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RhoC-GTPase and WISP3 Proteins

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

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

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13. ABSTRACT (Maximum 200 Words) 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.				
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Celina G. Kleer, M.D.

Annual Report for Award Number: DAMD17-02-1-0490

Introduction

This is an annual report for a project that aims at understanding the clinical utility of RhoC-GTPase and WISP3 proteins in breast cancer patients. These two genes were identified as key genetic determinants of inflammatory breast cancer (IBC). We believe that RhoC GTPase and WISP3 act in concert to determine a highly metastatic breast cancer phenotype, and that they may help identify which invasive breast carcinomas are aggressive from the outset and treat them more appropriately before the development of metastases. Specifically, we aim to determine whether detection of RhoC GTPase and WISP3 proteins in breast cancer tissue samples can identify aggressive tumors. A second goal of our award is to determine the effect of farnesyl transferase inhibitors (FTIs) in RhoC overexpressing xenografts. We have made significant progress in the last year, which is summarized below:

Body

The major thrusts of our year's work have been to better understand the functional significance of the WISP3 gene in Inflammatory Breast Cancer (IBC) and to develop key reagents to test the clinical utility of RhoC and WISP3 in breast cancer tissue samples. Below are brief descriptions of key accomplishments:

- a. 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.
- b. 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)	

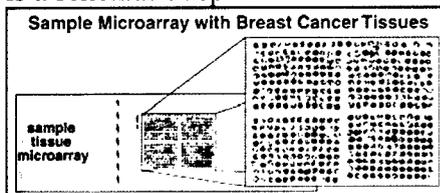
If margin positive:		Single focus		Multiple foci
If margin close:		Single focus		Multiple foci
Histopathological grade (Elton and Ellis):		1	2	3
Positive lymph nodes /total lymph nodes:		/		
Highest axillary node positive:		Yes	No	N/A
Extranodal extension:		Yes	No	N/A
Extensive DCIS:		Yes	No	N/A
DCIS > 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		

c. 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.

d. 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.

e. Immunohistochemical analysis for RhoC-GTPase

We are in the process of staining the TMAs using the RhoC antibody that we have developed in the laboratory (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.

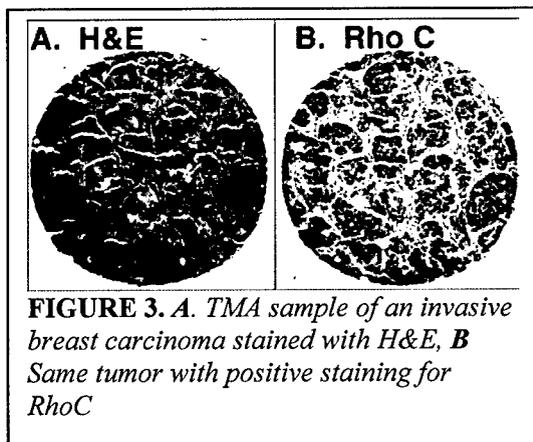


FIGURE 3. A. TMA sample of an invasive breast carcinoma stained with H&E, **B** Same tumor with positive staining for RhoC

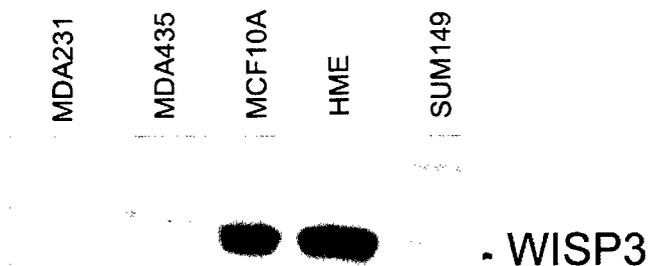
Am J of Pathol. 160(2), 579-584, 2002). We will use an internet-based tool (TMA profiler, University of Michigan, Ann Arbor, MI) that the PI is familiar with to evaluate the staining (Manley, S., Mucci, N.R., De Marzo, A.M. & Rubin, M.A. Relational database structure to manage high-density tissue microarray 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). Using this method the pathologist is blinded to tumor stage and clinical information.

f. Development of anti-WISP3 antibody.

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

Ac-CSGAKGGKKDSDQSN-CONH2
 Ac-CPEGRPGEVSDAPQRKQ-CONH2.

We are in the process of testing the specificity of this antibody and also staining some test tissues. The figure below is a 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.



g. Understanding WISP3 function– This work is seminal, as no other tumor suppressor gene has ever been defined specifically for Inflammatory Breast Cancer. We have demonstrated that WISP3 has tumor suppressor functions in IBC (*Oncogene*, 21, 3172-3180, 2002), and we have gained insight into WISP3 as a modulator of IGF signaling. A

manuscript detailing these findings is nearing completion, and is included in the appendix. This work was presented at the AACR meeting in Washington DC, July, 2003.

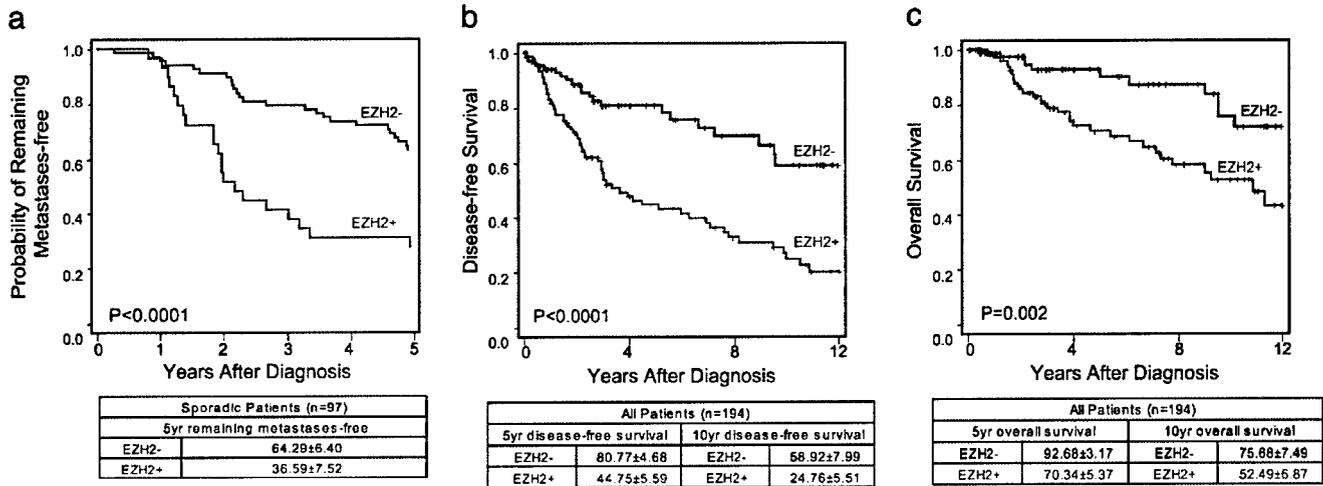
- h. We have also published a manuscript that for the first time reports that Epstein-Barr virus is associated with multiple breast adenomas in immunocompromised patients, and a report that describes the molecular, immunophenotypic, and clinicopathologic features of lymphocytic mastitis.
- i. We have also identified that EZH2 is a marker of aggressive breast cancer and that it promotes the neoplastic transformation of human mammary epithelial cells (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). 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.

Independent factors predictive of death from breast cancer

Parameter	<i>P</i> value	Hazard ratio	95% confidence interval for hazard ratio	
EZH2 positive (vs. negative)	0.01	2.04	1.17	3.57
Positive lymph nodes (≥ 4 , 1-3, 0)	<0.0001	1.9	1.4	2.57
PR positive (vs. negative)	0.02	0.54	0.32	0.91

Multivariate Cox Model with backward selection, $n = 161$, $P < 0.0001$ disease-free survival.

Kaplan-Meier curves of disease specific and overall survival according to the level of EZH2 expression.



In summary, we have had a very productive year where we have completed 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 a polyclonal antibody against WISP3
- Preliminary demonstration that RhoC could detect small invasive carcinomas with aggressive behavior
- Elucidation of WISP3 modulation of IGF-I signaling cascade in inflammatory breast cancer
- Discovered that Epstein-Barr virus is involved in breast adenomas, and that the lymphocytic infiltrate in lymphocytic mastitis is polyclonal in nature.
- 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 this year, the P.I. has learned how to develop tissues microarrays. She has also learned the technical aspects of its construction and design. The P.I. also learned how to design a relational database and how to query the database to link the immunohistochemical score with the clinical and pathological information. A significant learning opportunity was to direct and work closely with the statistician to perform survival analyses.

In the laboratory, the P.I. learned how to design and interpret the experiment conducted that revealed that WISP3 modulates IGF signaling pathways.

In developing the anti-WISP3 antibody, the P.I. learned how to design a peptide, and how to test and interpret the antibody at different points of the immune response.

Reportable Outcomes

We are in a position to definitively state that WISP3 is able to ameliorate the highly malignant features of inflammatory breast cancer. Specifically, WISP3 has growth and angiogenic inhibitory functions. In addition, we can report that RhoC over expression is an early marker of aggressive breast cancers, even when they are small.

Research

Manuscripts:

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.

Abstracts:

Kleer CG, Shen R, Chinnaiyan AM, Rubin MA. 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 CG, Zhang Y, Pan Q, Merajver SD. WISP3 is a secreted protein and modulates IGF signaling in inflammatory breast cancer. Minisymposium AACR Meeting, Washington DC, July 11-14, 2003.

Kleer CG, Chang T, Pan Q, Rubin MA, Merajver SD. Characterization of VEGF-C expression in Breast Tissues using High Density Tissue Microarrays. Oral presentation. International Academy of Pathology. Amsterdam, October 2002. *Histopathology* 41 (Suppl. 1), 1-63, 2002.

Sturtz DE, Schott AF, and Kleer CG. 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 CG, Zhang Y, Pan Q, Merajver SD. 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 CG, Shen R, Wolf J, Rubin MA. Characterization of platelet derived growth factor receptor expression in breast cancer identifies inflammatory breast cancer as a potential target for treatment with PDGFRb inhibitors. Poster presentation, USCAP meeting, Washington DC, 2003

Conclusion

We are encouraged by our progress. We want to move forward and test the clinical utility of WISP3 and RhoC, alone and in combination with other markers, in detecting highly aggressive breast cancer phenotypes before they develop metastases. We also wish to explore the relationship between WISP3 and the Wnt-1 pathway and IGF signaling. 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, 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.

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.

WISP3 is a Secreted Tumor Suppressor Protein that Modulates IGF
Signaling in Inflammatory Breast Cancer

Celina G. Kleer¹, Yanhong Zhang¹, Quintin Pan², and Sofia D. Merajver².

Departments of Pathology (1) and Internal Medicine, Division of Hematology/Oncology
(2), University of Michigan Medical Center, Ann Arbor, MI.

Corresponding authors: Sofia D. Merajver, M.D., Ph.D. and Celina G. Kleer, M.D.
Department of Internal Medicine
7217 CCGC
1500 E. Medical Center Dr.
Ann Arbor, MI 48108-0948
Phone (734) 936-6884
FAX (734) 936-7376
smerajve@umich.edu

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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 activation of the IGF-IR, and two of its main downstream signaling molecules, IRS1 and ERK-1/2 in SUM149 IBC cells. The modulation of IGF-IR activation was seen only in the presence of both WISP3 and IGF-I in the conditioned media. This work sheds light into the structure-function relationship of WISP3 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 in IBC.

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, likely causing 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 NH₂ 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 in the 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 tissue, and hyperactivation of IGF-IR in breast cancer has been linked with 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-positive breast cancer patients (17, 18). Based on the protein structure 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 WISP3 in IBC may be dependent, at least in part, upon 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. In the present paper, 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 in inflammatory breast cancer.

MATERIALS AND METHODS

Cell Culture and Transfections. SUM149 cells derive from a primary inflammatory breast cancer that has lost WISP3 expression (6, 19). SUM149 cells and their transfectants were cultured in Ham's F-12 media supplemented with 5% FBS, hydrocortisone (1 $\mu\text{g/ml}$), insulin (5 $\mu\text{g/ml}$), fungizone (2.5 $\mu\text{g/ml}$), Gentamycin (5 $\mu\text{g/ml}$) and Penicillin/streptomycin (100 $\mu\text{g/ml}$ each), at 37° C under 10% CO₂. SUM149 cells were transfected with HIS- tagged (pcDNA 3.1/V5-HIS TOPO TA expression vector, Invitrogen, 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 cell line with the empty vectors. The insert was confirmed by sequencing. 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, Carlsbad, CA).

Western Blotting and Immunoprecipitation. Conditioned media was generated by incubating wild-type SUM149 cells, SUM149 transfectants, and empty vector control transfectants in serum-free media. Three days later, the media were collected, cleared of cell debris by centrifugation, concentrated approximately ten-fold through a Centrplus YM-100 column (Millipore, Bedford, MA), and sterilized using a 0.45 μM PVDF Filter (Whatman, Clifton, NJ) before use. Subsequently, ten ml of the conditioned media from stable WISP3 transfectants and empty vector controls was tested by Western blot for the presence of WISP3 protein, using anti-WISP3 polyclonal antibody (gift from Dr. Warman), and anti-HIS tag antibody (Invitrogen, Carlsbad, CA). Approximately 10 ml of

conditioned media was used to examine the effects of WISP3 on IGF signaling in SUM149 cells. For this experiment, 70% confluent cultures of SUM149 cells were shifted to serum-free media. After 24 h, the cells were shifted to conditioned media containing WISP3 for 24 h. As controls, the conditioned media lacking detectable WISP3 (obtained from the empty vector transfectants) was used. Subsequently, the SUM149 cells were stimulated with 20 ng/ml human recombinant IGF-I (Upstate Biotechnology Inc., NY) for 15 minutes. Proteins were obtained by lysing the cells in a buffer composed of 50 mM HEPES (pH 7.5), 150 mM 1% Triton X-100, 1.5 mM MgCl₂, 5 mM EGTA, 10% glycerin, 0.2 mM Na₃VO₄, 1% phenylmethylsulfonyl fluoride, and 1% aprotinin. The IGF-IR was immunoprecipitated from 500 µg of protein lysate with anti-IGF-IR mAb (Calbiochem, San Diego, CA) and subsequently detected by immunoblot 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 ERK-1/2 and IRS-1 (phosphorylated and total) were measured with appropriate antibodies (Transduction Laboratories and Upstate Biotechnology). 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 < 0.05 was considered statistically significant.

Human breast tissues and immunohistochemistry. WISP3 protein expression was studied by immunohistochemistry in normal human breast tissues obtained from ten reduction

mammoplasty procedures. Immunohistochemical analysis was performed 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 counter stain. 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 multi-modular structure with a secretory signal peptide at the N-terminus. To investigate whether WISP3 is secreted by breast epithelial cells, SUM149 inflammatory breast cancer 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 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 1A**). 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 non-mammary cell, we repeated these experiments with the HEK293 cell line, derived from human embryonic kidney epithelial cells (gift from Dr. Koenig).

To examine the expression of WISP3 protein *in situ*, we performed immunohistochemical analysis of normal breast tissues. In all ten 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 1B**). In contrast, stromal fibroblast and myoepithelial cells did not express WISP3.

WISP3 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 inflammatory breast cancer (5, 6, 19, 23). The activation of the IGF signaling cascade plays a central role in breast cancer development and progression. Activation of the IGF-IR and downstream signaling molecules enhance growth in breast cancer. To investigate whether the growth inhibitory effects of WISP3 in inflammatory breast cancer (IBC) may be elicited by the modulation of IGF-IR signaling, phosphorylation of the 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. As shown in **Figure 2**, WISP3 containing conditioned media was able to attenuate IGF-induced phosphorylation of the IGF-IR, and of its major downstream substrate IRS1, and ERK-1/2.

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 in the 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.

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, 27-31). 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 investigated the function of WISP3 in relationship to its protein structure. We focused on determining whether WISP3 is secreted into the conditioned media and its relationship to IGF signaling pathways.

Analysis of the protein structure 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 (32-35). IGF-I is locally released by breast cancer cells and by stromal fibroblasts and it is involved in autocrine and paracrine stimulation of the mammary epithelium (36). 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 (37, 38). 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 lead 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. Taken together, 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.

One of the main IGF-IR downstream pathways that regulate breast cancer growth and survival is the ERK-1/2 cascade (39). 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 (40-42). 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 inflammatory breast cancer cell line markedly decreased the levels of cyclin E and of PCNA, a reliable marker of cellular proliferation (6). These data thus further strengthen the evidence that WISP3 may decrease the growth of inflammatory breast cancer by interfering with IGF-I signaling and blunting the activation of the IGF-IR and ERK-1/2

signaling cascade in the presence of IGF-I, thereby resulting in a decrease in cellular proliferation and tumor growth.

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 IGF-BPs, which may provide the proper protein folding to interact with IGF-like ligands and thereby enabling interference with IGF-signaling. Although initial studies reported that two other CCN proteins, CTGF (connective tissue growth factor) and Nov specifically bind to IGF-I (25, 43), these results have not been subsequently built upon and they remain to be duplicated by other investigators. Whether WISP3 physically binds to IGF-I warrants further investigation, in light of our 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 (44), 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 (44). 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. It is also possible, although less likely based on our experiments, that WISP3 binds to the IGF-IR independently of IGF-I.

The data presented here address for the first time, the structure-function relationship of WISP3 as we demonstrate that WISP3 is secreted, which in turn leads to modulation of IGF signaling in inflammatory breast cancer cells.

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Figure Legends

Figure 1. WISP3 protein is secreted. (A) Western immunoblot using anti-WISP3 polyclonal antibody detects WISP3 protein in the conditioned media of SUM149 cells stably transfected with WISP3 full-length cDNA. In contrast, WISP3 protein is not detected in the conditioned media of SUM149 cells transfected with the empty vector. (B) Immunohistochemical analysis of normal breast tissues shows WISP3 protein in the luminal secretions of ducts and acini (arrows). WISP3 is expressed at low levels in the normal epithelial cells. No WISP3 expression is detected in stromal fibroblasts or myoepithelial cells. (200X).

Figure 2. WISP3 decreases IGF-I-induced phosphorylation of the IGF-IR, IRS1 and ERK1/2. The effects of WISP3 in IGF signaling were studied in SUM149 cells incubated with WISP3 containing conditioned media. The conditioned media lacking detectable WISP3 protein was used as control. Experiments were performed in the presence and absence of IGF-I. WISP3 significantly decreases the activation of IGF-IR (A), IRS-1 (B), and ERK-1/2 (C) only in the presence of IGF-I. Graph bars show the percent inhibition of IGF-IR, IRS, and ERK-1/2 phosphorylation by WISP3 containing conditioned media in the presence of IGF-I. The measurements are an average of three separate experiments obtained by densitometry.

Figure 1 A

A Conditioned medium

**SUM149
control**

**SUM149/
WISP3**

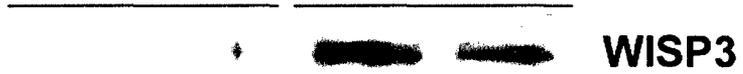


Figure 1 B

B

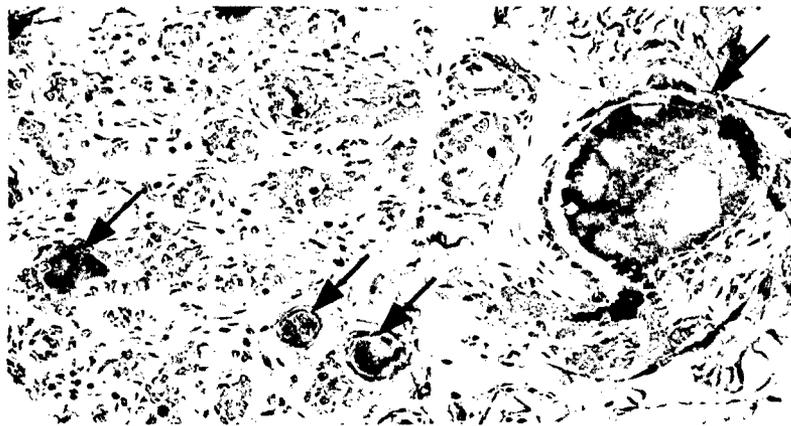


Figure 2 A

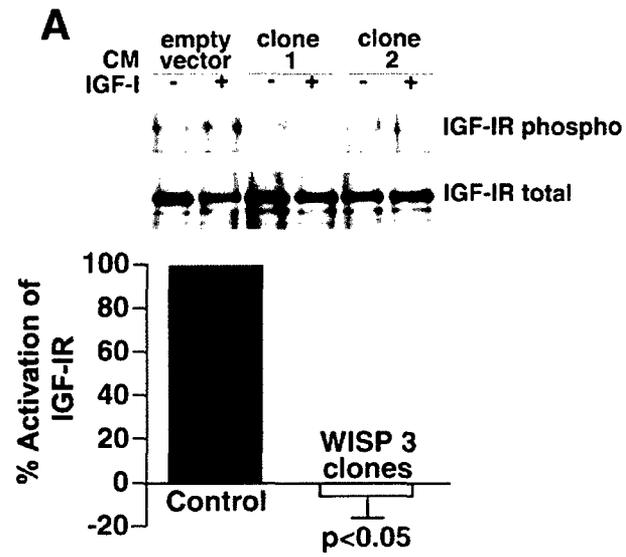


Figure 2 B

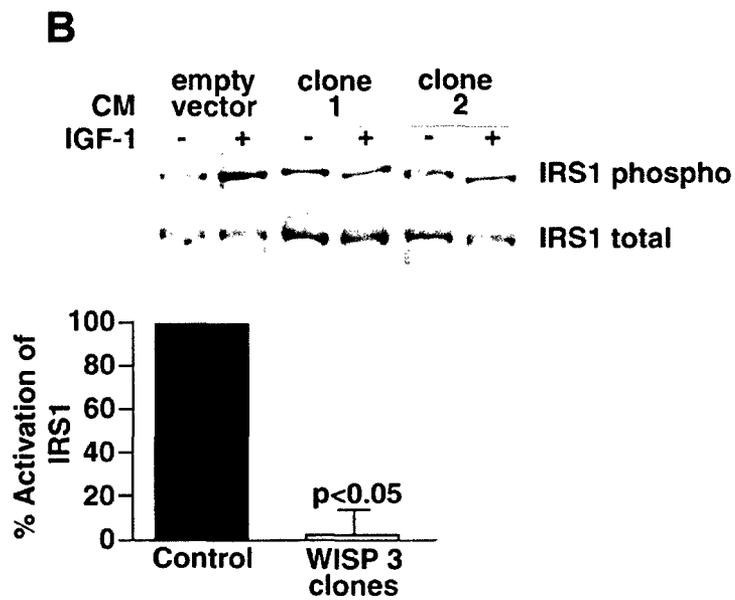
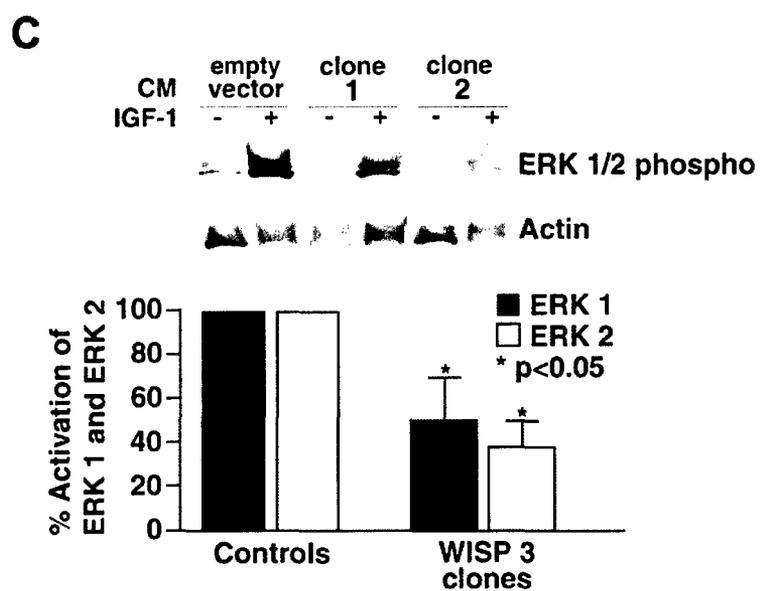


Figure 2 C



EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells

Celina G. Kleer^{*†§5}, Qi Cao^{**}, Sooryanarayana Varambally^{**}, Ronglai Shen^{*¶}, Ichiro Ota^{*¶}, Scott A. Tomlins^{*}, Debashis Ghosh^{*¶}, Richard G. A. B. Sewalt^{**}, Arie P. Otte^{**}, Daniel F. Hayes^{*¶}, Michael S. Sabel^{†,††}, Donna Livant^{†††}, Stephen J. Weiss^{¶¶}, Mark A. Rubin^{§§}, and Arul M. Chinnaiyan^{*†§¶¶}

Departments of ^{*}Pathology, [¶]Biostatistics, ^{¶¶}Internal Medicine, ^{††}Surgery, ^{**}Radiation Oncology, and ^{¶¶¶}Urology, and [†]Comprehensive Cancer Center, University of Michigan Medical School, Ann Arbor, MI 48109; ^{**}Swammerdam Institute for Life Sciences, Biocentrum Amsterdam, University of Amsterdam, 1018 TV Amsterdam, The Netherlands; and ^{§§}Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115

Edited by Lawrence H. Einhorn, Indiana University, Indianapolis, IN, and approved July 28, 2003 (received for review June 19, 2003)

The Polycomb Group Protein EZH2 is a transcriptional repressor involved in controlling cellular memory and has been linked to aggressive prostate cancer. Here we investigate the functional role of EZH2 in cancer cell invasion and breast cancer progression. EZH2 transcript and protein were consistently elevated in invasive breast carcinoma compared with normal breast epithelia. Tissue microarray analysis, which included 917 samples from 280 patients, demonstrated that EZH2 protein levels were strongly associated with breast cancer aggressiveness. Overexpression of EZH2 in immortalized human mammary epithelial cell lines promotes anchorage-independent growth and cell invasion. EZH2-mediated cell invasion required an intact SET domain and histone deacetylase activity. This study provides compelling evidence for a functional link between dysregulated cellular memory, transcriptional repression, and neoplastic transformation.

Breast cancer is a leading cause of cancer-related death in women, accounting for $\approx 40,000$ deaths per year in the United States (1). Despite advances in the early detection and treatment of breast cancer, mortality for those 20% of patients with recurrences and/or metastases is $\approx 100\%$ (2). Currently, the most important prognostic markers for patients with breast cancer that are used in the clinical setting are components of the staging system, such as primary tumor size and the presence of lymph node metastasis (3). However, the accuracy of these conventional indicators is not as precise as desired, leading to inefficient application of systemic therapy (4). Thus, there is a need for novel molecular predictors of tumor behavior at the time of diagnosis that will help guide clinical therapy decisions.

Few biomarkers of breast cancer progression have been proven to be clinically useful (4). Estrogen receptor (ER) and progesterone receptor (PR) are highly predictive of breast cancer patients that will benefit from endocrine therapy (5) but are weak prognostic factors (6). Other tumor markers that have been considered for prognostication in breast cancer include erbB2 amplification/overexpression, cathepsin D, and uPAR (4). The consensus, however, remains that new prognostic factors that are more precise and reliable are needed (7).

Through our gene expression profiling studies, we identified EZH2 as being overexpressed in metastatic prostate cancer (8). In clinically localized prostate cancer, EZH2 was found to be predictive of poor outcome postprostatectomy (i.e., biochemical recurrence or metastasis). EZH2 is a Polycomb Group (PcG) protein homologous to *Drosophila* Enhancer of Zeste and involved in gene silencing (9, 10). PcG proteins are presumed to function in controlling the transcriptional memory of a cell (9). Dysregulation of this gene silencing machinery can lead to cancer (9, 11, 12). In the context of prostate cancer, we provided evidence that EZH2 functions as a transcriptional repressor, and inhibition of EZH2 blocks prostate cell growth (8). Interestingly, several recent studies

demonstrated that EZH2 has enzymatic activity and functions as a histone H3 methyltransferase (13–15).

Biochemical analysis indicates that PcG proteins belong to at least two multimeric complexes, PRC1 (16) and EED-EZH2 (Enx1) (17). These complexes are thought to heritably silence genes by acting at the level of chromatin structure. The EED protein interacts directly with type 1 histone deacetylases (HDACs) in mammalian cells (18), and in *Drosophila* (19), and this has been suggested to be part of the silencing mechanism. Furthermore, recent studies have demonstrated that EED/EZH2 complexes methylate H3-K9 and K27 *in vitro*, with a strong preference for K27 (13–15). Methylation of both H3-K9 (20) and H3-K27 is thought to be involved in targeting the PRC1 complex to specific genetic target loci.

By interrogating publicly available gene expression data sets, we identified EZH2 as being dysregulated in breast cancer. In the present study, we examined EZH2 mRNA transcript and protein level in normal breast and in breast cancer progression. Immunohistochemical analyses performed on a spectrum of breast cancer specimens demonstrated that high EZH2 levels were strongly associated with poor clinical outcome in breast cancer patients. EZH2 was an independent predictor of breast cancer recurrence and death and provided prognostic information above and beyond known clinical, pathologic, and biomarkers studied. Overexpression of EZH2 in normal breast epithelial cell lines produced a neoplastic phenotype characterized by anchorage-independent growth and cell invasion. Neoplastic transformation mediated by EZH2 depended on both the SET domain as well as HDAC activity. Importantly, we propose a biologic basis for the association of EZH2 and tumor aggressiveness in that high levels of EZH2 promote the invasive potential of carcinomas.

Methods

Selection of Patients and Tissue Microarray Development. Breast tissues for tissue microarray construction were obtained from the Surgical Pathology files at the University of Michigan with Institutional Review Board approval. A total of 280 cases ($n = 917$ tissue microarray samples) were reviewed by the study pathologist (C.G.K.) and arrayed in three high-density tissue microarrays, as described (21, 22). See *Supporting Methods*, which are published as supporting information on the PNAS web site, www.pnas.org, for details.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; PcG, Polycomb Group; HDAC, histone deacetylase; TSA, trichostatin A; ECM, extracellular membrane; SU, sea urchin; CAM, chicken chorioallantoic membrane; SAHA, suberoylanilide hydroxamic acid.

[†]C.G.K., Q.C., and S.V. contributed equally to this work.

[§]To whom correspondence may be addressed. E-mail: kleer@umich.edu or arul@umich.edu.

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Immunohistochemical Studies. Immunohistochemistry was performed on the tissue microarrays (TMAs) by using standard biotin-avidin complex technique and a polyclonal antibody against EZH2 that was previously validated by immunoblot analysis (8). See *Supporting Methods* for detailed methodology. The TMAs were immunostained for ER and PR and for HER-2/neu by using well described and validated procedures (23). See *Supporting Methods* for details.

Statistical Analysis. Comparison of the intensity of EZH2 staining between normal breast, hyperplasia, ductal carcinoma *in situ*, invasive carcinoma, and metastases was carried out by calculating the median staining intensity for each case and applying the Wilcoxon rank test. A *P* value of <0.05 was considered significant. Overall survival was calculated from the date of surgical excision of the primary tumor to the date of death. Patients who died of or with the disease were included in the analysis. For disease-specific survival, data for patients who died from other causes were censored at the time of death. Overall survival and disease-specific survival curves were constructed by the Kaplan-Meier method. Clinical criteria for treatment failure were local recurrence and/or the development of metastases.

Univariate analyses of disease-specific survival were performed by using a two-sided log-rank test to evaluate EZH2 protein expression, age, tumor size, nodal status, stage, angiolymphatic invasion, ER status, PR status, and HER-2/neu status. To assess the influence of several variables simultaneously, a multivariable Cox proportional hazards model of statistically significant covariates was developed by removing nonsignificant parameters in a step-wise manner. Statistical significance in the Cox models was determined by Wald's test.

SYBR Green Quantitative Real-Time PCR. We performed SYBR green real-time quantitative PCR analysis on 19 laser-microdissected frozen breast tissues obtained from the frozen breast tissue bank in our institution with Institutional Review Board approval. See *Supporting Methods* for details.

Immunoblot Analysis. Protein extracts were prepared from normal and cancerous breast tissues and standard immunoblot analysis performed. See *Supporting Methods* for details.

Adenovirus Constructs. Adenoviral constructs were generated by *in vitro* recombination. In brief, the full-length EZH2 or SET domain deleted EZH2 (EZH2 Δ SET) were inserted in an adenoviral shuttle plasmid [pACCMVpLpA(-)loxP-SSP]. Viruses were generated by transfection into the 293-complementation cell line. Virus was propagated in 911 cells and purified on a CsCl gradient. Multiplicities of infection were calculated, and purified viruses were stored in 10 mM Tris-HCl (pH 7.4)/137 mM NaCl/5 mM KCl/1 mM MgCl₂ in 10% glycerol (by volume).

Cell Count. H16N2 were infected with EZH2 adenovirus. Cell counts were estimated by trypsinizing cells and analysis by Coulter counter at the indicated time points in triplicate.

Soft Agar Assay. A 0.6% (wt/vol) bottom layer of low melting point agarose in normal medium was prepared in six-well culture plates. On top, a layer of 0.6% agarose containing 1×10^5 stable transfected cells was placed (24). After 25 days, foci were stained with P-Iodonitrotetrazolium violet and counted.

HDAC Assay. HDAC activity assays were performed according to the manufacturer's instructions (Biomol, Plymouth Meeting, PA). See *Supporting Methods* for details.

Basement Membrane Matrix Invasion Assay. Cells were infected with vector, EZH2, and EZH2 Δ SET adenovirus. Forty-eight hours after infection, the cells were trypsinized and seeded at equal numbers onto the basement membrane matrix 24-well culture plates [extracellular membrane (ECM); Chemicon] in the presence or absence of HDAC inhibitors suberoylanilide hydroxamic acid (SAHA) (7.5

μ M) and trichostatin A (TSA) (0.5 μ M). FBS was added to the lower chamber to act as a chemoattractant. After 48-h incubation, the noninvading cells and ECM were removed gently by cotton swab. The cells that are invaded that are present on the lower side of the chamber were stained, air dried, and photographed. The invaded cells were counted under the microscope. For colorimetric assay, the inserts were treated with 150 μ l of 10% acetic acid, and absorbance was measured at 560 nm.

Sea Urchin (SU) Embryo Basement Membrane Invasion Assay. H16N2 cells were infected with vector, EZH2, and EZH2 Δ SET adenovirus and trypsinized after 48 h. The infected cells alone or treated with HDAC inhibitors SAHA (7.5 μ M) and TSA (0.5 μ M) and analyzed for invasiveness by using the SU embryo basement membrane invasion assay (25). See *Supporting Methods* for details.

Chick Chorioallantoic Membrane (CAM) Invasion Assay. EZH2- and control virus-infected H16N2 cells were labeled with Fluoresbrite carboxylated polystyrene nanospheres of 48 nm diameter (Polysciences) as described (26). See *Supporting Methods* for details.

Results

EZH2 Transcript and Protein Expression Are Elevated in Breast Cancer.

On the basis of our previous work characterizing EZH2 in prostate cancer (8), we were interested in determining whether EZH2 is dysregulated in breast cancer, which, similar to prostate cancer, is steroid hormone regulated. This was facilitated by our group's ongoing efforts to create a cancer microarray metaanalysis database (see www.ONCOMINE.org) stemming from our initial work in prostate (27). Of the five publicly available breast cancer gene expression datasets (28–32), only the Perou *et al.* (28) study had neoplastic and normal breast tissues to make comparisons between benign and cancer. Interestingly, in this dataset, we found that the EZH2 transcript was overexpressed significantly in invasive breast cancer and metastatic breast cancer relative to normal ($P = 0.002$, *t* test) (28).

To validate these DNA microarray results, we carried out SYBR green quantitative real-time PCR on 19 laser-capture microdissected normal and invasive breast cancers. As predicted, levels of EZH2 mRNA were increased an average of 7.5-fold in invasive carcinomas compared with normal breast epithelial cells (*t* test, $P = 0.0085$) (Fig. 1*a*). To confirm that EZH2 is elevated at the protein level in invasive breast cancer, we analyzed normal breast and breast cancer tissue extracts by immunoblot analysis. Consistent with the transcript data, invasive breast cancer expressed high levels of EZH2 protein relative to normal (Fig. 1*b*). Importantly, EED, a PcG protein that forms a complex with EZH2, did not exhibit similar protein dysregulation.

Using high-density tissue microarrays, we next evaluated the expression of EZH2 protein in a wide range of breast tissues (280 patients, $n = 917$ samples) to characterize its expression *in situ* by immunohistochemistry. EZH2 protein expression was observed primarily in the nucleus (Fig. 1*c*), as reported previously (33). Invasive breast cancer that expressed high levels of EZH2 (scores 3–4, EZH2+) and those that expressed low levels of EZH2 (scores 1–2, EZH2-) were readily apparent (Fig. 1*c* Center and Right). There was a remarkable staining difference between tumor cells that form intravascular emboli and adjacent normal breast epithelia (Fig. 1*c* Left). Consistent with our mRNA transcript data, EZH2 protein levels were elevated in invasive carcinoma relative to normal or atypical hyperplasia (Wilcoxon test, $P < 0.0001$, Fig. 1*d*). As in the case of metastatic prostate cancer (8), breast cancer metastases expressed high levels of EZH2 (Fig. 1*d*). Median EZH2 staining intensities of normal, atypical hyperplasia, ductal carcinoma *in situ* (DCIS), invasive carcinoma, and metastases were 1.47 (SE 0.61), 2 (SE 0), 2.38 (SE 0.52), 2.74 (SE 0.99), and 3.09 (SE 1.04), respectively (Fig. 1*d*). Interestingly, increased EZH2 protein and transcript were already present in DCIS, a precursor of invasive carcinoma (Fig. 1*d*).

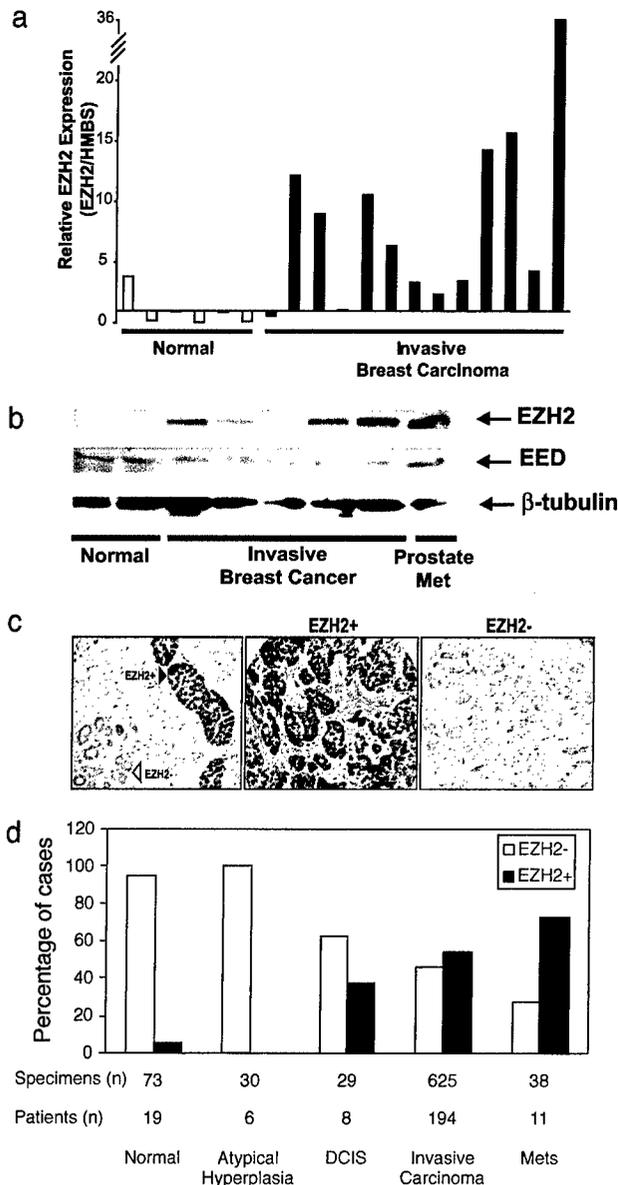


Fig. 1. EZH2 mRNA transcript and protein levels are elevated in breast cancer. (a) Quantitative SYBR green RT-PCR of EZH2 transcript in laser-capture microdissected normal and breast cancer epithelia. Each sample was performed in duplicate, and a ratio was calculated relative to the housekeeping gene hydroxymethylbilane synthase (HMBS). (b) Immunoblot analysis of EZH2 and EED in breast tissue extracts. Metastatic (Met) prostate cancer was used as a positive control. β -Tubulin was included as a loading control. (c) Representative breast tissue sections stained with an antibody to EZH2. (Left) Normal breast epithelia (open triangle) and adjacent intravascular breast cancer emboli (filled triangle). (Center) An invasive breast cancer expressing high levels of EZH2. (Right) An invasive breast cancer expressing low levels of EZH2. (d) Tissue microarray analysis of EZH2 expression in breast cancer progression. Tumor specimens were stratified into high EZH2 expressors (filled bars, scored 3 or 4) and low EZH2 expressors (open bars, scored 1 or 2). The y axis represents the percentage of patients in each category.

Prognostic Value of EZH2 in Breast Cancer. To investigate whether EZH2 mRNA expression levels are associated with outcome, we analyzed the published van't Veer *et al.* (30) breast cancer gene expression dataset, which contains outcome information on 78 sporadic invasive carcinomas <5 cm with negative lymph nodes. We found that the levels of EZH2 transcript expression were significantly

higher in invasive carcinomas that metastasized within 5 years of primary diagnosis when compared with invasive carcinomas that did not metastasize (Wilcoxon rank test $P = 0.01$, Fig. 2a). By Kaplan-Meier analysis, high EZH2 expression [>1.26 (log₂ ratio >0.1)] was associated significantly with the development of metastasis within 5 years of primary diagnosis (log rank $P < 0.0001$). Multivariable Cox hazards regression analysis showed that EZH2 mRNA expression was an independent predictor of the development of metastases with a hazard ratio of 2.02 (95% confidence interval 1.08–3.76, $P = 0.03$).

By using our breast cancer tissue microarray data, we were in the position to evaluate clinical and pathology associations of EZH2 protein levels in breast cancer. In our cohort of 236 consecutive breast cancer patients ($n = 712$ samples), 194 had complete follow-up information. Clinicopathologic characteristics of the patients can be found in Table 1. The median age of the study population was 56 years (ranging from 26 to 89 years). After a median follow-up of 3.2 years (range 17 days to 15.8 years), 42 of the 194 patients (21.6%) died of breast cancer. The 5- and 10-year disease-specific survival rates for the entire cohort of patients were 60.28% and 38.66%, respectively. The association between EZH2 protein levels and clinical characteristics is shown in Table 3, which is published as supporting information on the PNAS web site. EZH2 expression was strongly associated with standard pathology predictors of clinical outcome, including tumor diameter ($P = 0.002$) and stage of disease ($P < 0.0001$). Higher EZH2 levels were also significantly associated with decreasing age ($P = 0.0003$), negative ER status ($P = 0.0001$), negative PR status ($P < 0.0001$), and lymph node status ($P = 0.001$), but not HER2/neu overexpression. Hazard ratios of recurrence or metastasis according to EZH2 status were 2.92 ($P < 0.0001$).

The results of the univariate analysis are shown in Table 4, which is published as supporting information on the PNAS web site. As expected, at the univariate level, lymph node status, tumor diameter, and stage of disease were associated with disease-specific and overall survival. Hormone receptor status was inversely associated with outcome. We found a strong association between EZH2 protein levels and patient outcome. Higher EZH2 protein levels were associated with a shorter disease-free interval after initial surgical treatment, lower overall survival, and a high probability of disease-specific death (or death due to breast cancer) (Fig. 2b and c). The 10-year disease-free survival for patients with tumors expressing high EZH2 levels was 24.76% and, by contrast, 58.92% for low levels of EZH2 (log rank $P < 0.0001$, Fig. 2b). High EZH2 expression was associated with disease-specific survival in patients with lymph node-negative disease (log rank $P = 0.007$). EZH2 expression was associated with disease-specific survival in patients with stage I and II disease (log rank, $P = 0.037$ and $P = 0.048$, respectively), but not in patients with advanced stage (stages III and IV). EZH2 was not associated with survival in patients with positive lymph nodes. The strong inverse association between high EZH2 protein expression and negative ER status (Kruskal-Wallis test, $P = 0.001$, Table 3) prompted us to investigate whether the prognostic utility of EZH2 depends on ER status. Kaplan-Meier analysis showed that EZH2 levels were strongly associated with outcome in both ER-positive and -negative invasive carcinomas (see Fig. 5, which is published as supporting information on the PNAS web site). Thus, our data suggest that EZH2 has prognostic utility independently of ER status.

The best multivariable model predictive of disease-specific survival included positive lymph nodes, high EZH2 expression, and negative PR status (Table 2). High EZH2 expression was a strong independent predictor of outcome providing survival information above other independent prognostic features, with a hazard ratio of 2.04 and a 95% confidence interval of 1.17–3.57, $P = 0.01$. Tumor size, angiolymphatic invasion, and ER status, identified as having strong associations with EZH2 at the univariate level, were not independently associated with outcome at the multivariable level.

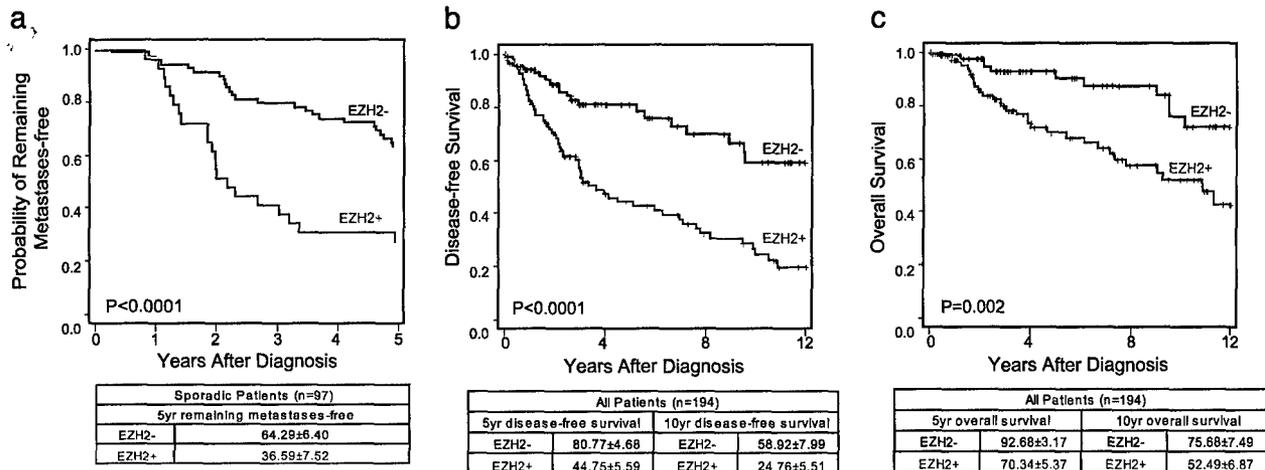


Fig. 2. High EZH2 levels are associated with aggressive breast cancer. (a) Kaplan–Meier analysis of metastasis-free survival according to EZH2 mRNA transcript levels as measured using DNA microarrays by van't Veer *et al.* (30). Kaplan–Meier analysis of disease-specific (b) and overall (c) survival according to EZH2 protein levels as assessed by immunohistochemical analysis. Patients grouped on the basis of high (+) or low (–) EZH2 expression levels. *P* values were calculated by using the log-rank test.

EZH2 Overexpression Promotes Anchorage-Independent Growth and HDAC Activity in Normal Breast Epithelial Cells. To study the function of dysregulated EZH2 expression in breast epithelial cells, we generated adenovirus constructs expressing EZH2. We also generated an adenovirus expressing a mutant version of EZH2 in which the C-terminal SET domain is truncated (EZH2ΔSET). Normal immortalized breast epithelial cells (H16N2) (34) were infected with EZH2 and EZH2ΔSET expressing viruses and protein expression demonstrated in Fig. 3a. Overexpression of EZH2 in breast epithelial cells did not significantly enhance cell proliferation in tissue culture (Fig. 3b). Interestingly, EZH2 overexpression markedly promoted colony formation in soft agar relative to EZH2ΔSET and vector controls (Fig. 3c and d). In fact, colonies were present only in EZH2-infected H16N2 cells, supporting the notion that EZH2 can facilitate anchorage-independent growth. As in our previous study with prostate cells (8), overexpression of EZH2 in breast carcinoma cells induced transcriptional repression of a cohort of target genes (data not shown). Previous studies have demonstrated that the EED–EZH2 complex recruits type I HDACs (18). To determine whether overexpression of EZH2

modulates HDACs, we measured HDAC enzymatic activity in breast epithelial cell lysates. Overexpression of EZH2 but not the EZH2ΔSET mutant increased total HDAC activity in breast epithelial cells. This activity was completely abrogated in the presence the HDAC inhibitor TSA.

Dysregulated EZH2 Orchestrates the Invasive Potential of Breast Epithelial Cells. We next assessed the biological function of EZH2 in the context of cancer cell invasion. We observed that overexpression of EZH2 in breast epithelial cells promotes invasion in a reconstituted basement membrane invasion chamber assay (Fig. 4a and b). The control experiments that included EZH2ΔSET mutant and vector did not exhibit similar proinvasive properties. Importantly, EZH2-mediated invasion was attenuated with inclusion of the HDAC inhibitors TSA and SAHA. Cell invasion was quantitated by both cell counting and colorimetry (Fig. 4b). Next, we used SU-ECM (25, 35) as invasion substrates to examine the invasive properties of EZH2 expressing breast epithelial cells. The SU-ECM assay has advantages over the reconstituted basement membrane assay in that it is a uniform, biological, serum-free basement membrane that closely mimics the type of extracellular matrix that cells encounter *in vivo*. As with the reconstituted basement membrane assay, EZH2 overexpression in the SU-ECM assay supported similar findings regarding the invasive potential of EZH2 and its requirement for HDAC activity (Fig. 4c).

To examine the role of EZH2-mediated invasion in an *in vivo* setting, we used a CAM assay. In this model, EZH2 overexpressing breast epithelial cells are labeled with fluorescent beads, seeded in duplicate on CAMs, of 10-day-old chicken embryos and incubated. At time of harvest, frozen sections were made from the CAM tissues and examined by fluorescent and light microscopy after hematoxylin/eosin staining. EZH2 overexpressing breast epithelial

Table 1. Demographics of patients with clinical follow-up used in this study

Parameter	Value
No. of patients	194
Median age, years (range)	56 (26–89)
Follow-up/years, median (range)	3.2 years (17 d–16 years)
Pathologic stage, no. (%)	
I	78 (40)
II	66 (34)
III	32 (16)
IV	18 (10)
Tumor size, cm (range)	2 (0.3–6.7)
Lymph node status, no. (%)	
Negative	99 (56)
Positive	78 (44)
ER status	
Negative, no. (%)	67 (36)
Positive, no. (%)	120 (64)
PR status	
Negative, no. (%)	86 (45)
Positive, no. (%)	107 (55)
HER-2/neu status	
Negative, no. (%)	163 (85)
Positive, no. (%)	28 (15)

Table 2. Independent factors predictive of death from breast cancer

Parameter	<i>P</i> value	Hazard ratio	95% confidence interval for hazard ratio	
			Lower	Upper
EZH2 positive (vs. negative)	0.01	2.04	1.17	3.57
Positive lymph nodes (≥4, 1–3, 0)	<0.0001	1.9	1.4	2.57
PR positive (vs. negative)	0.02	0.54	0.32	0.91

Multivariate Cox Model with backward selection, *n* = 161, *P* < 0.0001 disease-free survival.

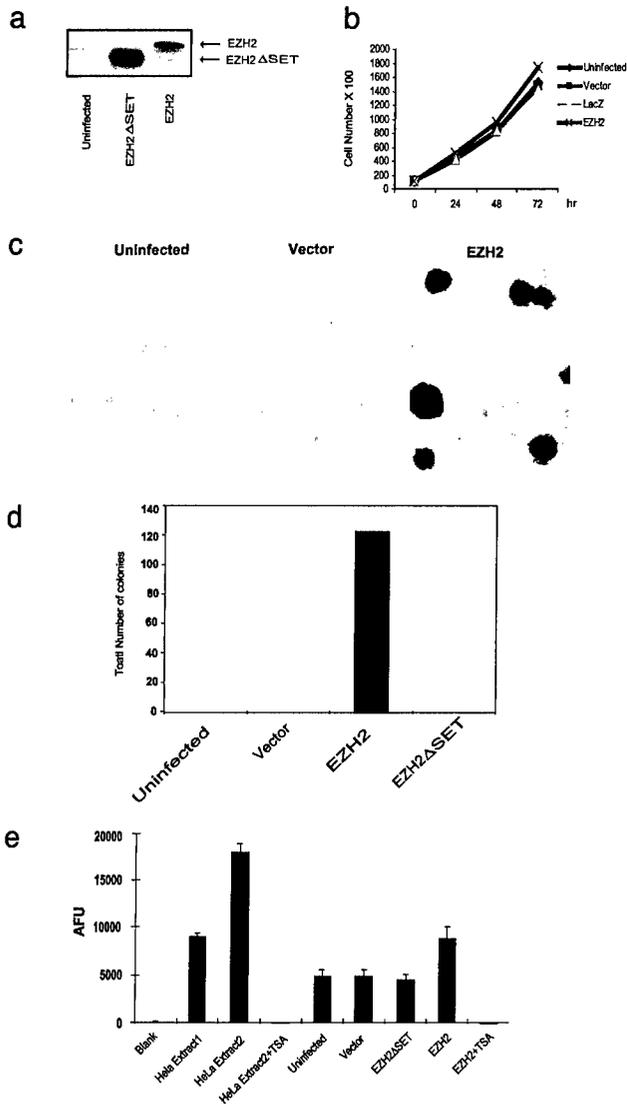


Fig. 3. Anchorage-independent growth mediated by EZH2. (a) Immunoblot analysis of breast cell line H16N2 infected with adenovirus encoding EZH2 or EZH2 Δ SET mutant. (b) Ectopic overexpression of EZH2 does not significantly enhance growth of breast epithelial cells in culture. H16N2 cells were infected with EZH2 adenovirus and controls, and cells were counted at indicated time points. LacZ adenovirus and vector adenovirus were used as controls. (c) EZH2 expression enhances anchorage-independent growth *in vitro*. H16N2 cells were infected with EZH2, EZH2 Δ SET, or vector adenoviruses. Anchorage-independent growth was determined by assaying colony formation in soft agar as described in *Methods*. After 25 days, the plates were stained and photographed. (d) Quantitation of soft agar colonies from experiments described in c. Colonies from three wells were quantitated for each condition. (e) EZH2 induces HDAC activity in breast epithelial cells. HDAC activity was measured in extracts from H16N2 cells infected with indicated viruses \pm treatment with TSA (1.0 μ M). As indicated by the manufacturer (Biomol), nuclear extracts from HeLa cells were used as positive controls. Extract 2 had 2-fold more HDAC activity than Extract 1. AFU, arbitrary fluorescence units.

cells consistently promoted invasion of the CAM (a representative experiment is shown in Fig. 4d).

Discussion

In the present study, we characterized the expression pattern of EZH2 transcript and protein in a wide spectrum of breast disease and assessed the utility of EZH2 as a prognostic marker in patients with breast cancer. EZH2 is significantly increased in invasive carcinoma and breast cancer metastases at both the

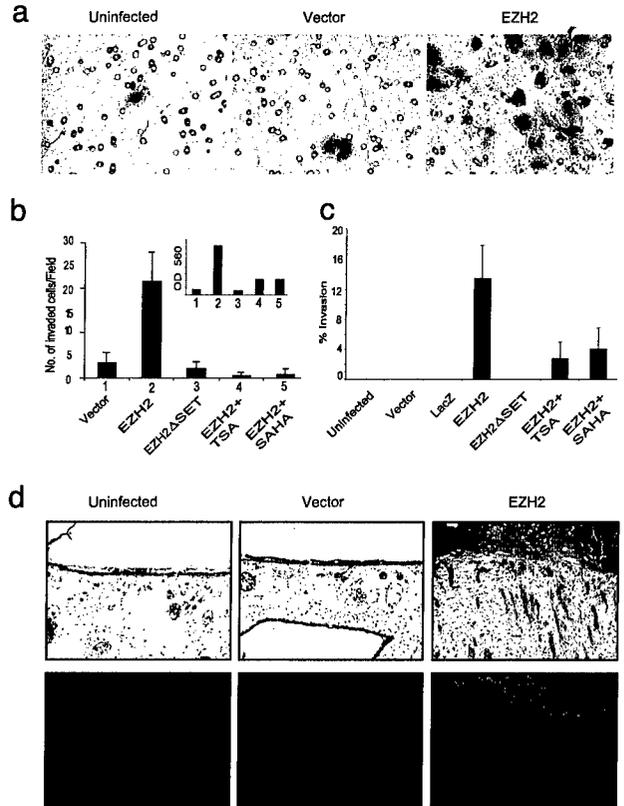


Fig. 4. EZH2 orchestrates cell invasion both *in vitro* and *in vivo*. (a) A reconstituted basement membrane invasion chamber assay (Chemicon) was used to assess breast epithelial cell lines infected with EZH2 and control adenoviruses. Representative fields of invaded and stained cells are shown. (b) The numbers of invaded cells were counted in six fields, and the mean values were determined. Quantitation by colorimetry (absorbance at 560 nm) is shown in *Inset*. (c) EZH2-mediated invasion of SU-ECM. H16N2 cells were infected with EZH2, EZH2 Δ SET, or control adenoviruses. (d) EZH2 overexpression mediates invasion of breast epithelial cells in a CAM assay. (Upper) CAM tissues stained with hematoxylin/eosin. Arrows indicate the cells that have invaded the CAM. Because cells were labeled with Fluoresbrite carboxylated polystyrene nanospheres, they could also be visualized by fluorescence (Lower).

transcript and protein levels when compared with normal breast tissues. Cells forming intravascular tumor emboli had strikingly increased EZH2 expression (Fig. 1c *Left*), suggesting that EZH2 may play a role in vascular invasion and breast cancer metastasis. *In vitro* and *in vivo* experiments in which EZH2 was ectopically overexpressed in normal mammary epithelial cell lines provide biological evidence that EZH2 can mediate anchorage-independent growth and cell membrane invasion, hallmarks of cancer (36). This is especially intriguing in that EZH2, which targets transcriptional repression of target genes, presumably mediates an invasive cancer phenotype.

To test the clinical utility of EZH2 protein expression as a prognostic biomarker of breast cancer progression, we evaluated the associations between EZH2 and survival after treatment. At the univariate level, EZH2, tumor stage, tumor size, the presence of axillary lymph node metastases, and hormone receptor status were all significantly associated with survival. In a multivariable Cox regression analysis, high EZH2 expression and lymph node metastasis were independent predictors of outcome. The single best multivariable model included high EZH2 levels, positive lymph nodes, and negative PR status. *In silico* analysis of the cDNA expression profiling of breast cancer performed by van't Veer *et al.* (30) showed that high EZH2 levels were associated with the development of metastasis within 5 years of primary diagnosis in

patients with sporadic invasive carcinomas. These findings support the potential clinical utility of incorporating EZH2 into clinical nomograms to help determine the risk of cancer progression.

A major limitation of our analysis is its retrospective nature, which precludes an accurate analysis of survival in the context of hormonal or adjuvant treatment. In our patient cohort, 88% ER-positive tumors received hormonal treatment. Thus, we critically evaluated the prognostic significance of EZH2, taking into account tumor ER status. EZH2 was strongly associated with clinical outcome in hormone-dependent and -independent breast cancer patients, indicating that the prognostic power of EZH2 is independent of ER status. Future studies will test the model developed in this study on a validation cohort to confirm these initial observations.

The prognostic significance of EZH2 as biomarker for aggressive breast cancer is likely linked to its biological functions. EZH2 is a member of a group of polycomb proteins that are involved in maintaining heritable gene expression profiles and thus regulate cell type identity. Thus, dysregulation of the transcriptional machinery of a cell may result in loss of cell type identity and neoplastic transformation. Here we provide biological evidence that dysregulated EZH2 promotes oncogenic transformation. Overexpression of EZH2 in breast epithelial cells induced anchorage-independent growth and cell invasion. Invasive properties of EZH2 overexpressing cells were demonstrated in both *in vitro* assays (i.e., basement membrane invasion chamber and SU-ECM assays) as well as in an *in vivo* assay (i.e., CAM). EZH2 overexpression induced HDAC enzymatic activity in breast epithelial cells. Interestingly, EZH2-mediated cell invasion are abrogated by the HDAC inhibitors TSA and SAHA, implying that EZH2-mediated invasion requires HDAC activity. Previous reports have shown that type I HDACs are recruited to the EZH2-EED PcG complex (18). Our group and other groups have found that EZH2-mediated gene silencing requires an intact SET domain and recruitment of HDAC activity (8), and that inhibition of HDAC activity blocked the transcriptional repressor functions of EZH2. Several HDAC inhibitors, including SAHA, have been shown to have promise clinically as antitumor agents (37). Thus, we suggest that inhibitors of HDAC may be useful therapeutic compounds in EZH2 overexpressing tumors. In addition, the HDAC activity induced by EZH2 may explain the intriguing strong association between EZH2 protein expression and negative ER, and one might speculate that

EZH2 may transcriptionally repress ER. Further investigation in this area may be warranted.

Several recent studies provide strong evidence that EZH2 has inherent activity as a histone H3 methyltransferase, which may represent the mechanism of PcG silencing (10, 13–15). Cao *et al.* (13) present evidence that the specific target of EZH2 is lysine 27 on the histone H3 N-terminal tail (13). If EZH2 plays a role in breast cancer progression, its inherent methyltransferase activity may serve as an attractive therapeutic target. Together, these studies suggest that the transcriptional memory machinery of a cell may have a role in cancer progression.

In summary, we discovered that EZH2 is a promising biomarker of aggressive breast cancer, not only extending our initial observations in prostate cancer but also suggesting that EZH2 (and thus the cell memory machinery) may have a role in carcinoma progression in malignancies from hormonally regulated tissues. Clinically, our retrospective studies suggest that EZH2 levels can be used to identify patients with breast cancer of a more aggressive phenotype, thereby enhancing our prognostic knowledge. Although our results are promising, EZH2 expression needs to be validated in relationship to outcome in the context of carefully controlled clinical trials. If confirmed, application of EZH2 immunohistochemical analysis should be technically straightforward and feasible. In addition to the potential prognostic utility of EZH2, we also provide a biologic mechanism for its association with aggressive cancers, by mediating anchorage-independent growth and cell invasion.

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