negative predictor of response to doxorubicin and cyclophosphamide. This work supports that RhoC may have a role in the genesis of a highly aggressive doxorubicin resistant breast cancer phenotype. Our finding that RhoC overexpression is an infrequent and specific marker of aggressive breast cancer with poor outcome despite treatment may have important clinical implications. Specifically, RhoC detection at the time of primary tumor diagnosis may, in the future, aid clinicians in guiding treatment and paves the way to the development of targeted treatments. While our results are promising, RhoC expression needs to be validated in relationship to outcome in the context of cohorts treated in controlled clinical trials where all patients are treated uniformly. If confirmed, application of RhoC immunohistochemical analysis would be technically straightforward and feasible.

Acknowledgements

Supported in part by Army grants DAMD17-02-1-0490 (CGK), DAMD17-02-1-491 (CGK), DAMD-17-00-1-0345 (SDM), NIH grants K08 CA 090876 (CGK), RO1CA10746 (CGK), RO1CA77612 (SDM), 1 P50-CADE97258 (SDM), and a grant from the John Suzanne Munn Endowed Research Fund of the University of Michigan Comprehensive Cancer Center (CGK), and NIH grant 5 P30 CA46592 (University of Michigan Cancer Center Support Grant).

References

1. Jemal A, Murray T, Samuels A, Ghafoor A, Ward E, Thun MJ: Cancer statistics CA Cancer J Clin 53: 5–26, 2003
2. Ellis M, Hayes D, Lippman M: Treatment of metastatic disease. In: Harris J, Lippman ME, Morrow M (eds) Diseases of the Breast. Lippincott-Raven, Philadelpha 2000, pp. 749–798
3. Hayes DF, Isaacs C, Stearns V: Prognostic factors in breast cancer: current and new predictors of metastasis J Mammary Gland Biol Neoplasia 6: 375–392, 2001
4. Hayes DF, Trock B, Harris AL: Assessing the clinical impact of prognostic factors: when is "statistically significant" clinically useful? Breast Cancer Res Treat 52: 305–319, 1998
5. Clark GM: Prognostic and predictive factors. In: Harris LM. J, Morrow M, Hellman S (eds) , Diseases of the Breast. Lipponcott-Raven Publishers, Philadephia 1996, pp. 461–485
6. Hayes : Do we need prognostic factors in nodal-negative breast cancer? Arbiter Eur J Cancer 36: 302–306, 2000
7. Golen KLvan, Davies S, Wu ZF, Wang Y, Bucana CD, Root H, Chandrasekharappa S, Strawderman M, Ethier SP, Merajver SD: A novel putative low-affinity insulin-like growth factor-binding protein, LIBC (lost in inflammatory breast cancer), and RhoC GTPase correlate with the inflammatory breast cancer phenotype Clin Cancer Res 5: 2511–2519, 1999

8. Golen KLvan, Wu ZF, Qiao XT, Bao L, Merajver SD: RhoC GTPase overexpression modulates induction of angiogenic factors in breast cells Neoplasia 2: 418–425, 2000
9. Golen KLvan, Wu ZF, Qiao XT, Bao LW, Merajver SD: RhoC GTPase, a novel transforming oncogene for human mammary epithelial cells that partially recapitulates the inflammatory breast cancer phenotype Cancer Res 60: 5832–5838, 2000
10. Kleer CG, Golen KLvan, Zhang Y, Wu ZF, Rubin MA, Merajver SD: Characterization of RhoC expression in benign and malignant breast disease : a potential new marker for small breast carcinomas with metastatic ability Am J Pathol 160: 579–584, 2002
11. Kleer CG, Cao Q, Varambally S, Shen R, Ota I, Tomlins SA, Ghosh D, Sewalt RG, Otte AP, Hayes DF, Sabel MS, Livant D, Weiss SJ, Rubin MA, Chinnaiyan AM: EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells Proc Natl Acad Sci USA 100: 11606–11611, 2003
12. Perrone EE, Theoharis C, Mucci NR, Hayasaka S, Taylor JM, Cooney KA, Rubin MA: Tissue microarray assessment of prostate cancer tumor proliferation in African-American and white men J Natl Cancer Inst 92: 937–939, 2000
13. Elston EW, Ellis IO: Method for grading breast cancer J Clin Pathol 46: 189–190, 1993
14. Manley S, Mucci NR, Marzo AMDe, Rubin MA: Relational database structure to manage high-density tissue microarray data and images for pathology studies focusing on clinical outcome: the prostate specialized program of research excellence model Am J Pathol 159: 837–843, 2001
15. Rhodes DR, Sanda MG, Otte AP, Chinnaiyan AM, Rubin MA: Multiplex biomarker approach for determining risk of prostate-specific antigen-defined recurrence of prostate cancer J Natl Cancer Inst 95: 661–668, 2003
16. Nobes CD, Hall A: Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia Cell 81: 53–62, 1995
17. Hall A, Nobes CD: Rho GT Pases molecular switches that control the organization and dynamics of the actin cytoskeleton Philos Trans R Soc Lond B Biol Sci 355: 965–970, 2000
18. Leung T, Chen XQ, Manser E, Lim L: The p160 RhoA-binding kinase ROK alpha is a member of a kinase family and is involved in the reorganization of the cytoskeleton Mol Cell Biol 16: 5313–5327, 1996
19. Kleer CG, Zhang Y, Pan Q, Gallagher G, Wu M, Wu ZF, Merajver SD: WISP3 and RhoC guanosine triphosphatase cooperate in the development of inflammatory breast cancer Breast Cancer Res 6: R110–R115, 2004
20. Wozniak MA, Desai R, Solski PA, CJDer, Keely PJ: ROCK-generated contractility regulates breast epithelial cell differentiation in response to the physical properties of a three-dimensional collagen matrix J Cell Biol 163: 583–595, 2003
21. Suwa H, Ohshio G, Imamura T, Watanabe G, Arii S, Imamura M, Narumiya S, Hiai H, Fukumoto M: Overexpression of the RhoC gene correlates with progression of ductal adenocarcinoma of the pancreas Br J Cancer 77: 147–152, 1998
22. Horiuchi A, Imai T, Wang C, Ohira S, Feng Y, Nikaido T, Konishi I: Up-regulation of small GTPases, RhoA and RhoC, is associated with tumor progression in ovarian carcinoma Lab Invest 83: 861–870, 2003
23. Kamai T, Tsujii T, Arai K, Takagi K, Asami H, Ito Y, Oshima H: Significant association of Rho/ROCK pathway with invasion and metastasis of bladder cancer Clin Cancer Res 9: 2632–2641, 2003
24. Kondo T, Sentani K, Oue N, Yoshida K, Nakayama H, Yasui W: Expression of RHOC is associated with metastasis of gastric carcinomas Pathobiology 71: 19–25, 2004
25. Shikada Y, Yoshino I, Okamoto T, Fukuyama S, Kameyama T, Maehara Y: Higher expression of RhoC is related to invasiveness in non-small cell lung carcinoma Clin Cancer Res 9: 5282–5286, 2003
26. Wang W, Yang LY, Yang ZL, Huang GW, Lu WQ: Expression and significance of RhoC gene in hepatocellular carcinoma World J Gastroenterol 9: 1950–1953, 2003
27. Ikoma T, Takahashi T, Nagano S, Li YM, Ohno Y, Ando K, Fujiwara T, Fujiwara H, Kosai K: A definitive role of RhoC in metastasis of orthotopic lung cancer in mice Clin Cancer Res 10: 1192–1200, 2004
28. Clark EA, Golub TR, Lander ES, Hynes RO: Genomic analysis of metastasis reveals an essential role for RhoC Nature 406: 532–535, 2000
29. Carr KM, Bittner M, Trent JM: Gene-expression profiling in human cutaneous melanoma Oncogene 22: 3076–3080, 2003.
30. Seabra MC: Membrane association and targeting of prenylated Ras-like GTPases Cell Signal 10: 167–172, 1998
31. Kirschmeier PT, Whyte D, Wilson O, Bishop WR, Pai JK: *In vivo* prenylation analysis of Ras and Rho proteins Methods Enzymol 332: 115–127, 2001
32. Adamson P, Marshall CJ, Hall A, Tilbrook PA: Post-translational modifications of p21rho proteins J Biol Chem 267: 20033–20038, 1992
33. Sinensky M: Recent advances in the study of prenylated proteins Biochim Biophys Acta 1484: 93–106, 2000
34. Bishop AL, Hall A: Rho GTPases and their effector proteins Biochem J 348 (Pt2): 241–255 2000
35. Omer CA, Chen Z, Diehl RE, Conner MW, Chen HY, Trumbauer ME, Gopal-Truter S, Seeburger G, Bhimnathwala H, Abrams MT, Davide JP, Ellis MS, Gibbs JB, Greenberg I, Koblan KS, Kral AM, Liu D, Lobell RB, Miller PJ, Mosser SD, O'Neill TJ, Rands E, Schaber MD, Senderak ET, Oliff A, Kohl NE: Mouse mammary tumor virus-Ki-rasB transgenic mice develop mammary carcinomas that can be growth-inhibited by a farnesyl:protein transferase inhibitor Cancer Res 60: 2680–2688, 2000
36. Prendergast GC, Khosravi-Far R, Solski PA, Kurzawa H, Lebowitz PF, CJDer: Critical role of Rho in cell transformation by oncogenic Ras Oncogene 10: 2289–2296, 1995
37. Prendergast GC, Davide JP, deSolms SJ, Giuliani EA, Graham SL, Gibbs JB, Oliff A, Kohl NE: Farnesyltransferase inhibition causes morphological reversion of ras-transformed cells by a complex mechanism that involves regulation of the actin cytoskeleton Mol Cell Biol 14: 4193–4202, 1994
38. Lebowitz PF, Casey PJ, Prendergast GC, Thissen JA: Farnesyltransferase inhibitors alter the prenylation and growth-stimulating function of RhoB J Biol Chem 272: 15591–15594, 1997
39. van Golen KL, Bao L, DiVito MM, Wu Z, Prendergast GC, Merajver SD: Reversion of RhoC GTPase-induced inflammatory breast cancer phenotype by treatment with a farnesyl transferase inhibitor Mol Cancer Ther 1: 575–583, 2002
40. Collisson EA, Kleer C, Wu M, De A, Gambhir SS, Merajver SD, Kolodney MS: Atorvastatin prevents RhoC isoprenylation, invasion, and metastasis in human melanoma cells Mol Cancer Ther 2: 941–948, 2003

Address for offprints and correspondence: C.G. Kleer, 2G332 University Hospital, 1500 E. Medical Center Dr., Ann Arbor, MI 48109-0054; *Tel.:* + 1-734-936-6775; *Fax:* + 1-734-615-3441; *E-mail:* Kleer@umich.edu

Research article

Open Access

Inhibition of CCN6 (WISP3) expression promotes neoplastic progression and enhances the effects of insulin-like growth factor-1 on breast epithelial cells

Yanhong Zhang[1], Quintin Pan[2,3], Hui Zhong[1], Sofia D Merajver[2,3] and Celina G Kleer[1,3]

[1]Department of Pathology, University of Michigan Medical Center, 1150 W Medical Center Drive, Ann Arbor, MI 48109, USA
[2]Department of Internal Medicine, Division of Hematology/Oncology, University of Michigan Medical Center, 1500 E. Medical Center Dr., Ann Arbor, MI 48109, USA
[3]Comprehensive Cancer and Geriatrics Center, University of Michigan Medical Center, 1500 E. Medical Center Dr. Ann Arbor, MI 48109, USA

Corresponding author: Celina G Kleer, kleer@umich.edu

Received: 29 Aug 2005 Revisions requested: 15 Sep 2005 Revisions received: 3 Oct 2005 Accepted: 13 Oct 2005 Published: 8 Nov 2005

Breast Cancer Research 2005, **7**:R1080-R1089 (DOI 10.1186/bcr1351)
This article is online at: http://breast-cancer-research.com/content/7/6/R1080

Abstract

Introduction CCN6/WISP3 belongs to the CCN (Cyr61, CTGF, Nov) family of genes that contains a conserved insulin-like growth factor (IGF) binding protein motif. CCN6 is a secreted protein lost in 80% of the aggressive inflammatory breast cancers, and can decrease mammary tumor growth *in vitro* and *in vivo*. We hypothesized that inhibition of CCN6 might result in the loss of a growth regulatory function that protects mammary epithelial cells from the tumorigenic effects of growth factors, particularly IGF-1.

Method We treated human mammary epithelial (HME) cells with a CCN6 hairpin short interfering RNA.

Results CCN6-deficient cells showed increased motility and invasiveness, and developed features of epithelial-mesenchymal transition (EMT). Inhibition of CCN6 expression promoted anchorage-independent growth of HME cells and rendered them more responsive to the growth effects of IGF-1, which was coupled with the increased phosphorylation of IGF-1 receptor and insulin receptor substrate-1 (IRS-1).

Conclusion Specific stable inhibition of CCN6 expression in HME cells induces EMT, promotes anchorage-independent growth, motility and invasiveness, and sensitizes mammary epithelial cells to the growth effects of IGF-1.

Introduction

Wnt-1-induced secreted protein 3 (CCN6/WISP3) is a cysteine-rich protein that belongs to the CCN (Cyr61, CTGF, Nov) family of genes, which also contains five other members: CCN1 (connective tissue growth factor or CTGF), CCN2 (Cyr61), CCN3 (Nov), CCN4 (WISP1) and CCN5 (WISP2) [1-3]. CCN4, the first cloned member of the WISP proteins was discovered because it was upregulated in the mouse mammary epithelial cell line C57MG transformed by Wnt-1 [2]. CCN6 shares high sequence homology to CCN4, although there is no evidence that CCN6 is induced by Wnt-1 signaling [2]. CCN growth factors are mostly secreted and extracellular matrix-associated proteins that regulate important functions including cellular differentiation and development, cell proliferation, survival, angiogenesis, cell migration and adhesion [2-6]. CCN proteins interact with key signaling molecules such as cell surface integrins, NOTCH1, fibulin C, S100A4, and ion channels [7-12].

CCN proteins contain an N-terminal secretory signal, followed by four distinct motifs with homology to insulin-like growth factor (IGF)-binding protein; von Willebrand factor type C; thrombospondin 1 (TSP1); and a C-terminal region with heparin-binding motifs and sequence similarities to the C termini of von Willebrand factor and mucins, the latter also being present in other growth factors such as transforming growth factor-β, platelet-derived growth factor, and nerve growth factor [1,2,9]. It is becoming increasingly evident that CCN proteins function in a cell-type-specific and tissue-type-specific manner. For example, increased expression of CCN3 (Nov) was correlated

bp = base pair; EGF = epidermal growth factor; EMT = epithelial-mesenchymal transition; FBS = fetal bovine serum; HME = human mammary epithelial; IGF = insulin-like growth factor; IGF-1R = insulin-like growth factor-1 receptor; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; RT-PCR = reverse transcriptase-mediated polymerase chain reaction; siRNA = short interfering RNA.

with reduced tumorigenicity in glioma, choriocarcinoma, and Ewing's sarcoma cell lines [13-16]. However, increased expression of CCN3 was associated with enhanced tumor growth in renal cell carcinomas [17]. CCN2 (Cyr61) stimulates the growth of breast and gastric adenocarcinomas [18,19], whereas its expression is downregulated in non-small cell lung cancer, in which CCN2 suppressed the growth of NSCLC cells by triggering a signal transduction pathway through β-catenin [20,21]. These data suggest that two members of the CCN family, CCN2 and CCN3, behave under certain circumstances as oncogenes or tumor suppressor genes in different tissue types (reviewed in [12,22]).

CCN6 maps to chromosome 6q21-22, a locus that displays high rates of loss of heterozygosity in breast cancer [23,24]. We have previously reported that CCN6 is lost in the majority of inflammatory breast cancers, a highly aggressive and metastatic form of breast cancer [25]. Restoration of CCN6 expression in inflammatory breast cancer cells results in growth inhibition *in vitro* and *in vivo* [6,26]. After synthesis, CCN6 protein is secreted and is able to modulate the effects of IGF-1 on IGF-1 receptor (IGF-1R) activation and its signaling pathways [27]. Here we investigate the function of CCN6 in the normal breast epithelium. We show that inhibition of CCN6 expression in human mammary epithelial cells strongly induces epithelial-mesenchymal transition (EMT) and leads to a highly motile and invasive phenotype. CCN6 loss promotes anchorage-independent growth in breast epithelial cells and increases cell proliferation, which is mechanistically driven, at least in part, by enhancement of the growth effects of IGF-1 and the activation of IGF-1R signaling pathways.

Materials and methods

Cell culture

Human mammary epithelial (HME) cells were immortalized with human papilloma virus E6/E7 and were characterized as being positive for keratin 19 [6,28]. Cells were cultured in Ham's F-12 medium supplemented with 5% fetal bovine serum (FBS), hydrocortisone (1 μg/ml), insulin (5 μg/ml), epidermal growth factor (EGF; 10 ng/ml), cholera toxin (100 μg/ml), fungizone (2.5 μg/ml) and gentamycin (5 μg/ml), at 37°C under 10% CO_2.

Generation of stable hairpin short interfering RNA-CCN6 HME cells

Hairpin short interfering RNA (siRNA) 5'-GATCCCGCCAGGGGAAATCTGCAATGTTCAAGAGA-CATTGCAGATTTCCCCTGGTTTTTTGGAAA-3' and the complementary strand 5'-AGCTTTTCCAAAAAACCAG-GGGAAATCTGCAATGTCTCTTGAACATTGCAGATTTC-CCCTGGCGG-3' were synthesized (Invitrogen, Carlsbad, CA, USA), and annealed hairpin siRNA-CCN6 inserts were cloned into pSilencer2.1-U6 hygro expression vector (Ambion, Austin, TX, USA). The sequence of siRNA-CCN6 (insert in) expression vector was confirmed by sequencing (University of Michigan DNA Sequencing Core). HME cells were transfected with pSilencer2.1-U6 negative control (siRNA-control; Ambion) or siRNA-CCN6 plasmids by using FuGene 6 transfection reagent (Roche-Boehringer Mannheim, Mannheim, Germany). Stable transfectants were established by culturing transfected cells in the described medium supplemented with 100 μg/ml hygromycin (Invitrogen) for 4 weeks.

RT-PCR analysis

Total RNA was isolated from HME cells and siRNA HME clones with a Trizol kit (Life Technologies, Inc., Gaithersburg, MD, USA). First-strand cDNA synthesis was performed by using 1 μg of total RNA with AMV reverse transcriptase (Promega, Madison, WI, USA) and oligo(dT) as a primer. A 2 μl portion of the reaction mixture was used for amplification by PCR. The following primers were used: CCN6 forward primer 5'-ATGCAGGGGCTCCTCTTCTCC-3' and CCN6 reverse primer 5'-CTTGAGCTCAGAAAATATATC-3' to amplify a 1,050 bp product; E-cadherin forward primer 5'-CCTTCCTC-CCAATACATCTCCC-3' and E-cadherin reverse primer 5'-TCTCCGCCTCCTTCTTCATC-3' to amplify a 600 bp product. PCR was performed under the following conditions: denaturing for 1 minute at 94°C, annealing for 1 minute at 58°C for CCN6 and at 65°C for E-cadherin, and elongation for 1 minute at 72°C, for 35 cycles.

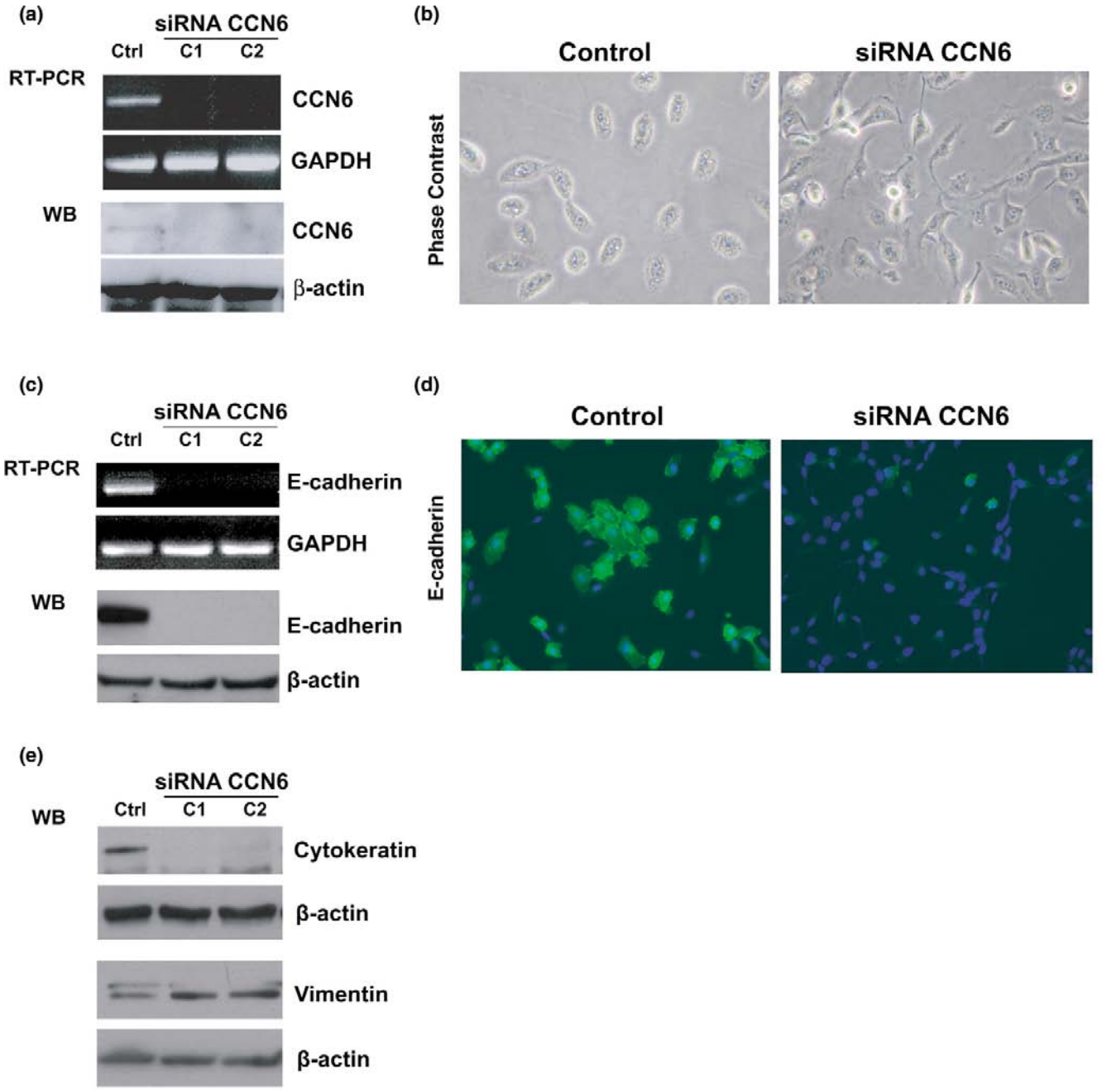
Immunofluorescence

Cells were grown on glass coverslips, fixed with methanol for 5 minutes at -20°C and then permeabilized with 0.2% Triton X-100 for 15 minutes at room temperature 25°C. The coverslips were then saturated for 30 minutes with 2% bovine serum albumin in PBS. After being washed in PBS, cells were incubated for 1 hour with a monoclonal antibody against E-cadherin (dilution 1:50, BD Transduction Laboratories, San Jose, CA, USA). Cells were exposed for 1 hour to Alexa Fluor anti-mouse Ig G (Molecular Probes, Eugene, OR, USA). After being washed with PBS three times, the coverslips were then mounted with prolong Gold anti-fade reagent with 4',6-diamidino-2-phenylindole and the borders were sealed with transparent nail polish.

Anchorage-independent growth

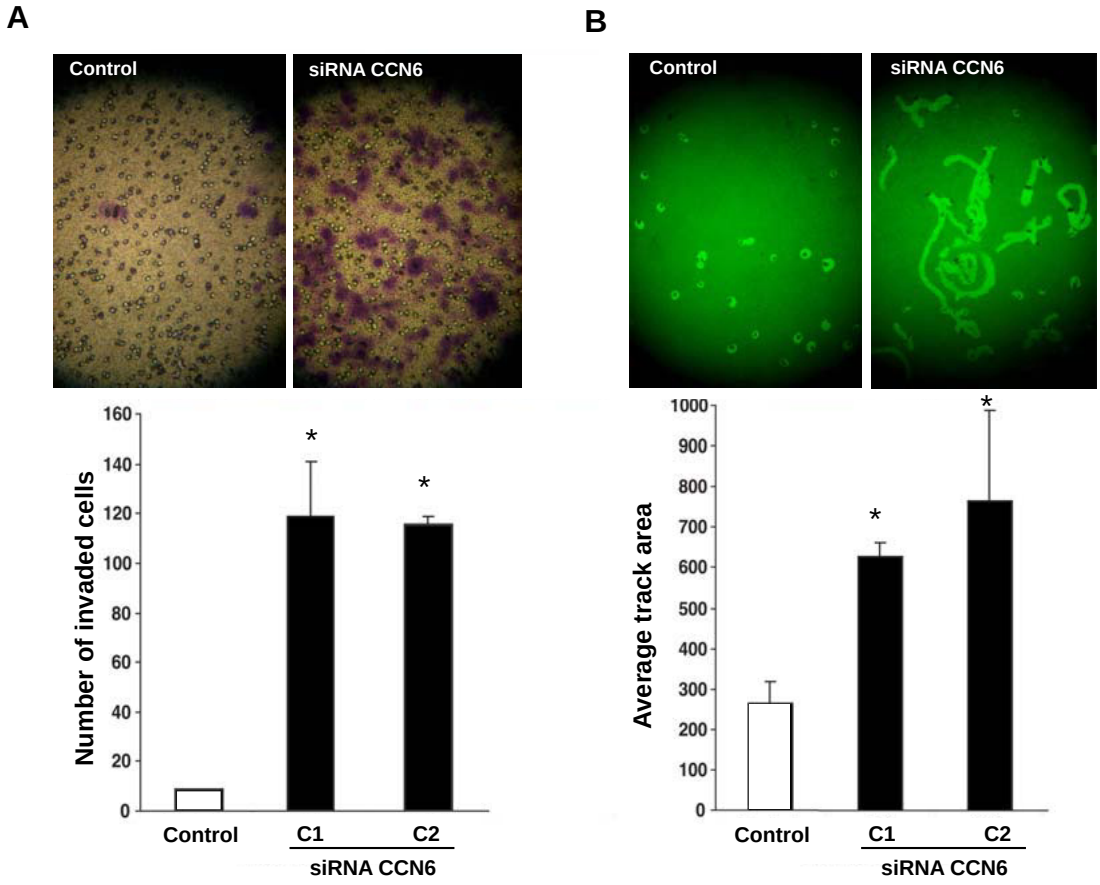
For studies of anchorage-independent growth we performed soft agar assays on stable clones of HME/CCN6 siRNA and on HME control cells. Each well of a six-well plate was first layered with 0.6% agar diluted with 5% FBS-supplemented Ham's F-12 medium complete with growth factors. The cell layer was then prepared by diluting agarose to concentrations of 0.3% with 5×10^3 cells in 2.5% FBS-supplemented Ham's F-12 (1.5 ml per well). Plates were maintained for 3 weeks at 37°C under 10% CO_2. Colonies were counted under a microscope with a grid. The experiment was performed three times independently.

Figure 1



Effect of CCN6 inhibition on the phenotype of mammary epithelial cells. **(a)** Human mammary epithelial (HME) cells were stably transfected with pSilencer2.1-U6 hygro expression vector expressing CCN6 hairpin siRNA. The suppression of CCN6 was confirmed by reverse transcriptase PCR and Western immunoblotting (WB). HME control cells express low levels of CCN6 protein. C1 and C2 (two stable HME CCN6 siRNA clones) show almost complete inhibition of CCN6. **(b)** Stable inhibition of CCN6 on HME cells results in a change from compact and oval-shaped cells to irregularly shaped cells with many protruding processes. **(c)** E-cadherin protein and mRNA were decreased in CCN6-deficient cells. **(d)** By immunofluorescence CCN6-deficient cells show a drastic decrease of membrane-associated E-cadherin protein. **(e)** Western immunoblots show that CCN6 inhibition results in a decrease in cytokeratin and an increase in vimentin proteins. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Figure 2



Effect of CCN6 inhibition on random motility and invasion. **(a)** A reconstituted basement membrane invasion chamber assay was used to investigate the effects of CCN6 inhibition on the invasion abilities of HME cells. Representative fields of invaded and stained cells are shown. *t* test $*p < 0.04$ vs. control. **(b)** Cell motility on a field of microscopic fluorescent beads. Representative photomicrographs show the clearing in the fluorescent bead field corresponding to phagokinetic cell tracks. $*p < 0.005$ vs. control. Experiments were performed independently three times. C1 and C2 are two stable CCN6 deficient clones.

Basement membrane matrix invasion

HME/CCN6 siRNA and HME controls were trypsinized and 300 µl of 10^6 cells/ml in serum-free medium were seeded at equal numbers onto the extracellular matrix layer (ECM; Chemicon, Temecula, CA, USA). Subsequently, we added 500 µl of medium containing 5% FBS to the lower chamber. After incubation for 24 hours, the non-invading cells and ECM gel from the interior of the insert were removed gently with a cotton swab. The invasive cells on the lower surface of membrane were stained, air dried, and photographed. The invaded cells were counted under the microscope. The experiment was performed three times independently.

Random motility

Random cell motility was determined as described in the motility assay kit (Cellomics Inc., Pittsburg, PA, USA). Cells were harvested and 500 cells per well were suspended in 2.5% FBS medium and plated on top of a field of microscopic fluorescent beads in a 96-well plate. After incubation for 16 hours, cells were fixed and areas of clearing in the fluorescent bead field corresponding to phagokinetic cell tracks were quantified with an NIH ScionImager. The experiment was performed three times independently.

Immunoprecipitation

HME cells and HME/CCN6 siRNA cells were serum-starved for 24 hours and subsequently stimulated for 15 minutes with 25 ng/ml IGF-1. Proteins were obtained by lysing the cells in

a buffer composed of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P40, 1 mM Na_3VO_4, 1 mM phenylmethylsulphonyl fluoride, 1 μg/ml leupeptin, and 10 μg/ml aprotinin. The IGF-1R was immunoprecipitated overnight from 500 μg of cell (protein) lysate with anti-IGF-1R monoclonal antibody (Oncogene, San Diego, CA, USA) at 4°C. After isolation with protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA), the immunocomplexes were separated by 7.5% SDS-PAGE. The proteins were transferred to a poly(vinylidene difluoride) membrane and subsequently detected by immunoblotting with anti-IGF-1R β subunit polyclonal antibody (Santa Cruz Biotechnology). Tyrosine phosphorylation of immunoprecipitated IGF-1R was assessed with anti-phosphotyrosine monoclonal antibody PY20 (Transduction Laboratories, Lexington, KY, USA).

Western blot analysis

Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P40, 1 mM Na_3VO_4, 1 mM phenylmethylsulphonyl fluoride, 1 μg/ml leupeptin, and 10 μg/ml aprotinin). Cell lysates (50 μg of protein) were fractionated by SDS-PAGE and transferred to Immobilon-P membrane (Millipore, Billerica, MA, USA). The membranes were blocked for 1 hour with Tris-buffered saline containing 5% non-fat milk and 0.1% Tween 20. Immobilized proteins were probed with antibodies specific for phosphorylated and total insulin receptor substrate-1 (IRS-1; Upstate Biotechnology), E-cadherin and vimentin monoclonal antibodies (BD Transduction Laboratories) and antibodies against cytokeratin 18 (c-04, ab668, Abcam Inc, Cambridge MA, USA). CCN6 was detected with a polyclonal anti-CCN6 antibody developed against an immunogenic peptide Ac-PEGRPGEVSDAPQRKQ-$CONH_2$ corresponding to amino acids 31 to 46 of human CCN6 with the assistance of Covance.

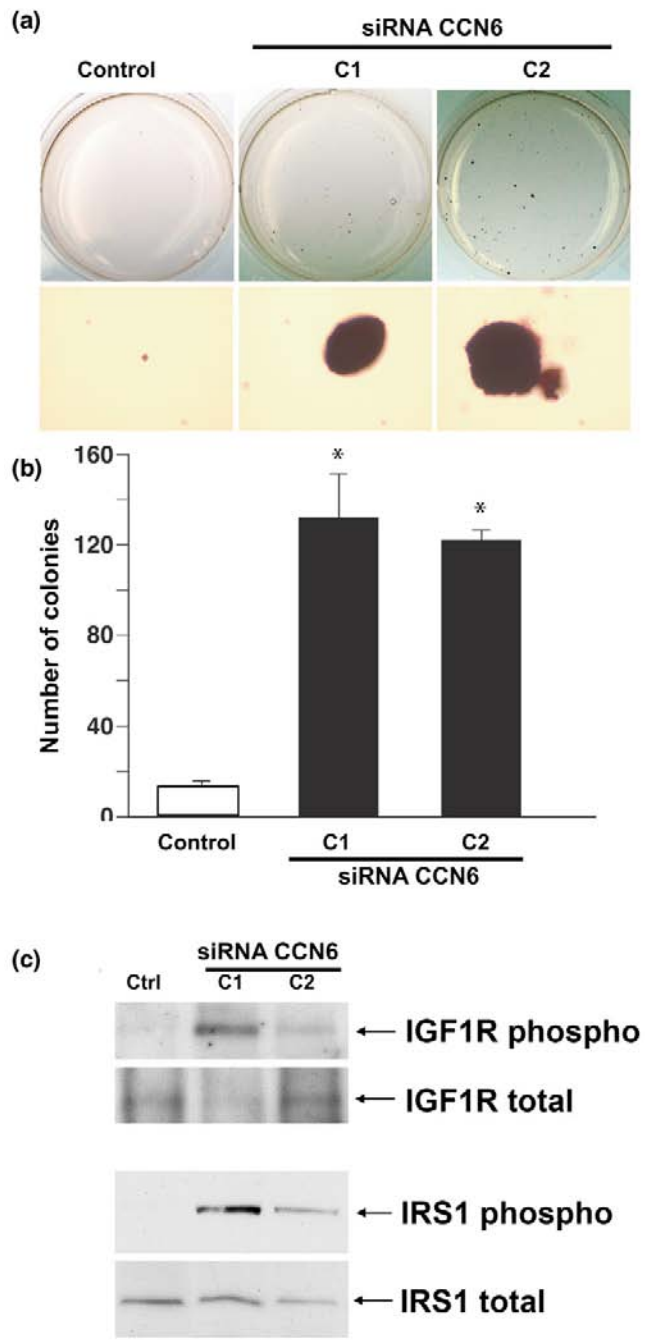
IGF-1 growth assay

HME/CCN6 siRNA and HME control cells were plated in 96-well plates at a concentration of 5 × 10^4 cells/ml and serum-starved for 24 hours. Subsequently, 25 ng/ml human recombinant IGF-1 (Upstate Biotechnology) was added. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) reagents were added 24 hours later in accordance with the manufacturer's protocol, and the plate was read at a wavelength of 595 nm. The experiment was performed with triplicate samples.

S-phase analysis

HME/CCN6 siRNA and HME control cells were plated at a concentration of 5 × 10^4 cells/ml and serum-starved for 24 hours. Subsequently, 25 ng/ml human recombinant IGF-1 (Upstate Biotechnology) was added. Serum-starved and IGF-1-treated HME control cells and HME/CCN6 siRNA cells were pulsed for 2 hours with 10 μM bromodeoxyuridine (BrdU; Roche, Indianapolis, IN, USA), harvested with trypsin-EDTA, and fixed with 70% ethanol at -20°C for at least 1 hour.

Figure 3



CCN6-deficient cells acquire anchorage-independent growth abilities. **(a)** Anchorage-independent growth of human mammary epithelial stable cell lines in soft agar. The upper panels show an assay in soft agar; the lower panels show the colony size under a × 100 magnification of the same wells after 3 weeks in culture. Three independent experiments yielded similar results. **(b)** Bar graph summarizing the results of three independent experiments in soft agar. **(c)** CCN6 inhibition induced an increase in phosphorylation of insulin-like growth factor-1 receptor (IGF-1R) and insulin receptor substrate-1 (IRS-1). C1 and C2 are two stable CCN6 deficient clones.

Cells were resuspended in 2 M HCl for 20 minutes at room temperature. After centrifugation, the cell pellets were resuspended in borax (pH 8.5) to neutralize any residual acid. After brief centrifugation the pellets were washed in 1 ml PBS and resuspended for 1 hour in fluorescein isothiocyanate-labeled anti-BrdU antibody (catalogue no. 347583; Becton Dickinson San Jose, CA, USA) in the dark. Cells were washed in PBS, resuspended in PBS containing propidium iodide, and analyzed for propidium iodide and BrdU staining on a Becton-Dickinson flow cytometer with the use of Cellquest software. The experiment was performed three times independently.

Results

Expression of CCN6 in immortalized human mammary epithelial cells

A CCN6-specific receptor has not yet been cloned; blockade of the receptor is therefore not yet feasible as a means of inhibiting CCN6 function. Thus, to investigate the role of CCN6 in the mammary epithelium and in breast tumorigenesis, HME cells were treated with a CCN6 hairpin siRNA. siRNAs silence gene expression in a sequence-specific post-translational way; they therefore constitute a useful strategy for identifying gene function [29].

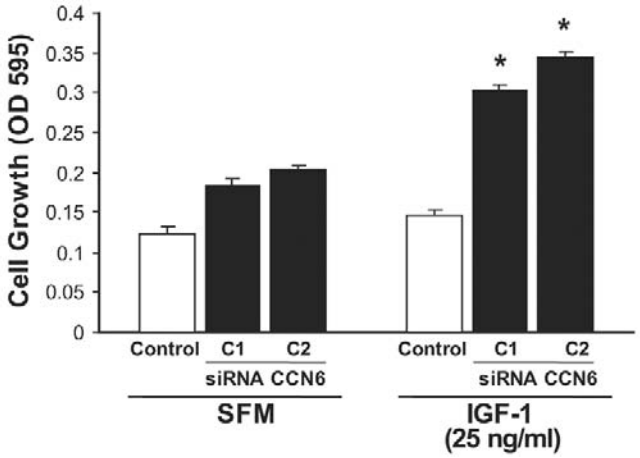
The expression of CCN6 transcript and protein in HME controls and HME cells stably transfected with CCN6 siRNA were studied by RT-PCR and Western immunoblotting, respectively (Fig. 1a). For subsequent experiments, two clones with CCN6 protein downregulation were selected to study the functional consequences of CCN6 inhibition. The hairpin inhibition of CCN6 protein levels was stable and remained stable 5 months after transfection, as tested by Western blotting.

CCN6 inhibition is a potent inducer of EMT and triggers motile and invasive properties on mammary epithelial cells

While the parental HME cells were compact, thick, and ovoid, with rare extended processes, CCN6 inhibition led to cells that were thin, spreading, with stellate shape and numerous cytoplasmic extended processes reminiscent of an epithelial to mesenchymal transition (EMT) (Fig 1b). Consistently, CCN6-deficient cells had loss of the epithelial markers E-cadherin and cytokeratin, whereas they exhibited increased vimentin protein, a characteristic marker for mensenchymal cells (Fig. 1c,d and 1e).

Because E-cadherin is crucial for epithelial cell-cell adhesion and its downregulation has been shown to enhance cellular motility and invasion, we tracked the movement of CCN6-deficient cells and their ability to invade a basement membrane. As shown in Fig. 2, CCN6 deficient cells were much more motile and invasive *in vitro* than the controls.

Figure 4



CCN6 inhibition increases the growth of human mammary epithelial cells in response to insulin-like growth factor-1 (IGF-1). Cells were serum starved for 24 hours, and subsequently stimulated with 25 ng/ml of IGF-1. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) reagents were added 24 hours later and the plate was read at a wavelength of 595 nm. Results are expressed as means ± SD for three independent experiments. Asterisks indicate statistical significance (*t* test); $p < 0.005$ for short interfering RNA (siRNA)/CCN6 after treatment with IGF-1 compared with serum free medium (SFM). C1 and C2 are two stable CCN6 deficient clones.

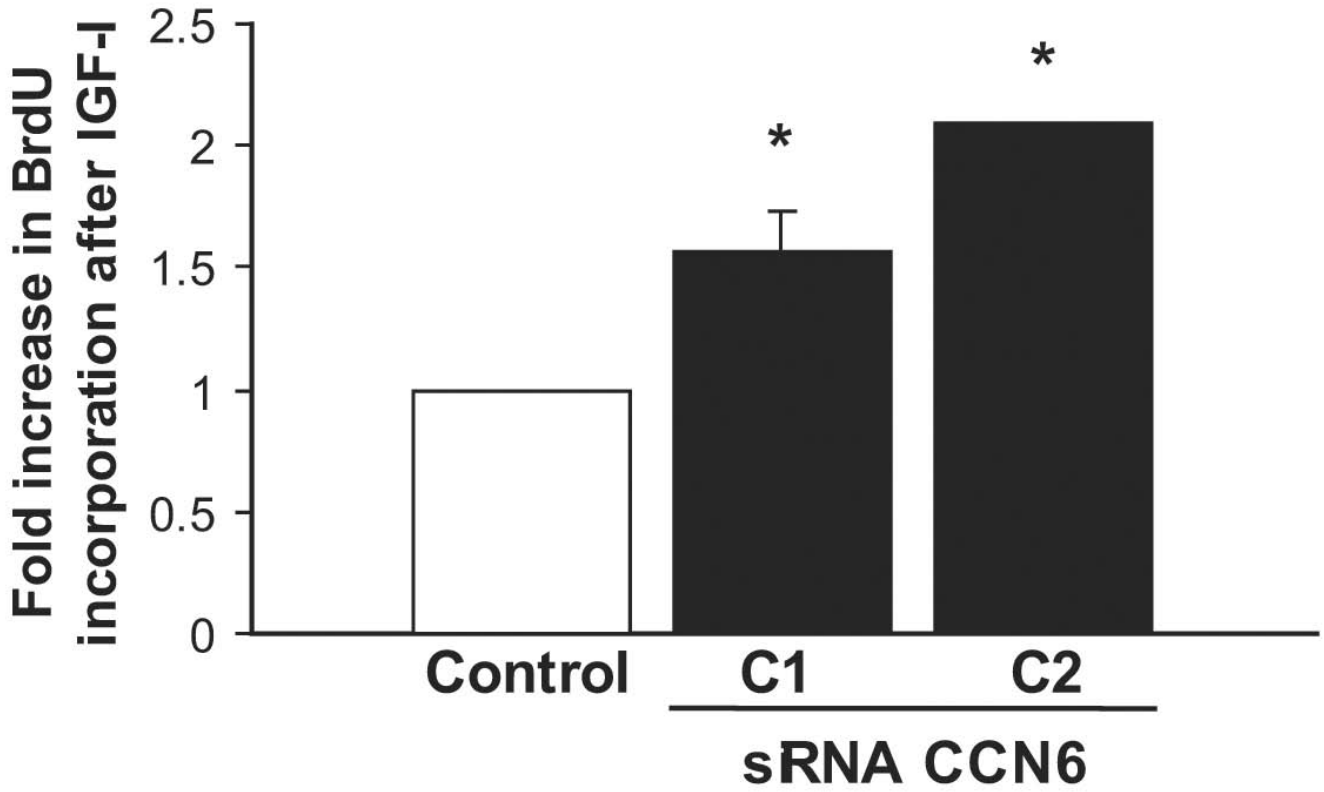
CCN6 inhibition promotes anchorage-independent growth, increases IGF-1R phosphorylation and potentiates the mitogenic effects of IGF-1

Stable inhibition of CCN6 by hairpin siRNA strongly promoted the growth of HME cells in soft agar (Fig. 3). CCN6-deficient cells were able to form colonies in medium supplemented with only 2.5% FBS, whereas HME control cells did not significantly form colonies under these conditions. CCN6-deficient cells formed about 10-fold more colonies than the control cells after 21 days. Furthermore, the colonies formed by the CCN6-deficient cells were much larger than the colonies formed by the control cells.

Our observation of the ability of CCN6-deficient cells to form colonies in soft agar in the presence of limiting serum suggested that these lines might have increased activation of growth receptor tyrosine kinase signaling pathways. This is a well-known mechanism for transformation that allows the cells to grow in the absence, or with minimal amounts, of growth factors [30,31]. Because CCN6 contains an IGFBP motif and once secreted it is able to modulate IGF signaling pathways [27], it was logical to begin our analysis by investigating whether inhibition of CCN6 might directly or indirectly induce the activation of IGF-1R. Stable CCN6 inhibition by hairpin siRNA led to an increase in the phosphorylation of IGF-1R and IRS-1 (Fig. 3c).

Next, we assayed CCN6-deficient cells and controls for their growth ability in serum free medium and in the presence of

Figure 5



CCN6 inhibition enhances the proliferative effects of insulin-like growth factor-1 (IGF-1) on mammary epithelial cells. The bar graph shows that CCN6-deficient cells exhibited a significant increase in bromodeoxyuridine (BrdU) incorporation in response to treatment with IGF-1 (*t* test, $p < 0.03$ for both clones). The human mammary epithelial (HME) control cells did not exhibit differences in the S-phase fraction after the addition of IGF-1. The experiment was performed three times independently. Results are expressed as means ± SD for three independent experiments. C1 and C2 are two stable CCN6 deficient clones.

IGF-1 in the condition media. In the complete absence of serum, CCN6-deficient cells were able to survive and were metabolically active when compared to controls (Fig. 4). Upon addition of IGF-1 we observed that inhibition of CCN6 enhanced the growth effects of IGF-1. Inhibition of CCN6 stimulated the growth of HME cells in serum free medium, however, when 25 ng of IGF-1 was added, we noted a 2.5-fold increase in cell growth, which did not increase significantly with increasing concentrations of recombinant IGF-1 in the medium.

While IGF-1 stimulation did not affect the proliferative activity of the control cells, CCN6-deficient cells exhibited a significant increase in the S-phase fraction (Fig. 5). Taken together these data support the hypothesis that loss of CCN6 in HME cells results in loss of a growth regulatory mechanism which enhances the proliferative and growth response to IGF-1.

Discussion

In this study we characterize the function of CCN6, a recently identified gene that is lost in inflammatory breast cancer, the most lethal form of breast cancer [6,25]. Here we show that inhibition of CCN6 expression induces the EMT of mammary epithelial cells and results in a highly motile and invasive phenotype. CCN6-deficient cells acquire anchorage-independent growth abilities and exhibit increased growth and proliferative response to IGF-1 through enhanced IGF-1R signaling.

To characterize the function of CCN6 in the breast epithelium, we first analyzed the phenotypic effects of stable inhibition of CCN6 expression by hairpin siRNA. Inhibition of CCN6 profoundly altered the phenotype of human mammary epithelial cells, leading to the acquisition of features of EMT. EMT involves the phenotypic alteration of epithelial cells to fibroblastoid, spindle, migratory, and more malignant cells, which show a mesenchymal gene expression program [32,33], and represents an important *in vitro* correlate of tumor progression [32,33]. Several proteins have been implicated in this process, which involves a dominant transcriptional repression that leads to a downregulation of E-cadherin expression and an increase in the expression of mesenchymal markers. The detailed mechanisms of EMT in breast epithelial cells remain elusive.

On the basis of our studies, inhibition of CCN6 results in a morphologic change towards a mesenchymal phenotype, which is accompanied by a marked downregulation of E-cadherin and cytokeratin, and an increase in vimentin, a mesenchymal marker. Functionally, CCN6 inhibition led to a pronounced increase in cell motility and invasion. The mechanism of E-cadherin downregulation brought about by the loss of CCN6 is intriguing. E-cadherin transcript expression was also inhibited in CCN6-deficient cells, suggesting that this effect is probably exerted at the transcriptional level, by epigenetic mechanisms such as hypermethylation and transcriptional silencing. These results were also obtained when CCN6 was knocked down in MCF10A cells (data not shown). It is possible that the loss of CCN6 might lead to increased methylation of the E-cadherin promoter, which has been shown to be correlated with a loss of E-cadherin expression in breast cancer cell lines and primary invasive ductal and lobular carcinomas of the breast [34,35]. It is also possible that CCN6 loss results in transcriptional silencing of E-cadherin, perhaps by the induction of transcriptional repressors, such as Snail [36,37] and SIP1/ZEB2 [36,37], known to inactivate the E-cadherin promoter. Supporting evidence has recently been reported by Sen and colleagues [38] showing that CCN6 is able to activate the SOX transcription factors by a similar mechanism.

CCN6 inhibition led to the acquisition of anchorage-independent growth abilities even under extremely low serum conditions. We therefore reasoned that loss of CCN6 might impair a central growth regulatory mechanism, rendering the cells more sensitive to the effect of even minimal amounts of growth factors such as EGF and IGF. Although CCN6 inhibition had no effect on the phosphorylation of the EGF receptor (data not shown), it markedly enhanced the growth effects of IGF-1, a potent mitogen for breast epithelial cells [39]. CCN6 inhibition led to an increase in the phosphorylation of IGF-1R and its main downstream target molecule IRS-1. This was coupled with an increase in the metabolic and proliferative activities of mammary epithelial cells. These data support a role for CCN6 a modulator of the growth effects of IGF-1 on the breast epithelium, and may have clinical implications, as CCN6 loss may mark an epithelium at increased risk of malignant transformation and might constitute a target for breast cancer prevention.

The IGF family of growth factors is crucial in the development and progression of breast cancer. Studies *in vitro* and *in vivo* have shown that IGFs promote the proliferation, survival, and metastatic ability of cancer cells [40-43]. The role of IGF-1R in promoting the proliferation of breast cancer cells is well established [39,44,45]. A role for IGF-1R in breast cancer metastases has been shown recently [40]. 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 [46]. High expression of IGF-1R has been demonstrated in most primary human breast cancers when compared with normal or benign breast tissue, and hyperactivation of IGF-1R in breast cancer has been linked with increased radioresistance and cancer recurrence at the primary site [39,44,45]. High levels of IRS-1, a major signaling molecule downstream of the IGF-1R, are correlated with tumor size and shorter disease-free survival in estrogen receptor-positive breast cancer patients [47,48].

The effect of CCN6 inhibition on the phosphorylation of the IGF-1R and IRS-1 is consistent with a growth regulatory effect on the mammary epithelium. The relationship between the IGF family and CCN6 emerges from our previous investigations as well as from another laboratory [14,27,38]. CCN6-rich medium induces a decrease in IGF-1-stimulated IGF-1R phosphorylation in breast cancer cells, and then results in an inhibition of cellular proliferation [27]. In another system, Sen and colleagues [38] reported that anti-IGF-1 neutralizing antibody partly blocks the CCN6-mediated upregulation of COL2A1 mRNA in immortalized chondrocyte cell lines. In view of these data, we postulate that CCN6 may modulate the availability of IGF-1 to the IGF-1R, conceivably by binding and sequestering IGF-1, and/or by interfering with other regulators of the IGF signaling pathway.

Conclusion

We propose a model whereby CCN6 serves as an important growth regulatory protein of the mammary epithelium and controls the cellular responses to the growth stimulatory effects of IGF-1. Loss of CCN6 expression alters the phenotype of the breast epithelium and promotes motility and invasion. The CCN6-deficient cells are extremely sensitive to the growth stimulatory effects of IGF-1. The uncontrolled growth may in turn predispose to neoplasia. It is possible that pharmacological restoration of the CCN6 autocrine regulatory loop could be targeted to prevent malignant transformation of mammary epithelial cells.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YZ carried out the cell culture, transfections, western blotting, RT-PCR, functional assays, and IGF-1 assays. She drafted the manuscript. QP assisted with the optimization of techniques, and was involved in the overall planning of experiments. HZ contributed her knowledge on flow cytometry and performed the BrdU incorporation assays. She assisted with the planning and interpretation of IGF-1 experiments, and manuscript writing. SDM conceived some ideas on the study, and contributed to the experimental plan, especially on the IGF-1 connection. She assisted in trouble shooting experiments. She helped planning the figures and edited the manuscript. CGK contributed ideas for the study and participated in the design and

coordination of the study. She wrote and edited the manuscript significantly. She was involved and directed all the aspects of the study. SDM and CGK share senior authorship. All authors read and approved the final manuscript.

Acknowledgements

We thank members of the Kleer and Merajver laboratories for critical reading and constructive suggestions throughout the execution of this project, and Karilynn Schneider and Robin Kunkel for artwork. We thank Dr Bernard Perbal (Université de Paris 7 – Denis Diderot) for helpful discussions. This work was supported in part by NIH grants K08CA090876 and R01CA107469 (CGK), RO1CA77612 (SDM), Department of Defense grants DAMD17-01-1-490 (CGK) and DAMD17-01-1-491 (CGK), a grant from the Breast Cancer Foundation and the Burrhows Wellcome fund (SDM).

References

1. Hurvitz JR, Suwairi WM, Van Hul W, El-Shanti H, Superti-Furga A, Roudier J, Holderbaum D, Pauli RM, Herd JK, Van Hul EV, *et al.*: Mutations in the CCN gene family member WISP3 cause progressive pseudorheumatoid dysplasia. *Nat Genet* 1999, 23:94-98.
2. Pennica D, Swanson TA, Welsh JW, Roy MA, Lawrence DA, Lee J, Brush J, Taneyhill LA, Deuel B, Lew M, *et al.*: **WISP genes are members of the connective tissue growth factor family that are up-regulated in wnt-1-transformed cells and aberrantly expressed in human colon tumors.** *Proc Natl Acad Sci USA* 1998, **95:**14717-14722.
3. Perbal B: **NOV (nephroblastoma overexpressed) and the CCN family of genes: structural and functional issues.** *Mol Pathol* 2001, **54:**57-79.
4. Thorstensen L, Holm R, Lothe RA, Trope C, Carvalho B, Sobrinho-Simoes M, Seruca R: **WNT-inducible signaling pathway protein 3, WISP-3, is mutated in microsatellite unstable gastrointestinal carcinomas but not in endometrial carcinomas.** *Gastroenterology* 2003, **124:**270-271.
5. Thorstensen L, Diep CB, Meling GI, Aagesen TH, Ahrens CH, Rognum TO, Lothe RA: **WNT1 inducible signaling pathway protein 3, WISP-3, a novel target gene in colorectal carcinomas with microsatellite instability.** *Gastroenterology* 2001, **121:**1275-1280.
6. Kleer CG, Zhang Y, Pan Q, van Golen KL, Wu ZF, Livant D, Merajver SD: **WISP3 is a novel tumor suppressor gene of inflammatory breast cancer.** *Oncogene* 2002, **21:**3172-3180.
7. Lin CG, Leu SJ, Chen N, Tebeau CM, Lin SX, Yeung CY, Lau LF: **CCN3 (NOV) is a novel angiogenic regulator of the CCN protein family.** *J Biol Chem* 2003, **278:**24200-24208.
8. Sakamoto K, Yamaguchi S, Ando R, Miyawaki A, Kabasawa Y, Takagi M, Li CL, Perbal B, Katsube K: **The nephroblastoma overexpressed gene (NOV/ccn3) protein associates with Notch1 extracellular domain and inhibits myoblast differentiation via Notch signaling pathway.** *J Biol Chem* 2002, **277:**29399-29405.
9. Perbal B, Martinerie C, Sainson R, Werner M, He B, Roizman B: **The C-terminal domain of the regulatory protein NOVH is sufficient to promote interaction with fibulin 1C: a clue for a role of NOVH in cell-adhesion signaling.** *Proc Natl Acad Sci USA* 1999, **96:**869-874.
10. Li CL, Martinez V, He B, Lombet A, Perbal B: **A role for CCN3 (NOV) in calcium signalling.** *Mol Pathol* 2002, **55:**250-261.
11. Wahab NA, Weston BS, Mason RM: **Connective tissue growth factor CCN2 interacts with and activates the tyrosine kinase receptor TrkA.** *J Am Soc Nephrol* 2005, **16:**340-351.
12. Brigstock D, Lau L, Perbal B: **CCN workshop.** *J Clin Pathol* 2005, **58:**463-465.
13. Gupta N, Wang H, McLeod TL, Naus CC, Kyurkchiev S, Advani S, Yu J, Perbal B, Weichselbaum RR: **Inhibition of glioma cell growth and tumorigenic potential by CCN3 (NOV).** *Mol Pathol* 2001, **54:**293-299.
14. Benini S, Perbal B, Zambelli D, Colombo MP, Manara MC, Serra M, Parenza M, Martinez V, Picci P, Scotlandi K: **In Ewing's sarcoma CCN3(NOV) inhibits proliferation while promoting migration and invasion of the same cell type.** *Oncogene* 2005, **24:**4349-61.
15. Fu CT, Bechberger JF, Ozog MA, Perbal B, Naus CC: **CCN3 (NOV) interacts with connexin43 in C6 glioma cells: possible mechanism of connexin-mediated growth suppression.** *J Biol Chem* 2004, **279:**36943-36950.
16. Gellhaus A, Dong X, Propson S, Maass K, Klein-Hitpass L, Kibschull M, Traub O, Willecke K, Perbal B, Lye SJ, *et al.*: **Connexin43 interacts with NOV: a possible mechanism for negative regulation of cell growth in choriocarcinoma cells.** *J Biol Chem* 2004, **279:**36931-36942.
17. Glukhova L, Angevin E, Lavialle C, Cadot B, Terrier-Lacombe MJ, Perbal B, Bernheim A, Goguel AF: **Patterns of specific genomic alterations associated with poor prognosis in high-grade renal cell carcinomas.** *Cancer Genet Cytogenet* 2001, **130:**105-110.
18. Tsai MS, Hornby AE, Lakins J, Lupu R: **Expression and function of CYR61, an angiogenic factor, in breast cancer cell lines and tumor biopsies.** *Cancer Res* 2000, **60:**5603-5607.
19. Babic AM, Kireeva ML, Kolesnikova TV, Lau LF: **CYR61, a product of a growth factor-inducible immediate early gene, promotes angiogenesis and tumor growth.** *Proc Natl Acad Sci USA* 1998, **95:**6355-6360.
20. Tong X, O'Kelly J, Xie D, Mori A, Lemp N, McKenna R, Miller CW, Koeffler HP: **Cyr61 suppresses the growth of non-small-cell lung cancer cells via the beta-catenin-c-myc-p53 pathway.** *Oncogene* 2004, **23:**4847-4855.
21. Tong X, Xie D, O'Kelly J, Miller CW, Muller-Tidow C, Koeffler HP: **Cyr61, a member of CCN family, is a tumor suppressor in non-small cell lung cancer.** *J Biol Chem* 2001, **276:**47709-47714.
22. Bleau AM, Planque N, Perbal B: **CCN proteins and cancer: two to tango.** *Front Biosci* 2005, **10:**998-1009.
23. Nessling M, Richter K, Schwaenen C, Roerig P, Wrobel G, Wessendorf S, Fritz B, Bentz M, Sinn HP, Radlwimmer B, *et al.*: **Candidate genes in breast cancer revealed by microarray-based comparative genomic hybridization of archived tissue.** *Cancer Res* 2005, **65:**439-447.
24. Chappell SA, Walsh T, Walker RA, Shaw JA: **Loss of heterozygosity at chromosome 6q in preinvasive and early invasive breast carcinomas.** *Br J Cancer* 1997, **75:**1324-1329.
25. van Golen KL, Davies S, Wu ZF, Wang Y, Bucana CD, Root H, Chandrasekharappa S, Strawderman M, Ethier SP, Merajver SD: **A novel putative low-affinity insulin-like growth factor-binding protein, LIBC (lost in inflammatory breast cancer), and RhoC GTPase correlate with the inflammatory breast cancer phenotype.** *Clin Cancer Res* 1999, **5:**2511-2519.
26. Kleer CG, Zhang Y, Pan Q, Gallagher G, Wu M, Wu ZF, Merajver SD: **WISP3 and RhoC guanosine triphosphatase cooperate in the development of inflammatory breast cancer.** *Breast Cancer Res* 2004, **6:**R110-R115.
27. Kleer CG, Zhang Y, Pan Q, Merajver SD: **WISP3 (CCN6) is a secreted tumor-suppressor protein that modulates IGF signaling in inflammatory breast cancer.** *Neoplasia* 2004, **6:**179-185.
28. Ethier SP, Mahacek ML, Gullick WJ, Frank TS, Weber BL: **Differential isolation of normal luminal mammary epithelial cells and breast cancer cells from primary and metastatic sites using selective media.** *Cancer Res* 1993, **53:**627-635.
29. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T: **Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells.** *Nature* 2001, **411:**494-498.
30. Di Fiore PP, Pierce JH, Kraus MH, Segatto O, King CR, Aaronson SA: **erbB-2 is a potent oncogene when overexpressed in NIH/3T3 cells.** *Science* 1987, **237:**178-182.
31. Di Fiore PP, Pierce JH, Fleming TP, Hazan R, Ullrich A, King CR, Schlessinger J, Aaronson SA: **Overexpression of the human EGF receptor confers an EGF-dependent transformed phenotype to NIH 3T3 cells.** *Cell* 1987, **51:**1063-1070.
32. Zhou BP, Deng J, Xia W, Xu J, Li YM, Gunduz M, Hung MC: **Dual regulation of Snail by GSK-3β-mediated phosphorylation in control of epithelial-mesenchymal transition.** *Nat Cell Biol* 2004, **6:**931-940.
33. Huber MA, Azoitei N, Baumann B, Grunert S, Sommer A, Pehamberger H, Kraut N, Beug H, Wirth T: **NF-κB is essential for epithelial-mesenchymal transition and metastasis in a model of breast cancer progression.** *J Clin Invest* 2004, **114:**569-581.
34. Nass SJ, Herman JG, Gabrielson E, Iversen PW, Parl FF, Davidson NE, Graff JR: **Aberrant methylation of the estrogen receptor**

and E-cadherin 5' CpG islands increases with malignant progression in human breast cancer. *Cancer Res* 2000, **60:**4346-4348.
35. Droufakou S, Deshmane V, Roylance R, Hanby A, Tomlinson I, Hart IR: **Multiple ways of silencing E-cadherin gene expression in lobular carcinoma of the breast.** *Int J Cancer* 2001, **92:**404-408.
36. Batlle E, Sancho E, Franci C, Dominguez D, Monfar M, Baulida J, Garcia De Herreros A: **The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells.** *Nat Cell Biol* 2000, **2:**84-89.
37. Cano A, Perez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG, Portillo F, Nieto MA: **The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression.** *Nat Cell Biol* 2000, **2:**76-83.
38. Sen M, Cheng YH, Goldring MB, Lotz MK, Carson DA: **WISP3-dependent regulation of type II collagen and aggrecan production in chondrocytes.** *Arthritis Rheum* 2004, **50:**488-497.
39. Surmacz E: **Function of the IGF-I receptor in breast cancer.** *J Mammary Gland Biol Neoplasia* 2000, **5:**95-105.
40. Sachdev D, Hartell JS, Lee AV, Zhang X, Yee D: **A dominant negative type I insulin-like growth factor receptor inhibits metastasis of human cancer cells.** *J Biol Chem* 2004, **279:**5017-5024.
41. Yee D, Lee AV: **Crosstalk between the insulin-like growth factors and estrogens in breast cancer.** *J Mammary Gland Biol Neoplasia* 2000, **5:**107-115.
42. Lee AV, Gooch JL, Oesterreich S, Guler RL, Yee D: **Insulin-like growth factor I-induced degradation of insulin receptor substrate 1 is mediated by the 26S proteasome and blocked by phosphatidylinositol 3'-kinase inhibition.** *Mol Cell Biol* 2000, **20:**1489-1496.
43. Lee AV, Hilsenbeck SG, Yee D: **IGF system components as prognostic markers in breast cancer.** *Breast Cancer Res Treat* 1998, **47:**295-302.
44. Resnik JL, Reichart DB, Huey K, Webster NJ, Seely BL: **Elevated insulin-like growth factor I receptor autophosphorylation and kinase activity in human breast cancer.** *Cancer Res* 1998, **58:**1159-1164.
45. Turner BC, Haffty BG, Narayanan L, Yuan J, Havre PA, Gumbs AA, Kaplan L, Burgaud JL, Carter D, Baserga R, *et al.*: **Insulin-like growth factor-I receptor overexpression mediates cellular radioresistance and local breast cancer recurrence after lumpectomy and radiation.** *Cancer Res* 1997, **57:**3079-3083.
46. Byrne C, Colditz GA, Willett WC, Speizer FE, Pollak M, Hankinson SE: **Plasma insulin-like growth factor (IGF) I, IGF-binding protein 3, and mammographic density.** *Cancer Res* 2000, **60:**3744-3748.
47. Lee AV, Jackson JG, Gooch JL, Hilsenbeck SG, Coronado-Heinsohn E, Osborne CK, Yee D: **Enhancement of insulin-like growth factor signaling in human breast cancer: estrogen regulation of insulin receptor substrate-1 expression in vitro and in vivo.** *Mol Endocrinol* 1999, **13:**787-796.
48. Rocha RL, Hilsenbeck SG, Jackson JG, VanDenBerg CL, Weng C, Lee AV, Yee D: **Insulin-like growth factor binding protein-3 and insulin receptor substrate-1 in breast cancer: correlation with clinical parameters and disease-free survival.** *Clin Cancer Res* 1997, **3:**103-109.

RhoC GTPase Expression as a Potential Marker of Lymph Node Metastasis in Squamous Cell Carcinomas of the Head and Neck

Celina G. Kleer,[1,5] Theodoros N. Teknos,[4,5] Mozaffarul Islam,[4,5] Benjamin Marcus,[4,5] Julia Shin-Jung Lee,[2,5] Quintin Pan,[3,5] and Sofia D. Merajver[3,5]

Abstract

Purpose: Survival rates for squamous cell carcinoma of the head and neck (SCCHN) have remained unchanged for several decades due to local tumor recurrences as well as regional and distant metastases. Recent evidence has shown that RhoC GTPase is overexpressed in stages III and IV regionally metastatic SCCHN compared with stages I and II localized disease. This study evaluated the expression of RhoC in head and neck carcinoma and investigated the prognostic use of this marker on a large cohort of previously untreated patients with SCCHN.

Experimental Design: Standard Western blot techniques were used to evaluate RhoC protein expression in nine established head and neck cancer cell lines and in normal oral epithelium. *In vivo* expression of RhoC in metastatic and nonmetastatic SCCHN was investigated using immunohistochemical analysis on a tissue microarray composed of 113 independent tumor samples. RhoC expression was analyzed as it related to clinical and pathologic variables of interest.

Results: Levels of RhoC protein were increased in the SCCHN cell lines compared with normal oral epithelium. The *in vivo* expression of RhoC correlated with advanced clinical stage and lymph node metastases for the entire patient cohort as well as in small primary tumors (T_1 and T_2).

Conclusions: This study is the first to examine the expression of RhoC GTPase protein in SCCHN and normal squamous epithelium. It is clear from the results that RhoC is a specific marker of lymph node metastases in patients with this challenging form of carcinoma. RhoC levels seem to identify a subset of patients with early tumor stage primary tumors and high metastatic potential that might benefit from more aggressive therapy. Through continued investigation, blockade of RhoC activity may be a potential target in the development of novel strategies for treating metastases of head and neck cancer.

Squamous cell carcinoma of the head and neck (SCCHN) accounts for >95% of all head and neck malignancies and is responsible for ~40,000 incident cases yearly in the United States. Unfortunately, the majority of SCCHN patients present with advanced-stage disease (stages III and IV), requiring multimodality therapy. Even with combinations of chemotherapy, radiotherapy, and surgery, cure rates are only 30% for advanced-stage disease and have remained unchanged for decades. This poor survival is due mainly to the development of local tumor recurrences as well as regional and distant metastases. Novel molecular predictors of regional and distant metastatic potential at the time of diagnosis are needed to help guide clinical therapy decisions.

Many of the factors necessary to convey the metastatic phenotype to cancer cells are controlled by the members of the Ras superfamily of small GTP-binding proteins. RhoC GTPase is a member of the Ras superfamily, and its activation results in the assembly of the actin-myosin contractile filaments into focal adhesion complexes that, ultimately, lead to cell polarity and facilitate motility (1–3). Our laboratory has detected overexpression of RhoC mRNA in advanced breast cancers by *in situ* hybridization and characterized RhoC as a transforming oncogene for human mammary epithelial cells. In addition, RhoC overexpression results in a highly motile and invasive phenotype that recapitulates the most lethal form of locally advanced breast cancer, inflammatory breast cancer. Further work showed that RhoC is specifically expressed in invasive breast carcinomas capable of metastasizing, and it may be clinically useful in patients with tumors <1 cm to guide treatment (4, 5). Subsequent investigations in other tumor types have shown that RhoC overexpression enhances tumor metastasis in melanoma (6), ovarian carcinoma (7), ductal adenocarcinoma of the pancreas (8), and lung carcinoma (9).

Authors' Affiliations: Departments of [1]Pathology, [2]Biostatistics, [3]Internal Medicine, and [4]Otolaryngology-Head and Neck Surgery and [5]Comprehensive Cancer Center, University of Michigan, Ann Arbor, Michigan

Received 2/20/06; revised 4/6/06; accepted 5/16/06.

Grant support: Specialized Programs of Research Excellence grant in Head and Neck NIH/National Institute of Dental and Craniofacial Research/National Cancer Institute 1P50CA/DE97248-01 (T.N. Teknos and S.D. Merajver), Army grants DAMD17-02-1-491 (C.G. Kleer) and DAMD-17-00-1-0345 (S.D. Merajver), and NIH grants K08 CA 090876 (C.G. Kleer), R01CA107469 (C.G. Kleer), RO1CA77612 (S.D. Merajver), and 1 P50-CADE97258 (S.D. Merajver).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: C.G. Kleer and T.N. Teknos are cofirst authors on this article.

Requests for reprints: Theodoros N. Teknos, Department of Otolaryngology-Head and Neck Surgery, University of Michigan, 1904 Taubman Center, 1500 East Medical Center Drive, Ann Arbor, MI 48109. Phone: 734-936-3172; Fax: 734-936-9625; E-mail: teknos@umich.edu.

doi:10.1158/1078-0432.CCR-06-0376

To date, no studies have investigated the role of RhoC in the metastatic phenotype of head and neck carcinoma. Abraham et al. (10) did immunohistochemical studies investigating other Ras superfamily motility-related gene expression but did not specifically stain for RhoC. Interestingly, however, they concluded that there was a significant difference in the expression of these proteins between normal epithelium and malignant cells and hypothesized that motility-related genes may prove to be a marker of malignancy and/or aggressiveness in head and neck cancer (10).

Through gene expression profiling studies, we have identified RhoC as being differentially overexpressed in stages III and IV regionally metastatic SCCHN compared with stages I and II localized disease (11). This finding led us to hypothesize that RhoC may be a clinically useful marker of metastatic potential in SCCHN. In this study, we have analyzed RhoC protein expression in normal squamous epithelium as well as in a panel of squamous cell carcinoma cell lines *in vitro*. We then investigated the prognostic use of detecting RhoC protein *in situ* by immunohistochemistry on human tissues from a large cohort of previously untreated patients with SCCHN.

Materials and Methods

Western blots. UMSCC and normal oral epithelium cell lines were homogenized in 0.2 mol/L Tris-HCl buffer (pH 7.4) containing 1 mmol/L EGTA, 2.5 mmol/L EDTA, 5 mmol/L DTT, 0.3 mol/L sucrose, 10 μg/mL leupeptin, 50 μg/ml aprotinin, 2 mmol/L Na_3VO_4, and 0.1% Triton X-100. The homogenate was centrifuged at 500 × *g* for 10 minutes at 4°C to remove all cell debris. Total proteins were estimated in clear supernatants by dye-binding method using Bradford reagent. Equal amounts of protein were boiled in Laemmli buffer for 5 minutes and then analyzed by 10% SDS-PAGE. After transferring the separated proteins onto nitrocellulose membranes, the membranes were blocked for 3 hours at room temperature with TBS-0.1% Tween 20 containing 5% nonfat dry milk. The membranes were incubated overnight with polyclonal RhoC antibody (1:1,200) at 4°C with mild shaking. After incubation with primary antibody, the membranes were blotted for 1 hour with a secondary horseradish peroxidase–conjugated antibody (1:5,000). Next, the membranes were washed and the proteins were visualized using enhanced chemiluminescence Western detection system (Amersham, Piscataway, NJ). The analysis of activated RhoC was done using the RhoC Activation Assay Biochem kit (Cytoskeleton, Denver, CO) following manufacturer's protocols. The cells were lysed using lysis buffer, and cell lysates were centrifuged at 10,000 rpm for 5 minutes to remove cell debris. The clear supernatants were taken out and incubated with 60 μL of pretreated beads for 1 hour at 4°C. The beads were separated from the supernatants by centrifuging at 10,000 rpm for 5 minutes at 4°C. The protein-bead complex was washed once with lysis buffer followed by a clear wash with the wash buffer (supplied). A small portion of this complex was used to determine the protein concentration (Bradford assay). The remaining portion was used to load exact amounts of proteins on 12% SDS gels following standard Western blot techniques as described previously above.

Selection of patients and tissue microarray development. *In vivo* expression of RhoC in metastatic and nonmetastatic SCCHN was investigated using immunohistochemical analysis on a tissue microarray composed of 113 independent tumor samples. All patients for this validation group were presented to the University of Michigan Hospital (Ann Arbor, MI) between 1997 and 2000 with newly diagnosed, previously untreated HNSCC. University of Michigan institutional review board approval and written consent were obtained. Patients underwent clinical and pathologic staging followed by surgical tumor resection and regional lymphadenectomy. A surgical pathologist used H&E staining to evaluate cryotome sections (5 μm) from each paraffin-embedded primary tumor block. Representative areas of primary tumor and normal squamous cell mucosa were marked. A high-density tissue microarray was constructed from the marked areas using three replicate tumor cores (0.6-mm diameter) and one normal mucosa core per patient. By obtaining three cores from different areas of each tumor, we accounted for tumor heterogeneity.

Immunohistochemical studies. Immunohistochemistry was done on the tissue microarray by using a standard biotin-avidin complex technique and a polyclonal antibody against RhoC that was developed and validated in our laboratory by immunoblot analysis and immunohistochemistry (12). RhoC expression was evaluated at least thrice for every tissue microarray element and at least nine times for each tumor. Using this method, the pathologist was blinded to tumor stage and clinical information. The mean value of all measurements from a single individual was used for subsequent analyses.

Because RhoC protein interacts with the contractile cytoskeleton of the cell and is localized to the submembrane space, cytoplasmic stain was expected. Not surprisingly, myoepithelial cells and vascular smooth muscle cells were strongly positive in all cases, serving as consistent internal positive controls. The intensity of cytoplasmic staining was scored using a four-tiered system by comparison to the positive internal controls (score 1 = negative, score 2 = weak, score 3 = moderate, and score 4 = strong staining). This scoring system has been previously validated (12). Based on the biological characterization of RhoC in SCCHN, we defined high RhoC expression when there was moderate or strong cytoplasmic staining (scores 3 and 4) and low RhoC expression when staining was negative or weak (scores 1 and 2). Furthermore, positive controls for staining consisted of head and neck cell lines known to overexpress RhoC and negative controls were done by omitting the primary antibody.

Statistical analysis. The goal of the analysis was to determine if RhoC expression in primary tumor specimen relates to clinical variables of interest. The clinical variables of interest included lymph node metastases, presence of nodal extracapsular spread, tumor stage, clinical stage, and presence of perineural invasion, primary tumor differentiation, disease-free survival, and overall survival. The mean score (rounded to the nearest integer) of all measurements from a single individual was used for analysis. To evaluate the association of RhoC with covariates of interest, generalized linear models were fit to the data using a generalized estimating equation approach to account for both within and between subject variations derived from repeated measurements. A multinomial distribution with cumulative logit link was used for the staining intensity of RhoC. The clinical variables of interest were tested as a main effect in these models. The relationship between RhoC and survival outcomes was explored by the Kaplan-Meier method. The log-rank test was used to compare the homogeneity of survival rate between each scoring category.

For exploratory purposes, a separate analysis was conducted by using only the maximum RhoC staining of each subject. Spearman rank correlation coefficient was used to assess the association between clinical variables and the maximum RhoC staining scores. All statistical analyses were done using SAS version 8.2 (SAS, Carey, NC). A two-tailed $P \leq 0.05$ was considered to be statistically significant.

Results

RhoC protein levels are elevated in SCCHN. To illustrate that RhoC is up-regulated in squamous carcinomas when compared with normal squamous epithelium, we did Western blots on a panel of squamous cell carcinoma cell lines and compared it with that of normal oral epithelium. As illustrated in Fig. 1, levels of RhoC and activated RhoC were increased in all the cancer cell lines tested compared with normal epithelial cells. This recapitulates and validates the findings of our previous gene array studies.

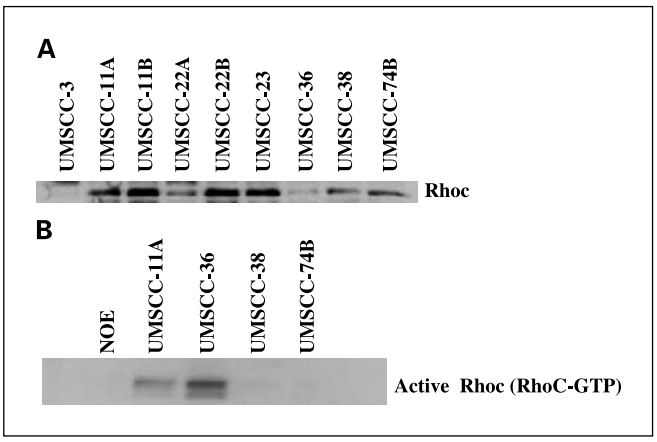


Fig. 1. Western blots illustrating the protein expression levels of RhoC (*A*) and activated RhoC (*B*) in a panel of UMSCC cell lines and normal oral epithelium (*NOE*).

RhoC expression correlates with clinical stage and lymph node metastasis. Of the 113 patients on the tissue microarray available for analysis, 23 were excluded from this analysis due to the presence of a synchronous or metachronous primary lesion, positive surgical margins, failure to receive indicated postoperative radiation therapy, and the use of immunosuppressive agents. Of the remaining 90 patients, 68 had evaluable tumor cores for histologic analysis and were the subjects of this study.

The clinical and pathologic features of the 68 patients in our cohort are summarized in Table 1. Of the 68 invasive carcinomas, 15 (22.1%) were early stage (stages I and II) and 53 (77.9%) were advanced stage (stages III and IV). As expected, based on our previous studies in breast cancer, RhoC was expressed mainly in the cytoplasm (Fig. 2). SCCHN that expressed high levels of RhoC and those that expressed low levels of RhoC were readily identifiable (Fig. 2). In our cohort of 68 tumors, RhoC protein was expressed in 82.35% (56 of 68) of patients and high RhoC protein was detected in 32 (47%) of the carcinomas, whereas low RhoC expression was found in 36 (53%) of the SCCHN tumors.

When evaluating the entire patient population, the mean RhoC score was significantly associated with the clinical stage ($P = 0.014$), as higher mean RhoC staining scores were seen in advanced-stage (stages III and IV) regionally metastatic tumors compared with early-stage (stages I and II) lesions. This finding is also consistent with the results of our gene expression studies (11) and validates these findings at the protein level. In our group of patients, 28 had small tumors (T_1 and T_2). In this group of small primary SCCHN, the mean RhoC score was significantly associated with the presence of lymph node metastasis ($P = 0.024$) and clinical stage ($P = 0.04$). Furthermore, for patients with T_1 or T_2 tumors, only 9 of 17 (53%) patients with low RhoC (scores 1 and 2) staining had evidence of regional metastases, whereas 9 of 11 (82%) patients with high RhoC (scores 3 and 4) expression developed metastases. In fact, all patients ($n = 4$) with small primary tumors and strong (4+) RhoC staining had pathologic evidence of lymph node metastasis (Table 2). For the entire group of early lesions, when using high RhoC levels (scores 3 and 4) as a predictor of pathologically proven lymph node metastasis, the sensitivity was 66.7% and the specificity was 83.3%, with a positive predictive value of 90% and a negative predictive value of 52.63%.

Based on these results, we undertook a similar statistical analysis on this patient cohort using the maximum RhoC score obtained from each patient. Due to the heterogeneity of head and neck tumors and the presence of RhoC "hotspots" in these tumors, we wanted to investigate whether the maximum RhoC score varied significantly from the mean scores in terms of relationships with known clinical and pathologic variables. This analysis revealed that the maximum RhoC score correlated with lymph node metastases for the entire patient cohort as

Table 1. Clinical and pathologic features of the evaluable patients in the head and neck tissue microarray

Clinical/pathologic variable	
Eligible patients	68
Positive nodes, yes/no (%)	42/68 (61.8)
Positive nodes 95% CI*	50.2-73.3
No. positive nodes	
0	26
1	12
2	8
3	8
>3	14
Extracapsular spread, presence/absence (%)	24/68 (35.3)
Extracapsular spread 95% CI	23.9-46.7
Early tumor stage, T_1, T_2 (%)	28/68 (41.2)
Tumor stage 95% CI	29.5-52.9
Tumor stage	
0	0
I	5/68 (7.4)
II	23/68 (33.8)
III	18/68 (26.5)
IV	22/68 (32.4)
Perineural invasion (%)	12/67 (17.9)
Perineural invasion 95% CI	8.7-27.1
Early clinical stage, I, II (%)	15/68 (22.1)
Clinical stage 95% CI	12.2-31.9
Clinical stage IV (%)	38/68 (55.9)
Clinical stage IV 95% CI	44.1-67.7
Clinical stage	
I	3/68 (4.4)
II	12/68 (17.7)
III	15/68 (22.1)
IV	38/68 (55.9)
Primary differentiation	
1 (Well)	10/66 (15.2)
2 (Moderate)	41/66 (62.1)
3 (Poor)	15/66 (22.7)
Median length of follow-up (mo)	41.1
Median follow-up 95% CI	36.0-46.0
3-y Survival (%)	74.7
Survival 95% CI	63.8-85.6

*95% confidence interval.

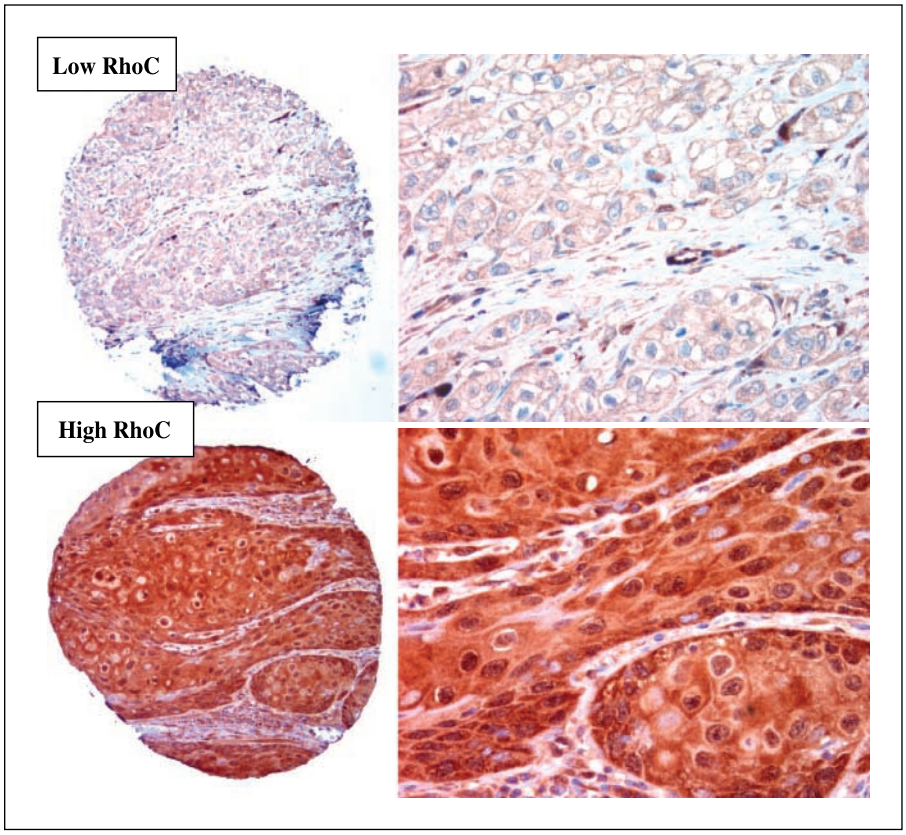


Fig. 2. Typical staining patterns seen with RhoC immunohistochemistry in the head and neck high-density tissue microarray. Low RhoC expression was defined as negative or weak cytoplasmic staining (scores 1 and 2), whereas high RhoC expression was defined as moderate or strong cytoplasmic staining (scores 3 and 4).

well as for the early-stage tumors (P = 0.04 and 0.04, respectively; Table 3). In addition, for both the RhoC mean and RhoC maximum analysis, there was a trend, in which the higher the RhoC staining, the more likely a tumor was to develop extracapsular spread of tumor in regional lymphatics, although this failed to reach statistical significance (Table 4).

No association was found between RhoC and other pathologic or clinical variables, including degree of differentiation, presence of perineural spread, tumor stage, disease-specific survival, and overall survival.

Discussion

The formation and growth of metastases is the principal cause of death for most cancer patients, particularly those with head and neck cancer (13, 14). An ever-expanding body of evidence is implicating RhoC GTPase as a critical determinant of metastasis for a variety of tumor types (1–9). Ours is the first study to investigate the role of RhoC in the behavior of SCCHN.

Through the *in vitro* portion of this study, we have illustrated that RhoC protein expression is present in all squamous cell carcinoma cell lines tested and at levels dramatically higher than normal squamous epithelium. This is consistent with our findings in a gene array analysis comparing normal squamous epithelium versus SCCHN, thus validating these data (11). It is also in line with our previous work in breast cancer where we showed that RhoC expression increases once breast cancer cells acquire the ability to invade the host tissues and metastasize (3). Finally, although previous authors did not specifically address RhoC, our findings confirm the work of Abraham et al. (10), who concluded that motility-related proteins are

Table 2. Distribution of RhoC staining intensity for T_1 and T_2 tumors

	Node positive	Node negative	Total
1 (No stain)	3	4	7
2 (Low)	6	4	10
3 (Median)	5	2	7
4 (High)	4	0	4
Total	18	10	28

Table 3. Proportion of subjects with positive node in each category of RhoC staining

Staining score	Proportion of patients with positive lymph nodes (%)	
	RhoC mean value	RhoC maximum value
1 (No stain)	4/12 (33.3)	4/11 (36.4)
2 (Low)	15/24 (62.5)	11/19 (57.9)
3 (Median)	17/24 (70.8)	17/26 (65.4)
4 (High)	6/8 (75.0)	10/12 (83.3)

NOTE: Mean was the subject-wise average score rounded to the nearest integer.

Table 4. Proportion of subjects with extracapsular spread presence in each category of RhoC staining

Staining score	Proportion of patients with extracapsular spread (%)	
	RhoC mean value	RhoC maximum value
1 (No stain)	2/12 (16.7)	2/11 (18.2)
2 (Low)	9/24 (37.5)	6/19 (31.6)
3 (Median)	9/24 (37.5)	10/26 (38.5)
4 (High)	4/8 (50.0)	6/12 (50.0)

overexpressed in squamous cell carcinoma relative to normal keratinocytes and may prove to be markers of metastasis.

To further evaluate the importance of RhoC in conferring a metastatic phenotype in SCCHN, we correlated *in vivo* levels of RhoC with known demographic and clinical variables using a large cohort of previously untreated head and neck cancer patients. Collectively, our data indicate that increasing levels of RhoC expression are associated with advanced-stage tumors and with lymph node metastasis even in small primary tumors. Furthermore, there was a trend noted, in which increasing RhoC expression was appreciated in patients with extracapsular spread in their lymph node metastases, indicating aggressive tumor behavior. Interestingly, although RhoC correlated with lymph node metastases and advanced tumor stage, a clear correlation with survival and distant metastases could not be illustrated. This may be due to the relatively short length of clinical follow-up (41 months) and could reach significance as the patient cohort matures. Furthermore, the bias of our institution toward aggressive postoperative treatment of patients with high-risk tumors (i.e., multiple lymph nodes and extracapsular spread) with combined chemoradiation therapy may have minimized the survival difference between the two groups. Regardless, our study clearly illustrates that RhoC expression is correlated with lymph node metastasis and clinical stage in head and neck cancer. Even with small primary tumors, RhoC may identify those small (T_1 and T_2) carcinomas with high metastatic potential and may assist clinicians in selecting patients who would benefit from aggressive surgical and adjuvant therapy. In this pilot study, high RhoC expression was able to identify a metastatic phenotype in small tumors, with a relatively high degree of specificity and positive predictive value.

Our findings in this study are consistent with observations made by others in different tumor types. In melanoma, for instance, genomic analysis of highly metastatic tumors revealed an essential role for RhoC by inducing a highly motile, invasive tumor phenotype, which was abrogated *in vitro* with the introduction of dominant-negative Rho (6). Similarly, Wang et al. (15) identified RhoC as strong predictor of lymph node metastasis in hepatocellular carcinoma using genomic analysis and tissue microarray immunohistochemistry. In an orthotopic model of non–small cell lung cancer, Ikoma et al. (9) found that metastasis, *in vitro* migration, and invasion were significantly enhanced by overexpression of RhoC. They further concluded that RhoC does not affect primary tumor growth but enhances the metastatic nature of lung cancer not only by stimulating cell motility but also by up-regulating the expression of certain matrix metalloproteinases. Similar data correlating RhoC expression with lymph node metastasis and tumor progression have been noted in pancreatic and ovarian carcinoma (7, 8).

The emerging role of RhoC in the metastatic process strongly suggests that attenuation of RhoC activity may be a potential target for novel strategies in the treatment of head and neck as well as other carcinomas and melanoma. To that end, Rho proteins are dependent on post-translational isoprenylation for their biological activity (16, 17) RhoC specifically depends on geranylgeranylation, and this can be inhibited by the statin class of drugs, which are commonly used for their cholesterol lowering affect (18). These drugs, by inhibiting 3-hydroxy-3-methylglutaryl CoA reductase, cause dramatic reductions not only in cholesterol but also in its isoprenoid precursors (farnesyl pyrophosphate and geranylgeranyl pyrophosphate). Recently, atorvastatin was found to inhibit RhoC isoprenylation and metastasis in a mouse model of human melanoma (19). Furthermore, clinical trials of statins for heart disease have noted antineoplastic effects of statins in general (20) and in melanoma in particular (21). Ongoing studies in our laboratory are investigating the effect of statin therapy on growth, invasion, and metastasis of head and neck cancer through RhoC inhibition. If the preliminary findings in this study and future investigations indicate efficacy of statin therapy, this may prove to be an important adjunct to existing therapies in head and neck cancer to prevent regional and distant metastasis.

In conclusion, this study is the first in examining the expression of RhoC GTPase protein in SCCHN and normal squamous epithelium. It is clear from the results that RhoC is a specific marker of lymph node metastases in patients with this challenging form of carcinoma. Importantly, although our data are preliminary, RhoC levels seem to identify a subset of patients with early tumor stage primary tumors and high metastatic potential that might benefit from more aggressive therapy. Through continued investigation, blockade of RhoC activity may be a potential target in the development of novel strategies for treating metastases of head and neck cancer.

References

1. Nobes CD, Hall A. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes with actin stress fibers, lamellipodia, and filopodia. Cell 1995;81:53–62.
2. Leung T, Chen XQ, Manser E, Lim L. The p160 RhoA-binding kinase ROK α is a member of a kinase family and is involved in the reorganization of the cytoskeleton. Mol Cell Biol 1996;16:5313–27.
3. Kimura K, Ito M, Amano M, et al. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). Science 1996;273:245–8.
4. van Golen KL, Wu ZF, Qiao XT, Bao LW, Merajver SD. RhoC-GTPase, a novel transforming oncogene for human mammary epithelial cells that partially recapitulates the inflammatory breast cancer phenotype. Cancer Res 2000;60:5832–8.
5. Kleer CG, van Golen KL, Zhang Y, Wu ZF, Rubin MA, Merajver SD. Characterization of RhoC expression in benign and malignant breast disease: a potential new marker for small breast carcinomas with metastatic ability. Am J of Pathol 2002;160:579–84.
6. Clark EA, Golub TR, Lander ES, Hynes RO. Genomic analysis of metastasis reveals an essential role for RhoC. Nature 2000;406:532–5.
7. Horiuchi A, Imai T, Wang C, et al. Up-regulation of small GTPases, RhoA and RhoC, is associated with tumor progression in ovarian carcinoma. Lab Investig 2003;83:861–70.
8. Suwa H, Ohshio G, Imamura T, et al. Overexpression of the rhoC gene correlates with progression of ductal adenocarcinoma of the pancreas. Br J Cancer 1998; 77:147–52.
9. Ikoma T, Takahashi T, Nagano S, et al. A definitive role

of RhoC in metastasis of orthotopic lung cancer in mice. Clin Cancer Res 2004;10:1192–200.

10. Abraham MT, Kuriakose MA, Sacks PG, et al. Motility-related proteins as markers for head and neck squamous cell cancer. Laryngoscope 2001;111:1285–9.

11. Schmalbach CE, Chepeha DB, Giordano TJ, et al. Molecular profiling and the identification of genes associated with metastatic oral cavity/pharynx squamous cell carcinoma. Arch Otolaryngol Head Neck Surg 2004;130:295–302.

12. Van den Eynden GG, Van der Auwera I, Van Laere S, et al. Validation of a tissue microarray to study differential protein expression in inflammatory and non-inflammatory breast cancer. Breast Cancer Res Treat 2004;85:13–22.

13. Lin M, van Golen KL. Rho-regulatory proteins in breast cancer cell motility and invasion. Breast Cancer Res Treat 2004;84:49–60.

14. Fidler IJ. Critical factors in the biology of human cancer metastasis: twenty eighth GHA Clowes Memorial Award Lecture. Cancer Res 1990;50:6130–8.

15. Wang W, Yang LY, Huang GW, et al. Genomic analysis reveals RhoC as a potential marker in hepatocellular carcinoma with poor prognosis. Br J Cancer 2004;90:2349–55.

16. Collisson EA, Carranza DC, Chen IY, Kolodney MS. Isoprenylation is necessary for the full invasive potential of RhoA overexpression in melanoma cells. J Invest Dermatol 2002;119:1172–6.

17. Allal C, Favre F, Couderc B, et al. Rho prenylation is required for promotion of cell growth and transformation and cytoskeleton organization but not for induction of serum response element transcription. J Biol Chem 2000;275:31001–8.

18. Bellosta S, Ferri N, Bernini F, Paoletti R, Corsini A. Non-lipid related effects of statins. Ann Med 2000;32:164–76.

19. Collisson EA, Kleer C, Wu M, et al. Atorvastatin prevents RhoC isoprenylation, invasion, and metastasis in human melanoma cells. Mol Cancer Ther 2003;2:941–8.

20. Pedersen TR, Wilhelmsen L, Faergeman O, et al. Follow-up study of patients randomized in the Scandinavian simvastatin survival study (4S) of cholesterol lowering. Am J Cardiol 2000;86:257–62.

21. Papadakis JA, Mikhailidis DP. Coronary events with lipid-lowering therapy: the AFCAPS/TexCAPS trial. Air Force/Texas Coronary Atherosclerosis Prevention Study. JAMA 1999;281:416–9.

Cells Tissues Organs

Cells Tissues Organs 2007;185:95–99
DOI: 10.1159/000101308

CCN6 (WISP3) as a New Regulator of the Epithelial Phenotype in Breast Cancer

Celina G. Kleer[a, c] Yanhong Zhang[a, c] Sofia D. Merajver[b, c]

Departments of [a]Pathology and [b]Internal Medicine, Division of Hematology/Oncology, and [c]Comprehensive Cancer Center, University of Michigan Medical School, Ann Arbor, Mich., USA

Key Words
Epithelial-mesenchymal transition · CCN · CCN6 · WISP3 · WISP · Breast cancer · Inflammatory breast cancer

Abstract
CCN6 (WISP3) is a cysteine-rich secreted protein that belongs to the CCN (Cyr61, CTGF, Nov) family of genes. We found that CCN6 mRNA is reduced in 80% of cases of the most lethal form of locally advanced breast cancer, inflammatory breast cancer. CCN6 contains four highly conserved motifs with sequence similarities to insulin-like growth factor binding proteins, von Willebrand type C, thrombospondin 1, and a carboxyl-terminal domain putatively involved in dimerization. CCN6 has tumor growth-, proliferation-, and invasion-inhibitory functions in breast cancer. Recently, by using a small infering RNA to downregulate CCN6 in immortalized human mammary epithelial cells, CCN6 was found to be essential to induce the process of epithelial-mesenchymal transition (EMT) with repression of E-cadherin gene expression and induction of a protein expression program characteristic of EMT. This review will focus on the current knowledge regarding the function of CCN6 in breast cancer with special emphasis on the emerging role of CCN6 as a regulator of the epithelial phenotype and E-cadherin expression in the breast.

Abbreviations used in this paper

CT	carboxy-terminal motif
EMT	epithelial-mesenchymal transition
HME	human mammary epithelial cells
IBC	inflammatory breast cancer
IGF	insulin-like growth factor
IGF-1R	insulin-like growth factor receptor 1
siRNA	small infering RNA

CCN6 Is Lost in Inflammatory Breast Cancer

Inflammatory breast cancer (IBC) is the most lethal form of breast cancer, characterized by a rapid onset of disease typically arising within 6 months, and high proclivity for the development of metastasis [Lee and Tannenbaum, 1924]. Despite aggressive multimodality treatment, the overall 5-year disease-free survival rate for IBC patients is less than 45% [Jaiyesimi et al., 1992]. The inflammatory designation in IBC is a misnomer, as no prominent host inflammatory response is elicited by these tumors. Instead, the skin redness and warmth are the result of lymphatic channel obstruction from tumor emboli [Lee and Tannenbaum, 1924]. Through a modified version of the differential display technique and in situ hybridization analysis of human breast tissues we

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

Accessible online at:
www.karger.com/cto

Dr. Celina G. Kleer
Department of Pathology
3510C Medical Sciences Research Building 1
1150 W. Medical Center Drive, Ann Arbor, MI 48109 (USA)
Tel. +1 734 615 3448, Fax +1 734 615 3441, E-Mail kleer@umich.edu

have identified CCN6 as being downregulated in 80% of IBC and in 20% of stage-matched, noninflammatory breast cancer tumors (non-IBC) [van Golen et al., 1999]. These data served as a springboard to encourage us to elucidate the biologic function of CCN6 in breast cancer and in benign breast epithelial cells.

CCN6 Has a Multimodular Architecture

CCN6 is a cysteine-rich protein that belongs to the CCN family of genes. CCN growth factors have intracellular and extracellular functions [Perbal, 2001; Brigstock et al., 2003]. Other members of this protein family are CCN1 (CTGF), CCN2 (Cyr61) CCN3 (Nov), CCN4 (WISP1) and CCN5 (WISP2). The CCN proteins have been shown to regulate cellular differentiation and development, cell proliferation, cell migration and adhesion, and angiogenesis [Perbal, 2001; Brigstock et al., 2003]. Notably, CCN proteins mediate epithelial and stromal cross talk, and have been shown to interact with key signaling molecules such as cell surface integrins, NOTCH1, fibulin C, and ion channels [Lau and Lam, 1999; Perbal et al., 1999a; Li et al., 2002; Sakamoto et al., 2002; Lin et al., 2003, 2005]. Dysregulation of this protein family can lead to cancer [Kleer et al., 2002; Manara et al., 2002; Benini et al., 2005]. Furthermore, certain CCN proteins behave under certain circumstances as oncogenes or tumor suppressor genes in different tissue types. For example, CCN2 (Cyr61) stimulates the growth of breast and gastric adenocarcinomas, whereas CCN2 suppresses the growth of lung cancer cells [Bleau et al., 2005; Brigstock et al., 2005].

The CCN6 gene maps to chromosome 6q21-22 and encodes for a 354-amino acid, 36.9-kDa protein with 57% homology to CCN1 (CTGF). The CCN proteins share a highly conserved structure with a multimodular organization, consisting of cysteine-rich motifs. Figure 1 shows the multimodular structure of CCN6. The N-terminal motif, which includes the first 12 cysteine residues, contains the highly conserved insulin-like growth factor (IGF) binding protein consensus sequence (GCGCCXXC) which may facilitate the binding to IGF [Imai et al., 2000; Byun et al., 2001; Grotendorst and Duncan, 2005]. This domain is followed by a von Willebrand factor-like motif (VWC), and the thrombospondin type 1 motif (TSP-1) which is involved in cell-cell interactions and possibly inhibition of angiogenesis. The carboxy-terminal motif (CT) is present in all CCN proteins described to date and forms a 'cysteine knot', since the protein is folded into two highly twisted antiparallel pairs of β strands and contains three disulfide bonds. The CT domain has been identified in several other signaling peptides (such as transforming growth factor-β, platelet-derived growth factor, and nerve growth factor) and may participate in dimerization and receptor binding [Pennica et al., 1998; Hurvitz et al., 1999; Perbal et al., 1999b]. Interestingly, most CCN proteins contain a cleavable signal peptide which participates in the secretion of these proteins into the extracellular matrix [Yang and Lau, 1991; Perbal, 2001].

The multimodular organization of the CCN proteins suggests that the different modules correspond to functional domains that can interact with other proteins, and that the final biological properties of the CCN proteins might be dependent upon combinatorial effects of these multiple interactions. Studies performed on CCN2 and CCN3 have provided valuable information about the structural basis for some biological properties of the CCN proteins. For example, CCN3 can be found as a full-length protein and as a truncated protein [Perbal, 2001]. It appears that the full-length protein, containing the signal peptide at the N-terminal region, is secreted. Full-length CCN3 has growth inhibitory effects in chicken nephroblastomas. In contrast, the truncated form of CCN3, which does not contain the signal peptide, accumulates in the cytoplasm of the cell and induces a morphological alteration without affecting tumor growth [Perbal, 2001]. These data suggest that the multimodular architecture of the CCN proteins allows them to interact with different positive and negative effectors and that truncations of the CCN proteins at either their N-terminus or the C-terminus may disrupt these regulatory interactions, thereby altering the balance of positive and negative regulatory signals. Furthermore, CCN proteins in general, and CCN6 in particular may exert their functions as secreted proteins as well as in the cell cytoplasm.

CCN6 Is a Tumor Suppressor for IBC

Our studies involving transcript and protein regulation of CCN6 in human breast tissue samples revealed that its levels are decreased in primary IBC and in a subset of aggressive non-IBC tumors [van Golen et al., 1999]. Further work revealed that restoration of CCN6 in an IBC cell line (SUM149), which lacks CCN6 expression, resulted in decreased anchorage-independent growth and impaired its invasiveness [Kleer et al., 2002]. Conversely, when CCN6 was inhibited in normal mammary epithelial cells,

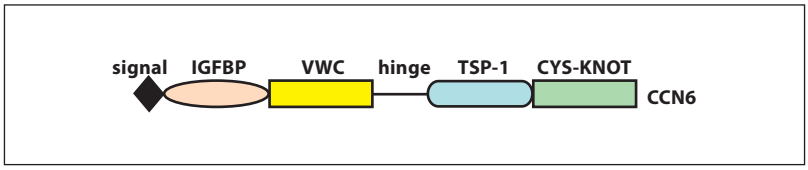


Fig. 1. Modular architecture of CCN6. The protein has four conserved motifs. Note the IGFBP (insulin-like growth factor binding protein) motif at the N-terminal region.

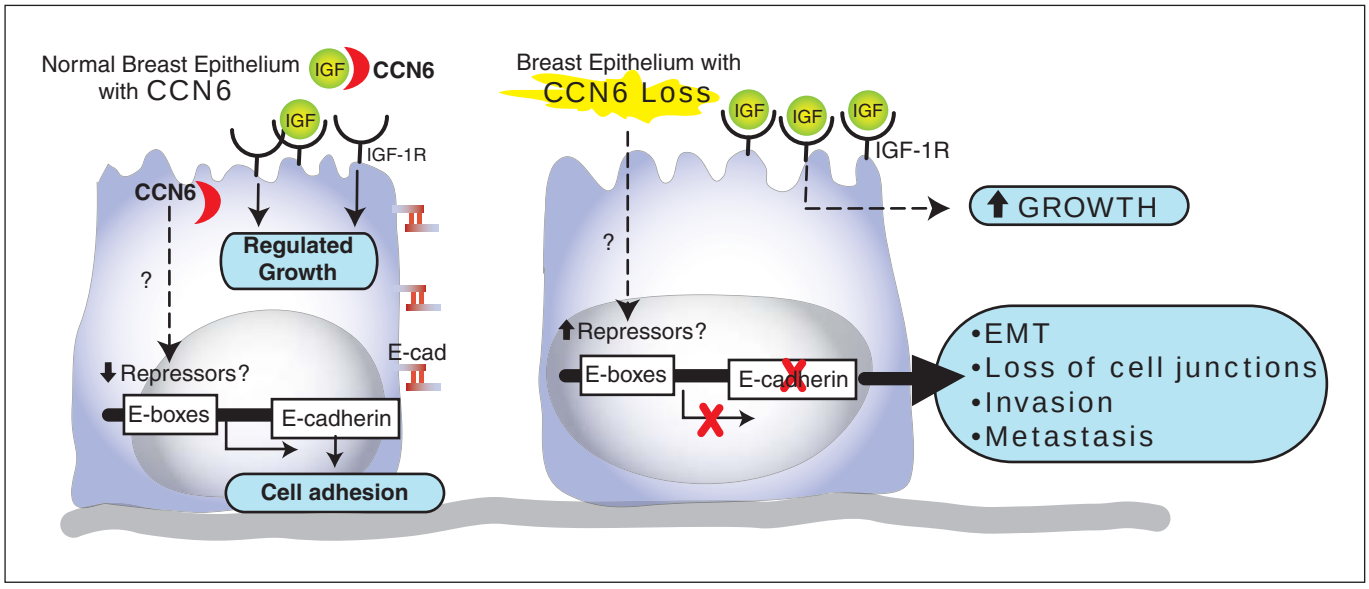


Fig. 2. Our working model of CCN6 function in breast cancer progression and in the EMT process. We postulate that CCN6 modulates the growth of normal breast epithelial cells by affecting IGF-1 signaling pathways, and contributes to the maintenance of the epithelial phenotype through regulation of EMT. Loss of CCN6 promotes the growth and invasiveness of breast cancer by enhancing the effects of IGF-1 on cancer cells, and by promoting EMT.

growth in soft agar and invasion through a basement membrane ensued [Zhang et al., 2005]. Furthermore, CCN6 had strong tumor growth-inhibitory functions in vivo [Kleer et al., 2002]. When injected in the mammary fat pad of athymic nude mice, SUM149 cells with restored CCN6 expression had a decreased rate of tumor uptake and formed smaller tumors. CCN6-expressing tumors were better differentiated than the control IBC tumors; in particular, they exhibited glandular formation, less atypia, and no necrosis. Taken together, these data strongly support a role for CCN6 as a tumor suppressor gene in IBC and perhaps in breast cancer in general.

The next main line of investigation we have pursued is the mechanism of CCN6's tumor suppressor function. Preliminary work from our own and another laboratory has shown that CCN6 is present in two pools: a cytoplasmic pool and an extracellular, secreted pool [Kleer et al., 2004; Davis et al., 2006]. This led us to postulate that once secreted in the extracellular medium, CCN6 may inhibit breast cancer growth at least in part by interfering with IGF-1 signaling, as IGF-1 is an important growth factor associated with increased breast cancer risk and tumor growth [Byrne et al., 2000; Allen et al., 2005; Diorio et al., 2005; Schernhammer et al., 2005]. We showed that CCN6 in the conditioned media of IBC cells is able to inhibit activation of the insulin-like growth factor receptor 1 (IGF-1R) and two of its main downstream signaling molecules, IRS-1 and ERK-1/2 [Kleer et al., 2004]. Furthermore, CCN6 expression knockdown in normal mammary epithelial cells triggered their proliferative activity [Zhang et al., 2005]. An important question which remains to be addressed is whether CCN6 directly interacts with IGF-1 or IGF-1R. If either possibility is true, it may have profound implications for the development and testing of targeted therapies against IGF-1 and IGF-1R activation in breast cancer.

CCN6 Emerges as a Novel Regulator of the Epithelial Phenotype in the Breast

To ascertain the function of CCN6 in normal breast epithelium, we stably inhibited CCN6 in human mammary epithelial cells (HME) by small infering RNA (siRNA) [Zhang et al., 2005]. This resulted in a profound morphological change and protein expression patterns characteristic of an epithelial-mesenchymal transition (EMT). While the parental HME cells and vector controls were compact, thick, and ovoid, with rare extended processes, CCN6-deficient cells were thin, spreading, and of stellate shape with numerous cytoplasmic extensions typical of an EMT. Consistent with EMT, CCN6-deficient HME cells exhibited decreased expression of the epithelial marker cytokeratin-18 and gain of the mesenchymal marker vimentin.

To begin to understand the molecular basis of CCN6-mediated EMT, we explored whether loss of CCN6 was accompanied by E-cadherin downregulation, one of the central proteins in the process of EMT [Oka et al., 1993; Rasbridge et al., 1993; Palacios et al., 1995; Kleer et al., 2001; Kowalski et al., 2003]. Indeed, CCN6 inhibition by siRNA consistently induced a pronounced downregulation of E-cadherin in two normal mammary epithelial cells, HME and MCF10A cells [Zhang et al., 2005]. This marked downregulation of E-cadherin protein was accompanied by a decrease in E-cadherin messenger RNA. Importantly, loss of CCN6 led to a marked decrease in the activity of the E-cadherin promoter (unpubl. data).

Clinical Implications, Future Directions, and Challenges

At present, we can conclude that CCN6 has tumor-inhibitory functions in breast cancer and that it may be a novel regulator of the process of EMT in the breast epithelium. Even though CCN6 appears to negatively interfere with the IGF-1 signaling pathway to suppress breast tumor growth, the effect of CCN6 on EMT is likely IGF-1-independent, based on emerging unpublished data from our laboratory. Nevertheless, because of the importance of this process in invasion and metastasis, further studies dissecting the mechanism of action of CCN6 will be of great interest. In particular, it should be investigated how CCN6 loss leads to decreased E-cadherin, whether CCN6 affects E-cadherin transcriptional repressors (e.g. Snail, Slug, SIP1), and/or interacts with E-cadherin repressor proteins. Given the central role that EMT plays in the metastatic process, understanding the molecular mechanisms of CCN6 function on EMT may enable the development of targeted therapies against metastases in breast cancer. Figure 2 illustrates our working model for CCN6 function in normal breast tissues and in breast tumorigenesis. We postulate that CCN6 expression in normal breast tissues affects (1) the growth of normal epithelial cells through modulating IGF-1 signaling, and (2) the epithelial phenotype by regulating E-cadherin expression and epithelial differentiation. Loss of CCN6 promotes the growth and invasiveness of breast cancer by modulating the growth effects of IGF-1 and its pathway on the mammary epithelium, and by repressing the E-cadherin complex and triggering EMT, thereby driving aggressive and metastatic breast cancer.

Finally, as is well known, new biomarkers of aggressive and metastatic breast cancer are needed. These markers will help clinicians to identify which patients are at risk of tumor metastasis and tailor treatment interventions based on the presence or absence of expression of the biomarker in tumor biopsy samples. Our data support the hypothesis that CCN6 may be a novel biomarker of invasive and metastatic carcinomas of the breast and opens the way to test its expression in human breast tissues in the context of controlled clinical trials.

Acknowledgments

The authors were supported by NIH grants K08 CA090876 and R01 CA107469 (CGK), R01 CA77612 (SDM), Department of Defense grants DAMD17-01-1-490 (CGK), a grant from the John and Suzanne Munn Endowed Research Fund of the University of Michigan Comprehensive Cancer Center (CGK), a grant from the Burroughs Wellcome Fund (SDM), and from the Breast Cancer Research Foundation (SDM).

References

Allen, N.E., A.W. Roddam, D.S. Allen, I.S. Fentiman, I. Dos Santos Silva, J. Peto, J.M. Holly, T.J. Key (2005) A prospective study of serum insulin-like growth factor-I (IGF-I), IGF-II, IGF-binding protein-3 and breast cancer risk. Br J Cancer *92:* 1283–1287.

Benini, S., B. Perbal, D. Zambelli, M.P. Colombo, M.C. Manara, M. Serra, M. Parenza, V. Martinez, P. Picci, K. Scotlandi (2005) In Ewing's sarcoma CCN3(NOV) inhibits proliferation while promoting migration and invasion of the same cell type. Oncogene *24:* 4349–4361.

Bleau, A.M., N. Planque, B. Perbal (2005) CCN proteins and cancer: two to tango. Front Biosci *10:* 998–1009.

Brigstock, D.R., R. Goldschmeding, K.I. Katsube, S.C. Lam, L.F. Lau, K. Lyons, C. Naus, B. Perbal, B. Riser, M. Takigawa, H. Yeger (2003) Proposal for a unified CCN nomenclature. Mol Pathol *56:* 127–128.

Brigstock, D., L. Lau, B. Perbal (2005) CCN workshop. J Clin Pathol *58:* 463–465.

Byrne, C., G.A. Colditz, W.C. Willett, F.E. Speizer, M. Pollak, S.E. Hankinson (2000) Plasma insulin-like growth factor (IGF) I, IGF-binding protein 3, and mammographic density. Cancer Res *60:* 3744–3748.

Byun, D., S. Mohan, D.J. Baylink, X. Qin (2001) Localization of the IGF binding domain and evaluation of the role of cysteine residues in IGF binding in IGF binding protein-4. J Endocrinol *169:* 135–143.

Davis, L., Y. Chen, M. Sen (2006) WISP-3 functions as a ligand and promotes superoxide dismutase activity. Biochem Biophys Res Commun *342:* 259–265.

Diorio, C., M. Pollak, C. Byrne, B. Masse, N. Hebert-Croteau, M. Yaffe, G. Cote, S. Berube, C. Morin, J. Brisson (2005) Insulin-like growth factor-I, IGF-binding protein-3, and mammographic breast density. Cancer Epidemiol Biomarkers Prev *14:* 1065–1073.

Grotendorst, G.R., M.R. Duncan (2005) Individual domains of connective tissue growth factor regulate fibroblast proliferation and myofibroblast differentiation. FASEB J *19:* 729–738.

Hurvitz, J.R., W.M. Suwairi, W. Van Hul, H. El-Shanti, A. Superti-Furga, J. Roudier, D. Holderbaum, R.M. Pauli, J.K. Herd, E.V. Van Hul, H. Rezai-Delui, E. Legius, M. Le Merrer, J. Al-Alami, S.A. Bahabri, M.L. Warman (1999) Mutations in the CCN gene family member WISP3 cause progressive pseudorheumatoid dysplasia. Nat Genet *23:* 94–98.

Imai, Y., A. Moralez, U. Andag, J.B. Clarke, W.H. Busby Jr, D.R. Clemmons (2000) Substitutions for hydrophobic amino acids in the N-terminal domains of IGFBP-3 and -5 markedly reduce IGF-I binding and alter their biologic actions. J Biol Chem *275:* 18188–18194.

Jaiyesimi, I.A., A.U. Buzdar, G. Hortobagyi (1992) Inflammatory breast cancer: a review. J Clin Oncol *10:* 1014–1024.

Kleer, C.G., K.L. van Golen, T. Braun, S.D. Merajver (2001) Persistent E-cadherin expression in inflammatory breast cancer. Mod Pathol *14:* 458–464.

Kleer, C.G., Y. Zhang, Q. Pan, K.L. van Golen, Z.F. Wu, D. Livant, S.D. Merajver (2002) WISP3 is a novel tumor suppressor gene of inflammatory breast cancer. Oncogene *21:* 3172–3180.

Kleer, C.G., Y. Zhang, Q. Pan, S.D. Merajver (2004) WISP3 (CCN6) is a secreted tumor-suppressor protein that modulates IGF signaling in inflammatory breast cancer. Neoplasia *6:* 179–185.

Kowalski, P.J., M.A. Rubin, C.G. Kleer (2003) E-cadherin expression in primary carcinomas of the breast and its distant metastases. Breast Cancer Res *5:* R217–R222.

Lau, L.F., S.C. Lam (1999) The CCN family of angiogenic regulators: the integrin connection. Exp Cell Res *248:* 44–57.

Lee, B.J., N.D. Tannenbaum (1924) Inflammatory carcinoma of the breast: a report of twenty-eight cases from the breast clinic of Memorial Hospital. Surg Gynecol Obstet *39:* 580–595.

Li, C.L., V. Martinez, B. He, A. Lombet, B. Perbal (2002) A role for CCN3 (NOV) in calcium signalling. Mol Pathol *55:* 250–261.

Lin, C.G., S.J. Leu, N. Chen, C.M. Tebeau, S.X. Lin, C.Y. Yeung, L.F. Lau (2003) CCN3 (NOV) is a novel angiogenic regulator of the CCN protein family. J Biol Chem *278:* 24200–24208.

Lin, C.G., C.C. Chen, S.J. Leu, T.M. Grzeszkiewicz, L.F. Lau (2005) Integrin-dependent functions of the angiogenic inducer NOV (CCN3): implication in wound healing. J Biol Chem *280:* 8229–8237.

Manara, M.C., B. Perbal, S. Benini, R. Strammiello, V. Cerisano, S. Perdichizzi, M. Serra, A. Astolfi, F. Bertoni, J. Alami, H. Yeger, P. Picci, K. Scotlandi (2002) The expression of ccn3(nov) gene in musculoskeletal tumors. Am J Pathol *160:* 849–859.

Oka, H., H. Shiozaki, K. Kobayashi, M. Inoue, H. Tahara, T. Kobayashi, Y. Takatsuka, N. Matsuyoshi, S. Hirano, M. Takeichi, et al. (1993) Expression of E-cadherin cell adhesion molecules in human breast cancer tissues and its relationship to metastasis. Cancer Res *53:* 1696–1701.

Palacios, J., N. Benito, A. Pizarro, A. Suarez, J. Espada, A. Cano, C. Gamallo (1995) Anomalous expression of P-cadherin in breast carcinoma. Correlation with E-cadherin expression and pathological features. Am J Pathol *146:* 605–612.

Pennica, D., T.A. Swanson, J.W. Welsh, M.A. Roy, D.A. Lawrence, J. Lee, J. Brush, L.A. Taneyhill, B. Deuel, M. Lew, C. Watanabe, R.L. Cohen, M.F. Melhem, G.G. Finley, P. Quirke, A.D. Goddard, K.J. Hillan, A.L. Gurney, D. Botstein, A.J. Levine (1998) WISP genes are members of the connective tissue growth factor family that are up-regulated in wnt-1-transformed cells and aberrantly expressed in human colon tumors. Proc Natl Acad Sci USA *95:* 14717–14722.

Perbal, B. (2001) NOV (nephroblastoma overexpressed) and the CCN family of genes: structural and functional issues. Mol Pathol *54:* 57–79.

Perbal, B., C. Martinerie, R. Sainson, M. Werner, B. He, B. Roizman (1999a) The C-terminal domain of the regulatory protein NOVH is sufficient to promote interaction with fibulin 1C: a clue for a role of NOVH in cell-adhesion signaling. Proc Natl Acad Sci USA *96:* 869–874.

Perbal, B., C. Martinerie, R. Sainson, M. Werner, B. He, B. Roizman (1999b) The C-terminal domain of the regulatory protein NOVH is sufficient to promote interaction with fibulin 1C: a clue for a role of NOVH in cell-adhesion signaling. Proc Natl Acad Sci USA *96:* 869–874.

Rasbridge, S.A., C.E. Gillett, S.A. Sampson, F.S. Walsh, R.R. Millis (1993) Epithelial (E-) and placental (P-) cadherin cell adhesion molecule expression in breast carcinoma. J Pathol *169:* 245–250.

Sakamoto, K., S. Yamaguchi, R. Ando, A. Miyawaki, Y. Kabasawa, M. Takagi, C.L. Li, B. Perbal, K. Katsube (2002) The nephroblastoma overexpressed gene (NOV/ccn3) protein associates with Notch1 extracellular domain and inhibits myoblast differentiation via Notch signaling pathway. J Biol Chem *277:* 29399–29405.

Schernhammer, E.S., J.M. Holly, M.N. Pollak, S.E. Hankinson (2005) Circulating levels of insulin-like growth factors, their binding proteins, and breast cancer risk. Cancer Epidemiol Biomarkers Prev *14:* 699–704.

van Golen, K.L., S. Davies, Z.F. Wu, Y. Wang, C.D. Bucana, H. Root, S. Chandrasekharappa, M. Strawderman, S.P. Ethier, S.D. Merajver (1999) A novel putative low-affinity insulin-like growth factor-binding protein, LIBC (lost in inflammatory breast cancer), and RhoC GTPase correlate with the inflammatory breast cancer phenotype. Clin Cancer Res *5:* 2511–2519.

Yang, G.P., L.F. Lau (1991) Cyr61, product of a growth factor-inducible immediate early gene, is associated with the extracellular matrix and the cell surface. Cell Growth Differ *2:* 351–357.

Zhang, Y., Q. Pan, H. Zhong, S.D. Merajver, C.G. Kleer (2005) Inhibition of CCN6 (WISP3) expression promotes neoplastic progression and enhances the effects of insulin-like growth factor-1 on breast epithelial cells. Breast Cancer Res *7:* R1080–R1089.