

# Functional cooperation of miR-125a, miR-125b, and miR-205 in entinostat-induced downregulation of erbB2/erbB3 and apoptosis in breast cancer cells

S Wang<sup>1,2</sup>, J Huang<sup>1,3</sup>, H Lyu<sup>1</sup>, C-K Lee<sup>4,5</sup>, J Tan<sup>2</sup>, J Wang<sup>\*3</sup> and B Liu<sup>\*1</sup>

We reported that the class I HDAC inhibitor entinostat induced apoptosis in erbB2-overexpressing breast cancer cells via downregulation of erbB2 and erbB3. Here, we study the molecular mechanism by which entinostat dual-targets erbB2/erbB3. Treatment with entinostat had no effect on *erbB2/erbB3* mRNA, suggesting a transcription-independent mechanism. Entinostat decreased endogenous but not exogenous erbB2/erbB3, indicating it did not alter their protein stability. We hypothesized that entinostat might inhibit erbB2/erbB3 protein translation via specific miRNAs. Indeed, entinostat significantly upregulated miR-125a, miR-125b, and miR-205, that have been reported to target *erbB2* and/or *erbB3*. Specific inhibitors were then used to determine whether these miRNAs had a causal role in entinostat-induced downregulation of erbB2/erbB3 and apoptosis. Transfection with a single inhibitor dramatically abrogated entinostat induction of miR-125a, miR-125b, or miR-205; however, none of the inhibitors blocked entinostat action on erbB2/erbB3. In contrast, co-transfection with two inhibitors not only reduced their corresponding miRNAs, but also significantly abrogated entinostat-mediated reduction of erbB2/erbB3. Moreover, simultaneous inhibition of two, but not one miRNA significantly attenuated entinostat-induced apoptosis. Interestingly, although the other HDAC inhibitors, such as SAHA and panobinostat, exhibited activity as potent as entinostat to induce growth inhibition and apoptosis in erbB2-overexpressing breast cancer cells, they had no significant effects on the three miRNAs. Instead, both SAHA- and panobinostat-decreased erbB2/erbB3 expression correlated with the reduction of their mRNA levels. Collectively, we demonstrate that entinostat specifically induces expression of miR-125a, miR-125b, and miR-205, which act in concert to downregulate erbB2/erbB3 in breast cancer cells. Our data suggest that epigenetic regulation via miRNA-dependent or -independent mechanisms may represent a novel approach to treat breast cancer patients with erbB2-overexpressing tumors.

*Cell Death and Disease* (2013) 4, e556; doi:10.1038/cddis.2013.79; published online 21 March 2013

Subject Category: Cancer

The erbB receptor tyrosine kinase (RTK) family, including the epidermal growth factor receptor (EGFR), erbB2 (*HER2/neu*), erbB3, and erbB4, is arguably the most important receptor family in the context of development and tumorigenesis.<sup>1,2</sup> *ErbB2* amplification and/or overexpression occur in ~25–30% of invasive breast cancer and are significantly associated with a worse prognosis in breast cancer patients.<sup>3,4</sup> Although erbB2-targeted therapy, such as Herceptin (or trastuzumab) has been successfully used in breast cancer patients with erbB2-overexpressing tumors,<sup>5</sup> resistance to Herceptin frequently occur and currently represent a significant clinical problem.<sup>6,7</sup> However, erbB2 does not act in isolation, and it

often interacts with other RTKs, such as erbB3, to activate cell signaling. Numerous studies have established the critical role of erbB3 as a co-receptor of erbB2, and the expression of erbB3 is a rate-limiting factor for erbB2-induced breast cancer cell survival and proliferation.<sup>8,9</sup> Thus, novel strategies/agents targeting both erbB2 and erbB3 receptors should be more effective to treat the breast cancer patients whose tumors overexpress erbB2.

Numerous studies indicate that deregulation of histone acetylation and deacetylation has an important role in aberrant gene expression in human cancers.<sup>10,11</sup> Histone deacetylases (HDACs) are relatively easier tractable

<sup>1</sup>Department of Pathology, School of Medicine, University of Colorado Anschutz Medical Campus, Aurora, CO, USA; <sup>2</sup>Fujian Key Laboratory of Transplant Biology, Fuzhou General Hospital, Fuzhou, Fujian, China; <sup>3</sup>State Key Laboratory of Experimental Hematology, Institute of Hematology and Blood Disease Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin, China and <sup>4</sup>Division of Medical Oncology, Department of Medicine, School of Medicine, University of Colorado Anschutz Medical Campus, Aurora, CO, USA

\*Corresponding author: J Wang, State Key Laboratory of Experimental Hematology, Institute of Hematology and Blood Disease Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, 288 Nanjing Road, Tianjin 300020, China. E-mail: wangjx@ihcams.ac.cn or B Liu, Department of Pathology, School of Medicine, University of Colorado Anschutz Medical Campus, MS-8104, 12801 E. 17th Avenue, Aurora, CO 80045, USA. E-mail: bolin.liu@ucdenver.edu

<sup>5</sup>Current address: San Juan Cancer Center, Montrose Memorial Hospital, Montrose, CO, USA.

**Keywords:** entinostat; HDAC; miRNA; erbB2; erbB3; breast cancer

**Abbreviations:** RTK, receptor tyrosine kinase; EGFR, epidermal growth factor receptor; IGF-1R, insulin-like growth factor-I receptor; miRNA, microRNA; HDAC, histone deacetylase; HDACi, HDAC inhibitor; HH3, histone H3; Ac-HH3, acetylated histone H3; TGF $\beta$ , transforming growth factor  $\beta$ ; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; DNMT1, DNA methyltransferase I; PI-3K, phosphoinositide 3-kinase; PARP, poly(ADP-ribose) polymerase; ELISA, enzyme-linked immunosorbent assay; DMEM, Dulbecco's Modified Eagle's Medium; PAGE, polyacrylamide gel electrophoresis; Entinostat, *N*-(2-Aminophenyl)-4-(*N*-(pyridine-3-ylmethoxycarbonyl)aminomethyl)benzamide; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt

Received 20.8.12; revised 31.1.13; accepted 18.2.13; Edited by G Ciliberto

enzymes, and have recently become attractive therapeutic targets. Inhibitors of HDACs exhibit anticancer activity in a variety of tumor cell models via influencing cell cycle progression, apoptosis, differentiation, and tumor angiogenesis.<sup>12,13</sup> Many HDAC inhibitors (HDACi) are currently under clinical investigations as potential anticancer agents.<sup>14,15</sup> Entinostat (also known as MS-275, SNDX-275, Syndax Pharmaceuticals, Inc., Waltham, MA, USA) is a synthetic benzamide derivative class I HDACi. It inhibits cancer cell growth with an IC<sub>50</sub> in the submicromolar range, and exhibits both *in vitro* and *in vivo* activities against various cancer types, including solid tumors and hematologic malignancies.<sup>16</sup> In breast cancers, entinostat has been shown to inhibit cell proliferation and/or promote apoptosis.<sup>17–21</sup> Recent studies suggest that entinostat exerts different effects towards distinct subtypes of human breast cancers. Entinostat increases expression of estrogen receptor  $\alpha$  (ER $\alpha$ ) and aromatase, and restores the responsiveness of letrozole mainly in triple-negative breast cancer cells.<sup>22,23</sup> In studying whether entinostat may also target the erbB receptors, we have shown that entinostat selectively induces apoptosis in erbB2-overexpressing breast cancer cells via downregulation of erbB2/erbB3, but not EGFR expression,<sup>24</sup> and it enhances trastuzumab efficacy and exhibits the potential to overcome trastuzumab resistance.<sup>25</sup> Our results may provide a new treatment option against the aggressive erbB2-overexpressing breast cancers. Nonetheless, the molecular mechanism through which entinostat induces downregulation of erbB2/erbB3 receptors and apoptosis in breast cancer cells remains unknown.

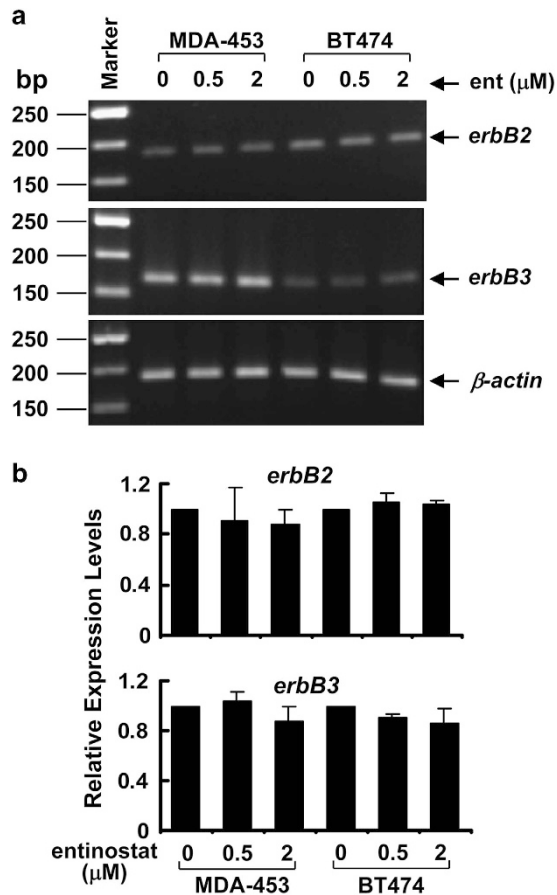
MicroRNAs (miRNAs) are endogenous, small noncoding RNAs that regulate gene expression by targeting mRNAs for degradation or translational repression via sequence-specific recognition.<sup>26,27</sup> Recent studies demonstrate that miRNAs can function as oncogenes or tumor suppressors to regulate tumor cell proliferation and apoptosis, and have highly diverse roles in the development of human cancers,<sup>28–30</sup> including breast cancer.<sup>31</sup> Nonetheless, the underlying mechanisms of miRNA deregulation in human cancer remain unclear. Several mechanisms contributing to miRNA deregulation have been proposed,<sup>32</sup> among which, epigenetic alterations have been found to exhibit profound effects on miRNA-aberrant expression in human cancer.<sup>33,34</sup> For instance, the broad-spectrum HDACi LAQ824 rapidly alters the expression of multiple miRNAs in SKBR3 breast cancer cells.<sup>35</sup> To date, there is no study indicating whether the selective class I HDACi entinostat may modulate erbB2/erbB3 expression through a miRNA-dependent mechanism. In the current study, we test the hypothesis that entinostat downregulates erbB2/erbB3 receptors via induction of specific miRNAs that target *erbB2* and/or *erbB3* in erbB2-overexpressing breast cancer cells.

## Results

**Entinostat does not affect the mRNA levels of *erbB2* and *erbB3* in breast cancer cells.** To explore the molecular mechanism by which entinostat downregulates erbB2 and erbB3 in breast cancer cells, we first studied whether entinostat might modulate *erbB2* and *erbB3* mRNA levels. While treatment with 1  $\mu$ mol/l of entinostat for 24 h clearly

reduced erbB2/erbB3 protein levels in erbB2-overexpressing breast cancer cells,<sup>24</sup> both conventional reverse transcription-PCR and quantitative real-time (qRT)-PCR assays revealed that entinostat when used at similar condition had no significant effect on the mRNA levels of *erbB2* and *erbB3* in MDA-MB-453 and BT474 breast cancer cells (Figure 1). To confirm the results, we designed additional primers amplifying distinct cDNA fragments of human *erbB2* and *erbB3*. With the PCR primers, we again did not observe any change in *erbB2/erbB3* mRNA expression upon entinostat treatment in both SKBR3 and BT474 cells (Supplementary Figure S1). Thus, our findings suggested that entinostat downregulated erbB2/erbB3 receptors through a transcription-independent mechanism.

**Entinostat reduces the protein levels of endogenous, but not exogenous, erbB2 and erbB3.** We next investigated whether entinostat might alter erbB2/erbB3 protein stability. In our previous report, we observed an interesting phenomenon that entinostat specifically reduced the levels of endogenous, but not exogenous, erbB3 in breast cancer cells.<sup>24</sup> Additional studies confirmed that entinostat did not lower the expression of exogenous erbB3 via transient transfection, although the levels of endogenous erbB2 and erbB3 were clearly reduced by entinostat in both MDA-MB-453 and BT474 cells (Figure 2a). Similar results were also observed in SKBR3 cells (Supplementary Figure S2). We then reasoned if entinostat might possess the similar discrimination effects on endogenous and exogenous erbB2. MDA-MB-435 is a human cancer cell line with erbB2 low expression. We generated its erbB2-high expressing clone (435.eB1) in our previous studies.<sup>36</sup> Entinostat reduced the levels of endogenous erbB3 in both lines; however, it did not reduce exogenous erbB2 in 435.eB1 cells (Figure 2b). In fact, the expression levels of exogenous erbB3 and erbB2 were clearly increased upon treatment with entinostat (Figures 2a and b). This is possibly because both *erbB3* and *erbB2* cDNAs are driven by the CMV promoter in the expression vectors,<sup>24,36</sup> as recent studies show that HDAC inhibitors are capable of enhancing CMV promoter activity.<sup>37,38</sup> Furthermore, the mammary tumor cell lines 85815 and 85819 derived from MMTV-*neu* transgenic mice were used to examine the effects of entinostat on endogenous mouse erbB3 and the transgene rat *erbB2/neu*-encoded protein. Cell growth assays showed that entinostat exhibited potent inhibitory effects on cell proliferation/survival (Figure 2c), similar to that we had observed in human breast cancer cells.<sup>24</sup> Interestingly, entinostat reduced the expression levels of endogenous mouse erbB3 in a dose- and time-dependent manner, and increased the levels of the transgene *erbB2/neu*-encoded protein (Figure 2d), consistent with the results we had obtained from human breast cancer cells (Figures 2a and b). The transgene rat *erbB2/neu* (containing a wild-type rat *neu/erbB2* coding sequence, but no original 5'-UTR and 3'-UTR) is driven by a MMTV promoter whose activity may be enhanced or repressed by HDAC inhibition.<sup>39,40</sup> A more detailed study suggest that moderate acetylation of core histones generated with low concentrations of HDACi enhances transcription from MMTV promoter, whereas HDACi at higher concentrations suppress MMTV



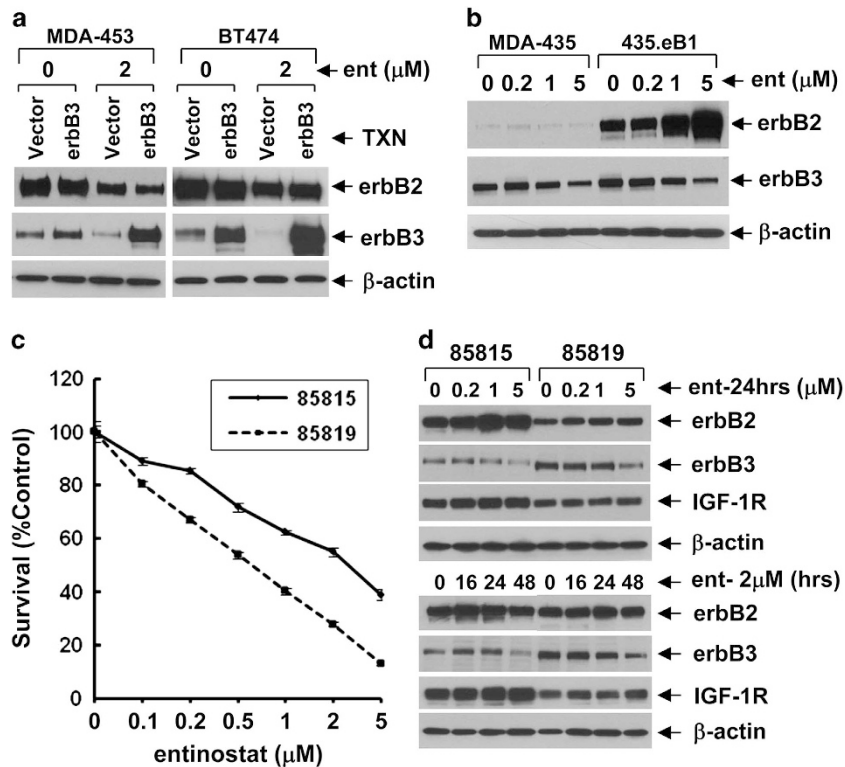
**Figure 1** Treatment with entinostat does not affect mRNA levels of both *erbB2* and *erbB3* in breast cancer cells. MDA-MB-453 (MDA-453) and BT474 cells untreated or treated with entinostat (ent) at indicated concentrations for 24 h were subjected to total RNA extraction. (a) First-strand cDNA was synthesized using a reverse transcription kit from Applied Biosystems. The partial coding sequence of *erbB2*, *erbB3*, or  $\beta$ -actin was amplified with specific primers. The PCR products were separated on a 2% agarose gel containing ethidium bromide and visualized under a UV light. (b) The mRNA levels of *erbB2* and *erbB3* were measured by qRT-PCR. Bars, S.D. The data are representative of three independent experiments

transcription.<sup>41</sup> In our study, entinostat at 0.2, 1, 2, or 5  $\mu$ mol/l might have enhanced MMTV activity, and therefore increased the expression of the transgene *erbB2/neu* (Figure 2d). It appeared that entinostat specifically targeted *erbB3* receptor, as it had no effect on another endogenous, membrane protein IGF-1R (Figure 2d). To confirm this observation, we performed similar studies in human lines, and found no change of IGF-1R in MDA-MB-453, BT474, and SKBR3 cells upon entinostat treatment (data not shown). Taken together, these data provide compelling evidence suggesting that entinostat does not promote *erbB2/erbB3* protein degradation in human and mouse breast/mammary cancer cells. The specific downregulation of endogenous *erbB2/erbB3* induced by entinostat may involve a mechanism of impaired protein translation.

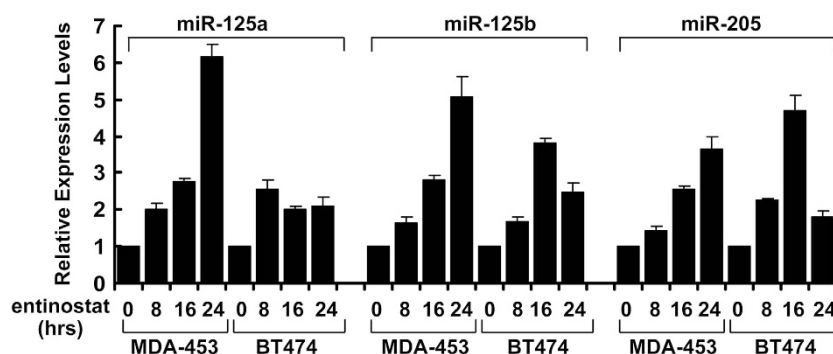
**Entinostat treatment induces expression of the *erbB2/erbB3*-targeting miRNAs in *erbB2*-overexpressing breast cancer cells.** It is well known that miRNAs generally regulate gene expression by two mechanisms: targeting

messenger RNA (mRNA) for degradation or translational repression.<sup>26,27</sup> The mRNA levels of *erbB2/erbB3* remain unchanged upon entinostat treatment (Figure 1a). We therefore hypothesize that entinostat may induce expression of specific miRNAs that target *erbB2/erbB3* to repress their protein translation. It has been shown that HDAC inhibition leads to rapid alteration of miRNA expression,<sup>35</sup> and overexpression of miR-125a or miR-125b results in coordinate suppression of *erbB2* and *erbB3*.<sup>42</sup> Several studies report that miR-205 targets *erbB3* to exert antitumor activity.<sup>43–45</sup> To provide direct evidence supporting our hypothesis, we examined the effects of entinostat on expression of miR-125a, miR-125b, and miR-205 in *erbB2*-overexpressing breast cancer cells. The expression levels of these miRNAs were assessed by qRT-PCR using TaqMan (Applied Biosystems, Foster City, CA, USA) miRNA assays. After normalization with the internal control RNU6B, we found that treatment of MDA-MB-453 and BT474 cells with entinostat upregulated the levels of all three miRNAs in a time-dependent manner (Figure 3). It appeared that the induction of all three miRNAs reached to their highest levels by 16–24 h. Similar results were also observed in SKBR3 cells (data not shown). These studies revealed that entinostat-mediated downregulation of *erbB2/erbB3* correlated with the concomitant induction of miR-125a, miR-125b, and miR-205, which have been reported to target *erbB2* and/or *erbB3* mRNAs.<sup>42–45</sup> Supplementary Figure S3 shows the schematic representation of the binding sites of miR-125a, miR-125b, and miR-205 on 3'-UTRs of human *erbB2* and *erbB3*.

**Entinostat downregulates *erbB2/erbB3* receptors and induces apoptosis in breast cancer cells via functional cooperation of miR-125a, miR-125b, and miR-205.** We next studied whether the induction of these miRNAs had a causal role in entinostat-induced downregulation of *erbB2/erbB3* and apoptosis. Specific miRIDIAN hairpin inhibitors were used in the following studies. We first transfected single inhibitor into MDA-MB-453 or BT474 cells, and the cells were then left untreated or treated with entinostat. Although the miR-125a, miR-125b, or miR-205 inhibitor dramatically abrogated entinostat-induced upregulation of miR-125a, miR-125b, or miR-205, respectively (Figure 4a), none of the inhibitors altered entinostat-induced downregulation of *erbB2/erbB3* (Figure 4b). These data suggest that inactivation of one miRNA may not be sufficient to block entinostat action on *erbB2/erbB3*. To test whether the three miRNAs might work cooperatively to reduce the protein levels of *erbB2* and *erbB3*, we combined two inhibitors and performed co-transfection in MDA-MB-453 and BT474 cells. Similar results were obtained from qRT-PCR on the expression of miR-125a, miR-125b, and miR-205, that is, two specific inhibitors were able to dramatically reduce their corresponding miRNAs and showed less effectiveness towards the third miRNA (Figure 5a), suggesting a high specificity of the inhibitors. As all three of the miRNAs are able to target *erbB3*,<sup>42–45</sup> any two of the miRNA inhibitors exhibited almost equal efficiency to block entinostat-induced downregulation of *erbB3* in both lines (Figure 5b). However, only miR-125a and miR-125b have been reported to target *erbB2*,<sup>42,46</sup> and none of the databases (miRanda, Pictar, and TargetScan)



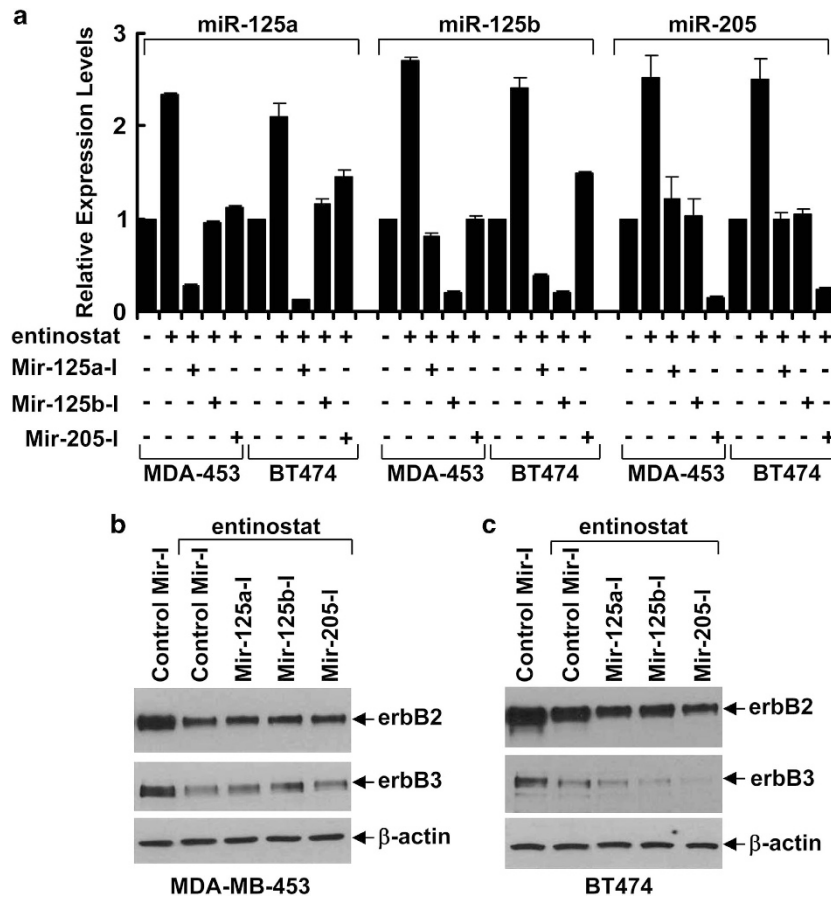
**Figure 2** Entinostat reduces the protein levels of endogenous, but not exogenous, *erbB2* and *erbB3* in human and mouse breast/mammary cancer cells. (a) MDA-MB-453 (MDA-453) and BT474 cells transiently transfected (TXN) with either control vector (Vector) or pDsRed-*erbB3* (*erbB3*) were cultured in the presence or absence of entinostat for 24 h. Cells were collected and subjected to western blot analyses with specific antibody directed against *erbB2*, *erbB3*, or  $\beta$ -actin. (b) MDA-MB-435 (MDA-435) and 435.eB1 cells were treated with entinostat at the indicated concentrations for 24 h. Cells were collected and subjected to western blot analyses with specific antibody directed against *erbB2*, *erbB3*, or  $\beta$ -actin. (c) Mammary tumor cell lines 85815 and 85819 were plated onto 96-well plates. After 24 h incubation, cells were grown in either control medium, or the same medium containing indicated concentrations of entinostat for another 72 h. The percentages of surviving cells relative to controls, defined as 100% survival, were determined by reduction of MTS. Bars, S.D. The data are representative of three independent experiments. (d) The cells treated with entinostat (ent) at indicated concentrations for 24 h or with 2  $\mu$ mol/l of entinostat (ent) for different time points were collected and subjected to western blot analyses with specific antibody directed against *erbB2*, *erbB3*, IGF-1R, or  $\beta$ -actin



**Figure 3** Entinostat upregulates the expression levels of miR-125a, miR-125b, and miR-205 in *erbB2*-overexpressing breast cancer cells. MDA-MB-453 (MDA-453) and BT474 cells untreated or treated with entinostat (0.5 and 2  $\mu$ mol/l, respectively) for 8, 16, 24 h were collected and subjected to total RNA extraction, inclusive of the small RNA fraction. The expression levels of miR-125a, miR-125b, and miR-205 were measured by qRT-PCR using TaqMan miRNA assays. All results were normalized with the internal control RNU6B. Bars, S.D. The data are representative of three independent experiments

predict that miR-205 targets *erbB2*. Thus, the combination of miR-125a and miR-125b inhibitors elicited strong blockade on entinostat-mediated reduction of *erbB2*, whereas the other two combinations had less effects on *erbB2* (Figure 5b). Moreover, specific apoptotic ELISA and western

blot analyses showed that single miRNA inhibitor did not alter entinostat-induced DNA fragmentation and PARP cleavage, the hallmarks of apoptosis (Figures 6a and b). In contrast, simultaneous inhibition of two miRNAs significantly attenuated entinostat-induced apoptosis (Figure 6c) and PARP



**Figure 4** Inhibition of single miRNA has no effect on entinostat-mediated downregulation of *erbB2* and *erbB3* in breast cancer cells. MDA-MB-453 (MDA-453) and BT474 cells were transfected with either control miRNA inhibitor no. 1 or the indicated miRNA inhibitor (60 nmol/l each). After 24 h, the cells were then untreated or treated with entinostat (1.5  $\mu$ mol/l) for another 24 h. (a) Half of the cells were collected and subjected to total RNA extraction, inclusive of the small RNA fraction. The expression levels of miR-125a, miR-125b, and miR-205 were measured by qRT-PCR using TaqMan miRNA assays. RNU6B was used as an internal control. Bars, S.D. Data shows the representative of three independent experiments. (b) Another half of the cells were collected and subjected to western blot analyses with specific antibody directed against *erbB2*, *erbB3*, or  $\beta$ -actin

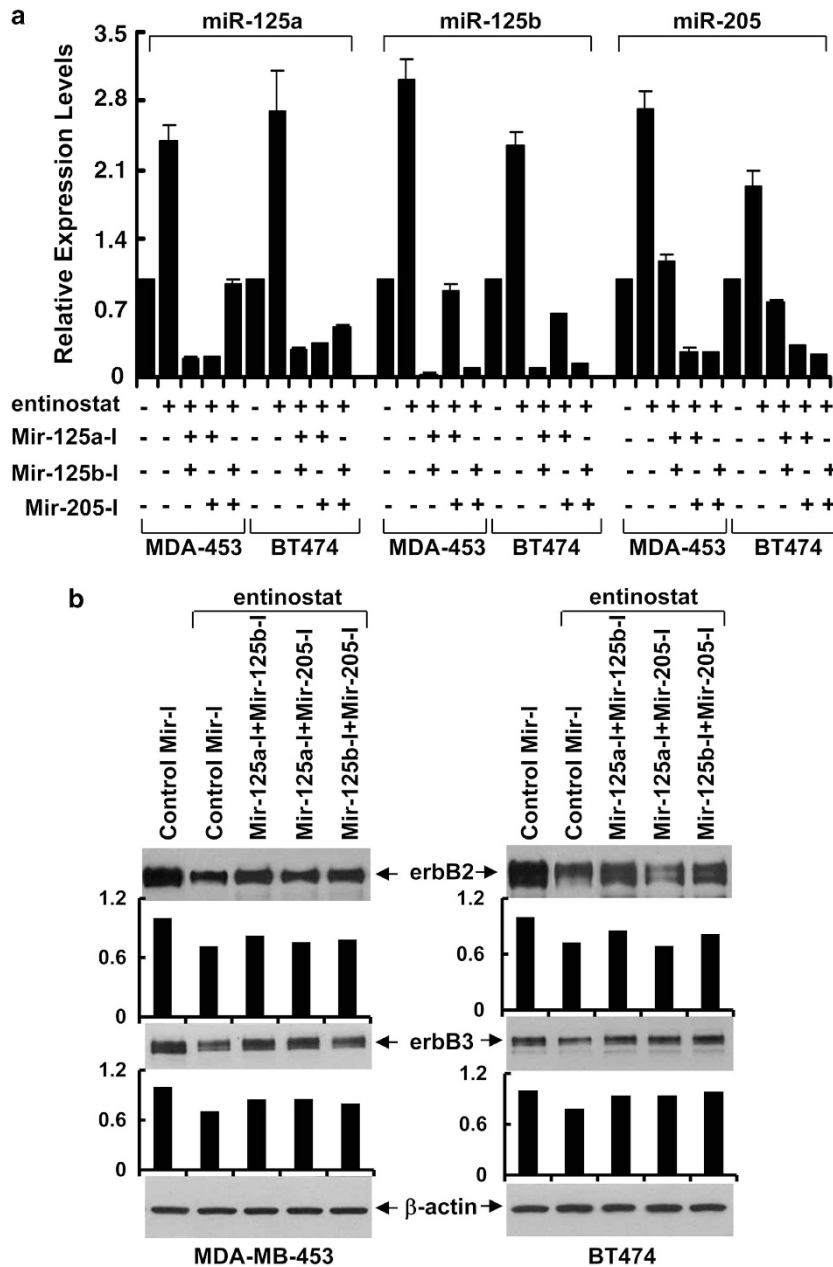
cleavage (Figure 6d) in both MDA-MB-453 and BT474 cells. Further studies found that the combination of all three miRNA inhibitors displayed a similar activity as two miRNA inhibitors to block entinostat action (data not shown). Collectively, these data indicate that at least two of the three miRNAs induced by entinostat are required and sufficient to downregulate *erbB2/erbB3* and promote apoptosis in *erbB2*-overexpressing breast cancer cells.

**SAHA and panobinostat inhibit cell growth and induce apoptosis in *erbB2*-overexpressing breast cancer cells correlated with the reduction of mRNA and protein expression of *erbB2/erbB3*.** To test whether the induction of *erbB2/erbB3*-targeting miRNAs is a mechanism of action of all HDACis in *erbB2*-overexpressing breast cancer cells, we studied the anti-proliferative/anti-survival effects of vorinostat (SAHA) and panobinostat (LBH589), both are class I and II HDACis,<sup>47</sup> on MDA-MB-453 and BT474 cells. Our data showed that both SAHA and panobinostat exhibited a similar activity as entinostat to strongly inhibit proliferation of MDA-MB-453 and BT474 cells (Figure 7a). Further studies with the concentrations close to their IC<sub>50</sub>s (SAHA and

panobinostat were used at 300 and 8 nmol/l, respectively, for MDA-MB-453 cells. SAHA and panobinostat were used at 1.5  $\mu$ mol/l and 20 nmol/l, respectively, for BT474 cells) indicated that both HDACis were able to reduce the protein levels of *erbB2* and *erbB3*, and induce apoptosis as evidenced by PARP cleavage and increased DNA fragmentation (Figure 7b). However, unlike entinostat, after treatment of MDA-MB-453 and BT474 cells for 16 h, neither SAHA nor panobinostat altered the expression levels of miR-125a, miR-125b, and miR-205 (Figure 7d). In contrast, both SAHA and panobinostat significantly decreased the *erbB2/erbB3* mRNA levels (Figure 7c). Similar miRNA and mRNA results were obtained when the cells were treated with the HDACis for 24 h (data not shown). These data suggest that SAHA and panobinostat may downregulate *erbB2/erbB3* expression in breast cancer cells via a miRNA-independent and transcription-dependent mechanism.

## Discussion

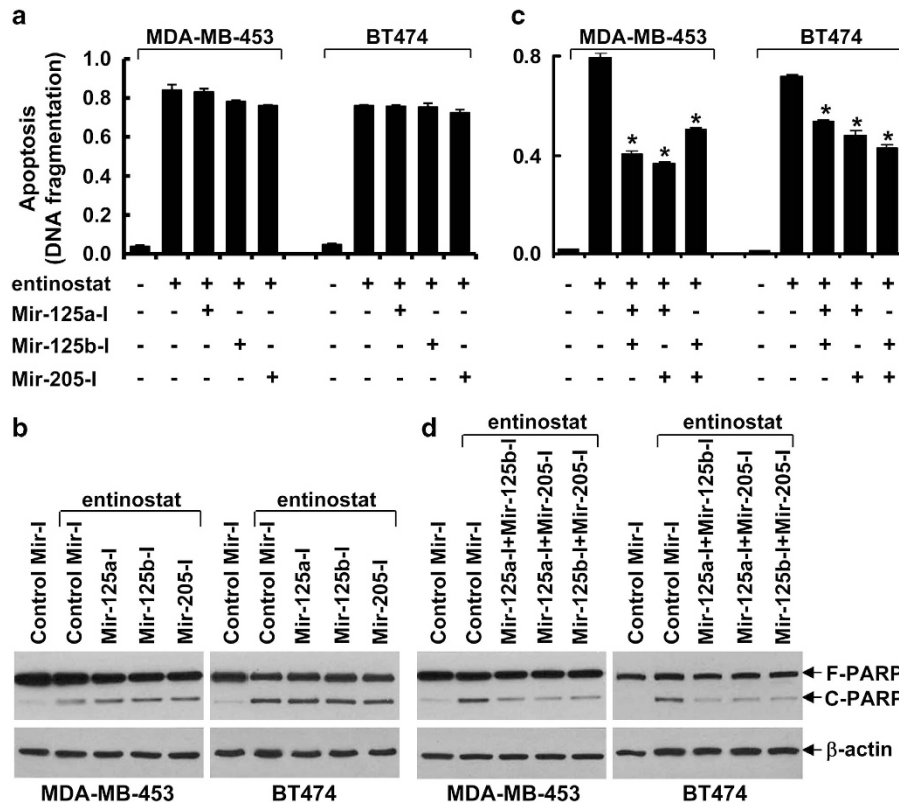
In this report, we provide evidence indicating that entinostat dual-targets *erbB2/erbB3* receptors in breast cancer cells



**Figure 5** Simultaneous inhibition of two miRNAs reduces entinostat-mediated downregulation of *erbB2* and *erbB3* in breast cancer cells. MDA-MB-453 (MDA-453) and BT474 cells were transfected with combinations of either the two control miRNA inhibitors no. 1 and no. 2 or the two indicated miRNA inhibitors (60 nmol/l each). After 24 h, the cells were then untreated or treated with entinostat (1.5  $\mu$ mol/l) for another 24 h. (a) Half of the cells were collected and subjected to total RNA extraction, inclusive of the small RNA fraction. The expression levels of miR-125a, miR-125b, and miR-205 were measured by qRT-PCR using TaqMan miRNA assays. RNU6B was used as an internal control. (b) Another half of the cells were collected and subjected to western blot analyses with specific antibody directed against *erbB2*, *erbB3*, or  $\beta$ -actin. The bar graph underneath was obtained by densitometry analysis. The relative signal intensities of *erbB2* or *erbB3* were measured by the Bio-Rad Gel Documentation System. Bars, S.D. The data are representative of three independent experiments

via a mechanism independent of transcription. As this HDACi exhibits inhibitory activity specifically towards the endogenous, but not exogenous, *erbB2/erbB3*, we believe that entinostat action on *erbB2/erbB3* involves miRNA-mediated translational suppression. Indeed, entinostat upregulates the expression of three *erbB2/erbB3*-targeting miRNAs. Further studies with specific inhibitors reveal a

functional cooperation of miR-125a, miR-125b, and miR-205 in entinostat-induced downregulation of *erbB2/erbB3* and apoptosis in breast cancer cells. The expression vectors we used contain only the coding sequences of human *erbB2* and *erbB3*, but not the 3'UTR of their mRNAs.<sup>24,36</sup> Similarly, in the MMTV-*neu* transgenic model, only the coding sequence of the transgene rat *erbB2/neu*



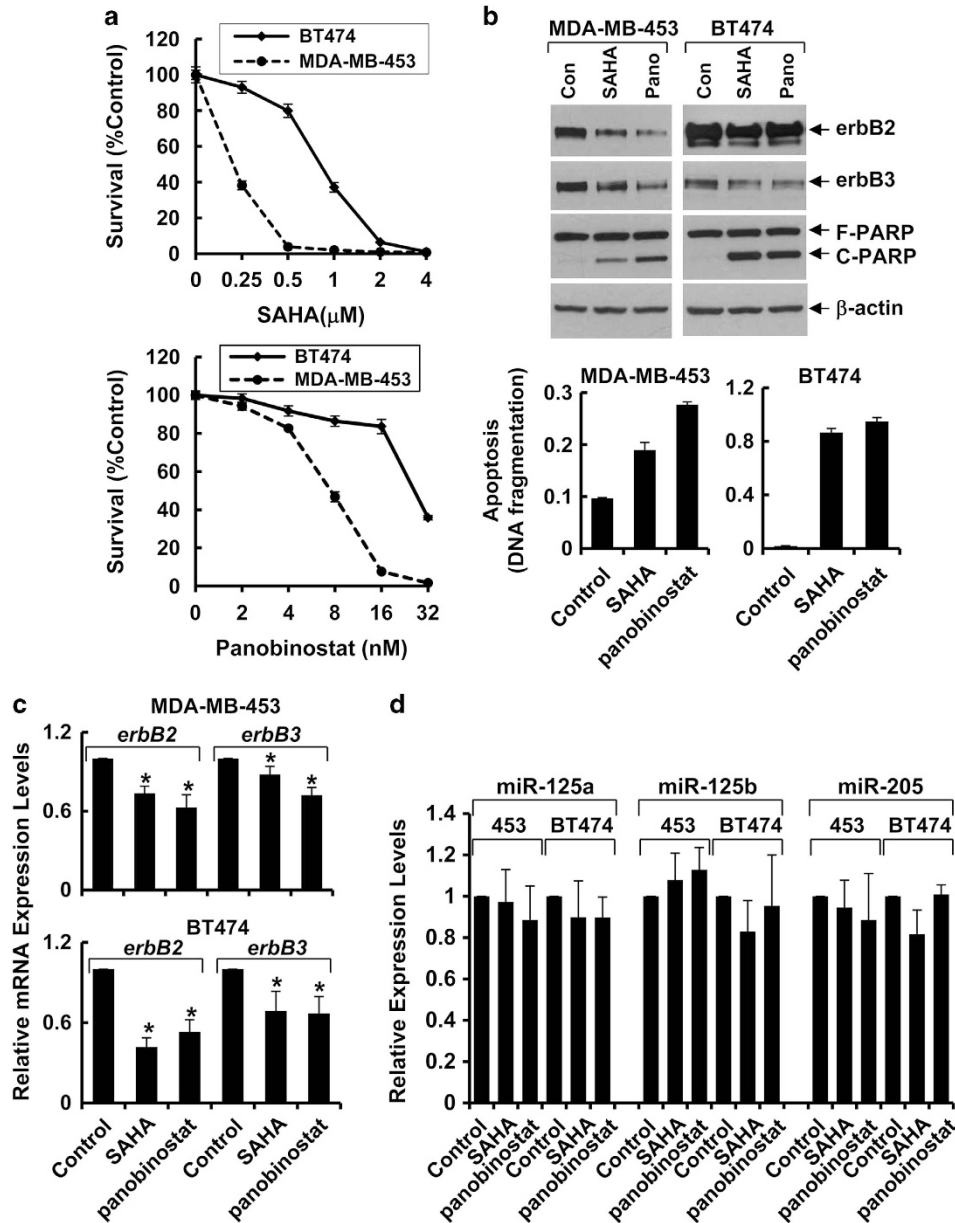
**Figure 6** Inactivation of two, but not one miRNA significantly attenuates entinostat-induced apoptosis in erbB2-overexpressing breast cancer cells. MDA-MB-453 and BT474 cells were similarly transfected and then treated with entinostat as described in Figure 4. All cells were collected and subjected to a specific apoptotic ELISA (a) and western blot analyses with specific antibody directed against PARP (F-PARP, full length PARP; C-PARP, cleaved PARP) or  $\beta$ -actin (b). MDA-MB-453 or BT474 cells were simultaneously transfected with two miRNA inhibitors and then treated with entinostat, same as described in Figure 5. All cells were collected and subjected to a specific apoptotic ELISA (c) and western blot analyses with specific antibody directed against PARP or  $\beta$ -actin (d). Bars, S.D. The data are representative of three independent experiments. \* $P < 0.001$  versus entinostat treatment with control transfection

was inserted downstream of MMTV promoter.<sup>48</sup> Thus, the exogenously expressed erbB2 (or rat erbB2/neu) and erbB3 may not be regulated by miRNAs. Further studies with additional HDACis indicated SAHA and panobinostat might not induce miRNA expression to downregulate erbB2/erbB3 receptors in breast cancer cells, instead they reduced the mRNA levels of *erbB2/erbB3* (Figure 7). These data suggest that the functional cooperation of miR-125a, miR-15b, and miR-205 elicited by entinostat is not a general phenomenon in the action of all HDACis. Some HDACis may regulate erbB2/erbB3 expression via a transcription-dependent mechanism.

While it has been shown that miRNA cluster may function through the cooperation between individual miRNAs to regulate the specific signaling pathway,<sup>49</sup> a recent study is probably the only defined example that experimentally confirms that multiple miRNAs target the same gene.<sup>50</sup> To the best of our knowledge, we are the first providing experimental data to demonstrate that miR-125a, miR-15b, and miR-205 act in concert to regulate the expression of erbB2/erbB3 in breast cancer cells. Our studies may further our understanding of the roles of miRNA networks in cancer biology. As compared with simultaneous inhibition of two miRNAs, inactivation of all three miRNAs showed equal

efficiency to block entinostat action in our studies, suggesting that functional cooperation between any two of miR-125a, miR-125b, and miR-205 might already reach the threshold that was required to reduce erbB2/erbB3 in the breast cancer cells tested. Additional experiments are warranted to further elucidate this notion.

The erbB receptor tyrosine kinase (RTK) family members are often aberrantly activated in a wide variety of cancers, particularly breast cancer, and are excellent targets for selective anticancer therapies. The currently used erbB-targeted therapies in clinic can be divided into two strategies: blocking antibody (Ab), such as trastuzumab targeting erbB2, and tyrosine kinase inhibitor, such as lapatinib against EGFR and erbB2. For erbB3 receptor, because of its lack of or low kinase activity,<sup>51,52</sup> blocking Ab is the only strategy currently under preclinical<sup>53,54</sup> and clinical studies (<http://www.clinicaltrials.gov/>). In fact, many anti-erbB3 Abs as novel therapeutics have shown promise for cancer treatment.<sup>55</sup> Co-expression of erbB family members is common in human breast cancer. It has been reported that erbB2 requires erbB3 to promote breast cancer cell proliferation,<sup>8</sup> and erbB3 has an important role in *erbB2*-altered breast cancers.<sup>9</sup> Our recent studies indicate that expression of erbB3 contributes to erbB2-mediated therapeutic resistance to tamoxifen<sup>56</sup> and



**Figure 7** SAHA and panobinostat inhibit proliferation and induce apoptosis in *erbB2*-overexpressing breast cancer cells associated with the reduction of mRNA levels and protein expression of *erbB2/erbB3*. (a) MDA-MB-453 or BT474 cells were plated onto 96-well plates. After 24 h incubation, cells were grown in either control medium, or the same medium containing indicated concentrations of SAHA or panobinostat for another 72 h. The percentages of surviving cells relative to controls, defined as 100% survival, were determined by reduction of MTS. Bars, S.D. The data are representative of three independent experiments. (b) MDA-MB-453 cells were treated with SAHA (300 nmol/l) or panobinostat (8 nmol/l) for 24 h. BT474 cells were treated with SAHA (1.5 μmol/l) or panobinostat (20 nmol/l) for 24 h. All cells were collected and subjected to western blot analyses with specific antibody directed against *erbB2*, *erbB3*, PARP, or β-actin (top) and a specific apoptotic ELISA (bottom). Bars, S.D. The data are representative of three independent experiments. (c) MDA-MB-453 or BT474 cells were treated as described in (b) for 16 h. All cells were collected and subjected to total RNA extraction. First-strand cDNA was synthesized using a reverse transcription kit from Applied Biosystems. The mRNA levels of *erbB2* and *erbB3* were measured by qRT-PCR. Bars, S.D. The data are representative of three independent experiments. \* $P < 0.03$  versus control. (d), MDA-MB-453 (453) or BT474 cells were treated as described in (c). All cells were collected and subjected to total RNA extraction, inclusive of the small RNA fraction. The expression levels of miR-125a, miR-125b, and miR-205 were measured by qRT-PCR using TaqMan miRNA assays. All results were normalized with the internal control RNU6B. Bars, S.D. The data are representative of three independent experiments

paclitaxel.<sup>57</sup> Thus, novel strategies or agents simultaneously targeting both *erbB2* and *erbB3* may have a broader impact on treatment of breast cancer. Here, we propose a novel approach to target *erbB2/erbB3*—reducing their protein levels rather than just inhibiting the signaling, which may reduce the

opportunity for tumor cells to develop resistance after initial response. Our data may facilitate the development of novel therapeutics with the ability to induce *erbB2/erbB3*-targeting miRNAs against the aggressive *erbB2*-overexpressing breast cancers.



As a selective class I HDACi, entinostat inhibits cell proliferation and induces apoptosis in breast cancer cells.<sup>17–20</sup> Because of its low toxicity and easy administration (taken orally), entinostat is currently in clinical trials of human cancers, including estrogen receptor-positive and triple-negative breast cancer patients (<http://www.clinicaltrials.gov/ct2/results?term=entinostat>). To date, the clinical activity of entinostat against *erbB2*-overexpressing breast cancer remains unclear. Our previous report<sup>24</sup> and the current studies suggest that entinostat may exert more potent effects on this subtype of breast cancers uniquely utilizing the functional cooperation of *erbB2/erbB3*-targeting miRNAs. In addition, we have also tested this HDACi's activity in the trastuzumab-resistant breast cancer model and shown that entinostat has a potential to overcome trastuzumab resistance.<sup>25</sup> Our data may provide a strong rationale for a recently initiated clinical study testing the activity of entinostat in combination with lapatinib in breast cancer patients that are *erbB2*-overexpressing and progressed on trastuzumab.

The molecular mechanism by which entinostat induces expression of miR-125a, miR-125b, and miR-205 in breast cancer cells is unclear. Epigenetic alterations, including DNA methylation and histone modifications, emerges as one of the major mechanisms regulating miRNA expression. Both acetylated histone H3 (Ac-HH3) and some methylated histone H3 are associated with open chromatin structure and active gene, including miRNA, expression.<sup>58,59</sup> We show that entinostat enhances Ac-HH3 correlated with reduced HDAC1.<sup>24</sup> It also induces degradation of DNA methyltransferase I (DNMT1), an enzyme responsible for maintaining DNA methylation patterns in eukaryotic cells,<sup>59</sup> in breast cancer cells.<sup>21</sup> It is possible that both increased Ac-HH3 and reduced promoter methylation contribute to entinostat-induced upregulation of miR-125a, miR-125b, and miR-205 in *erbB2*-overexpressing breast cancer cells.

In summary, we demonstrate that the class I HDACi entinostat induces expression of miR-125a, miR-125b, and miR-205, which work cooperatively to downregulate *erbB2/erbB3* receptors and subsequently promote the *erbB2*-overexpressing breast cancer cells undergoing apoptosis. Our data suggest that epigenetic approaches, such as HDAC inhibition with entinostat and the combinations of *erbB2/erbB3*-targeting miRNAs may be developed as novel strategies to treat the breast cancer patients whose tumors overexpress *erbB2*.

## Materials and Methods

**Reagents and antibodies.** The miRIDIAN miRNA hairpin inhibitors and negative control inhibitors (IN-001005-01-05 no.1 and IN-002005-01-05 no. 2) were purchased from Dharmacon, Inc. (Chicago, IL, USA). Entinostat was kindly provided by Syndax Pharmaceuticals, Inc. (Waltham, MA, USA), dissolved in DMSO to make a 20 mmol/l stock, and stored at -20 °C. Vorinostat (SAHA) and panobinostat (LBH589) were obtained from LC Laboratories (Woburn, MA, USA). The vector pDsRed-Monomer-N1 and human *erbB3*-expressing vector pDsRed-*erbB3* were described previously.<sup>24,57</sup>

Antibodies were obtained as follows: *erbB2* (Ab3) (EMD Chemicals, Inc., Gibbstown, NJ, USA); *erbB3* (Ab7) (LabVision Corp., Fremont, CA, USA); IGF-1R/ $\beta$  and PARP (Cell Signaling Technology, Inc., Beverly, MA, USA);  $\beta$ -actin (clone AC-75) (Sigma Co., St. Louis, MO, USA). All other reagents were from Sigma Co. unless otherwise specified.

**Cells and cell culture.** The human breast cancer cell lines MDA-MB-453, BT474, SKBR3, and MDA-MB-435 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The *erbB2*-transfectant 435.eB1 cell line<sup>36</sup> was kindly provided by Dr. Dihua Yu (MD Anderson Cancer Center). The mouse mammary tumor cell lines 85815 and 85819 were derived from MMTV-*neu* transgenic model.<sup>60</sup> All cells were maintained in DMEM/F-12 (1:1) medium supplemented with 10% fetal bovine serum (FBS), and cultured in a 37 °C humidified atmosphere containing 95% air and 5% CO<sub>2</sub> and were split twice a week.

**Reverse transcription (RT)-PCR and quantitative real-time (qRT)-PCR.** Total RNA was extracted using a modified chloroform/phenol procedure (TRIZOL, Invitrogen, Carlsbad, CA, USA). First-strand cDNA was generated using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's instructions. The analysis of human *erbB2* and *erbB3* mRNA expression was examined by conventional RT-PCR as we had described previously.<sup>60</sup> To quantify the mRNA levels, qRT-PCR was performed using the Absolute<sup>+</sup> Blue QPCR Master Mixes (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's protocol. The expression of  $\beta$ -actin was used as an internal control for both conventional RT-PCR and qRT-PCR. All qRT-PCR reactions were carried out on a 7500 Fast Real-Time PCR system (Applied Biosystems). Sequences of specific primers used are listed in Supplementary Table 1.

**Analysis of miRNA expression.** Total RNA, including small RNA, was extracted and purified using the miRNeasy Mini Kit (QIAGEN Inc., Valencia, CA, USA) following the manufacturer's instructions. For miRNA analysis, TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems) was first used to generate cDNA with the hairpin primers, which are specific to the mature miRNA and will not bind to the precursors. The expression levels of miR-125a-5p, miR-125b, and miR-205 were then measured by real-time PCR using TaqMan MicroRNA Assays (assay ID: 002198, 000449, 000509, respectively; Applied Biosystems) according to the manufacturer's protocol. RNU6B was used as an internal control to normalize all data using the TaqMan RNU6B Assay (assay ID: 001093; Applied Biosystems). RNU6B levels were unaffected by entinostat treatment. The relative miRNA levels were calculated using the comparative Ct method ( $-\Delta\Delta Ct$ ).

**Transfection of cells with expression vector.** Cell transfection with either the control vector pDsRed-Monomer-N1 or pDsRed-*erbB3* was performed with the FuGENETM-6 transfection kit (Roche Diagnostics Corp., Indianapolis, IN, USA) according to the manufacturer's instructions.

**Transfection of cells with miRNA inhibitor.** Cell transfection with the miRNA hairpin inhibitors or negative controls was carried out using HiPerFect Transfection Reagent (QIAGEN) according to the manufacturer's protocol.

**Cell proliferation assay.** The CellTiter96 AQ non-radioactive cell proliferation kit (Thermo Fisher Scientific, Inc.) was used to determine cell viability.<sup>25,57,60</sup> In brief, cells were plated onto 96-well plates with complete medium for 24 h, and then grown in either control medium or the same medium containing a series of doses of entinostat for another 72 h. After reading all wells at 490 nm with a microplate reader, the percentages of surviving cells from each group relative to controls, defined as 100%, were determined by reduction of MTS.

**Quantification of apoptosis.** A specific apoptotic ELISA kit (Roche Diagnostics Corp.) was used to quantitatively measure cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes), as we had reported.<sup>24,25,57</sup>

**Western blot analysis.** Protein expression levels were determined by western blot analysis as described previously.<sup>24,25,57</sup> Equal amounts of total cell lysates were boiled in Laemmli SDS-sample buffer, resolved by SDS-PAGE, transferred to nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA), and probed with the primary antibodies described in the figure legends. After the blots were incubated with horseradish peroxidase-labeled secondary antibody, the signals were detected using the enhanced chemiluminescence reagents (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA).

**Statistical analysis.** All experiments were performed at least in duplicate. Statistical analyses of the experimental data were determined using a two-sided Student's *t*-test. A *P*-value < 0.05 was deemed statistically significant.

### Conflict of Interest

The authors declare no conflict of interest.

**Acknowledgements.** We are grateful to Dr. Peter Ordentlich (Syndax Pharmaceuticals, Inc., Waltham, MA, USA) for providing etinostat, to Dr. Dihua Yu (MD Anderson Cancer Center) for providing 435.eB1 cells, and to Ms. Lisa Litzberger for her excellent assistance in arts preparation. This work was supported in part by research grants (AWD-102888 and AWD-122695) from the Cancer League of Colorado (to BL).

- Baselga J, Swain SM. Novel anticancer targets: revisiting ERBB2 and discovering ERBB3. *Nat Rev Cancer* 2009; **9**: 463–475.
- Hynes NE, MacDonald G. ErbB receptors and signaling pathways in cancer. *Curr Opin Cell Biol* 2009; **21**: 177–184.
- Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 1987; **235**: 177–182.
- Thor AD, Schwartz LH, Koerner FC, Edgerton SM, Skates SJ, Yin S et al. Analysis of c-erbB-2 expression in breast carcinomas with clinical follow-up. *Cancer Res* 1989; **49**: 7147–7152.
- Hudis CA. Trastuzumab—mechanism of action and use in clinical practice. *N Engl J Med* 2007; **357**: 39–51.
- Nahta R, Esteva FJ. Trastuzumab: triumphs and tribulations. *Oncogene* 2007; **26**: 3637–3643.
- Nahta R, Yu D, Hung MC, Hortobagyi GN, Esteva FJ. Mechanisms of Disease: understanding resistance to HER2-targeted therapy in human breast cancer. *Nat Clin Pract Oncol* 2006; **3**: 269–280.
- Holbro T, Beerli RR, Maurer F, Koziczak M, Barbas 3rd CF, Hynes NE. The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires ErbB3 to drive breast tumor cell proliferation. *Proc Natl Acad Sci USA* 2003; **100**: 8933–8938.
- Lee-Hoeflich ST, Crocker L, Yao E, Pham T, Munroe X, Hoeflich KP et al. A central role for HER3 in HER2-amplified breast cancer: implications for targeted therapy. *Cancer Res* 2008; **68**: 5878–5887.
- Cress WD, Seto E. Histone deacetylases, transcriptional control, and cancer. *J Cell Physiol* 2000; **184**: 1–16.
- Mahlknecht U, Hoelzer D. Histone acetylation modifiers in the pathogenesis of malignant disease. *Mol Med* 2000; **6**: 623–644.
- Bolden JE, Peart MJ, Johnstone RW. Anticancer activities of histone deacetylase inhibitors. *Nat Rev Drug Discov* 2006; **5**: 769–784.
- Minucci S, Pellicci PG. Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. *Nat Rev Cancer* 2006; **6**: 38–51.
- Mottet D, Castronovo V. Histone deacetylases: target enzymes for cancer therapy. *Clin Exp Metastasis* 2008; **25**: 183–189.
- Yang XJ, Seto E. HATs and HDACs: from structure, function and regulation to novel strategies for therapy and prevention. *Oncogene* 2007; **26**: 5310–5318.
- Knipstein J, Gore L. Etenostat for treatment of solid tumors and hematologic malignancies. *Expert Opin Investig Drugs* 2011; **20**: 1455–1467.
- Lee BI, Park SH, Kim JW, Sausville EA, Kim HT, Nakanishi O et al. MS-275, a histone deacetylase inhibitor, selectively induces transforming growth factor beta type II receptor expression in human breast cancer cells. *Cancer Res* 2001; **61**: 931–934.
- Park SH, Lee SR, Kim BC, Cho EA, Patel SP, Kang HB et al. Transcriptional regulation of the transforming growth factor beta type II receptor gene by histone acetyltransferase and deacetylase is mediated by NF- $\kappa$ B in human breast cancer cells. *J Biol Chem* 2002; **277**: 5168–5174.
- Singh TR, Shankar S, Srivastava RK. HDAC inhibitors enhance the apoptosis-inducing potential of TRAIL in breast carcinoma. *Oncogene* 2005; **24**: 4609–4623.
- Xu J, Zhou JY, Wei WZ, Philipsen S, Wu GS. Sp1-mediated TRAIL induction in chemosensitization. *Cancer Res* 2008; **68**: 6718–6726.
- Zhou Q, Agoston AT, Atadja P, Nelson WG, Davidson NE. Inhibition of histone deacetylases promotes ubiquitin-dependent proteasomal degradation of DNA methyltransferase 1 in human breast cancer cells. *Mol Cancer Res* 2008; **6**: 873–883.
- Chumsri S, Sabnis GJ, Howes T, Brodie AM. Aromatase inhibitors and xenograft studies. *Steroids* 2011; **76**: 730–735.
- Sabnis GJ, Goloubeva O, Chumsri S, Nguyen N, Sukumar S, Brodie AM. Functional activation of the estrogen receptor- $\alpha$  and aromatase by the HDAC inhibitor etinostat sensitizes ER-negative tumors to letrozole. *Cancer Res* 2011; **71**: 1893–1903.
- Huang X, Gao L, Wang S, Lee CK, Ordentlich P, Liu B. HDAC inhibitor SNDX-275 induces apoptosis in erbB2-overexpressing breast cancer cells via down-regulation of erbB3 expression. *Cancer Res* 2009; **69**: 8403–8411.
- Huang X, Wang S, Lee CK, Yang X, Liu B. HDAC inhibitor SNDX-275 enhances efficacy of trastuzumab in erbB2-overexpressing breast cancer cells and exhibits potential to overcome trastuzumab resistance. *Cancer Lett* 2011; **307**: 72–79.
- Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell* 2009; **136**: 215–233.
- Winter J, Jung S, Keller S, Gregory RI, Diederichs S. Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nat Cell Biol* 2009; **11**: 228–234.
- Esquela-Kerscher A, Slack FJ. Oncomirs—microRNAs with a role in cancer. *Nat Rev Cancer* 2006; **6**: 259–269.
- Nelson KM, Weiss GJ. MicroRNAs and cancer: past, present, and potential future. *Mol Cancer Ther* 2008; **7**: 3655–3660.
- Zhang B, Pan X, Cobb GP, Anderson TA. microRNAs as oncogenes and tumor suppressors. *Dev Biol* 2007; **302**: 1–12.
- Adams BD, Guttilla IK, White BA. Involvement of microRNAs in breast cancer. *Semin Reprod Med* 2008; **26**: 522–536.
- Deng S, Calin GA, Croce CM, Coukos G, Zhang L. Mechanisms of microRNA deregulation in human cancer. *Cell Cycle* 2008; **7**: 2643–2646.
- Yang N, Coukos G, Zhang L. MicroRNA epigenetic alterations in human cancer: one step forward in diagnosis and treatment. *Int J Cancer* 2008; **122**: 963–968.
- Zhang L, Volinia S, Bonome T, Calin GA, Greshock J, Yang N et al. Genomic and epigenetic alterations deregulate microRNA expression in human epithelial ovarian cancer. *Proc Natl Acad Sci USA* 2008; **105**: 7004–7009.
- Scott GK, Mattie MD, Berger CE, Benz SC, Benz CC. Rapid alteration of microRNA levels by histone deacetylase inhibition. *Cancer Res* 2006; **66**: 1277–1281.
- Yu D, Liu B, Tan M, Li J, Wang SS, Hung MC. Overexpression of c-erbB-2/neu in breast cancer cells confers increased resistance to Taxol via mdr-1-independent mechanisms. *Oncogene* 1996; **13**: 1359–1365.
- Kasman L, Onicescu G, Voelkel-Johnson C. Histone deacetylase inhibitors restore cell surface expression of the coxsackie adenovirus receptor and enhance CMV promoter activity in castration-resistant prostate cancer cells. *Prostate Cancer* 2012; **2012**: 137163.
- Lai MD, Chen CS, Yang CR, Yuan SY, Tsai JJ, Tu CF et al. An HDAC inhibitor enhances the antitumor activity of a CMV promoter-driven DNA vaccine. *Cancer Gene Ther* 2010; **17**: 203–211.
- Astrand C, Belikov S, Wrangé O. Histone acetylation characterizes chromatin presetting by NF1 and Oct1 and enhances glucocorticoid receptor binding to the MMTV promoter. *Exp Cell Res* 2009; **315**: 2604–2615.
- Mulholland NM, Soeth E, Smith CL. Inhibition of MMTV transcription by HDAC inhibitors occurs independent of changes in chromatin remodeling and increased histone acetylation. *Oncogene* 2003; **22**: 4807–4818.
- Bartsch J, Truss M, Bode J, Beato M. Moderate increase in histone acetylation activates the mouse mammary tumor virus promoter and remodels its nucleosome structure. *Proc Natl Acad Sci USA* 1996; **93**: 10741–10746.
- Scott GK, Goga A, Bhaumik D, Berger CE, Sullivan CS, Benz CC. Coordinate suppression of ERBB2 and ERBB3 by enforced expression of micro-RNA miR-125a or miR-125b. *J Biol Chem* 2007; **282**: 1479–1486.
- Gandellini P, Folini M, Longoni N, Pennati M, Binda M, Colecchia M et al. miR-205 exerts tumor-suppressive functions in human prostate through down-regulation of protein kinase Cepsilon. *Cancer Res* 2009; **69**: 2287–2295.
- Iorio MV, Casalini P, Piovano C, Di Leva G, Merlo A, Triulzi T et al. microRNA-205 regulates HER3 in human breast cancer. *Cancer Res* 2009; **69**: 2195–2200.
- Wu H, Zhu S, Mo YY. Suppression of cell growth and invasion by miR-205 in breast cancer. *Cell Res* 2009; **19**: 439–448.
- Hofmann MH, Heinrich J, Radziwill G, Moelling K. A short hairpin DNA analogous to miR-125b inhibits C-Raf expression, proliferation, and survival of breast cancer cells. *Mol Cancer Res* 2009; **7**: 1635–1644.
- Dokmanovic M, Clarke C, Marks PA. Histone deacetylase inhibitors: overview and perspectives. *Mol Cancer Res* 2007; **5**: 981–989.
- Guy CT, Webster MA, Schaller M, Parsons TJ, Cardiff RD, Muller WJ. Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc Natl Acad Sci USA* 1992; **89**: 10578–10582.
- Mestdagh P, Bostrom AK, Impens F, Fredlund E, Van Peer G, De Antonellis P et al. The miR-17-92 microRNA cluster regulates multiple components of the TGF-beta pathway in neuroblastoma. *Mol Cell* 2010; **40**: 762–773.
- Wu S, Huang S, Ding J, Zhao Y, Liang L, Liu T et al. Multiple microRNAs modulate p21Cip1/Waf1 expression by directly targeting its 3' untranslated region. *Oncogene* 2010; **29**: 2302–2308.
- Citri A, Skaria KB, Yarden Y. The deaf and the dumb: the biology of ErbB-2 and ErbB-3. *Exp Cell Res* 2003; **284**: 54–65.
- Shi F, Telesco SE, Liu Y, Radhakrishnan R, Lemmon MA. ErbB3/HER3 intracellular domain is competent to bind ATP and catalyze autophosphorylation. *Proc Natl Acad Sci USA* 2010; **107**: 7692–7697.
- Schoeberl B, Faber AC, Li D, Liang MC, Crosby K, Onsum M et al. An ErbB3 antibody, MM-121, is active in cancers with ligand-dependent activation. *Cancer Res* 2010; **70**: 2485–2494.
- Schoeberl B, Pace EA, Fitzgerald JB, Harms BD, Xu L, Nie L et al. Therapeutically targeting ErbB3: a key node in ligand-induced activation of the ErbB receptor-PI3K axis. *Sci Signal* 2009; **2**: ra31.

55. Aurisicchio L, Marra E, Roscilli G, Mancini R, Ciliberto G. The promise of anti-ErbB3 monoclonals as new cancer therapeutics. *Oncotarget* 2012; **3**: 744–758.
56. Liu B, Ordonez-Ercan D, Fan Z, Edgerton SM, Yang X, Thor AD. Downregulation of erbB3 abrogates erbB2-mediated tamoxifen resistance in breast cancer cells. *Int J Cancer* 2007; **120**: 1874–1882.
57. Wang S, Huang X, Lee CK, Liu B. Elevated expression of erbB3 confers paclitaxel resistance in erbB2-overexpressing breast cancer cells via upregulation of Survivin. *Oncogene* 2010; **29**: 4225–4236.
58. Chuang JC, Jones PA. Epigenetics and microRNAs. *Pediatr Res* 2007; **61**: 24R–29R.
59. Veeck J, Esteller M. Breast cancer epigenetics: from DNA methylation to microRNAs. *J Mammary Gland Biol Neoplasia* 2010; **15**: 5–17.
60. Liu B, Ordonez-Ercan D, Fan Z, Huang X, Edgerton SM, Yang X *et al*. Estrogenic promotion of ErbB2 tyrosine kinase activity in mammary tumor cells requires activation of ErbB3 signaling. *Mol Cancer Res* 2009; **7**: 1882–1892.



**Cell Death and Disease** is an open-access journal published by **Nature Publishing Group**. This work is licensed under a **Creative Commons Attribution-NonCommercial-NoDerivs 3.0 Unported License**. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-nd/3.0/>

Supplementary Information accompanies this paper on Cell Death and Disease website (<http://www.nature.com/cddis>)