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

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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.

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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.1,2 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.3–5 Although erbB2-targeted therapy, such as Herceptin (or trastuzumab) has been successfully used in breast cancer patients with erbB2-overexpressing tumors,9 resistance to Herceptin frequently occur and currently represent a significant clinical problem.6–7 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.8,9 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.10,11 Histone deacetylases (HDACs) are relatively easier tractable

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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/β, transforming growth factor β; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; DNMT1, DNA methyltransferase 1; PI-3K, phosphoinositide 3-kinase; PARP, poly(ADP-ribose) polymerase; ELISA, enzyme-linked immunosorbent assay; DEMEM, Dulbecco’s Modified Eagle’s Medium; PAGE, polyacrylamide gel electrophoresis; Entinostat, N-(2-Aminophenyl)-4-(N-(pyridine-3-yl)methoxy-carbonyl)aminomethyl)benzamide; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt

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Enzymes, and have recently become attractive therapeutic targets. Inhibitors of HDACs exhibit antitumor activity in a variety of tumor cell models via influencing cell cycle progression, apoptosis, differentiation, and tumor angiogenesis.\textsuperscript{12,13} Many HDAC inhibitors (HDACi) are currently under clinical investigations as potential anticancer agents.\textsuperscript{14,15} 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\textsubscript{50} in the submicromolar range, and exhibits both in vitro and in vivo activities against various cancer types, including solid tumors and hematologic malignancies.\textsuperscript{16} In breast cancers, entinostat has been shown to inhibit cell proliferation and/or promote apoptosis.\textsuperscript{17–21} 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.\textsuperscript{22,23} In studying whether entinostat restores the responsiveness of letrozole mainly in triple-negative breast cancer cells via downregulation of erbB2/erbB3, but not EGFR expression,\textsuperscript{24} and it enhances trastuzumab sing breast cancer cells via downregulation of erbB2/erbB3, entinostat selectively induces apoptosis in erbB2-overexpressing breast cancer cells.\textsuperscript{24} 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 breast cancer cells (Figure 1a). Similar results were also observed in SKBR3 cells (Supplementary Figure S2). We then reasoned that 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.\textsuperscript{36} 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.\textsuperscript{24,36} As recent studies show that HDAC inhibitors are capable of enhancing CMV promoter activity.\textsuperscript{37,38} 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.\textsuperscript{24} 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.\textsuperscript{39,40} 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 transcription.
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 mRNA for degradation or translational repression. 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, and over-expression of miR-125a or miR-125b results in coordinate suppression of erbB2 and erbB3. Several studies report that miR-205 targets erbB3 to exert antitumor activity. 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. 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, 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, and none of the databases (miRanda, Pictar, and TargetScan)
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 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 β-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 β-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 μmol/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 β-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 μmol/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.
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, 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 IC50s (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 μmol/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.
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.24,36 Similarly, in the MMTV-neu transgenic model, only the coding sequence of the transgene rat erbB2/neu

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 μmol/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 β-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.
was inserted downstream of MMTV promoter. 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, a recent study is probably the only defined example that experimentally confirms that multiple miRNAs target the same gene. 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, blocking Ab is the only strategy currently under preclinical and clinical studies (http://www.clinicaltrials.gov). In fact, many anti-erbB3 Abs as novel therapeutics have shown promise for cancer treatment. 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, and erbB3 has an important role in erbB2-altered breast cancers. Our recent studies indicate that expression of erbB3 contributes to erbB2-mediated therapeutic resistance to tamoxifen and

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 β-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 β-actin (d). Bars, S.D. The data are representative of three independent experiments. *P<0.001 versus entinostat treatment with control transfection
paclitaxel.\(^7\) 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.

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.
As a selective class I HDACi, entinostat inhibits cell proliferation and induces apoptosis in breast cancer cells. 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. To date, the clinical activity of entinostat against erbB2-overexpressing breast cancer remains unclear. Our previous report 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. 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. We show that entinostat enhances Ac-HH3 correlated with reduced HDAC1. It also induces degradation of DNA methyltransferase I (DNMT1), an enzyme responsible for maintaining DNA methylation patterns in eukaryotic cells, in breast cancer cells. 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 miRDIAN 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. Vincristine (SAHA) and panobinostat (LBH589) were obtained from LC Laboratories (Woburn, MA, USA). The vector pDsRed-Monimer-N1 and human erbB3-expressing vector pDsRed-erbB3 were described previously. Antibodies were obtained as follows: erbB2 (Ab3) (EMD Chemicals, Inc., Gibbstown, NJ, USA); erbB3 (Ab7) (LabVision Corp., Fremont, CA, USA); IGF-1R/ and PARP (Cell Signaling Technology, Inc., Beverly, MA, USA); β-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 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. 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% CO2 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. To quantify the miRNA levels, qRT-PCR was performed using the Absolute Blue OPCR Master Mixes (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer’s protocol. The expression of β-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 (1.0/ΔCt).

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. 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.

Western blot analysis. Protein expression levels were determined by western blot analysis as described previously. 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.

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