Published in final edited form as:

Breast Cancer Res Treat. 2014 June ; 145(3): 593–604. doi:10.1007/s10549-014-2979-6.

Suppression of triple-negative breast cancer metastasis by pan-DAC inhibitor panobinostat via inhibition of ZEB family of EMT master regulators

Lyndsay V. Rhodes¹, Chandra R. Tate¹, H. Chris Segar¹, Hope E. Burks¹, Theresa B. Phamduy³, Van Hoang¹, Steven Elliott¹, Diari Gilliam¹, F. Nell Pounder¹, Muralidharan Anbalagan², Douglas B. Chrisey^{3,4}, Brian G. Rowan², Matthew E. Burow¹, and Bridgette M. Collins-Burow¹

¹Department of Medicine, Section of Hematology and Medical Oncology, Tulane University Health Sciences Center, 1430 Tulane Ave, New Orleans, LA, 70112, USA

²Department of Structural and Cellular Biology, Tulane University Health Sciences Center, 1430 Tulane Ave, New Orleans, LA, 70112, USA

³Department of Biomedical Engineering, Tulane University, 5050 Percival Stern Hall, New Orleans, LA, 70118, USA

⁴Department of Physics and Engineering Physics, Tulane University, 5050 Percival Stern Hall, New Orleans, LA, 70118, USA

Abstract

Triple-negative breast cancer (TNBC) is a highly aggressive breast cancer subtype that lacks effective targeted therapies. The epithelial-to-mesenchymal transition (EMT) is a key contributor in the metastatic process. We previously showed the pan-deacetylase inhibitor LBH589 induces CDH1 expression in TNBC cells, suggesting regulation of EMT. The purpose of this study was to examine the effects of LBH589 on the metastatic qualities of TNBC cells and the role of EMT in this process. A panel of breast cancer cell lines (MCF-7, MDA-MB-231, BT-549), drugged with LBH589, were examined for changes in cell morphology, migration, and invasion *in vitro*. The effect on *in vivo* metastasis was examined using immunofluorescent staining of lung sections. EMT gene expression profiling was used to determine LBH589-induced changes in TNBC cells. *ZEB* overexpression studies were conducted to validate requirement of *ZEB* in LBH589-mediated proliferation and tumorigenesis. Our results indicate a reversal of EMT by LBH589 as demonstrated by altered morphology and altered gene expression in TNBC. LBH589 was shown to be a more potent inhibitor of EMT than other HDAC inhibitors, SAHA and TMP269. Additionally, we found that LBH589 inhibits metastasis of MDA-MB-231 cells *in vivo*. These

Corresponding author: Bridgette M. Collins-Burow, 1430 Tulane Ave, SL-78, New Orleans, LA 70112, Office: (504) 988-6879, Fax: (504) 988-5483, bcollin1@tulane.edu.

Ethical Standards

The authors declare that all experiments reported in this publication were performed in compliance with all current laws and regulations of the United States of America.

Conflict of interest

The authors declare that they have no conflict of interest.

effects of LBH589 were mediated in part by inhibition of *ZEB*, as overexpression of *ZEB1* or *ZEB2* mitigated the effects of LBH589 on MDA-MB-231 EMT-associated gene expression, migration, invasion, CDH1 expression, and tumorigenesis. These data indicate therapeutic potential of LBH589 in targeting EMT and metastasis of TNBC.

Keywords

HDAC inhibitor; epithelial-to-mesenchymal transition; metastasis; ZEB1; ZEB2

Introduction

Triple-negative breast cancer (TNBC) is a heterogeneous subset of neoplasms defined by the absence of estrogen receptor and progesterone receptor, and non-amplification of Her2/neu [1–3], representing approximately 15% of diagnosed breast cancer cases [3–4]. Studies have shown that tumors of this aggressive subtype are of higher histological grade and affect a disproportionate number of young women [1–3,5,6]. Although TNBC primary tumor display a better initial response to chemotherapy compared to estrogen receptor-positive tumors, TNBC patients have a worse prognosis regardless of tumor stage at diagnosis [3]. The poor prognoses of TNBC appear to be linked to the presence of residual or disseminated disease, as many TNBC patients present with metastatic disease much earlier than their estrogen receptor-positive counterparts, typically within the first 3 years after diagnosis [3]. The high rate of metastasis coupled with the fact that these disseminated cells frequently display multidrug resistance [7] make treatment of metastatic disease difficult [1–3,5,6]. The lack of classical therapeutic targets (ER, Her2/neu) and the increased risk of metastasis in the TNBC subtype make identifying and developing novel therapeutics critical for the treatment of these patients.

Histone deacetylase inhibitors (HDACis) have emerged as a promising new class of multifunctional anticancer agents [8–9]. That promise lies in the ability of HDACis to affect multiple epigenetic changes in aberrant cells. Panobinostat (LBH589) is a potent pan-deacetylase inhibitor that can block multiple cancer related pathways and reverse epigenetic events implicated in cancer progression [10]. In addition to the anti-cancer effects of LBH589 observed in hematologic, lung, thyroid, and prostate malignancies, our previous results show significant effects in the triple-negative subtype of breast cancer [11]. Our studies revealed decreased cell proliferation and viability, increased apoptosis, as well as inhibition of primary tumor volumes of TNBC cells. Significant increases in the epithelial cell marker CDH1 following treatment with LBH589 both *in vitro* and *in vivo* led us to question the effects of LBH589 on the epithelial-mesenchymal transition of TNBC and, thus, the effects on cell motility and metastasis.

The epithelial-to-mesenchymal transition (EMT) has been recognized as a major player in cancer cell invasion and metastasis [12]. EMT is characterized by the loss of epithelial cell markers, namely epithelial-cadherin (CDH1), enhanced expression of mesenchymal cell markers, including neuronal-cadherin (CDH2) and vimentin (VIM), and increased expression of CDH1 transcriptional repressors such as zinc finger E-box-binding homeobox

1 (ZEB1) and 2 (ZEB2) [13–16]. The expression of EMT markers, including *CDH1*, *CDH2*, *VIM*, *ZEB1* and *ZEB2*, has been associated with poor prognosis of breast cancer patients [17–18]. As such, regulators of EMT have become attractive targets for the development of anti-metastasis therapies. Here we demonstrate the ability of LBH589 to inhibit the migration, invasion, and metastasis of TNBC cell lines via suppression of EMT. Furthermore, we reveal a role for ZEB expression in the LBH589-mediated anti-EMT effects in TNBC cells. Together our data support the development of LBH589 as a novel therapeutic to target the aggressive and metastatic nature of TNBC.

Materials and Methods

Cell lines and reagents

Human TNBC (MDA-MB-231, BT-549) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). MDA-MB-231 and BT-549 cells are characterized as triple-negative/basal-B mammary carcinoma [19]. Liquid nitrogen stocks were made upon receipt and maintained until the start of each study. MCF-7 cells, characterized as ER-positive/PgR-positive luminal mammary carcinoma, were obtained from frozen stocks routinely used and previously published [20]. Cells were used for no more than six months after being thawed with periodic recording of morphology and doubling times to ensure maintenance of phenotype. Cells were maintained at 37°, 5% CO₂ in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Hyclone, Salt Lake City, UT, USA) and 1% penicillin/streptomycin (Invitrogen). Panobinostat was generously provided by Novartis Pharmaceutical Inc. (East Hanover, NJ, USA). SAHA was purchased from Cayman Chemicals (Ann Arbor, MI, USA) and TMP269 from Xcess Biosciences Inc. (San Diego, CA, USA). Panobinostat (LBH589), SAHA, and TMP269 were dissolved in dimethyl sulfoxide (DMSO) (Invitrogen) as a 10 mM stock solution and stored at –20°C. Drugs were diluted in culture media and used at concentrations indicated.

Crystal violet assay

Cells were plated at 7.5×10^3 cells per well in a 96-well plate format in 100µl 5% charcoal/dextran treated fetal bovine serum (Atlanta Biologicals, Atlanta, GA, USA) phenol red-free DMEM (5% CS-DMEM) and allowed to attach overnight at 37°C, 5% CO₂. Cells were treated the next day at concentrations indicated. After 3 days of treatment, plates were harvested by the addition of 10µl glutaraldehyde per well incubated at room temperature for 30 minutes. Cells were washed and allowed to dry. Cells were stained with 50µl of 0.1% crystal violet (in 20% methanol) for 30 minutes, washed and allowed to dry. Cells were imaged on a Nikon TE2000 inverted microscope with IPLab software (BD Biosciences, Rockville, MD, USA). Original magnification, 200×. Cells were lysed with 100µl 33% acetic acid and absorbance read at 630 nm.

Transwell migration and invasion assays

Migration assays were performed following the manufacturer's instructions (BD Biosciences) and as previously published [21]. Cells were treated at time of plating. After 6 or 24 hours (as noted in the legend), migrated cells were fixed to the membranes and stained

with crystal violet (0.1% in 20% methanol), and migrated cells visualized and quantified by microscopy. Invasion assays were conducted in the same manner as the migration assays using Matrigel® (BD Biosciences) coated transwell membranes and a seeding density of 5×10^4 cells per well for 24 hours. Data represented as number of migrated or invaded cells per field of view $\pm$ SEM for triplicate experiments.

Immunohistochemical staining and quantitation

Staining was conducted as previously published [11, 22]. *RFP staining*: rabbit anti-RFP (Abcam, Cambridge, UK) 1:30 dilution, Alexa Fluor® 488 goat anti-rabbit secondary (Invitrogen); *CDH1 staining*: rabbit E-cadherin antibody (24E10; Cell Signaling Technology, Boston, MA, USA) 1:400 dilution. Internal negative controls were exposed to rabbit IgG or 10% goat serum rather than primary antibody. Images were captured on a Nikon TE2000 inverted microscope with IPLab software (BD Biosciences, Rockville, MD, USA), original magnification at 200 $\times$ (RFP) or 400 $\times$ (CDH1).

Metastases were calculated as a ratio of the number of RFP-positive cells versus the total amount of cells (determined by DAPI nuclear stain) per field of view. Points represent the ratio of RFP-positive cells versus DAPI for each section examined with mean for each group represented as horizontal black bar. SEM for each group indicated in red.

RNA isolation

Cells were plated in 10% DMEM at 70% confluency and treated with 100nM LBH589 or vehicle for 24 hours. Cells were harvested by trypsinization and total RNA was isolated using the RNeasy kit, according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). The quantity and quality of the RNA were determined by absorbance at 260 and 280nm using the NanoDrop ND-1000 (NanoDrop, Wilmington, DE, USA).

Human EMT quantitative reverse transcription real-time PCR array

Human Epithelial to Mesenchymal Transition (EMT) RT² Profiler™ PCR Arrays (PAHS-090 and 090Z) were obtained from SABiosciences (Frederick, MD, USA) and assayed according to manufacturer's protocol. Biological triplicates were run for each sample.

Quantitative reverse transcription real-time PCR

Total RNA was reverse-transcribed using the BioRad First Strand cDNA synthesis kit, following the manufacturer's protocol (BioRad) and then assayed via quantitative real-time PCR (qPCR) to assess gene expression changes as previously published [21]. Primers are available in supplemental materials. Data represented as normalized Ct (fold expression) compared to control samples of biological triplicate samples $\pm$ SEM.

Western blot analysis

Western blot analyses were conducted as previously published [21]. Primary antibodies were used at a concentration of 1:100. ZEB1 (H-102) was purchased from Santa Cruz Biotechnology (Dallas, TX) and ZEB2/SIP1 (6E5) from Millipore (Billerica, MA).

Secondary antibodies (IRDye) were used at 1:10,000 and purchased from Licor Bioscience (Lincoln, NE).

MTT cell proliferation assay

Proliferation of MDA-MB-231-ZEB1, ZEB2, and vector cells was measured by MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Cell Proliferation Assay, according to the manufacturer's protocol (Invitrogen) as previously published [11]. 5×10^3 cells/well were plated in 96-well format in 10% DMEM. Cells were treated with LBH589 (100nM) or vehicle for 24 hours. Data represented as mean percent vehicle treated MDA-MB-231-vector cell proliferation $\pm$ SEM of biological triplicate experiments with internal duplicates.

CDH1 ELISA

MDA-MB-231-vector, -ZEB1, and ZEB2 were treated with LBH589 or vehicle (DMSO) for 24 hours, and used for ELISA to determine CDH1 protein changes. CDH1 ELISA was purchased from R&D Systems (Minneapolis, MN) conducted per manufacturer's instructions and as previously published [11].

Animal xenograft studies

Xenograft tumor studies were conducted as previously described [20]. At necropsy, animals were euthanized by cervical dislocation following CO₂ exposure. Tumors, livers, lungs, and brains were removed and snap frozen or fixed in 10% formalin for future analysis. All procedures involving animals were conducted in compliance with State and Federal laws, the U.S. Department of Health and Human Services, and guidelines established by Tulane University Animal Care and Use Committee. The facilities and laboratory animals programs of Tulane University are accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care.

Quantification of brain metastases

At necropsy mouse brains were removed and half of each brain was placed in 1 mL saline containing 0.2% collagenase for 20 minutes. The resulting cell suspensions were dissociated via 70 μ m sterile cell strainers (BD Falcon, Sparks, MD, USA). To determine percentage of RFP positive cells, filtrates were counted on a Cellometer Vision automated cell counter (Nexcelom Bioscience, Lawrence, MA, USA) according to the manufacturer's protocol. Data represented as average number of tRFP-positive cells per sample $\pm$ SEM of triplicate samples.

ZEB1 and ZEB2 stable cell generation

MDA-MB-231 cells were plated at approximately 50% confluency in 10cm² culture dishes in 10% DMEM and allowed to adhere overnight in a 37°C incubator. The following day cells were transfected with 5 μ g of CMV-eGFP vector, CMV-eGFP-ZEB1, or CMV-eGFP-ZEB2 (GeneCopoeia, Rockville, MD) plasmids using Attractene (Qiagen, Germantown, MD) per manufacturer's instructions. After 24 hours, the transfection media was replaced with fresh culture media and cells treated with neomycin to select for transfected cells. Cells

were also monitored for GFP expression. Upon stable selection, pooled populations were confirmed by qPCR (Supplemental Figure 2a–b) and western blot (Supplemental Figure 2c).

Statistical analyses

Statistical analyses were carried out with GraphPad Prism software (Graph-Pad Software, Inc., San Diego, CA, USA). Studies involving more than two groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc multiple comparison tests. All others were subjected to unpaired Student's t-test, with $p < 0.05$ considered statistically significant.

Results

LBH589 alters TNBC morphology and inhibits EMT-associated gene expression

Our previously published findings suggest a partial reversal of EMT in TNBC following LBH589 treatment as indicated by increased CDH1 expression and cell morphology changes [11]. We further examined this effect in a long-term morphological crystal violet (CV) assay to compare the effects of LBH589 to two other known HDACi, SAHA (class I and II inhibitor) and TMP269 (class IIa inhibitor). Figure 1a depicts representative images of MDA-MB-231, BT-549, and MCF-7 cells treated with LBH589, SAHA, TMP269 (10nM) or vehicle control (DMSO) for 3 days. Altered morphology suggestive of the reversal of EMT or mesenchymal-to-epithelial transition (MET) is evident in the majority of cells treated with LBH589; similar effects were not observed in the other treatment groups until much higher doses (SAHA 100nM, TMP269 1–10 μ M; data not shown). Quantitation of the CV assay dose response (10nM–10 μ M) in MDA-MB-231 cells demonstrated a similar effect on cell proliferation by LBH589 as previously observed [11], while SAHA and TMP269 were much less effective (Figure 1b). Similar results were observed in BT-549 and MCF-7 cells (Supplemental Fig 1a–b). Gene expression changes of known EMT-regulated genes were also examined following treatment with HDACi. LBH589 shows a significant increase in CDH1 and a decrease in VIM, ZEB1 and ZEB2, while SAHA and TMP269 show no significant effects in EMT-related gene expression in MDA-MB-231 (100nM for 24 hours) (Figure 1c). A similar trend in EMT-regulated gene expression was observed in the BT-549 cells when treated with LBH589, while no effect or a reversed effect was observed with the other HDACi (Supplemental Fig 1c). Additionally, MCF-7 cells showed no effect on EMT gene expression when treated with LBH589 (Supplemental Fig 1d). The effect of LBH589 on breast cancer cell morphology were further examined using the electrical cell-substrate impedance sensing (ECIS) system, which monitors the flow of alternating current under and between cell culture monolayers to determine changes in surface coverage and cell-cell junctions in real time. ECIS studies revealed a nearly immediate increase in resistance (~4900 to ~6800 Ohm) and decrease in capacitance (~3.5 to ~2.2 nF) of MDA-MB-231 cell monolayers following LBH589 treatment (Supplemental Figure 2a–b). The resistance and capacitance of MCF-7 cells treated with LBH589 remained unchanged (Supplemental Figure 2c–d). These data indicate a rapid increase in cell-cell tight junctions (resistance) and surface coverage (capacitance) of MDA-MB-231 cells treated with LBH589, consistent with the morphology changes observed and a more epithelial phenotype.

LBH589 reduces the migratory and invasive potential of TNBC cells *in vitro*

To determine if these changes translated to altered biological responses, the effects of LBH589 on cell migration and invasion were tested. Treatment with LBH589 for 24 hours reduced both the migratory (Figure 1d) and invasive (Figure 1e) abilities of all cell lines tested; however, the most dramatic effects were observed in the MDA-MB-231 and BT-549 TNBC cell lines, with the ER-positive MCF-7 cells being affected to a lesser extent.

LBH589 reduces metastasis of MDA-MB-231 cells to the lung and brain *in vivo*

The EMT phenotype, cell migration and invasion have all been linked to enhanced metastatic potential of cancer cells [12]. Our observation of increased expression of the epithelial cell marker CDH1 as well as inhibition of the migratory and invasive responses of TNBC cell led us to examine the tissues of animals from our previously published *in vivo* study for distant metastases [11]. SCID/beige mice injected in the mammary fat pad with MDA-MB-231-tRFP cells were treated with i.p. injections of LBH589 (10mg/kg) or vehicle control for 28 days. At necropsy, lungs were harvested and fixed in 10% formalin for 2 days and then processed and embedded in paraffin. Sections were cut and stained for H&E (Figure 2a, first column), or anti-RFP (Figure 2a, second column) with DAPI nuclear stain (Figure 2a, third column), and the number of RFP-positive cells quantified. Higher magnification inserts are shown of the red-bordered area and white arrows indicate areas of metastatic cells within the lung. As shown in Figure 2b, LBH589 treatment significantly reduced the number of metastatic cells present in the lung ($p<0.05$). Additionally, the LBH589 treated animals also demonstrated a significant reduction in metastatic cells present in the brain compared to vehicle treated cells (Figure 2c, $p<0.001$).

LBH589 treatment alters the expression of EMT-associated gene reflective of inhibition of EMT

These results along with our previously observed LBH589-induction of CDH1 expression in TNBC cells [11] led us to further examine the effects of LBH589 treatment on EMT. Using qPCR superarrays, 84 EMT-associated genes were examined following treatment with LBH589 for 24 hours in two triple-negative cell lines (MDA-MB-231, BT-549) and an estrogen receptor-positive, luminal cell line (MCF-7). Table 1 summarizes the significant changes in gene expression common to the triple-negative cell lines (full array data available in Supplemental Tables 1–3). Genes differentially expressed between subtypes (TNBC vs. luminal) are indicated in bold print. LBH589 treatment significantly induced expression of 24 genes while significantly repressing 4 genes in the TNBC cell lines. Of those genes upregulated by LBH589, eight are known to be downregulated during EMT or metastasis (*CDH1*, *ERBB3*, *F11R*, *FGFBP1*, *KRT19*, *RGS2*, *TFPI2*, *TSPAN13*) [23–36]. Additionally, all of the genes significantly downregulated by LBH589 in TNBC are commonly upregulated during EMT (*FZD7*, *WNT5B*, *ZEB1*, *ZEB2*) [17, 37–40]. These data suggest that LBH589 induces an anti-EMT gene expression profile in TNBC cell lines. Interestingly, LBH589 treatment differentially affected expression of EMT genes in luminal A (MCF-7) cells compared to the triple-negative subtype, again suggesting subtype-specific effects of LBH589 for the more aggressive TNBC subtype noted in our previous publication [11].

Forced expression of ZEB1 or ZEB2 abrogates the effects of LBH589 on markers of EMT

Again, we found *CDH1* expression levels to be significantly elevated following LBH589 treatment in the two triple-negative lines, while no change was observed in the ER-positive line. Additionally, expression of *ZEB1* and *ZEB2*, direct regulators of *CDH1* expression [17, 40], were significantly suppressed by LBH589 in both the MDA-MB-231 and BT-549 TNBC cell lines, while no change was detected in the MCF-7 samples. To determine if the effects of LBH589 on EMT in TNBC could be overcome by exogenous overexpression of *ZEB1* or *ZEB2*, MDA-MB-231 cells were transfected with *ZEB1*, *ZEB2*, or vector expression plasmids. Following selection, stable expression of *ZEB1* and *ZEB2* was confirmed by qPCR (Supplemental Figure 3a and b, respectively) and western blot (Supplemental Figure 3c). Furthermore, treatment with LBH589 for 24 hours further stimulated the expression of *ZEB1* in the MDA-MB-231-ZEB1 cells (Supplemental Figure 3d) and *ZEB2* in the MDA-MB-231-ZEB2 cells (Supplemental Figure 3e). Forced expression of either *ZEB1* or *ZEB2* modestly, but significantly, reversed the inhibitory effects of LBH589 on MDA-MB-231 cell proliferation (Supplemental Figure 3f).

ZEB1/2 expression inhibits LBH589-induced tumor suppressive activity

Most profound, however, were the effects of ZEB overexpression on LBH589-mediated regulation of migration, invasion, and *CDH1* gene expression in the MDA-MB-231 cells. The role of ZEBs in LBH589 regulation of cell motility and invasion was tested via migration and invasion assays. MDA-MB-231 –vector, *ZEB1*, or *ZEB2* cells were plated in transwell migration (Figure 3a) or Matrigel® coated invasion (Figure 3b) assays. Cells were treated with vehicle or LBH589 (100nM) for 6 and 24 hours, respectively. The overexpression of either *ZEB1* or *ZEB2* in MDA-MB-231 cells significantly reversed the ability of LBH589 to inhibit the *in vitro* migration of MDA-MB-231 cells as well as invasion through Matrigel®. *ZEB1* or *ZEB2* expression also inhibited LBH589-induced *CDH1* gene expression to near control levels (Figure 3c). These changes were confirmed at the protein level by *CDH1* ELISA, which demonstrated a significant reduction of *CDH1* expression following LBH589 treatment in the MDA-MB-231-ZEB1 and –ZEB2 cell lines (Figure 3d).

An *in vivo* model was next used to determine if the anti-tumor effects of LBH589 on TNBC cells previously reported were mediated by regulation of ZEBs. MDA-MB-231-ZEB1 or –ZEB2 cells were injected orthotopically into the mammary fat pad of female SCID/beige mice. Following tumor formation (day 6 post injection), animals were randomized into treatment groups to receive daily injections of either LBH589 (10mg/kg/day, five days/week) or vehicle for 28 days. While LBH589 treatment significantly inhibited tumor growth in the MDA-MB-231-ZEB1 injected mice (Figure 4a) to similar levels observed in MDA-MB-231 parental cells treated with LBH589, *ZEB2* overexpression mitigated the inhibitory effects of LBH589 on MDA-MB-231 tumorigenesis (Figure 4b). We previously demonstrated that LBH589 treatment induced *CDH1* protein expression in MDA-MB-231 tumors [11]. However, immunohistochemical staining for *CDH1* in tumors from the present study revealed no observable changes in the LBH589 treated *ZEB1* or *ZEB2* overexpressing tumors compared to their vehicle-treated counterparts (Supplemental Figure 5). Positive staining for *CDH1* in the normal luminal ducts within the mammary fat pad tissue adjacent

to tumors served as a positive control (bottom panels). These results indicate the effects of ZEB overexpression on CDH1 expression observed *in vitro* translates to the *in vivo* setting as well.

Expression of **ZEB2** significantly suppresses the effects of LBH589 on EMT gene expression

ZEB2 expression appeared to be more affected by LBH589 treatment compared to *ZEB1* at the mRNA level, and appeared to have a greater effect on mediating the LBH589-induced effects on migration, invasion, CDH1 gene expression, and most apparently xenograft tumorigenesis. Therefore, MDA-MB-231-ZEB2 cells were examined by qPCR EMT arrays to determine the overall impact of *ZEB2* expression on EMT-related gene changes induced by LBH589. As seen in figure 5, *ZEB2* overexpression significantly inhibited LBH589-induced expression of *CDH1*, *ERBB3*, *TSPAN13*, and *WNT11* (Figure 5a) and LBH589-repressed expression of *WNT5B*, *ZEB1*, and *ZEB2* (Figure 5b; full array data available in Supplemental Table 4). These data demonstrate that the effects of LBH589 on TNBC cell biology are regulated at least in part through suppression of ZEB expression, specifically *ZEB2*.

Discussion

Despite our knowledge of the highly aggressive and metastatic nature of the triple-negative subtype of breast cancer, we have yet to discover truly effective therapies to target this subset of disease. Previously, development of many new therapies was centered on targeting the primary tumor; however, the majority of breast cancer deaths are due to distant metastasis [41]. Once thought to be a late stage event, metastasis has been documented to occur quite early in the progression of breast cancer, particularly in the more aggressive subtypes including TNBC [42]. Therefore, more focus should be placed on targeting the early pro-metastatic events. The epithelial-to-mesenchymal transition is thought to be one such event, critical to the ability of cancer cells to escape the primary tumor and aid in the development of distant metastases [12]. Identifying agents that can inhibit or reverse this process would be beneficial in the development of anti-metastatic therapeutics.

There have been conflicting findings as to the effects of HDAC inhibitors on cancer metastasis [43–45] and EMT [46–48]. Our previous research and the work presented here demonstrate the ability of the pan-DAC inhibitor LBH589 (Panobinostat) to inhibit the expression of classical EMT-associated genes and induce expression of CDH1 in TNBC cell lines but not in luminal (Table 1) or basal A breast cancer cell lines [11]. These findings are supported by recent work by Fortunati et al. that demonstrated LBH589 induction of CDH1 in MDA-MB-231 cells independent of estrogen receptor expression [49]. We also showed LBH589 to be much more potent in its anti-EMT effects than other known HDAC inhibitors, SAHA and TMP269. Additionally, these alterations in EMT gene expression correlate to diminished cell migration and invasion of TNBC *in vitro*, as well as significant inhibition of TNBC cell metastasis to both brain and lung in a xenograft model.

Though LBH589 induced expression of many genes in our qPCR array, we saw significant inhibition of several predominant pro-EMT genes including *ZEB1* and *ZEB2*. Furthermore,

forced exogenous expression of *ZEB1* or *ZEB2* in MDA-MB-231 cells was able to abrogate the inhibitory effects of LBH589 treatment on cell migration, invasion, and CDH1 expression. Known to be significant in direct repression of CDH1 gene expression by binding to the *CDH1* promoter, it has recently been shown that the ZEBs also recruit Class I HDAC complexes as co-repressors to inhibit the expression of CDH1 [48, 50–51]. LBH589 is classified as a pan-DAC inhibitor with potent activity against Class I, II and IV HDAC enzymes, making it likely that, in addition to inhibition of ZEB expression, LBH589 is able to prevent ZEB-mediated repression of *CDH1* by inhibiting the Class I HDACs co-repressors. While ZEB2 expression was able to overcome the anti-tumor effects of LBH589 *in vivo*, ZEB1 expression did not have the same effect. Additionally, no change in CDH1 expression was observed in the tumors of either cell line following treatment with LBH589. These results indicate that while LBH589 inhibition of ZEB1 may be involved in the stimulation of CDH1 *in vitro*, ZEB1 expression alone may not be sufficient to overcome the effects of LBH589 on CDH1 expression *in vivo*. Rather, the effects of LBH589 on tumorigenesis appear to be regulated more through the repression of ZEB2. Our work here coupled with our previous work support the anti-cancer effects of LBH589 in solid tumors, specifically the triple-negative subtype of breast cancer. Through our studies we have shown that not only does LBH589 work to inhibit TNBC primary tumorigenesis, but LBH589 also inhibits EMT and thus the metastatic potential of TNBC cells. These findings combined with its low nanomolar activity, and favorable clinical responses with limited toxicity in both hematological and solid cancers including breast, make LBH589 an ideal candidate for further development as an anti-cancer and anti-metastasis therapy in TNBC disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by: The Department of Defense Breast Cancer Research Program BC085426 (BM Collins-Burow); The National Institutes of Health/National Center for Research Resources P20RR020152 (BM Collins-Burow) and CA125806 (ME Burow); The National Science Foundation CMMI-1200201/1200247 (DB Chrisey). The funders did not have any involvement in study design; the collection, analysis, or interpretation of the data; the writing of the manuscript; or the decision to submit the manuscript for publication.

References

1. Schneider BP, Winer EP, Foulkes WD, Garber J, Perou CM, Richardson A, et al. Triple-negative breast cancer: risk factors to potential targets. *Clin Cancer Res.* 2008; 14:8010–18. [PubMed: 19088017]
2. Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, et al. Triple-negative breast cancer: clinical features and patterns of recurrence. *Clin Cancer Res.* 2007; 13:4429–34. [PubMed: 17671126]
3. Cleere DW. Triple-negative breast cancer: a clinical update. *Community Oncol.* 2010; 7:203–11.
4. Anders CK, Carey LA. Biology, metastatic patterns, and treatment of patients with triple-negative breast cancer. *Clin Breast Cancer.* 2009; 9:S73–81. [PubMed: 19596646]
5. De Laurentiis M, Cianniello D, Caputo R, Stanzione B, Arpino G, Cinieri S, et al. Treatment of triple negative breast cancer (TNBC): current options and future perspectives. *Cancer Treat Rev.* 2010; 36:S80–6. [PubMed: 21129616]

6. Chacon RD, Costanzo MV. Triple-negative breast cancer. *Breast Cancer Res.* 2010; 12:S3. [PubMed: 21050424]
7. O'Driscoll L, Clynes M. Biomarkers and multiple drug resistance in breast cancer. *Curr Cancer Drug Targets.* 2006; 6:365–84. [PubMed: 16918307]
8. Drummond DC, Noble CO, Kirpotin DB, Guo Z, Scott GK, Benz CC. Clinical development of histone deacetylase inhibitors as anticancer agents. *Annu Rev Pharmacol Toxicol.* 2005; 45:495–528. [PubMed: 15822187]
9. Liu T, Kuljaca S, Tee A, Marshall GM. Histone deacetylase inhibitors: multifunctional anticancer agents. *Cancer Treat Rev.* 2006; 32:157–65. [PubMed: 16516391]
10. Atadja P. Development of the pan-DAC inhibitor Panobinostat (LBH589): successes and challenges. *Cancer Lett.* 2009; 280:233–41. [PubMed: 19344997]
11. Tate CR, Rhodes LV, Segar HC, Driver JL, Pounder FN, Burow ME, Collins-Burow BM. Targeting triple-negative breast cancer cells with the histone deacetylase inhibitor panobinostat. *Breast Cancer Research.* 2012; 14(3):R79. [PubMed: 22613095]
12. Guarino M, Rubino B, Ballabio G. The role of epithelial mesenchymal transition in cancer pathology. *Pathology.* 2007; 39:305–18. [PubMed: 17558857]
13. Thiery JP, Sleeman JP. Complex networks orchestrate epithelial-mesenchymal transitions. *Nat Rev Mol Cell Biol.* 2006; 7:131–42. [PubMed: 16493418]
14. Schmalhofer O, Brabletz S, Brabletz T. E-cadherin, beta-catenin, and ZEB1 in malignant progression of cancer. *Cancer Metastasis Rev.* 2009; 28:151–66. [PubMed: 19153669]
15. Bex G, Raspe E, Christofori G, Thiery JP, Sleeman JP. Pre-EMTing metastasis? Recapitulation of morphogenetic processes in cancer. *Clin Exp Metastasis.* 2007; 24:587–97. [PubMed: 17978854]
16. Kong D, Li Y, Wang Z, Sarkar FH. Cancer stem cells and epithelial-to-mesenchymal transition (EMT) – phenotypic cells: are they cousins or twins? *Cancers.* 2011; 3:716–29. [PubMed: 21643534]
17. Eger A, Aigner K, Sonderegger S, Dampier B, Oehler S, Schreiber M, et al. DeltaEF1 is a transcriptional repressor of E-cadherin and regulates epithelial plasticity in breast cancer cells. *Oncogene.* 2005; 24:2375–85. [PubMed: 15674322]
18. Comijn J, Bex G, Vermassen P, Verschueren K, van Grunsven L, Bruyneel E, et al. The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. *Mol Cell.* 2001; 7:1267–78. [PubMed: 11430829]
19. Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, et al. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell.* 2006; 10:515–27. [PubMed: 17157791]
20. Rhodes LV, Muir SE, Elliott S, Guillot LM, Antoon JW, Penfornis P, et al. Adult human mesenchymal stem cells enhance breast tumorigenesis and promote hormone independence. *Breast Cancer Res Treat.* 2010; 121:293–300. [PubMed: 19597705]
21. Zhou C, Zhong Q, Rhodes LV, Townley I, Bratton MR, Zhang Q, et al. Proteomic analysis of acquired tamoxifen resistance in MCF-7 cells reveals expression signatures associated with enhanced migration. *Breast Cancer Res.* 2012; 14(2):R45. [PubMed: 22417809]
22. Marsden CG, Wright MJ, Carrier L, Moroz K, Pochampally R, Rowan BG. A novel in vivo model for the study of human breast cancer metastasis using primary breast tumor-initiating cells from patient biopsies. *BMC Cancer.* 2012; 12:10. [PubMed: 22233382]
23. Cowin P, Rowlands TM, Hatsell SJ. Cadherins and catenins in breast cancer. *Curr Opin Cell Biol.* 2005; 17(5):499–508. [PubMed: 16107313]
24. Nozato M, Kaneko S, Nakagawara A, Komuro H. Epithelial-mesenchymal transition-related gene expression as a new prognostic marker for neuroblastoma. *Int J Oncol.* 2013; 42(1):134–40. [PubMed: 23135478]
25. Feng MY, Wang K, Shi QT, Yu XW, Geng JS. Gene expression profiling in TWIST-depleted gastric cancer cells. *Anat Rec.* 2009; 292(2):262–70.
26. Fuchs BC, Fujii T, Dorfman JD, Goodwin JM, Zhu AX, Lanuti M, et al. Epithelial-to-mesenchymal transition and integrin-linked kinase mediate sensitivity to epidermal growth factor receptor inhibition in human hepatoma cells. *Cancer Res.* 2008; 68(7):2391–9. [PubMed: 18381447]

27. Naik MU, Naik TU, Suckow AT, Duncan MK, Naik UP. Attenuation of junctional adhesion molecule-A is a contributing factor for breast cancer cell invasion. *Cancer Res.* 2008; 68(7):2194–203. [PubMed: 18381425]
28. Abuharbeid S, Czubyko F, Aigner A. The fibroblast growth factor-binding protein FGF-BP. *Int J Biochem Cell Biol.* 2006; 38(9):1463–8. [PubMed: 16324873]
29. Kokkinos MI, Wafai R, Wong MK, Newgreen DF, Thompson EW, Waltham M. Vimentin and epithelial-mesenchymal transition in human breast cancer—observations in vitro and in vivo. *Cells Tissues Organs.* 2007; 185(13):191–203. [PubMed: 17587825]
30. Lorient C, Burnichon N, Gadessaud N, Vescovo L, Amar L, Libé R, et al. Epithelial to mesenchymal transition is activated in metastatic pheochromocytomas and paragangliomas caused by SDHB gene mutations. *J Clin Endocrinol Metab.* 2012; 97(6):E954–62. [PubMed: 22492777]
31. Zhou C, Nitschke AM, Xiong W, Zhang Q, Tang Y, Bloch M, et al. Proteomic analysis of tumor necrosis factor-alpha resistant human breast cancer cells reveals a MEK5/Erk5-mediated epithelial-mesenchymal transition phenotype. *Breast Cancer Res.* 2008; 10(6):R105. [PubMed: 19087274]
32. Jiang Z, Wang Z, Xu Y, Wang B, Huang W, Cai S. Analysis of RGS2 expression and prognostic significance in stage II and III colorectal cancer. *Biosci Rep.* 2010; 30(6):383–90. [PubMed: 20001967]
33. Takada H, Wakabayashi N, Dohi O, Yasui K, Sakakura C, Mitsufuji S, et al. Tissue factor pathway inhibitor 2 (TFPI2) is frequently silenced by aberrant promoter hypermethylation in gastric cancer. *Cancer Genet Cytogenet.* 2010; 197(1):16–24. [PubMed: 20113832]
34. Hibi K, Goto T, Shirahata A, Saito M, Kigawa G, Nemoto H, et al. Detection of TFPI2 methylation in the serum of gastric cancer patients. *Anticancer Res.* 2011; 31(11):3835–8. [PubMed: 22110206]
35. Huang H, Sossey-Alaoui K, Beachy SH, Geradts J. The tetraspanin superfamily member NET-6 is a new tumor suppressor gene. *J Cancer Res Clin Oncol.* 2007; 133(10):761–9. [PubMed: 17486367]
36. Huang H, Groth J, Sossey-Alaoui K, Hawthorn L, Beall S, Geradts J. Aberrant expression of novel and previously described cell membrane markers in human breast cancer cell lines and tumors. *Clin Cancer Res.* 2005; 11(12):4357–64. [PubMed: 15958618]
37. Vincan E, Swain RK, Brabletz T, Steinbeisser H. Frizzled7 dictates embryonic morphogenesis: implications for colorectal cancer progression. *Front Biosci.* 2007; 12:4558–67. [PubMed: 17485397]
38. Morioka K, Tanikawa C, Ochi K, Daigo Y, Katagiri T, Kawano H, et al. Orphan receptor tyrosine kinase ROR2 as a potential therapeutic target for osteosarcoma. *Cancer Sci.* 2009; 100(7):1227–33. [PubMed: 19486338]
39. Bradley EW, Drissi MH. Wnt5b regulates mesenchymal cell aggregation and chondrocyte differentiation through the planar cell polarity pathway. *J Cell Physiol.* 2011; 226(6):1683–93. [PubMed: 21413026]
40. Vandewalle C, Comijn J, De Craene B, Vermassen P, Bruyneel E, Andersen H, et al. SIP1/ZEB2 induces EMT by repressing genes of different epithelial cell-cell junctions. *Nucleic Acids Res.* 2005; 33(20):6566–78. [PubMed: 16314317]
41. Weigelt B, Peterse JL, van 't Veer LJ. Breast cancer metastasis: markers and models. *Nat Rev Cancer.* 2005; 5:591–602. [PubMed: 16056258]
42. Scully OJ, Bay BH, Yip G, Yu Y. Breast Cancer Metastasis. *Cancer Genomics and Proteomics.* 2012; 9:311–20. [PubMed: 22990110]
43. Wang F, Qi Y, Li X, He W, Fan QX, Zong H. HDAC inhibitor trichostatin A suppresses esophageal squamous cell carcinoma metastasis through HADC2 reduced MMP-2/9. *Clin Invest Med.* 2013; 36(2):E87–94. [PubMed: 23544610]
44. Shan Z, Feng-Nian R, Jie G, Ting Z. Effects of valproic acid on proliferation, apoptosis, angiogenesis and metastasis of ovarian cancer in vitro and in vivo. *Asian Pac J Cancer Prev.* 2012; 13(8):3977–82. [PubMed: 23098503]
45. Lin KT, Wang YW, Chen CT, Ho CM, Su WH, Jou YS. HDAC inhibitors augmented cell migration and metastasis through induction of PKCs leading to identification of low toxicity

- modalities for combination cancer therapy. *Clin Cancer Res.* 2012; 18(17):4691–701. [PubMed: 22811583]
46. Jiang GM, Wang HS, Zhang F, Zhang KS, Liu ZC, Fang R, et al. Histone deacetylase inhibitor induction of epithelial-mesenchymal transitions via up-regulation of Snail facilitates cancer progression. *Biochim Biophys Acta.* 2013; 1833(3):663–71. [PubMed: 23246564]
47. Kong D, Ahmad A, Bao B, Li Y, Banerjee S, Sarkar FH. Histone deacetylase inhibitors induce epithelial-to-mesenchymal transition in prostate cancer cells. *PLoS One.* 2012; 7(9):e45045. [PubMed: 23024790]
48. Wang Y, Shang Y. Epigenetic control of epithelial-to-mesenchymal transition and cancer metastasis. *Exp Cell Res.* 2013; 319(2):160–9. [PubMed: 22935683]
49. Fortunati N, Marano F, Bandino A, Frairia R, Catalano MG, Boccuzzi G. The pan-histone deacetylase inhibitor LBH589 (panobinostat) alters the invasive breast cancer cell phenotype. *Int J Oncol.* 2014; 44(3):700–8. [PubMed: 24366407]
50. Aghdassi A, Sendler M, Guenther A, Mayerle J, Behn CO, Heidecke CD, et al. Recruitment of histone deacetylases HDAC1 and HDAC2 by the transcriptional repressor ZEB1 downregulates E-cadherin expression in pancreatic cancer. *Gut.* 2012; 61(3):439–48. [PubMed: 22147512]
51. Kakhana M, Ohira T, Chan D, Webster RB, Kato H, Drabkin HA, et al. Induction of E-cadherin in lung cancer and interaction with growth suppression by histone deacetylase inhibition. *J Thorac Oncol.* 2009; 4(12):1455–65. [PubMed: 20009910]

Fig. 1. LBH589 alters morphology, EMT-associated gene expression, and attenuates the migratory and invasive properties of triple-negative breast cancer cells *in vitro*

a. 7.5×10^3 cells were seeded in 96-well plate and treated with LBH589, SAHA, TMP269 (10nM) or vehicle (DMSO). After 3 days, cells were stained with crystal violet and morphology examined. Original magnification, 200 $\times$. **b.** MDA-MB-231 cells seeded at 7.5×10^3 in a 96-well plate were treated with LBH589, SAHA, TMP269 (10nM–10 μ M) or vehicle (DMSO). After 3 days, cells were stained with CV and lysed. Points indicate mean absorbance at 630nm $\pm$ SEM. Similar results were observed in BT-549 but not MCF-7 cells (Supplemental Fig. 1a–b). **c.** EMT-related gene expression analysis (qPCR) of MDA-MB-231 cells treated with LBH589, SAHA, TMP269 (100nM) or vehicle (DMSO) or 24 hours. Similar trends were seen in BT-549 cells but not MCF-7 cells (Supplemental Fig. 1c–d). **d.** 2.5×10^4 cells or **e.** 5×10^4 cells were seeded in the upper chamber of a transwell insert (8 μ M pore size) alone (**d.** migration) or coated with Matrigel® (**e.** invasion). Cells were treated with LBH589 (100nM) or vehicle control at time of plating. Lower wells contained DMEM supplemented with 10% FBS. After 24 hours, migrated or invaded cells were fixed and stained for visualization. Bars represent relative migration or invasion compared to MCF-7 cell vehicle treatment set to $1 \pm$ SEM. Experiments were conducted in triplicate. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Fig. 2. LBH589 inhibits *in vivo* metastasis of triple-negative breast cancer cells

MDA-MB-231-tRFP cells were injected into the MFP of 4–6 week old SCID/beige female mice. After 41 days, animals were sacrificed and organs removed for further analysis. **a.** Representative sections of FFPE lung tissue stained for H&E or IF against RFP with DAPI nuclear stain. Original magnification, 100 $\times$. **b.** Quantification of RFP positive cells in lung samples. Points represent ratio of RFP positive cells to number of DAPI nuclei. Black bars represent mean of each group, red bars represent SEM. $N=5$ mice per group with 3 fields of view per mouse. **c.** Following necropsy, brains were immediately dissociated and examined by flow cytometry for RFP. Bars represent average number of RFP positive cells per sample $\pm$ SEM for triplicate samples. *, $p<0.05$, ***, $p<0.001$.

Fig. 3. Overexpression of ZEB1 or ZEB2 inhibits the anti-invasive effects of LBH589 on MDA-MB-231 cells

a. 2.5×10^4 cells or **b.** 5×10^4 cells were seeded in the upper chamber of a transwell insert (8μM pore size) alone (**a.** migration) or coated with Matrigel® (**b.** invasion). Cells were treated with LBH589 (100nM) or vehicle control at time of plating. Lower wells contained DMEM supplemented with 10% FBS. After 6 hours (**a.**) or 24 hours (**b.**), migrated or invaded cells were fixed and stained for visualization. Bars represent mean normalized migration or invasion compared to vehicle treated cells set to 100% ± SEM for each cell line.

Experiments were conducted in triplicate. **c.** qPCR analysis of CDH1 expression in the MDA-MB-231 stable cell lines treated with LBH589 (100nM) for 24 hours compared to vehicle (DMSO) treated cells (set to one) for each cell line. Bars represent average normalized fold change ± SEM of triplicate experiments. **d.** CDH1 protein expression changes in MDA-MB-231-vector, -ZEB1, and -ZEB2 following treatment with LBH589 (100nM) for 24 hours determined by ELISA. Data represented as average mean (normalized to vehicle treated control) ± SEM of triplicate experiments. ***, $p < 0.001$ compared to control; δ, $p < 0.05$, δδ, $p < 0.01$, δδδ, $p < 0.001$ compared to LBH589 treated vector cells.

Fig. 4. ZEB2 overexpression in MDA-MB-231 cells negates the anti-tumor effects of LBH589 *in vivo*

a. MDA-MB-231-ZEB1 or **b.** MDA-MB-231-ZEB2 cells were injected bilaterally into the MFP of 4–6 week old SCID/beige female mice. Animals were treated 5days/week with injections of LBH589 (10mg/kg/mouse/day) or vehicle control for 28 days beginning at day 6 post cell injection. Data points represent mean tumor volume normalized to first day of treatment $\pm$ SEM. N=10 tumors/group. **, $p < 0.01$ compared to vehicle treated group on day 34 post cell injection. **c.** FFPE tumor sections (from a–b) were stained with human anti-CDH1 and representative sections from each treatment group shown. Bottom row, images of positive CDH1 staining in normal duct structures. Original magnification, 400 $\times$. Scale bar, 50 μ m.

Fig. 5. ZEB2 expression inhibits the effects of LBH589 on EMT-associated genes in MDA-MB-231 cells

MDA-MB-231-ZEB2 cells were treated with LBH589 (100 nM) or vehicle (DMSO) for 24 hours and analyzed for alterations in EMT-associated genes using the human EMT RT² ProfilerTM PCR Arrays. Data from ZEB2 overexpressing cell line was compared to previously generated parental data (Table 1, Supplemental Table 1). Graphs represent average fold change of LBH589 treated samples compared to corresponding vehicle treated samples for a select subset of significantly altered genes (**a–b**, up- and down-regulated genes, respectively). Full data available in Supplemental Table 4. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared to LBH589 treated parental data. Arrays were conducted in triplicate.

Table 1

EMT-associated gene changes induced by LBH589.

Gene	MDA-MB-231	BT-549	MCF-7
CDH1	27.2	5.6	(-1.1)
ERBB3	4.4	8.8	-2.3
F11R	2.5	3.4	2.0
FGFBP1	15.0	3.1	23.3
FN1	2.2	1.5	2.6
FZD7	-1.6	-3.4	(-1.5)
GSC	1.5	4.8	-3.9
GSK3B	2.2	1.6	(1.6)
KRT19	3.9	4.1	(2.0)
MITF	5.0	2.7	(1.6)
MMP9	19.2	1.4	(1.1)
NODAL	5.4	5.8	6.1
PDGFRB	3.6	11.4	3.1
PTP4A1	2.0	3.3	(1.5)
RGS2	6.5	5.7	5.6
SERPINE1	4.2	6.5	51.0
SNAI1	11.9	6.8	18.1
SNAI2	2.4	2.1	2.2
SNAI3	6.1	23.8	7.2
SPP1	15.3	15.7	42.2
STEAP1	1.6	3.1	(43.7)
TFPI2	8.2	4.5	6.5
TSPAN13	59.5	4.8	(1.2)
VPS13A	2.1	1.7	(1.2)
WNT11	42.3	24.9	4.4
WNT5B	-4.1	-4.1	(9.4)
ZEB1	-1.8	-1.6	7.6
ZEB2	-3.2	-1.7	(1.2)

Cells treated with LBH589 (100nM) for 24 hours, conducted in triplicate. Values represent fold regulation compared to vehicle treated controls for each cell line, $p < 0.05$. Parentheses indicate non-significant changes. Bolded genes indicate differential regulation between basal-like (MDA-MB-231, BT-549) and luminal (MCF-7) subtypes.