

SUPPLEMENTAL MATERIALS AND METHODS

Human Microarray Datasets

Survival data based on ZNF217 expression levels was determined using our previously published dataset (1), published studies (2-4). The meta-analysis was completed using previously published studies (5-13).

Metastasis-free survival was determined using the dataset from (14). Patients with ZNF217 expression data were separated into high and low ZNF217 expression.

Chemotherapy resistance was determined using datasets compiled from (2-4, 15). Patients were sorted by response to chemotherapy and by ZNF217 expression levels. If the tumor became smaller following neoadjuvant treatment, then patients were considered responsive and otherwise nonresponsive.

Meta-Analysis

Studies Included:

Study	GSE	Total samples	ER+/LN-/untreated*/outcome	Duplicates removed	ER+/HER-array	10 yr relapse	10 yr no relapse
Wang_2005	GSE2034	286	209	209	173	67	29
Loi_2007	GSE6532	327	170	43	40	10	5
Desmedt_2007	GSE7390	198	135	135	116	42	60
Symmans_2010	GSE17705	298	175	110	102	12	41
Zhang_2009	GSE12093	136	136	136	125	15	24
Schmidt_2008	GSE11121	200	200**	200	155	25	46
Sotiriou_2006	GSE2990	189	113	48	45	12	15
Miller_2005	GSE3494	251	132	115	100	30	52
Ivshina_2006	GSE4922	290	133	2	2	0	2
9 studies		2175	1403	998	858	213	274

*allows endocrine therapy alone, radiation

**ER status not available, filtered later based on array expression data

Study selection criteria

- N > 100
- ER+ (clinical or determined by array if unavailable)
- LN-
- HER2- (determined by array)
- no systemic therapy; endocrine-therapy-only (e.g., tamoxifen) acceptable

- outcome available: RFS, DMFS, or DFS
- Event time must be greater than 0 yrs.

Data pre-processing

Duplicates were removed if they had the same GSM number, were indicated as having the same sample or patient identifier, or displayed a perfect correlation (>0.99) with another sample in correlation analysis. Cel files were downloaded from GEO and processed in R/Bioconductor using the 'affy' and 'gcrma' libraries. All samples were normalized together using GCRMA and mapped to Entrez gene symbols using the standard affy CDF. ESR1 expression status was determined using probe "205225_at" which was found to be the most useful probe by visual inspection (it also has by far the greatest variance). Similarly, 4 probes were chosen from the ERBB2 amplicon for the genes ERBB2, GRB7, STARD3, PGAP3. Other genes in the amplicon (e.g., NEUROD2, TCAP, PNMT, IKZF3) either did not have probes or did not show useful expression. Expression values for the 4 probes in the ERBB2 amplicon were combined using a ranksum approach. ESR1- and ERBB2+ cutoff values were then chosen by mixed model clustering of their expression values. A total of 858 samples passed all filtering steps. Of these, 371 had insufficient follow-up for 10 yr analysis but were included in the dataset for use in survival analysis.

All filtered data were re-normalized together as above. Mapping was again performed with standard CDF files but also with custom CDFs from Dai et al (2005). Custom and standard CDF files were then combined into a single file for use in analysis. There was one probeset for each CDF (standardCDF: 203739_at; customCDF: 7764_at). Their expression was virtually identical (Spearman $r=0.998$, $p<0.000$). Therefore, only one was used in subsequent stats (7764_at).

Statistics

Association between ZNF217 expression and 5yr/10yr relapse status was determined by Mann-Whitney U-test (MU). Patients were also divided into expression groups (tertiles: low, medium, high) based on ZNF217 expression levels. Kaplan-Meier survival analysis was then performed for relapse-free-survival (RFS) with these expression groups as a factor. Significant survival differences between the groups were determined by log rank (Mantel-Cox) test (linear trend for factor levels). Events beyond 10 years were censored. Correlation between ZNF217 and other genes was determined by Spearman and Pearson correlation coefficient. All statistics were performed with the R programming language (version 2.11.0).

Microarray Gene Expression Analysis

For microarray gene expression analysis, total RNA was isolated from the samples indicated. RNA was also harvested from each cell line expressing a vector control to use as a reference for each microarray sample analyzed. Total RNA was hybridized to mouse MEEBO arrays as described (<http://www.microarray.org/sfgf/meebo.do>). Samples were verified to have strong overexpression of ZNF217 by qRT-PCR. To identify genes induced or repressed by ZNF217 expression, we averaged the

expression values for each cell type and then sorted and isolated genes that were induced or repressed 1.5 standard deviations from the mean. Gene sets were created of these induced or repressed genes. We used DAVID (<http://david.abcc.ncifcrf.gov>) to look for enrichment of Gene Ontology terms in each of the gene sets ($P < 0.01$; Table S2) (16). To visualize the expression patterns of the samples, a select set of genes were analyzed by unsupervised hierarchical clustering.

Mice Used in This Study

All mice used in this study were maintained on the FVB/n background and maintained under pathogen-free conditions in the UCSF barrier facility. Our animal protocols were reviewed and approved by the UCSF Institution Animal Care and Use Committee.

MMTV-PyMT mice on the FVB/n background (MGI:2679595) were genotyped using the following primers: 5'-GGA AGC AAG TAC TTC ACA AGG G and 5'-GGA AAG TCA CTA GGA GCA GGG. This reaction generated a 556 bp PCR fragment for PyMT positive mice and no band for wildtype mice.

Tissue Culture Cell Lines And Culture Conditions

Primary mouse mammary epithelial cells were isolated from wild type mammary glands and MMTV-PyMT tumors following collagenase treatment and differential centrifugation by methods described previously (17, 18). The cell lines used in this study all were carefully maintained in culture. Quality control was maintained by continual analysis of morphology and growth rate. The cell lines used include mouse mammary epithelial cell lines NMuMG (source: Rik Derynck, University of California, San Francisco, who got them from ATCC) (19), SCp2 (source: Mina Bissell, LBNL) (20), EpH4 (source: Mina Bissell, LBNL) (21) and Vo-PyMT-Luc (source: Conor Lynch, Vanderbilt) (22) and human mammary epithelial cell lines MCF7, ZR-75-1 and HBL100 (source: ATCC for all three lines) (23-25). MCF7 was authenticated by comparison of its SNP6.0 copy number profile to the corresponding sample available as part of the Cancer Cell Line Encyclopedia at the Broad (PMID: 22460905). The other cell lines cells used in this study have not been authenticated by additional methods.

The following medium conditions were used for the indicated cell lines. Primary mammary epithelial cells from tumors were grown in growth medium containing 10% FBS, 5 µg/ml insulin, 1 µg/ml hydrocortisone, 10 ng/ml EGF, penicillin/streptomycin, 50 µg/ml gentamicin, and glutamine in DMEM/F12 media and has been described previously (17, 18). SCp2 and EpH4 were grown in 2% fetal bovine serum (FBS), 5 µg/ml insulin, and 50 µg/ml gentamicin in DMEM/F12 media. NIH3T3 cells were grown in 10% FBS in DME H-21 media. NMuMG cells were grown with 10% FBS, penicillin/streptomycin and 10 µg/ml insulin in DME H-21 medium. Doxycycline was used on indicated cells at 1-5 µg/ml.

Virus production

Virus was produced either as described previously (17, 18) or by the UCSF Sandler Lentiviral Core. Virus was titered and equal MOIs (MOI=1-5) of vector and ZNF217 virus were used per experiment.

Motility Assays

Movies were taken using a brightfield setting using OpenLab 4.0.3 software for image collection and movie assembly.

Additional Constructs Used in This Study

Retroviral and lentiviral vector backbones have been described previously (17, 18). All mouse Znf217 constructs were untagged within the open reading frame but expressed a downstream internal ribosome entry site (IRES) upstream of a fluorescent reporter gene. The following vectors were used: pMIG (retroviral construct; GFP fluorescent reporter)(26), HIV-ZsGreen (pEiZ) (HIV-based self inactivating lentiviral construct; ZsGreen fluorescent reporter gene) and HIV-Tomato (pEiT) (based on pEiZ and generated by replacing ZsGreen with Tomato red fluorescent reporter gene). Znf217 constructs were generated by subcloning Znf217 from the TRE-Tight-Znf217 construct. Constructs include: Znf217-pMIG, Znf217-pEiT, and rtTA-pEiZ. Cloning details are available upon request.

Transplants

Primary cells from wildtype mammary glands and MMTV-PyMT tumors were isolated as described previously (18). In two experiments these cells were infected with lentivirus overexpressing Znf217 or vector (pEiT) alone upstream of an IRES-Tomato red fluorescent reporter gene. After one month of expression these cells were sorted by flow cytometry for collection of fluorescent cells. Cells resuspended in 10 μ l PBS were injected orthotopically on contralateral mammary fat pads cleared of epithelium on the same day following FACS sorting. 1×10^4 cells were injected per gland in the first experiment and 1.7×10^4 cells injected in the second experiment with both experiments yielding similar results. Vo-PyMT transplants: For transplants using the Vo-PyMT cells described below for triciribine treatment 1×10^5 cells were injected orthotopically in mammary glands of adult female mice.

Triciribine Treatment in vivo

Vo-PyMT cells were infected with lentivirus Znf217 or vector (pEiT) alone carrying a Tomato red fluorescent reporter gene (MOI=3) (22). Within one week of expression these cells were sorted by flow cytometry for collection of fluorescent cells. The sorted cells were expanded and injected (1×10^6 cells in 15 μ l) orthotopically on contralateral mammary fat pads cleared of epithelium in two separate experiments. For both experiments, tumors formed by two weeks post-transplant. For one experiment, at three

weeks post-transplant mice were injected with triciribine or DMSO solution for five days per week at a concentration of 1 mg/kg/day until 42 days post-transplant. (Note: In clinical trials in cancer patients, the dosage range was 20-48 mg/m²) (27, 28). This dosage is equivalent to 6.2-16 mg/kg in mouse, using an FDA-recommended conversion formula described previously (29). This is considerably higher (~6.2-16 times more) than the amount used in our studies. Five mice with contralateral transplants were included in the DMSO cohort, and eight mice were included in the triciribine cohort. In a second experiment, beginning at 10 days post-transplant, mice were injected i.p. daily for two weeks with triciribine or DMSO solution with seven mice per cohort and collected at 28 days post-transplant. Tissues were processed and scored for cell death by morphology by H&E. In both experiments, tumor burden was measured by a caliper.

For human xenograft experiments, 1×10^7 MCF7-M1 cells/0.1 ml were injected subcutaneously in nude (nu/nu) mice in five cohorts with five mice per cohort for a total of 40 mice. Estradiol pellets (Innovative Research, 0.72 μ g, 60 day release) were implanted at the time of transplant. The target starting volume was 200 mm⁻³. Doxorubicin was used at a dosage of 5 mg/kg once per week for three weeks alone or in combination with triciribine. A control (no drug) or triciribine dosage (50 mg/kg) were given bid for five days. These xenografts were completed in the Preclinical Therapeutics Core, UCSF Helen Diller Family Comprehensive Cancer Center.

RNA Isolation and qPCR

Tissues used for RNA isolation first were crushed in liquid nitrogen using a mortar and pestle and then resuspended in RNA-Bee (Tel-Test). RNA for tissue and cell lines was isolated following manufacturer's instructions and both quantified and tested for purity using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, www.nanodrop.com). Reactions were assayed on an Eppendorf Mastercycler EP Realplex machine.

qPCR primers

The qPCR primers used include:

Znf217:

5'-AGTGAGCCACATCCCAAAGA

5'-ACACTGGGTGACTCCACCTC

GAPDH:

5'-TGTAGACCATGTAGTTGAGGTCA

5'-AGGTCGGTGTGAACGGATTTG

TBP:

5'-ACAGCCTTCCACCTTATGCTCAG

5'-AACGGTGCAGTGGTCAGAGTT

HPRT:

5'- GTTGGATACAGGCCAGACTTTGTTG

5'- GAGGGTAGGCTGGCCTATAGGCT

Snail1:

5'- CACACGCTGCCTTGTGTCT

5'- GGTCAGCAAAGCACGGTT

Snail2:

5'- TGGTCAAGAAACATTTCAACGCC

5'- GGTGAGGATCTCTGGTTTTGGTA

Twist: from (30)

5'- CGGGTCATGGCTAACGTG

5'- CAGCTTGCCATCTTGGAGTC

Vimentin:

5'- CGTCCACACGCACCTACAG

5'- GGGGGATGAGGAATAGAGGCT

Tissue Processing

Mammary glands were collected for whole mounts, RNA and tissue lysates, and paraffin-embedded tissue blocks for sectioning. Tissues used for histology were fixed overnight in 4% paraformaldehyde, processed for embedding, and embedded in paraffin blocks.

Soft Agar Assay

For soft agar assay, we plated 1% agar on bottom layer of 6-well plate and plated per well 5000 cells resuspended in 0.7% agar in DMEM + 10% FBS + Pen/Strep. Media was added on top of cell suspension daily to prevent dehydration. Cells used were NIH3T3 cells infected with retrovirus (vector pMIG or Znf217-pMIG) and sorted for GFP+ cells. GFP is a reporter gene on the pMIG plasmid. Cells were incubated for two weeks before counting samples. Assay was repeated twice in triplicate with similar results for each experiment and replicate.

Mammosphere Assay

Primary cells were isolated as described previously and infected overnight on non-adherent plates (17, 18). Culture medium had a final concentration of 10 µg/ml insulin, 5 µg/ml transferrin, 30 µg/ml Bovine Pituitary Extract (13028-014; Gibco/Invitrogen), 30 ng/ml HGF (2207-HG; R&D Systems), 50 ng/ml FGF10 (345-FG; R&D Systems), 10 µM Roc inhibitor (688000; Calbiochem) in DMEM/F12 medium. The indicated cells were plated as single cells at the indicated density in culture medium mixed 2 parts Matrigel (354234; BD Biosciences) to 1 part culture media. After drying, the cell/Matrigel/culture

media sample was covered with culture medium,. Samples were analyzed, imaged and counted at one week after plating. The data presented is representative of at least three experiments, with each experiment performed in triplicate. Triciribine was tested at a concentration of 1 μ M-10 μ M with similar sphere inhibition at each concentration.

Triciribine and Doxorubicin Treatment of Cell Lines

HBL100 cells were stably transfected with ZNF217 as described previously (31). Cells were treated with either buffer or 100 ng/ml of doxorubicin and/or triciribine at the indicated concentrations for 72 hr prior to analysis of cell death (Figure S7G). MCF7 cells infected with either lentiviral scrambled control shRNA or ZNF217-shRNA (two different shRNAs tested) were treated with triciribine at the indicated concentrations for 48 hr prior to analysis of cell death (Figure 7H). In both experiments, cell death was assayed by Annexin V staining using either Annexin V-PE (556422; BD Biosciences) or Annexin V-Alexa Fluor 647 (A23204; Invitrogen) for staining and detected by flow cytometry following manufacturer's instructions.

Antibodies used in this study

Antibody name	Antigen	Ab source	Company	Catalog #	Dilution	Stock	Special Methods	Use of Ab in this study
Actin	human actin peptide	Goat	Santa Cruz	sc-1616	1/500	200 µg/ml		Western (43 kDa)
AKT		Rabbit	Cell Signaling	9272	1/1000			Western analysis
Cdc2 p34 PSTAIRE	peptide from the PSTAIRE region of human Cdc2	Rabbit	Santa Cruz	sc-053	1/200-1/1000			Western (34 kDa)
E-cadherin	human E-cadherin C-terminal protein	Mouse	BD Biosciences	610181	1/250	250 µg/ml		Immunofluorescence
ErbB3 (C-17)	C-terminus of human ERBB3	Rabbit	Santa Cruz	sc-285	1/1000			Western analysis
HDAC1		Rabbit	Cell Signaling	2062	1/1000			Western analysis
Keratin 8	cytokeratin Endo-A	Rat	Developmental Studies Hybridoma Bank, NICHD, Univ. of Iowa; originally developed by Philippe Brulet and Rolf Kemler	TROMA-1	1/500		Na Citrate	Immunofluorescence on paraffin embedded tissues
Keratin 14	peptide sequence derived from the C-terminus of the mouse keratin 14 protein	Rabbit	Covance	PRB-155P	1/5000	1 mg/ml	Na Citrate	Immunofluorescence on paraffin embedded tissues
MAPK	p44/42 MAPK (Erk1/2) (137F5)	Rabbit	Cell Signaling	4695	1/1000			Western analysis
Phospho-AKT (Ser473)(587F11)	phosphopeptide corresponding to residues around Ser473 of mouse Akt	Mouse	Cell Signaling	4051S	1/100 (IHC)		Na Citrate; Avidin-Biotin Blocking (Vector); ABC-Elite (Vector); MOM (BMK-2202; Vector)	IHC on paraffin embedded tissues; western analysis (60 kDa)
Phospho-AKT (Ser473)	phosphopeptide corresponding to residues around Ser473 of mouse Akt	Rabbit	Cell Signaling	9271	1/1000			Western analysis (60 kDa)
Phospho-ErbB3 (Tyr1289) (21D3)		Rabbit	Cell Signaling	4791	1/1000			Western (185 kDa)
Phospho-MAPK (Thr202/Tyr204)	phosphopeptide corresponding to residues around Thr202/Tyr204 of human Erk	Rabbit	Cell Signaling	9101	1/100		Na Citrate; Avidin-Biotin Blocking (Vector); ABC-Elite (Vector)	IHC on paraffin embedded tissues; western analysis
Phospho-Tyrosine (PY20)		Mouse	BD Transduction Labs	610000	1/1000			Western analysis
Smooth muscle Actin (SMA) Clone 1A4 Cy3 conjugate	peptide of SMA fused to KLH	Mouse	Sigma	C6198	1/1000	0.2 ml	Na Citrate	Immunofluorescence on paraffin embedded tissues
Tubulin (ascites)		Mouse	Sigma	T5168	1/25,000			Western analysis
ZNF217	peptide from human ZNF217	Rabbit	S. Krig (UC Davis)		1/1000			Western analysis
Phalloidin 568	STAIN--recognizes actin		Molecular Probes	A12380	1/40			Immunofluorescence on tissue culture cells
Anti-rabbit, Fluor 488	Alexa SECONDARY	Goat	Molecular Probes	A11008	1/250	2 mg/ml		Immunofluorescence
Anti-rabbit, Fluor 568	Alexa SECONDARY	Goat	Molecular Probes	A11036	1/250	2 mg/ml		Immunofluorescence
Anti-rabbit, Fluor 594	Alexa SECONDARY	Goat	Molecular Probes	A11012	1/250	2 mg/ml		Immunofluorescence
Anti-rabbit, biotinylated	SECONDARY	Goat	Vector	BA-1000	1/250	1.5 mg/ml		Immunohistochemistry
Anti-rat, Alexa Fluor 488	SECONDARY	Goat	Molecular Probes	A11006	1/250	2 mg/ml		Immunofluorescence
Anti-rat, biotinylated	SECONDARY	Goat	Vector	BA-9400	1/250	3 mg/ml	Avidin-Biotin Blocking (Vector); ABC-Elite (Vector)	Immunohistochemistry
Anti-Rat, peroxidase-conjugated	SECONDARY	Donkey	Jackson ImmunoResearch	712-035-150	1/500	0.8 mg/ml		Immunohistochemistry

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