The transcription factor ZNF217 is a prognostic biomarker and therapeutic target during breast cancer progression

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Abstract

The transcription factor ZNF217 is a candidate oncogene in the amplicon on chromosome 20q13 that occurs in 20-30% of primary human breast cancers and that correlates with poor prognosis. We show Znf217 overexpression drives aberrant differentiation and signaling events, promotes increased self-renewal capacity, mesenchymal marker expression, motility and metastasis, and represses an adult tissue stem cell gene signature downregulated in cancers. By in silico screening, we identified candidate therapeutics that at low concentrations inhibit growth of cancer cells expressing high ZNF217. We demonstrate that the nucleoside analog triciribine inhibits ZNF217-induced tumor growth and chemotherapy resistance, and inhibits signaling events (e.g., P-AKT, P-MAPK) in vivo. Our data suggest ZNF217 is a biomarker of poor prognosis and a therapeutic target in breast cancer patients, and triciribine may be part of a personalized treatment strategy in patients overexpressing ZNF217. Since ZNF217 is amplified in numerous cancers, these results have implications for other cancers.

Significance

This study finds that ZNF217 is a poor prognostic indicator and therapeutic target in breast cancer patients and may be a strong biomarker of triciribine treatment efficacy in patients. Because previous clinical trials for triciribine did not include biomarkers of treatment efficacy, this study provides a rationale for revisiting triciribine in the clinical setting as a therapy for breast cancer patients who
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overexpress ZNF217.
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**Introduction**

In the most aggressive breast tumors, neoplastic cells activate or amplify oncogenes, or inactivate or delete tumor suppressor genes to promote invasive growth and poor prognosis in patients. Amplification of the human chromosomal region 20q13 occurs in 20-30% of primary human breast cancers, as well as in other cancers, and its amplification correlates with poor patient prognosis (1, 2).

The ZNF217 gene on human 20q13.2 encodes a transcription factor that is overexpressed in all breast tumors and cell lines in which the gene is amplified, as compared to normal mammary tissue and epithelial cells (1, 2). The ZNF217 protein is a member of the C2H2 family of transcription factors and contains eight predicted Kruppel-like C2H2 zinc finger motifs and a proline-rich region. It is a component of a human histone deacetylase complex (CoREST-HDAC) and is found in complexes with the transcriptional co-repressor C-terminal binding protein (CtBP), the histone demethylases LSD1 (H3K4, H3K9) and KDM5B/JARID1B/PLU-1 (H3K4) and the methyltransferases G9a (H3K9, H3K27) and EZH2 (H3K27) (3-8). Its overexpression in human mammary epithelial cells (MECs) overcomes senescence and promotes immortalization accompanied by increased telomerase activity, increased resistance to TGFβ-induced growth inhibition and amplification of c-Myc (9). ZNF217 binds to the promoters of genes involved in differentiation and is repressed following retinoic acid treatment of pluripotent embryonal cells (10).

In this study we investigated whether and how Znf217 promotes tumor progression and poor prognosis using cultured cells, in vivo transplant models...
ZNF217 overexpression promotes malignancy and human patient expression datasets.

Results

ZNF217 is Prognostic of Poor Survival in Breast Cancer Patients

Using microarray expression data from primary breast tumors and corresponding clinical data (11, 12), we found that high ZNF217 amplification and expression correlate with shorter overall survival, disease-specific survival and relapse-free survival (Figures 1A-B, S1A-B). To determine if ZNF217 overexpression overlapped with another poor prognostic subtype, we compared ZNF217 expression levels across patient subtypes (e.g., ER+, ER-, ERBB2/HER2+, ERBB2/HER2-, luminal and basal patient cohorts) and found that ZNF217 expression levels are highest in ER+ tumors and lowest in basal subtype tumors (data not shown).

We next determined if ZNF217 had prognostic value across breast cancer subtypes. We compared survival and ZNF217 expression by univariate analysis across ER+, ER-, HER2+, luminal and basal subtypes. Patients with tumors expressing high ZNF217 consistently had reduced survival compared to patients with tumors expressing lower ZNF217 across multiple breast cancer subtypes (Figure 1C and data not shown). For example, in a meta-analysis of relapse-free survival across nine published studies that included 858 patients (ER+HER2-LN-) with ZNF217 expression, we found that ZNF217 expression was significantly associated with 5 yr ($P=0.012$) and 10 yr ($P=0.023$) relapse status (Mann-
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Whitney), and patients with relapse had higher ZNF217 expression. Similarly, patients grouped into the high expression tertile had significantly worse survival than low expression groups. These data show that ZNF217 is prognostic of poor survival in patients by univariate analysis. Moreover, ZNF217 was a better predictor of survival than ER status by multivariate analysis (Figure S1C).

**Overexpression of Znf217 Accelerates Loss of Adhesion and Increased Motility in Mouse Mammary Epithelial Cells**

To determine the consequences of Znf217 overexpression, we generated mouse mammary epithelial cell lines that overexpressed Znf217 by retroviral and lentiviral infection. Mouse mammary epithelial cell lines (SCp2, NMuMG, EpH4) overexpressing Znf217 had altered motility showing a more scattered phenotype than adherent, clustered control cells (Figures 2A-C, S2A-C). In a wound healing/scratch assay, individual SCp2 cells (Figure 2D) and NMuMG cells (data not shown) overexpressing Znf217 showed increased motility, forward extended lamellipodia and independent migration (i.e., separate from other cells) towards the middle of the scratch (Figure 2D, Movie S1). In contrast, vector-treated cells migrated as a sheet predominantly with a single leading edge. In keeping with the increased motility, cells overexpressing Znf217 reorganized their actin cytoskeleton with reduced cortical actin and increased actin stress fibers (Figure 2E) and upregulated EMT markers including Snail1 and Twist (Figure 2F). Consistently, we found ZNF217 expression levels correlated with expression of EMT markers, including Snail1, Snail2 and Vimentin, genes that have ZNF217
ZNF217 overexpression promotes malignancy enriched at their promoters in human breast cancer cell lines and tumors (Table S1). Taken together, these results indicate that the early effects of increased ZNF217 expression would lead to premalignant changes of enhanced mammary epithelial migration.

We used gene expression microarrays of the mouse SCp2 MECs overexpressing Znf217 to identify altered processes and molecular targets of Znf217 (Figure 2G). In these cells, 176 genes were upregulated and 243 genes downregulated following Znf217 overexpression. We used DAVID software to classify the gene sets with gene ontology (GO) terms (Table S2). The GO terms suggested that Znf217 overexpression promoted increased cell motility, decreased epithelial differentiation, increased vasculature development and changes at the membrane.

We also assayed for clonogenicity and transformation potential in vitro. Znf217 overexpression in NIH3T3 cells stimulated anchorage independent growth in a soft agar assay, with increased number and size of colonies (Figure 3A-C).

Znf217 Overexpression in Normal Primary Mammary Epithelium Promotes Increased Mammosphere Formation in Culture

Because we found that Znf217 overexpression in culture promoted increased motility, decreased epithelial marker expression/increased mesenchymal marker expression and increased clonogenicity/transformation potential in vitro, we reasoned that these changes were consistent with a change in differentiation
ZNF217 overexpression promotes malignancy towards a less differentiated or more mesenchymal phenotype. Znf217 gene expression was enriched in CD24^{Med}CD49^{f\text{High}} cell population, which includes basal, myoepithelial and progenitor cells, compared to CD24^{\text{High}}CD49^{f\text{Low}} cells, which include luminal and luminal progenitor cells (Figure 3D-E).

To determine if Znf217 could promote progenitor cell phenotypes, we overexpressed Znf217 in normal primary MECs and analyzed clonogenicity potential in the mammosphere assay. Primary MECs infected with Znf217-overexpressing lentivirus and grown in serum-free nonadherent culture conditions demonstrated increased self-renewal capacity (Figure 3F-G).

**Gene Expression Analysis of Mammary Epithelial Cells Following Znf217 Overexpression Predicts Changes in Epithelial Proliferation, Cell Adhesion and Motility**

We next determined the impact of Znf217 overexpression on global gene expression in normal primary mouse MECs by gene expression microarrays (Figure 3H). Primary MECs were infected with a Znf217-overexpressing lentivirus, passaged to expand the population, and confirmed to overexpress Znf217 by reverse transcription quantitative PCR (rt-qPCR) (Figure 3I). In these MECs, 340 genes were upregulated and 401 genes downregulated following Znf217 overexpression (Figure 3H). The GO terms classified by DAVID software (Table S2) suggested that Znf217 overexpression altered the gene expression profile of genes involved in cell proliferation, cell adhesion, cell migration, G-protein coupled receptor signaling pathways and ribosomal function.
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Genes identified by microarray analysis suggested that overexpressing Znf217 in vivo would promote increased epithelial growth or progenitor cell expansion (Figure 3H, Table S2). These genes included a number of TGFβ and Wnt pathway genes (Axud1/Csrnp-1, Bcl9l, Bmper, Bmpr2, follistatin, Samd9l, Sfrp1, Tcf4, Tgfβ2, Tgfβr3, Wnt5a). We validated selected genes including Wnt5a and Sfrp1 by rt-qPCR (Figure 3J). These results are consistent with Znf217 promoting differentiation towards a less differentiated cell-like phenotype via aberrant signaling in the TGFβ and Wnt pathways. We also found that ZNF217 was required for cell proliferation (Figure S2D).

We also examined epithelial marker expression in our microarray dataset. We found increased expression of both K17 (myoepithelial marker) and K18 (luminal epithelial marker) expression (Figure 3H). Consistent with these results, we found ZNF217 expression levels correlated with expression of K19 and K8/18, genes with ZNF217 enriched at their promoters in human breast cancer cell lines and tumors and with downregulated expression after ZNF217 knockdown in MCF7 cells (Tables S1, S3).

Znf217 Overexpression in Primary Mammary Epithelial Cells Represses an Adult Stem Cell Expression Signature Downregulated in Cancers

Because the aberrant differentiation markers seen both in vivo and in culture suggest that Znf217 may push mammary epithelial cells to a more progenitor cell-like phenotype, we compared our microarray dataset with our previously defined gene expression signature that we found expressed in adult stem cells...
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(13). Many of these adult stem cells are slowly cycling and show balanced differentiation versus self-renewal during normal homeostasis. Adult stemness as defined by this adult stem cell signature is not correlated with self-renewal. This signature has high expression in normal tissues, where cells predominantly are quiescent and have limited self-renewal, but low expression in cancerous tissues, where cells can self-renew. Therefore, reduced expression of the signature correlates with increased self-renewal (13).

Similar to that seen in cancers, primary MECs overexpressing Znf217 significantly repressed genes of the adult stem cell signature (Table S4; P=1.89x10^{-10}), thus making normal cells like self-renewing cancer cells at the expression level. Consistent with the increased clonogenicity by mammosphere assay, this alteration in the expression signature suggests that Znf217 may block differentiation and/or promote self-renewal.

**Tumors Overexpressing Znf217 Have a More Basal Pathology with Increased Dual Positive Luminal and Basal Cell Marker Expression**

Our finding that Znf217 overexpression promoted decreased differentiation in normal and immortalized mammary epithelium prompted us to determine if these changes are also followed in tumorigenic mammary epithelium both in culture and in vivo. We overexpressed Znf217 by lentiviral delivery of Znf217 into primary luminal-type mammary epithelial tumor cells isolated from 18-week-old MMTV-PyMT mice (PyMT MEC) or a MMTV-PyMT cell line (Vo-PyMT), sorted the cells for the IRES-Tomato reporter gene and confirmed Znf217
ZNF217 overexpression promotes malignancy overexpression by rt-qPCR (Figure 4A). In culture, Znf217 induced mesenchymal marker expression, reduced expression of E-cadherin, increased expression of EMT markers Snail2 and Twist (Figure 4A), and produced a more scattered phenotype (Figure S2C). In addition, cells overexpressing Znf217 readily formed increased numbers of mammospheres compared to vector control cells (Figure 4B-C).

When the sorted cells were transplanted into syngeneic mouse mammary fat pads cleared of epithelium, Znf217 overexpression accelerated the rate of tumor formation, reduced the tumor-free survival and increased both tumor volume and final tumor weight (Figures 4D-F, S3A). Tumors overexpressing Znf217 had a markedly altered, heterogeneous histology compared to tumors from vector-treated cells (Figure 4G). Control tumors had little to no smooth muscle actin (SMA) staining with predominantly luminal K8+ epithelium, while Znf217-overexpressing tumors expressed higher levels of myoepithelial and myofibroblast SMA protein (Figure 4H).

Tumor cells from vector-treated cohorts were predominantly K8+ with few K14+ cells; most of the K14+ cells were K8- and were located basal to the K8+ tumor cells. Znf217 overexpression increased numbers of K14+ cells in tumors, with many double-positive K8+K14+ cells (Figures 4I-J and data not shown). The K8+K14+ cells may be a bipotent progenitor population capable of forming both luminal and myoepithelial cells, also seen by others as K18+K19+ cells (14-18).

We also assayed for epithelial E-cadherin expression in these tumors. In vivo Znf217 overexpression resulted in heterogeneous staining of E-cadherin
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with a large number of regions containing a marked reduction in E-cadherin expression, whereas control tumors had E-cadherin localized to the plasma membrane throughout the epithelium (Figures 5A, S3B).

Taken together, these phenotypic changes seen within tumors are consistent with Znf217 promoting the acquisition of a mesenchymal/progenitor cell phenotype.

**Overexpression of Znf217 Promotes Metastasis**

Since metastasis in vivo often is accompanied by increased motility, invasive, and mesenchymal/basal phenotypes, we next asked if Znf217 promotes metastasis in vivo. In mice transplanted with either PyMT or Vo-PyMT cells, Znf217 overexpression significantly increased the percentage of mice with lung metastases, increased metastatic burden and increased the number of spontaneous lung metastases per mouse (Figures 5B-D, S3C). In keeping with these results, high ZNF217 expression was prognostic of reduced metastasis-free survival in breast cancer patients (Figure 5E) (19).

**ZNF217 Promotes Resistance to Chemotherapy**

Patients with tumors expressing stem cell-like/progenitor cell markers have increased resistance to chemotherapy (20). We determined if ZNF217 was a prognostic predictor of treatment response by comparing clinical data to expression data from patients who received neoadjuvant chemotherapy. In one dataset, patients received either doxorubicin or a combination of 5-fluorouracil
ZNF217 overexpression promotes malignancy and mitomycin (FUMI) before surgical removal and microarray gene expression analysis of the tissue (12). Tumors that responded to treatment (i.e., became smaller) expressed less ZNF217 than did nonresponsive tumors (Figure 6A).

We also found that tumors with low ZNF217 expression responded better to treatment (pathological complete response) than did tumors with high ZNF217 expression. In a second dataset, fine needle aspirate samples were collected prior to treatment and used for gene expression (21). All patients received similar preoperative treatments (paclitaxel and fluorouracil-doxorubicin-cyclophosphamide). We found ZNF217 expression levels higher in nonresponsive tumors that did not respond to therapy than in tumors that responded (Figure 6A). Therefore, ZNF217 is a prognostic predictor of patient outcome in response to chemotherapy.

**ZNF217 Levels are Related to Levels of Activated AKT, MAPK and ERBB3**

ERBB3 is a direct target of ZNF217 (22). We found significant correlation between ZNF217 and ERBB3 expression levels in human breast tumors (Figure 6B). To determine the mechanism underlying the effects of ZNF217, we analyzed several downstream signaling molecules downstream of ERBB3 in two human breast cancer cell lines (MCF7, ZR-75-1) that express high levels of ZNF217. Using both wild type cells and cells after knockdown for ZNF217 expression by siRNA, as described previously (22), we treated serum-starved cells with the growth factor ligand for ERBB3, heregulin/neuregulin (HRG). Cells containing ZNF217-siRNAs consistently required higher concentrations of HRG to induce
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ERBB3 signaling, phospho-AKT and phospho-MAPK (Figures 6C, S4A-D). These data indicate that these pathways are downstream of ZNF217 and that ZNF217 sensitizes cells to HRG.

**ZNF217 is a Drug Target for Individualized Therapy**

Since ZNF217 is overexpressed in poor prognostic and chemoresistant breast cancer patients, we sought to identify drugs that kill tumor cells overexpressing ZNF217. We first used a candidate approach to determine if AKT pathway inhibitors promoted cell death in a ZNF217-dependent manner, since ZNF217 is required for and promotes AKT activation [Figure 6C and (23)]. MCF7 cells were infected with virus expressing shRNAs to ZNF217 and validated for reduced protein expression (Figure S4E-F). Assaying cell death of MCF7 cells infected with lentivirus expressing scrambled or ZNF217 shRNA, we observed that the PI3K inhibitor GDC0941 and the AKT inhibitor MK2206 did not induce ZNF217-dependent cell death (Figure 6D).

We next used an in silico screening approach to identify candidate therapeutics that inhibit growth of cancer cells expressing high ZNF217 at low drug concentrations. We determined ZNF217 expression by rt-qPCR in the NCI60 panel of cell lines. We then used a drug dataset from a panel of ~50,000 drugs generated by the NCI Developmental Therapeutics Program (dtp.nci.nih.gov). Correlation of ZNF217 expression in the cell line panel with the drug panel identified 15 drugs that selectively inhibited growth of cells expressing high levels of ZNF217, as assessed by GI50, with a low drug concentration...
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To determine if ZNF217 contributes to the drug-induced growth inhibition, we assayed cell death of MCF7 cells infected with lentivirus expressing stably integrated scrambled or ZNF217 shRNA knockdown constructs. As proof-of-concept, we tested bisacodyl, triciribine, nogalamycin and 2E3E for their ability to influence cell death in culture in a ZNF217-dependent manner. Cells expressing reduced levels of ZNF217 (shRNA-ZNF217) required higher concentrations of bisacodyl or triciribine for cell killing (Figure 6E-F). Three different ZNF217-shRNA constructs gave similar results (Figure 6 and data not shown). Nogalamycin or 2E2E treatment did not promote ZNF217-dependent cell death at a therapeutically possible concentration range (data not shown).

We focused on triciribine (also known as API-2), which is a nucleoside analog and DNA synthesis inhibitor that has been tested in Phase I clinical trials in cancer patients as well as in one Phase II clinical trial in metastatic breast cancer patients (24-27). Cancer cells expressing high levels of ZNF217 required lower concentrations of triciribine to inhibit growth than cells with low ZNF217 expression (Figure 6G-H). We then assayed triciribine on a panel of breast cancer cell lines that we previously analyzed for gene expression (28). The GI50s significantly correlated with ZNF217 expression levels ($r=-0.39$, $P=0.035$ Spearman) (Figure 6I), consistent with a selective effect of triciribine on cell lines that express the highest ZNF217 levels.

**Triciribine Kills Cells that Overexpress Znf217 in vivo**
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To test the effects of triciribine in vivo, we transplanted vector control and Znf217-overexpressing tumorigenic Vo-PyMT cells orthotopically to contralateral mammary fat pads cleared of epithelium. At three weeks, we injected mice with either triciribine or vehicle solution for five days per week. Triciribine treatment significantly reduced to baseline levels the increase in tumor burden seen as a result of Znf217 overexpression, and led to reduced phospho-AKT expression, reduced phospho-MAPK expression and increased cell death in vivo (Figures 7A-C, S4G). In culture, triciribine inhibited only phospho-AKT and did not inhibit phospho-MAPK or ErbB3 activation after heregulin stimulation (Figure 7D). The observed differences between signaling events inhibited by triciribine in culture versus in vivo suggest that cells within a tumor microenvironment respond differently to triciribine than do cells in culture.

Triciribine also was effective in vivo at inhibiting tumor growth in mice xenografted with the human tumorigenic cell line MCF7 compared to control treated mice (Figure 7E).

Triciribine Overcomes ZNF217-induced Doxorubicin Resistance

Tumor cells overexpressing ZNF217 are resistant to doxorubicin-induced cell death (23). Several groups have found triciribine to be an effective, synergistic therapy in combination with other drugs (e.g., trastuzumab, farnesyltransferase inhibitors) to reduce tumor burden (29, 30). Similarly, we found that the addition of triciribine with doxorubicin to cells in culture generated a synthetic lethality in which cells overexpressing ZNF217 were no longer resistant to doxorubicin and
instead were killed (Figure 7F). Interestingly, the parent mammary epithelial cell line HBL100 expresses low levels of adenosine kinase, which is required for the phosphorylation and activation of triciribine in patients. This suggests that ZNF217 may be a sufficiently predictive biomarker of triciribine efficacy, even in patients with low adenosine kinase, if patients are also treated in combination with a drug such as doxorubicin.
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Discussion

ZNF217 is a Biomarker of Disease Progression and Treatment Response and is a Therapeutic Target Inhibited by Triciribine

In this study we identified ZNF217 as a prognostic biomarker of reduced survival, metastasis and chemoresistance in breast cancer patients. Using both cultured cells and in vivo mouse transplant models, we found that ZNF217 overexpression contributes to multiple aspects of carcinogenesis including increased proliferation/decreased cell death, increased invasiveness, increased motility, immortalization, chemotherapy resistance, metastasis and progenitor cell expansion. Our data demonstrate that Znf217 promotes carcinogenesis by driving a differentiation gene expression signature towards a less differentiated/progenitor state indicative of expanding a multipotent progenitor population.

We identified a panel of drugs that inhibit the growth of cell lines that overexpress ZNF217 and validated two that induced ZNF217-dependent cell death.

Triciribine: a Component of Therapy for Poor Prognostic Breast Cancer Patients

Triciribine is a nucleoside analog and DNA synthesis inhibitor that has been tested in Phase I clinical trials in cancer patients as well as in one Phase II clinical trial in metastatic breast cancer patients (24-27). In the Phase II study,
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one of 14 patients had stable disease and the other patients progressed (27). The Phase II studies and subsequent studies found that triciribine was not readily bioactive in all patients, possibly due to the requirement for expression of multiple genes for triciribine bioactivation. Triciribine is also an allosteric inhibitor of AKT activation: it physically interacts with AKT to prevent AKT recruitment to the plasma membrane and to block the phosphorylation and activation of AKT (31, 32).

While several studies identified triciribine as an AKT inhibitor, triciribine is not always redundant with other AKT pathway inhibitors in treating tumors: often triciribine is more effective in vivo than other PI3K/AKT pathway inhibitors at inhibiting tumor progression (29). Indeed, we found that triciribine inhibited not only AKT activation but also MAPK activation in vivo (Figures 7B-C). We hypothesize that triciribine inhibits both AKT and MAPK pathways, both of which are downstream of ErbB3/ErbB2 activation. This could provide a rationale for inhibition of ZNF217-induced tumor burden by triciribine, since ZNF217 drives the overexpression of ErbB3 and leads to the activation of both AKT and MAPK pathways. Alternatively, triciribine could inhibit these pathways after activation of other receptor tyrosine kinases (RTKs) (Figure 7G).

Our study suggests that ZNF217 may be a sufficiently predictive biomarker of triciribine efficacy if patients are also treated in combination with a drug such as doxorubicin or another drug that offers synergy with triciribine. In part, ZNF217 may act by inducing upregulation of its target ERBB3 (22). Thus, cells resistant to triciribine treatment might independently activate multiple
ZNF217 overexpression promotes malignancy signaling pathways, making them less responsive to inhibitors that act upstream in the signaling pathway.

Combinatorial pathway activation may be therapeutically important in treating patients with high ZNF217 expression, as concurrent activation of the PI3K/AKT and RAS/MAPK pathways causes resistance to AKT inhibition in cells (33). Interestingly, in the panel of immortal cell lines expressing ZNF217 and tested for triciribine sensitivity, all outlier cell lines (i.e., lines with high GI50s and high ZNF217 expression) contained previously identified mutations in the PI3K/AKT and/or RAS/MAPK pathways (34-36). Since triciribine does not inhibit upstream activators PI3K or PDK1 or related family members directly (31), future studies will be required to sort out mechanistically how ZNF217 activates and triciribine inhibits signaling. Whether combination therapies will be more effective in vivo remains to be tested.

ZNF217 Reprograms Tumor Cells to Express Luminal and Myoepithelial Cell Markers

We found that Znf217 promotes phenotypes suggestive of expansion of progenitor cells in vivo and in culture and drives repression of an adult stem cell gene expression signature that is also downregulated in many epithelial cancers. Consistent with a progenitor phenotype, ZNF217 promotes increased telomerase, resistance to TGFβ growth inhibition, amplified c-MYC (9, 37), as well as chemotherapy resistance (23). That Znf217 may drive a less differentiated gene expression signature is supported by the observation that
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Znf217 is upregulated in the somite following the transition from the presomitic mesoderm and prior to the differentiation into the skeleton, muscle and dermis (38). Moreover, Znf217 is repressed concurrently with Oct4 following differentiation of a teratocarcinoma cell line to neuronal cells and binds to the promoters of a number of genes involved in differentiation (10). Thus, in tumors ZNF217 may promote transdifferentiation to or expansion of a pool of progenitor-like cells by aberrantly suppressing differentiation pathways.

Znf217 overexpression in tumor cells derived from mice expressing the oncogene PyMT switched their phenotype from a predominantly luminal to a more heterogeneous pathology characterized by expression of both luminal and myoepithelial epithelial cell markers. This phenotype is similar to that seen following Wnt1 overexpression or activation of the AKT pathway by PTEN deletion in vivo (39, 40). Interestingly, the PyMT oncogene can give rise to tumors expressing both luminal and myoepithelial markers, depending on the cell type into which it is introduced. Expression of PyMT by intraductal injection of avian retrovirus (RCAS-PyMT) induces tumors with markers of luminal, myoepithelial and progenitor cells (41). Recently a connection has emerged between the undifferentiated, stem cell-like phenotype in breast cancer cells and transdifferentiation of the tumor cells towards a mesenchymal phenotype [reviewed in (42-44)]. Induction of epithelial-to-mesenchymal transition (EMT) in cultured MECs not only increases the population of cells with mesenchymal markers but also increases those with progenitor cell characteristics (CD44\textsuperscript{high}/CD24\textsuperscript{low}) (45).
Conclusion

We used an integrated biological approach to model the multiple contributions of ZNF217 to carcinogenesis during tumor progression, metastasis and neoadjuvant treatment. We propose that ZNF217 is a biomarker that is prognostic of disease progression and is a therapeutic target. Our data suggest that triciribine may be a component of an effective treatment strategy in patients who have tumors expressing high ZNF217, possibly by targeting a progenitor population and reducing signaling in the AKT and MAPK pathways. Since ZNF217 is amplified in numerous cancers, this work has implications for other cancers as well.
Materials and Methods

Additional descriptions of materials and methods, including cell lines, antibodies, and staining procedures used, are in the Supplementary Data.

Cell Lines

Cell lines used in this study include mouse mammary epithelial cells NMuMG (source: Rik Derynck), SCp2 (source: Mina Bissell), EpH4 (source: Mina Bissell) and Vo-PyMT-Luc (source: Conor Lynch) and human mammary epithelial cell lines MCF7, ZR-75-1 and HBL100 (source: ATCC for all three lines). MCF7 was authenticated by SNP6.0 copy number analysis. Other cell lines were not authenticated.

Mouse Lines

Mice used in this study were maintained on the FVB/n background 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.

Metastasis analysis

Both PyMT MEC and Vo-PyMT transplants were analyzed for lung metastasis. To determine metastasis frequency, lung tissue blocks were sectioned into 5-μm
sections and stained by hematoxylin and eosin (H&E). For each mouse analyzed, one section was scored for number of metastases seen at 100x magnification in 3 (PyMT MECs) or 5 (Vo-PyMT cell line) high-powered fields in regions of the tissue section with the highest density of metastases. Each cohort had 6-11 mice analyzed.

**Statistical analysis**

Statistical analysis was performed using Prism 4 software (GraphPad Software, Inc.) or SPSS Statistics software (IBM) for Cox proportional hazard tests. Cohorts of three or more samples were compared using one-way analysis of variance (ANOVA). All tests used and P values are specified in the figure legends. P<0.05 was considered significant.

**Supplementary Data**

Supplementary Data include Supplemental Materials and Methods, 4 Supplemental Figures, one movie and 5 Supplemental Tables.

**Accession Numbers**

Microarray data were deposited to the NCBI’s GEO Repository and are accessible to reviewers through GEO series accession number GSE24727.

**Acknowledgments**


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References


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**Figure Legends**

**Figure 1.** ZNF217 overexpression is a prognostic indicator in breast cancer patients. (A) Patients (n=118) were separated into high (n=59) vs. low (n=59) ZNF217 expression and analyzed for overall survival (P=0.003; Logrank). (B) Relapse-free survival based on high (n=40) vs. low (n=41) ZNF217 expression (P=0.01; Logrank). (C) Patients (n=858) were separated into low (n=286), intermediate (n=286) and high (n=286) ZNF217 expression and analyzed for relapse-free survival. Patients with high ZNF217 expression had worse survival than low ZNF217 patients (P=0.03; Logrank).

**Figure 2.** Znf217 overexpression promotes increased cell motility and aberrant epithelial marker expression. (A) Relative Znf217 expression levels by rt-qPCR in SCp2 mammary epithelial cell lines infected with virus to overexpress vector or Znf217 with comparable results in three experiments. Each sample was tested by rt-qPCR in triplicate relative to the reference TBP, with similar results for other reference genes. Graphs show the mean ± S.E.M. (B) Western blot analysis of Znf217 protein (anti-Znf217) and loading control (anti-HDAC1) in SCp2 cells. Images are representative of multiple experiments using retrovirus or lentivirus overexpression of Znf217. Arrows mark the indicated proteins. (C) Brightfield images of SCp2 cells ± Znf217 display increased cell scattering in culture after Znf217 overexpression. (D) Frames from movies of SCp2 cells infected with vector or Znf217 following a scratch with a pipette tip. The movies ran 20.25 hours. Note the lamellipodia (arrow) extending from the cells by 5.5 hours and the increased number of Znf217-expressing cells in the middle of the scratch by 10.5 hours (arrow). (E) Phalloidin staining of SCp2 cells ± Znf217. (F) Relative expression of Znf217 and selected genes by rt-qPCR from NMuMG (top) and SCp2 (bottom) cells ± Znf217 in vitro. Graph shows the mean ± S.E.M., relative to the reference GAPDH. Similar results were seen with the reference HPRT. For each gene, samples for Znf217 were compared to vector by Mann-Whitney tests,
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and significant $P$ values $<0.02$ were marked with *. (G) Heat map of selected genes enriched following Znf217 overexpression in SCp2 cells.

**Figure 3.** Znf217 overexpression causes an increase in soft agar colonies and in mammosphere formation. (A) Western blot analysis of Znf217 protein in NIH3T3 cells infected with vector or Znf217 retrovirus. (B) Znf217 overexpression increases the number of colonies by anchorage-independent growth in soft agar assay. Relative number of colonies per well by soft agar with vector or Znf217 overexpression ($P=0.001$; Student’s t test). Graph compiles results from three experiments, each done in triplicate. (C) Brightfield images of anchorage-independent colonies from soft agar assay ± Znf217 in (B). Arrows mark examples of colonies. The large colonies were only seen with the Znf217-overexpressing cells, while much smaller colonies were seen with vector-expressing cells. (D) Relative expression of Znf217 by rt-qPCR from normal adult mammary gland (FVB/n), relative to the reference HPRT with line marking the mean. Glands were sorted by flow cytometry for CD24$^{\text{Med}}$CD49f$^{\text{High}}$ (basal/myoepithelial/progenitor cells) and CD24$^{\text{High}}$CD49f$^{\text{Low}}$ (luminal/luminal progenitor) fractions. RNA was isolated and used to generate cDNA from each population. Each dot represents one mouse sorted, collected and processed by rt-qPCR. Graph shows relative epithelial Znf217 expression in the CD24$^{\text{Med}}$CD49f$^{\text{High}}$ vs. CD24$^{\text{High}}$CD49f$^{\text{Low}}$ populations. Similar results were seen with the reference GAPDH. (E) Relative expression of Znf217 expression by rt-qPCR in primary mouse mammary epithelial cells following lentiviral infection with either pEiT vector or Znf217-pEiT in three separate samples. Each sample was tested by qPCR in triplicate relative to the reference TBP. These samples were used for microarray analysis. Graph shows the mean ± S.E.M. (F) Quantification and (G) brightfield images of mammosphere assay of Vo-PyMT cells overexpressing vector or Znf217. (H) Heat map of selected genes from gene expression microarray analysis enriched in primary MECs overexpressing Znf217. (I) Relative expression of Znf217 expression by rt-qPCR in primary mouse mammary epithelial cells following lentiviral infection with either vector.
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vector or Znf217 in three separate samples. Each sample was tested by qPCR in triplicate relative to the reference TBP and used for microarray analysis. Graph shows the mean ±S.E.M. (J) Rt-qPCR to validate microarray targets using the same samples used in (H) with HPRT as a reference in qPCR reactions. Similar results were obtained with GAPDH used as a reference (data not shown).

Figure 4. Znf217 overexpression in vivo increases rate of tumor progression, tumor heterogeneity and differentiation state. (A) Relative expression of Znf217 and EMT genes by RT-qPCR in the Vo-PyMT cell line overexpressing either vector or Znf217. Assay used the reference GAPDH. Similar results were seen using HPRT or TBP references. The cells used in this experiment had previously been sorted for fluorescent marker expression and were used for the Vo-PyMT transplants throughout this study. (B) Mammosphere assay of primary MECs infected with vector or Znf217-overexpressing lentivirus. (C) Quantification of mammosphere formation in primary MECs expressing vector or Znf217 after one week. Graph shows mean ± S.E.M., and samples were compared by unpaired t test. (D) Tumor-free survival over time in Vo-PyMT transplants (P=0.01; Logrank). (E) Tumor volume over time in Vo-PyMT transplants of Znf217 (n=8) versus vector (n=10) (P=0.007; ANOVA, repeated measures). (F) Final tumor weight in Vo-PyMT transplants (P=0.02; Mann-Whitney). Line represents median of vector (n=9) versus Znf217 (n=8). (G) H&E staining of MMTV-PyMT (PyMT MEC) tumors from transplants overexpressing vector (top) or Znf217 (center, bottom panels). Inserts are enlarged images of boxed regions and demonstrate heterogeneous pathology. (H) Immunofluorescence staining with anti-Keratin-8 (green), anti-alpha-smooth muscle actin (red)(arrows) and DNA (Hoechst; blue) in tumors derived from PyMT MEC transplants. (I) Immunofluorescence staining with anti-Keratin-8 (green), Keratin-14 (red) and DNA (blue) from PyMT MEC transplants. Arrows mark cells double positive for K8 and K14. (J) Quantification of a progenitor cell population: K8+K14+ (P=0.002), % K8+K14+ (P=0.0002)(unpaired t-tests). Bar graphs show mean representation
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(number/percentage) of K8+K14+ cells ± S.E.M. per HPF. HPF= 3 high-powered fields.

**Figure 5.** Znf217 overexpression in vivo increases lung metastasis. (A) Immunofluorescence with anti-E-cadherin (green) and DNA (blue) from Vo-PyMT transplants. Arrows mark regions with low E-cadherin expression. (B) Number of lung metastases per three (a) or five (b) high powered fields from (a) PyMT MEC \( (P=0.008) \) or (b) Vo-PyMT transplants \( (P=0.01; \) Mann-Whitney) with vector or Znf217 overexpression. Bar graph shows the mean ± S.E.M. (C) Metastatic burden from PyMT and Vo-PyMT transplants. Number of lung metastases per three (PyMT) or five (Vo-PyMT) high-powered fields divided by tumor weight from (a) PyMT MEC \( (P=0.003; \) Mann-Whitney) or (b) Vo-PyMT \( (P=0.32; \) Mann-Whitney) transplants with vector or Znf217 overexpression. Bar graph shows the mean ± S.E.M. Similar results were obtained using final tumor volume (data not shown). (D) H&E staining of lung metastases from PyMT MEC transplants. Arrows mark examples of metastases. (E) Metastasis-free survival based on high \( (n=41) \) vs. low \( (n=41) \) ZNF217 expression \( (P=0.01; \) Logrank) from (19).

**Figure 6.** Identification of triciribine as a candidate inhibitor of ZNF217-induced growth. (A) Response to neoadjuvant chemotherapy in breast cancer patients with high vs. low ZNF217 expression in tumors (a) from (12). Patients had responsive \( (n=27) \) or nonresponsive (stable/progressive) disease \( (n=28) \) in response to treatment \( (P=0.01; \) Mann-Whitney). Lines mark means. (b) from (21) \( (P<0.001; \) Mann-Whitney). Tumors were responsive (pathological complete response; \( n=34 \)) or nonresponsive (residual disease; \( n=34 \)) to treatment. Lines mark means. (B) ZNF217 and ERBB3 expression levels in human breast tumors \( (n=118) \) from (11). ZNF217 and ERBB3 strongly correlate \( (Pearson r=0.47; R^2=0.22; \) by linear regression, \( P<0.0001) \). (C) MCF7 cells (left) or ZR-75-1 cells (right) were transiently transfected with scrambled or ZNF217 siRNA. 48 hours after transfection, cells were serum starved 24 hours and treated for 15 minutes with heregulin. Lysates were blotted for the indicated proteins. (D) PI3K and AKT
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inhibitors do not promote ZNF217-dependent cell death. FACS analysis of cell death by Annexin V staining in MCF7 cells ± ZNF217-shRNA or scramble control and treated for two days with control, 2 μM GDC0941, or 10 μM MK2206. (E) Treatment of MCF7 cells ± ZNF217-shRNA with bisacodyl in triplicate at the indicated concentrations (P=0.001; ANOVA). Similar results were obtained in at least three experiments. (F) Treatment of MCF7 cells ± ZNF217-shRNA with 10 μM triciribine at the indicated concentrations (P=0.001; ANOVA). Similar results were obtained with a second shRNA and in at least three experiments. (G) ZNF217 expression levels and (H) related triciribine GI50 concentrations in NCI60 panel breast cancer cell lines. Inset: Chemical structure of triciribine. (I) ZNF217 expression levels across triciribine GI50s in 30 breast cancer cell lines (15 each of cell lines expressing highest/lowest ZNF217)(r=-0.39; P=0.035; Spearman correlation). Two outliers circled are identified by cell type and relevant mutations.

Figure 7. Triciribine inhibits Znf217 in vivo and in human cells. (A) Tumor burden growth rate of Vo-PyMT transplants treated with DMSO solution (solid lines) or triciribine (dotted line) (P<0.0001 by genotype; P=0.02, genotype over time; ANOVA). Vo-PyMT transplants overexpressed vector (blue) or Znf217 (orange). Shown are the mean ± S.E.M. (B) Phospho-AKT (left) and phospho-MAPK (right) protein expression by immunohistochemistry in tissues from Vo-PyMT transplants treated with either control or triciribine. (C) Model of pathways downstream from Znf217. Znf217 overexpression promotes phospho-AKT and phospho-MAPK. This activation is associated with increased tumor burden, chemotherapy resistance and mammosphere formation. Triciribine can block these phenotypes of Znf217 overexpression. (D) MCF7 cells ± triciribine were serum starved overnight and stimulated with heregulin/neuregulin-1ß for the indicated times. Cell lysates were blotted for the indicated proteins. (E) Human MCF7-M1 subcutaneous xenografts treated with control or triciribine (50 mg/kg) at the indicated time post-transplant. Ticks show mean tumor burden ± standard deviation. (F) Triciribine induces synthetic lethality with doxorubicin in culture.
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Stable HBL100 MECs (low ZNF217, low adenosine kinase expression) (± ZNF217) were treated with triciribine and doxorubicin at the indicated concentrations and monitored for cell death using Annexin V staining ($P=0.0002$; ANOVA). All doxorubicin-treated samples were statistically different ($P<0.05$; Bonferroni posttest), whereas triciribine treatment alone did not promote statistically significant results. Graph shows mean ± S.E.M. (G) Model of Znf217 function. Increased Znf217 promotes increased ErbB3 expression and activation of downstream signaling events during tumor progression. Znf217 may also activate other receptor tyrosine kinases (RTKs) that in turn lead to activation of AKT or MAPK pathways. In vivo during tumor progression, triciribine can block signaling events downstream of Znf217 overexpression.
A Overall survival  

B Relapse-Free Survival  

C ER+ Her2- LN- patients

Chin 2006  

Sortie 2006  

Meta-Analysis  

858 patients
**A** rt-qPCR

Relative Expression

**B** Western

vector Znf217

Znf217

**C** Brightfield

vector

Znf217

**D** Scratch assay

vector

Znf217

t=0

5.5 hours

10.5 hours

20.25 hours

**E** Phalloidin

vector

Znf217

**F** rt-qPCR

NMuMG

**G** Microarray

Fold change over vector

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

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Figure 3: Znf217 Expression in Primary MECs

A. Western blot showing Znf217 expression in Vector and Znf217-transfected MECs.

B. Bar graph showing relative number of colonies per well for Vector and Znf217.

C. Photomicrograph showing Znf217 expression.

D. Graph showing relative Znf217 expression (CD24^High/CD44^Low).

E. Bar graph showing relative expression (TBP).

F. Bar graph showing number of mammospheres per 5000 cells for Vector and Znf217.

G. Photomicrographs showing control and Znf217-transfected MECs.

H. Heat map showing fold change of gene expression over vector.

I. Bar graph showing relative expression (TBP).

J. Heat map showing relative expression of various genes (HPR1).
**Figure 4**

**A** Relative expression of Znf217 and its target genes in adult mammary epithelial cells. **B** Vo-PyMT tumors stained for Znf217 expression. **C** Number of mammospheres per 1000 cells. **D** Fraction of tumor-free mice over time post-transplantation. **E** Tumor volume over time. **F** Final tumor weight. **G** Hematoxylin and eosin staining of tumor sections. **H** Immunohistochemistry for K8 and SMA DNA in tumors. **I** Immunohistochemistry for K8 and K14 DNA in tumors. **J** Quantification of K8* and K14* cells per HPF.
Figure 5

A

B Lung metastasis
   a PyMT MEC
   b Vo-PyMT

C Metastatic burden
   a PyMT MEC
   b Vo-PyMT

D

E Metastasis-Free Survival

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Littlepage_Figure 6

A  Chemoresistance

- **Sorlie 2006**
  - $P = 0.01$

- **Hess 2006**
  - $P < 0.0001$

B  Expression

- **Chin 2006**
  - $P < 0.0001$

C  MCF7 cells

- siRNA-scramble
- siRNA-ZNF217

ZNF217, P-AKT, P-MAPK, ErbB3, P-Y20, tubulin

D  Cell death

- shRNA-scr
- shRNA-ZNF217 (F9)
- shRNA-ZNF217 (G4)

G  ZNF217 expression

- MDA-MB-361
- MDA-MB-468
- T47D

H  Tricirbine

- Tricirbine concentration (μM)

I  Tricirbine GI50s

- MDAMB361 (PK3CA mutation)
- ZR751 (PTEN mutation)
**Figure 7**

**A** Tumor Burden

![Graph showing tumor growth](image)

- **Start treatment** indicated at day 0.
- Linear regression line with slope significance indicated (P<0.0001).

**B** Phospho-AKT and Phospho-MAPK

![Images of phosphorylated proteins](image)

- Vector and Znf217 conditions compared.
- DMSO and Triciribine treatments shown.

- **C** ZNF217 and Triciribine

![Diagram of signaling pathways](image)

- Phospho-AKT and Phospho-MAPK indicated.
- Tumor burden and mammosphere formation shown.

- **D** Triciribine + HRG

![Western blot images](image)

- Control and + Triciribine conditions.
- 0-45 min time points.

**E** MCF-7 xenografts

![Graph showing tumor volume](image)

- Tumor volume over days after transplantation.
- Control and Triciribine groups.

- **F** Triciribine and Doxorubicin

![Cell death Annexin V assay](image)

- Control and ZNF217 treatments.
- 0-100 μM concentrations.

- **G** Other RTKs, HER2/NEU, HER3/ERBB3

![Schematic diagram](image)

- AKT and MAPK pathways.
- SRC involvement.
- Triciribine effects on survival and proliferation.
- Nucleus indicated.
The transcription factor ZNF217 is a prognostic biomarker and therapeutic target during breast cancer progression

Laurie E. Littlepage, Adam S Adler, Hosein Kouros-Mehr, et al.

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