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HDAC inhibitor suppresses proliferation and invasion of breast cancer cells through regulation of miR-200c targeting CRKL

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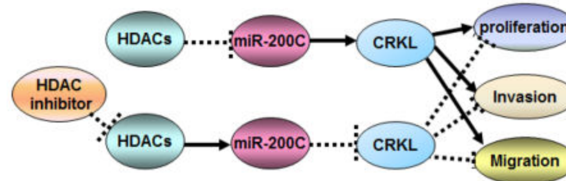
Abstract

Although histone deacetylase (HDAC) inhibitors have been shown to effectively induce the inhibition of proliferation and migration in breast cancer, the anticancer mechanism remains poorly understood. Our studies show that miR-200c was significantly downregulated in breast cancer cell lines compared to normal cell lines and inversely correlated with the levels of class IIa HDACs and CRKL. HDAC inhibitors and the ectopic expression of miR-200c as tumor suppressors inhibited the proliferation, invasion, and migration of breast cancer cells by downregulating CRKL. These results indicate that the anticancer mechanism of HDAC inhibitor was realized partially by regulating miR-200c via CRKL targeting. Our findings suggest that the HDAC-miR200c-CRKL signaling axis could be a novel diagnostic marker and potential therapeutic target in breast cancer.

Graphical Abstract



Keywords

Breast cancer; HDAC inhibitor; SAHA; MiR-200c; CRKL

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Conflict of interest

There is no potential conflict of interest to disclose.

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1. Introduction

Histone deacetylases (HDACs) have demonstrated to play vital roles in key steps associated with proliferation, invasion and migration in several cancers [1–3]. The recent studies have indicated that HDAC inhibitors are considered promising novel anticancer drugs, and prove useful in preclinical and preliminary clinical trials [4, 5]. A number of HDAC inhibitors have been characterized that inhibit tumor growth both *in vitro* and *in vivo* at amounts that have little or no toxicity. HDAC inhibitors have been shown to induce the inhibition of proliferation, apoptosis, invasion, and migration in breast cancer cell lines *in vitro* [6–9]. Nevertheless, little is known about the anticancer mechanism of HDAC inhibitors in breast cancer and whether the coregulatory signaling might involve microRNA (miRNA).

MiRNAs are small non-coding RNAs of approximately 19–25 nucleotides that can negatively regulate gene expression at both transcriptional and post-transcriptional levels [10, 11], and play important roles in diverse biological processes. MiRNAs have been shown to be aberrantly expressed in breast cancer while also functioning as regulators of tumor behavior and progression [12, 13]. The miR-200 family has been shown to suppress epithelial-mesenchymal transition (EMT) and invasion and to govern many biologic processes essential to tumor survival such as apoptosis, proliferation, and chemoresistance [14–16]. MiR-200c has been reported to serve as a potential tumor suppressor to regulate tumor angiogenesis, proliferation, invasion and migration in breast cancer [15, 17, 18]. Recently, HDAC inhibitors have been shown to reverse PELP1-mediated suppression of miR-200 family members [6]. The finding demonstrated that there was a direct relationship between HDAC inhibitor and miR-200 family members.

Crk-like (CRKL) is an adapter protein that has crucial roles in multiple biological processes, including cell proliferation, adhesion, and migration. The CRKL gene is located on chromosome 22, band q11, and is strikingly similar to the cellular component of the v-crk oncogene product[19]. CRKL is a key substrate and effective downstream molecule of the BCR-ABL1 oncogenic tyrosine kinase in chronic myelogenous leukemia [20, 21]. Accumulating studies show frequent upregulation of CRKL in several malignant tumors, including breast, lung, pancreatic, and colon cancers, as well as B-cell lymphoma and chronic lymphocytic leukemia [21–24]. Overexpression of CRKL was demonstrated in human breast cancers and showed direct correlation with tumor stage, with the ability to promote breast cancer cell proliferation [25, 26]. CRKL as a primary genetic driver of tumorigenesis may play an important role in tumor proliferation and migration in breast cancer, with implications as a possible novel diagnostic marker and potential therapeutic target.

In this study, we investigated the anticancer mechanism of HDAC inhibitors and more specifically, significance of the HDAC-miR200c-CRKL signaling axis in the regulation of proliferation, migration, and invasion of breast cancer cells. Our results showed that the anticancer mechanism of HDAC inhibitor in breast cancer cells was achieved in part by regulating miR-200c through downregulating CRKL. These novel findings suggest that the HDAC-miR200c-CRKL axis may be a critical component for inhibiting proliferation,

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migration, and invasion of breast cancer cells, and that HDAC inhibitors serve as attractive therapeutic agents for breast cancer.

2. Materials and methods

2.1. Cell culture, cell transfection, and cell treatment with SAHA

Breast cancer cell lines MDA-MB-231, HCC-1143, HCC-1395, MCF-7, SKBR3, HCC-1419, and MDA-MB-361, as well as two normal mammary epithelial cell lines, MCF-10A and A1N4, were purchased from the American Type Culture Collection (Mediatech Inc, Manassas, VA, USA) and freshly recovered from liquid nitrogen. The breast cancer cells were maintained in RPMI-1640 (Mediatech Inc, Manassas, VA, USA) supplemented with 10% fetal bovine serum. MCF-7 was grown in the medium along with 5 mg/mL insulin. The MCF-10A and A1N4 cells were cultured in IMEM (Mediatech Inc, Manassas, VA, USA) supplemented with 0.5% fetal calf serum, 0.5 mg/mL hydrocortisone, 5 mg/mL insulin, and 10ng/mL epidermal growth factor. All cells were grown and maintained at 37°C in a 5% CO_2 humidified incubator.

The hsa-miR-200c-3p mimics (Catalog No. 4464066), hsa-miR-200c-3p inhibitors (Catalog No. 4464084), and CRKL siRNA (Catalog No. 4392420) were purchased from ThermoFisher Scientific (Waltham, MA, USA). These mimics or inhibitors were transfected into the cells. All cell transfections were introduced by Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. For each cell transfection, three replicate experiments were performed.

For proliferation, invasion, and Western blot assays, MDA-MB-231 breast cancer cells were treated for 24 hrs with 1 μM Vorinostat (Suberoylanilide Hydroxamic Acid, SAHA) (Shelleckchem, Houston, USA).

2.2. Quantitative RT-PCR assays

Total RNA was isolated from the cells using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). The sequences of all primers used in this study are outlined in Table 1. *U6 snRNA* was used as the endogenous control for miR-200c amplification and *β-Actin* as the endogenous control for CRKL and HDAC1-11 respectively. Total RNA was quantified by detection of absorbance at 260nm. 2μg of each RNA sample was reverse-transcribed into complementary DNA. Quantitative PCR analysis was performed using the Prism 7900HT Sequence Detection System (ABI, Foster City, CA, USA) with the comparative threshold method. Thermal cycling conditions were as follows: 95°C for 10 minutes, followed by 36 cycles of 95°C for 20 seconds and 60°C for 30 seconds. SYBR Green quantitative PCR reaction was performed in a 20 μl reaction volume containing 10 μl of 2× SYBR Green PCR Master Mix (Life technologies, Grand Island, NY, USA). The relative expression levels of each sample were measured using the $2^{-\ \ Ct}$ method. All experiments were performed in triplicate and repeated once more.

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2.3. Western blotting analysis

Total proteins from treated cells were extracted using cell RIPA buffer. Proteins were loaded and separated on 10% SDS-PAGE gels, and then transferred onto PVDF membranes (Bio-Rad, Hercules, CA, USA). The membranes were incubated overnight at 4°C with monoclonal antibodies against CRKL (Millipore, Billerica, MA, USA) at a 1:10000 dilution with 5% non-fat dried milk. After washing with TBST, the membranes were incubated for 1 h with peroxidase-conjugated anti-mouse IgG (Bio-Rad, Hercules, CA, USA) at 1:20000 dilution, and bound proteins were visualized using ECL and detected using the SPX-101A BioImaging Systems (Konica Minolta, Inc., Tokyo, Japan). The relative protein levels were calculated by normalizing to β-Actin (Sigma–Aldrich, St. Louis, MO, USA) protein as the endogenous control. All experiments were performed in duplicate for each sample and the process was repeated once more. Quantification of protein bands was measured using the ImageJ software.

2.4. Cell proliferation assays

The viability and proliferation of MDA-MB-231 cells were determined by Cell Titer 96 AQueous Non-Radioactive Cell Proliferation Assay (MTS) Kit (Promega, Madison, WI, USA). The cells were plated in 96-well plates at 1×10^4 per well in a final volume of 100μl culture medium and treated with HDAC inhibitor. After incubation for 24, 48, 72, and 96 hours, 20μl of 96 AQueous solution was added to each well and incubated for an additional 2h at 37 °C, and the absorbance was measured at 490nm by ELISA plate reader Synergy HT Microplate Reader (Biotek, Winooski, VT, USA). The cell viability was normalized to that of cells cultured in the culture medium without treatment.

2.5. Matrigel invasion assays

After transfecting with miR-200c mimics or treating with HDAC inhibitors, the cells (5×10^4 per well) were seeded in BioCoat Matrigel invasion chamber 24-well plates (Becton Dickinson Bioscience, Franklin Lakes, NJ, USA). Cells in the upper chambers were allowed to invade overnight at 37°C with 5% CO_2. Non-invasive cells remaining on the upper side of the inserts were scratched away with a cotton swab. Invasive cells were fixed with 100% ethanol for 15 min, stained with hematoxylin-eosin, counted in 10 microscopic fields at 40x magnification, and averaged.

2.6. Wound healing assays

To analyze cell migration, the transfected cells were seeded in 6-well plates. When the cells reached 80% confluence, the supernatant was removed and a scratch was made using a sterilized 200 μl pipette tip. The debris was removed by washing with PBS. Serial photographs were obtained at different time points using the inverted microscope (Olympus IX51, Tokyo, Japan). The distances between the cell fronts on either side of the scratch were measured and recorded.

2.7. Computational target

For the identification of miR-200c targets, TargetScan 6.2 software was used to find conserved target sites throughout mammalian transcriptomes. We scanned the genome for

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putative miR-200 binding sites and 1057 transcripts with conserved sites, with a total of 1202 conserved sites and 525 poorly conserved sites. We focused on the theoretical genes that had high (>0.80) probability of conserved targeting (Targetscan aggregate P_{CT}) scores, and those that might contribute to the proliferation and invasiveness of breast cancer.

2.8. Statistical analysis

Three replications were performed for each experiment. Statistical differences among groups were analyzed using either Student's t-test or χ^2 test as appropriate. $P<0.05$ was considered significant; $P<0.01$ was considered highly significant. SPSS version 19.0 (SPSS Inc., IL, USA) will be used for statistical analysis.

3. Results

3.1. MiR-200c expression levels are downregulated in breast cancer cells and inversely correlate with expression levels of HDAC class IIa members

The expression levels of HDAC1-11 in MDA-MB-231, HCC1143, HCC1395, MCF-7, SBKR3, HCC1419, and MDA-MB-361 breast cancer cell lines, and two normal-like cell lines, A1N4 and MCF-10A, were profiled with qRT-PCR analysis. Several breast cancer cell lines demonstrated similar HDAC expression profiles; HDAC2 and class IIa HDACs including HDAC9, HDAC4, HDAC5, and HDAC7 were significantly elevated (Fig. 1A) in breast cancer cell lines compared to two normal-like cell lines. Particularly, HDAC9 was expressed in much higher levels in all breast cancer cell lines compared to normal-like cell lines A1N4 and MCF-10A (**Fig. A**).

To validate the role of miR-200c in HDACs-mediated breast cancer cell growth and invasion, we analyzed the expression of miR-200c in breast cancer cells lines by qRT-PCR. The qPCR results revealed that expression levels of miR-200c were downregulated in all seven breast cancer cell lines compared to the two immortalized normal mammary epithelial cell lines. Notably, miR-200c expression levels were consistently lower in triple-negative cell lines, MDA-MB-231, HCC-1143, and HCC-1395 (Fig. 1B). Additionally, our data show that miR-200c expression levels are inversely correlated with the levels of HDAC2 and class IIa HDACs.

3.2. The ectopic expression of miR-200c inhibits the proliferation, invasion, and migration of breast cancer cells

To investigate the potential role of miR-200c in breast cancer cells proliferation, invasion, and migration, we transfected miR-200c mimics into the breast cancer cell line MDA-MB-231 to overexpress miR-200c (Fig 2A). Cell proliferation assays showed that the ectopic expression of miR-200c could markedly inhibit the proliferation and growth of MDA-MB-231 cells compared to the mimic control (Fig. 2B). Moreover, Matrigel invasion assays demonstrated that miR-200c-transfected MDA-MB-231 cells were significantly less invasive than the cells transfected with mimic control (Fig. 2C). Wound healing assays demonstrated that the ectopic expression of miR-200c inhibited the cells' migration (Fig. 2D). Collectively, our results support the role of miR-200c as an important contributor to the inhibition of breast cancer proliferation, invasion, and migration as a tumor suppressor.

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3.3. HDAC inhibitor inhibits proliferation and invasion in breast cancer cells by modulating miR-200c and CRKL

Vorinostat (SAHA) is the most advanced HDAC inhibitor which is known to inhibit MCF-7, MDA-MB 231, and SKBr-3 breast cancer cell lines by inducing G1 and G2-M arrest and apoptosis [27]. Moreover, SAHA has entered clinical phase I-II trials in breast cancers, showing the potential for clinical benefit in terms of stable disease [28, 29]. Thus we focused on SAHA as the prototypical HDAC inhibitor of this study. To confirm whether miR-200c is the direct functional mediator contributing to the anticancer mechanism of HDAC inhibitor, breast cancer MDA-MB-231 cells, after SAHA treatment, were transfected with miR-200c inhibitors. Our results showed that miR-200c expression levels were upregulated in SAHA-treated breast cancer cells (Fig. 3A), while CRKL expression at protein levels were downregulated with SAHA treatment (Fig. 3B). Moreover, the miR-200c inhibitors could partially abrogate the SAHA induction to miR-200c expression in breast cancer cells (Fig. 3A and B).

More importantly, the introduction of SAHA significantly decreased the proliferation of breast cancer cells and blocked their invasion (Fig. 3C and D). The downregulation of miR-200c by transfecting with 100 nM miR-200c inhibitor could impair HDAC inhibitor's ability to induce the inhibition of proliferation and invasion in breast cancer cells. Cell proliferation assays showed that miR-200c inhibitors could partially abrogate the SAHA-treated effects in breast cancer cells, and restore the proliferation and growth of MDA-MB-231 cells at 48h (Fig. 3C). The results of Matrigel invasion assays demonstrated that the invasion of the breast cancer cells could partially be restored by the introduction of miR-200c inhibitors (Fig. 3D). The knockdown of CRKL with transient siRNA (Fig. 3B) significantly inhibited the proliferation and invasion of MDA-MB-231 breast cancer cells (**Fig. C** and **D**). Thus, the above results indicate the anticancer mechanism of HDAC inhibitor was partially realized by regulating miR-200c through targeting CRKL.

3.4. MiR-200c levels are inversely consistent with those of CRKL that is directly targeted by miR-200c in breast cancer cells

The expression profiles of miR-200c and CRKL were analyzed with qRT-PCR. The results show that expression levels of CRKL were upregulated in all seven breast cancer cell lines compared with the two immortalized normal mammary epithelial cell lines while miR-200c was down-regulated in breast cancer. This demonstrates that miR-200c levels were inversely consistent with CRKL in breast cancer cells (Fig. 4A).

To identify miR-200c target genes, we scanned the genome for putative miR-200 binding sites with bioinformatic analysis and identified CRKL as predicted target gene of miR-200 (Fig. 4B). To verify whether the expression of CRKL is modulated by miR-200c in breast cancer cells, MDA-MB-231 cells were transfected with miR-200c mimics. We further used western blotting to analyze the protein expression of CRKL. Fig. 4C shows that the ectopic expression of miR-200c downregulated CRKL expression by approximately 68%. Therefore, we conclude that miR-200c directly targets CRKL.

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4. Discussion

Breast cancer is the most commonly diagnosed cancer and is the leading cause of cancer death among females worldwide [30]. The study of epigenetic changes associated with the development and progression of breast cancer is rapidly emerging as an attractive field of study. HDACs exert their targeted action via posttranslational acetylation of core nucleosome histones, thereby regulating gene expression [28]. MiRNAs can play a critical role in HDAC regulation and control in cancer [31]. The anticancer mechanism of HDAC inhibitor in breast cancer remains poorly understood. In this report, our findings, which demonstrate the involvement of miR-200c through CRKL downregulation, provide novel evidence in mechanisms of HDAC inhibitor treatment.

It was reported that one third of human genes appear to be conserved miRNA targets, which are regulators of many functional genes [32]. Aberrant expression of some miRNAs occurs and plays critical roles in various cancers [31, 33–35]. Some miRNA deregulation has been demonstrated to play important roles in breast cancer proliferation, migration, metastasis, chemoresistance, and radioresistance through directly regulating expression of targeted genes [12, 36–38]. Previous studies have shown that the downregulation of specific members of the miR200 family members, which act as potential suppressors, was associated with the growth, migration, and invasion of breast cancer cells [16, 39–41]. Our studies indicated that miR-200c was downregulated in breast cancer cells. Upon transfection with miR-200c mimics, the ectopic expression of miR-200c significantly inhibited the proliferation, invasion, and migration of these cells. Furthermore, suppression of miR-200c expression could partially abrogate the miR-200c-mediated effects in breast cancer cells to restore proliferation and migration. Therefore, these results indicate that repression of miR-200c may induce tumor development and progression in breast cancer carcinogenesis.

We identified CRKL as a direct target of miR-200c in breast cancer cells. The ectopic expression of miR-200c led to a significant reduction in CRKL at the protein level. The upregulation of CRKL protein expression was presented after repression of miR-200c in MDA-MB-231 cells transfected with miR-200c inhibitors. MiR-200c expressions levels were inversely correlated with CRKL protein levels. As an oncogene, CRKL is overexpressed in a number of human malignant tumors [22, 42], and its overexpression contributes to malignant transformation in multiple aspects of tumorigenesis. Young *et al.* [23] demonstrated that amplification and resultant overexpression of CRKL contribute to diverse oncogenic phenotypes in lung cancer, and knockdown of CRKL in lung cancer cell lines has led to a significant decrease in cell proliferation and invasiveness; these findings suggest that altered expression of CRKL by gene amplification may contribute to the oncogenic phenotype in lung cancer. In regards to breast cancer, it was reported that the overexpression of CRKL protein played a role in integrating signals for migration and invasion of highly malignant breast cancer cell lines, and revealed a significant association between highly proliferative breast tumors and poor outcome [26]. Our results show that CRKL protein levels were up-regulated in breast cancer cells, and the overexpression of CRKL protein promoted breast cell proliferation, invasion, and migration. Our findings are the first to identify a significant inverse correlation between CRKL protein levels and

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miR-200c expression, which provided support for a novel diagnostic marker and potential therapeutic target in breast cancer.

Interestingly, our data revealed that the expression levels of miR-200c were significantly upregulated in breast cancer cell lines after HDAC inhibitor treatment, whereas, CRKL expressions levels at both mRNA and protein were downregulated. Previous studies have shown that HDAC inhibitors could reactivate gene expression and inhibit the growth and survival of tumor cells, underlying the potential of HDAC inhibitors as exciting new agents for the treatment of cancer [43]. HDAC inhibitors were identified to induce the inhibition of proliferation and clonogenic growth in several cancer cell lines *in vitro* [44, 45]. Vorinostat was the first histone deacetylase inhibitor approved by FDA for the treatment of CTCL [46]. Subsequently, three more HDAC inhibitors, Romidepsin [47], Panobinostat [48], and Belinostat [49], have been evaluated for safety and efficacy, and were approved by the U.S. Food and Drug Administration (FDA) for clinical treatment. HDAC inhibitors have been shown to rapidly induce p21Waf1/Cip1 mRNA and activation expression of this critical tumor suppressor gene is necessary for mediating the anti-proliferative effect *in vitro* and *in vivo* [50, 51]. Recently, SAHA has been shown to significantly promote *in vitro* trafficking of MDA-MB-231 and BT-549 cells via the induction of epithelial-mesenchymal transition (EMT) [52]. Nevertheless, the downstream anticancer mechanism of HDAC inhibitor and whether the coregulatory signaling involves an additional layer of epigenetic mechanisms of action remain poorly understood in breast cancer. More importantly, recent studies revealed that HDAC inhibitors can reverse PELP1-mediated suppression of miR-200 family members [6], suggesting a direct relationship between HDAC inhibitors and miR-200 family members in breast cancer. Our findings demonstrated that the decreased expression of miR-200c weakened the inhibitory functions of HDAC inhibitors in breast cancer cells, suggesting that the interactions of miR-200c with HDAC inhibitors played a critical role in the anticancer process. Furthermore, miR-200c inhibits cell proliferation, migration, and invasion by downregulating CRKL. We realize that our miR-200c inhibitors were unable to completely neutralize SAHA effect on inhibiting the proliferation and invasion of breast cancer cells. One possible reason is that our miR-200c inhibitors fail to completely block the increasing levels of miR-200c induced via SAHA. Moreover, altered signaling via introduction of either SAHA or miR-200c is likely connected to several downstream divergent pathways. Thus, we think that SAHA anticancer action realizes at least partly through the miR-200c-CRKL axis. It is imperative that more investigations explore the mechanism of HDAC inhibitor action.

In conclusion, this study demonstrates that the forced expression of miR-200c inhibited proliferation, invasion, and migration of human breast cancers by downregulating CRKL. We identified that HDAC inhibitors inhibited breast cancer cell proliferation and migration by regulating miR-200c through direct CRKL targeting. HDAC-miR200c-CRKL signaling axis may play an important role in the anticancer mechanism functions of HDAC inhibitor in breast cancer cells. Our novel findings partially revealed theories for breast cancer clinical treatment of HDAC inhibitor, and suggest that HDAC-miR200c-CRKL signaling axis could be a novel diagnostic marker and potential therapeutic target in breast cancer.

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Abbreviations

HDAC	histone deacetylase
SAHA	Suberoylanilide Hydroxamic Acid (Vorinostat)
miRNA	microRNA
CRKL	Crk-like
EMT	epithelial-mesenchymal transition

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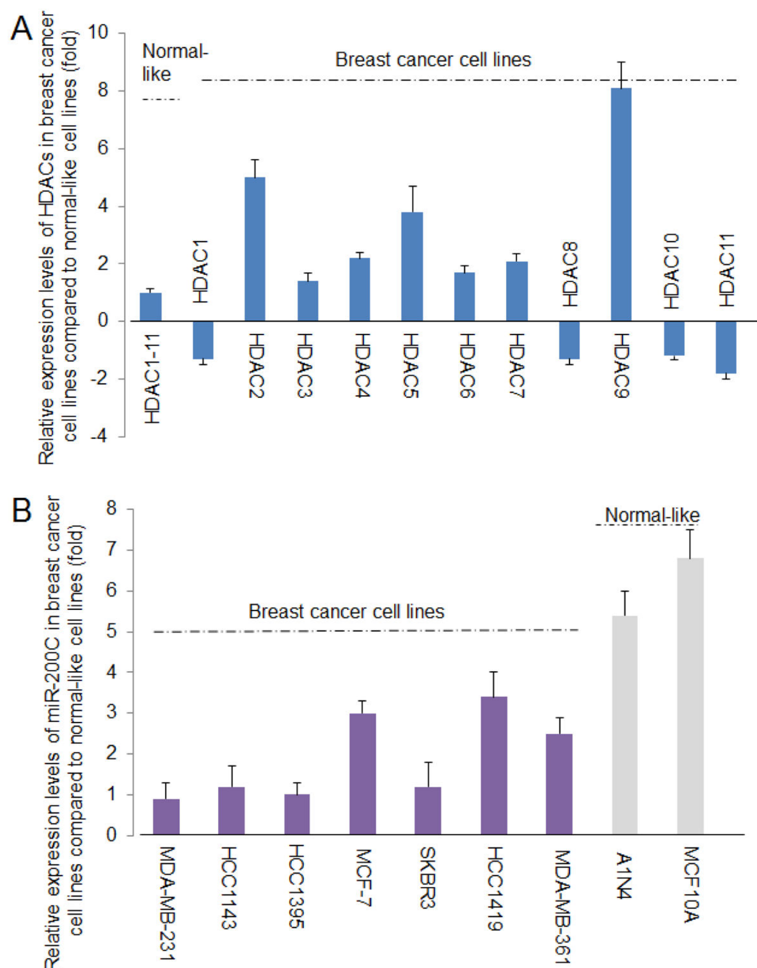

Fig. 1.

HDACs and miR-200c relative expression levels in breast cancer cell lines compared to those in normal-like cell lines. (A) Relative expression levels of HDAC1-11 in breast cancer cell lines, MDA-MB-231, HCC1143, HCC1395, MCF-7, SKBR3, HCC1419, and MDA-MB-361, compared to those (mean value as 1) in normal-like cell lines, A1N4 and MCF-10A, by quantitative RT-PCR analysis ($P<0.01$). (B) All seven breast cancer cell lines expressed lower miR-200c levels compared to two normal-like cell lines by quantitative RT-PCR analysis($P<0.01$). All samples were performed in triplicate. All experiments were then repeated once more.

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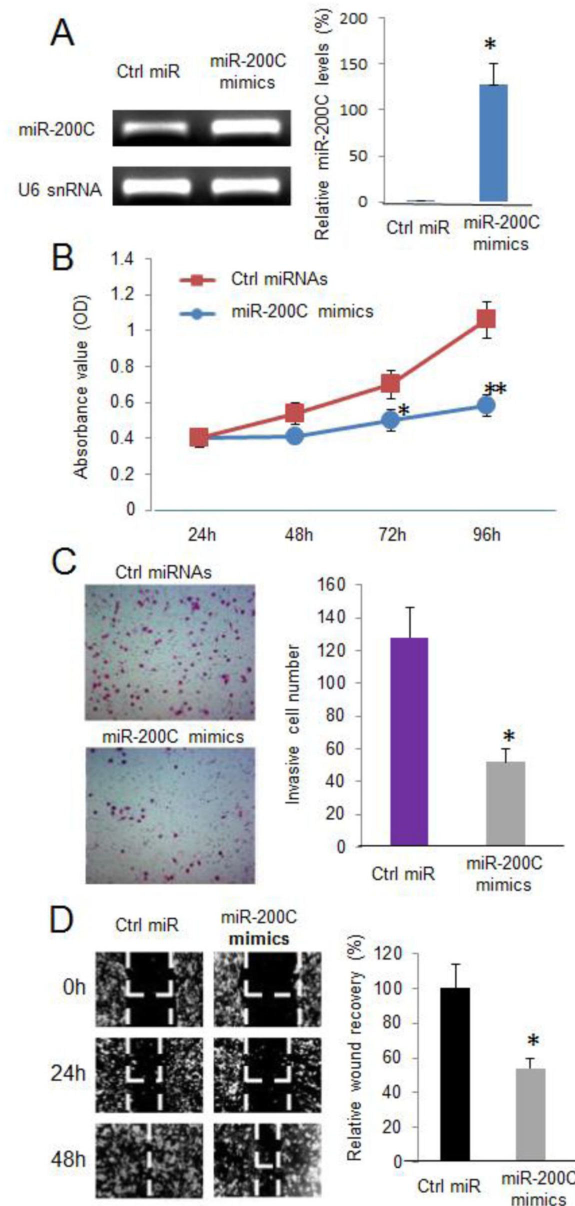

Fig. 2.

The ectopic expression of miR-200c inhibited the proliferation, invasion and migration of breast cancer cells. (A) miR-200c overexpression in MDA-MB-231 breast cancer cells at 24hr post transfection of miR-200c mimics determined quantitative PCR analysis. MDA-MB-231 cells were treated with 100 nM of miR-200c mimics or control oligonucleotides for 24 hours prior to qPCR. *, $P < 0.01$ compared to control. (B) Proliferation analysis of breast cancer cells treated with miR-200c mimics. MDA-MB-231 cells were treated with miR-200c mimics or control oligonucleotides for 24, 48, 72, or 96 hours before proliferation assays were performed. *, $P < 0.05$ and **, $P < 0.01$ compared to control, (C) Invasion of ectopically miR-200c-expressed breast cancer cells. MDA-MN-231 cells were with treated with 100 nM miR-200c mimics or control oligonucleotides for 16 hours prior to Matrigel

invasion assays. *, $P < 0.01$ compared to control. (D) Migration of ectopically miR-200c-expressed breast cancer cells. MDA-MB-231 cells were treated with 100 nM miR-200c mimics or control oligonucleotides for 24 hours before a wound-healing assays were performed. *, $P < 0.01$ compared to control.

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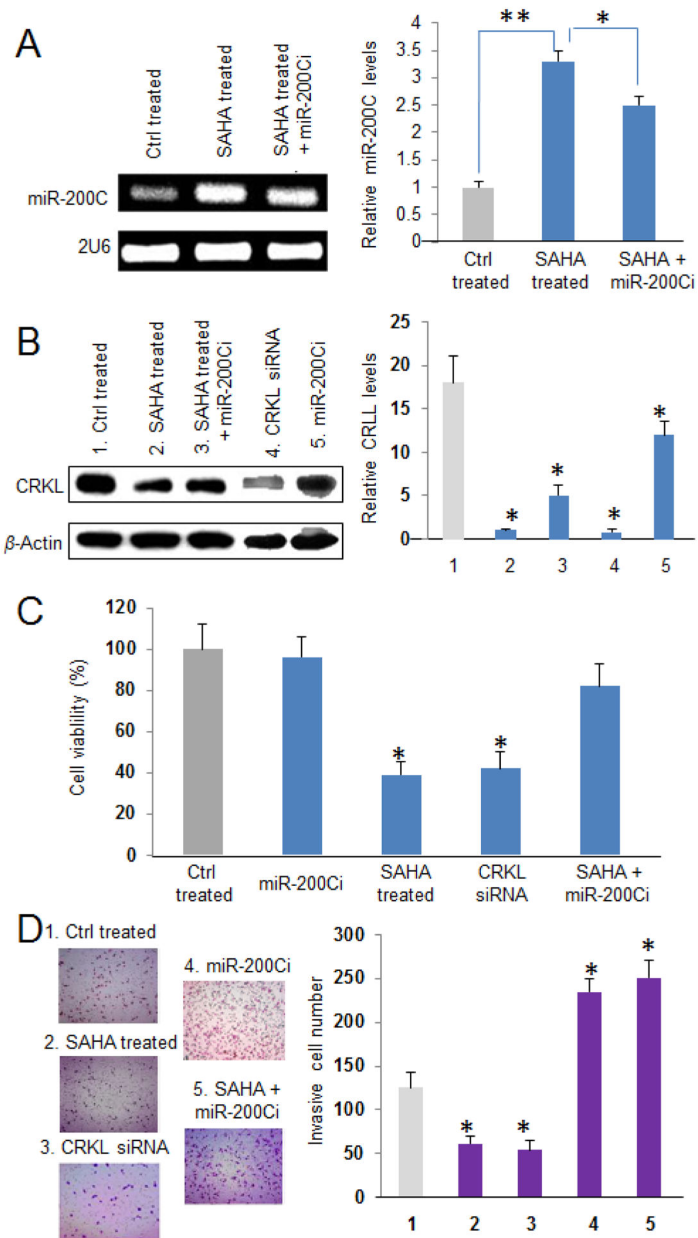

Fig. 3.

HDAC inhibitor inhibited proliferation and invasion in breast cancer cells by modulating miR-200c and CRKL. (A) Expression levels of miR-200c of breast cancer cells treated with SAHA. MDA-MB-231 cells were treated with 1 μM SAHA alone or a combination of SAHA and 100 nM miR-200C inhibitors for 24 hour before a quantitation RT-PCR assays were performed. *, $P < 0.05$ and **, $P < 0.01$ compared to the control. (B) Expression levels of CRKL in breast cancer cells treated with the inhibitors. Breast cancer MDA-MB-231 cells were treated with 1 μM SAHA, 100 nM miR-200c, or 100 nM CRKL siRNA alone or a combination of SAHA and miR-200c inhibitor for 24 hours before Western blot assays were performed. The experiments were performed in duplicates for each sample. The process was repeated once more. *, $P < 0.01$ compared to the control. (C) Proliferation of breast cells

treated with the inhibitors. MDA-MB-231 breast cancer cells were treated with 1 μM SAHA, 100 nM miR-200C, or 100 nM CRKL siRNA alone or a combination of SAHA and miR-200c for 24 hours before a proliferation assay was performed. *, $P < 0.01$ compared to the control. (D) Invasion of breast cancer cells treated with the inhibitors. MDA-MB-231 cells were treated with 1 μM SAHA, 100 nM miR-200c, or 100 nM CRKL siRNA alone or a combination of SAHA and 100 nM miR-200C inhibitors for 24 hours before Matrigel Invasion assays were performed. *, $P < 0.01$ compared to the control.

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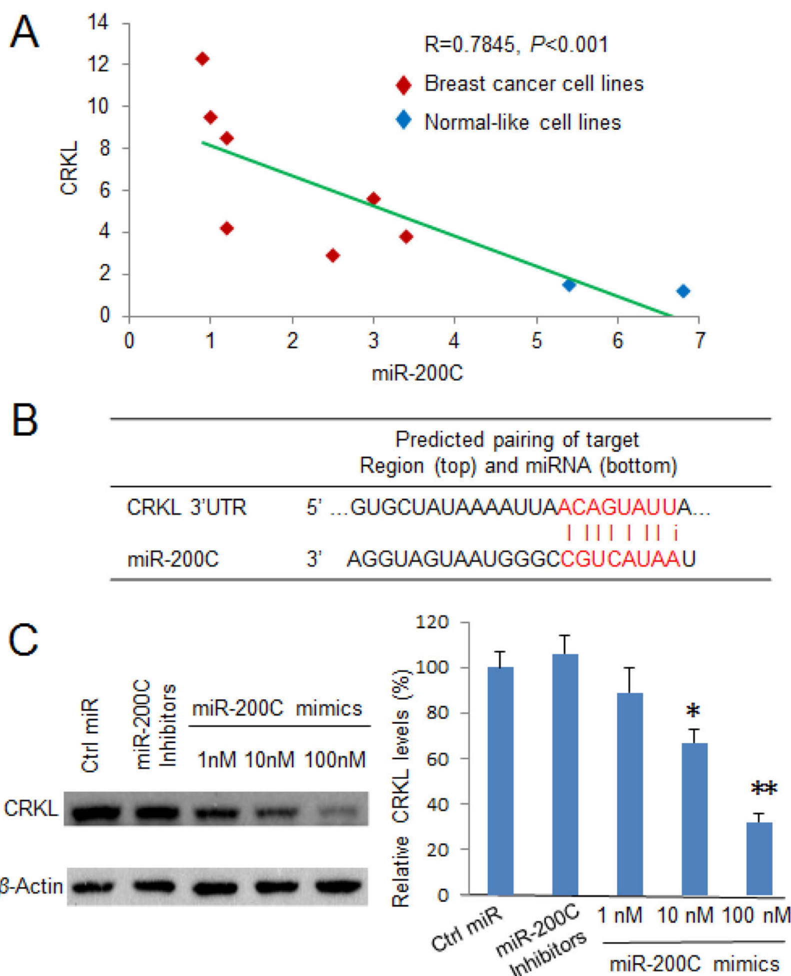

Fig. 4.

MiR-200c directly targeted at CRKL. (A) CRKL expression levels are conversely correlated with miR-200c levels in breast cancer cell lines and normal-like cell lines by qRT-PCR. Seven breast cancer cell lines (labeled with read in the graph) are MDA-MB-231, HCC1143, HCC1395, MCF-7, SKBR3, HCC1419, and MDA-MB-361. Two normal-like cell lines (labeled with blue in this graph) are A1N4 and MCF-10A. (B) A conserved targeted site of miR-200c high (>0.80) probability of conserved targeting (Targetscan aggregate P_{CT}) scores in the 3′UTRs of CRKL indentified by TargetScan 6.2 software. (C) Expression levels of CRKL of ectopically miR-200c-expressed breast cancer cells by Western blot assays. MDA-MB-231 cells were treated with 100 nM miR-200c inhibitors or control oligonucleotides of 1, 10, and 100 nM miR-200c mimics respectively for 24 hrs prior to Western blot assays. β-actin was used as a loading control. The experiments were performed in duplicates for each sample. The process was repeated once more. Quantification of protein bands was measured using the ImageJ software. *, $P < 0.05$ and **, $P < 0.01$ compared to control.

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Table 1

PCR primers used in this study

Gene	Forward Primer 5′-3′	Reverse Primer 5′-3′	Gene-Bank Accession No.
miR-200c	CCCTCGTCTTACCCAGCAGT	CCATCATTACCCGGCAGTAT	NR_029779
CRKL	ACTGCCTTGGCATTAGAGGT	ACAAAGCAGCAGGAAACAGG	NM_005207
HDAC1	TCGATCTGCTCCTCTGACAA	GCTTCTGGCTTCTCCTCCTT	AY627042
HDAC2	TTCCCTCAGCCCTTTTCTCT	ATGAGGCTTCATGGGATGAC	BC148797
HDAC3	GGAGCTGGACACCCTATGAA	GACTCTTGGTGAAGCCTTGC	AF039703
HDAC4	TGAAGAATGGCTTTGCTGTG	ACGCTAGGGTCGCTGTAGAA	AF132607
HDAC5	AGTGACACCGTGTGGAATGA	AGTTTTGCGGTGATGGCTAC	AF132608
HDAC6	AAGTAGGCAGAACCCCCAGT	GTGCTTCAGCCTCAAGGTTC	AF132609
HDAC7	CCCAGCAAACCTTCTACCAA	TATCCTGAAAGCAGCCAGGT	AY302468
HDAC8	GGCCAGTATGGTGCATTCTT	GGCTGGGCAGTCATAACCTA	AF245664
HDAC9	CAGCAACGAAAGACACTCCA	CAGAGGCAGTTTTTCGAAGG	BC152405
HDAC10	TCCACCCGAGTACCTTTCAC	GTGTTTCTGCTTGGCATGTG	AF426160
HDAC11	TCCGCACAGAACTCAGACAC	AGTAGAGGAAGGGCCAGCTC	BC009676
U6 snRNA	ACAACGTTGCAGTCATTGGA	CTGCTCGAATATTCCCCAAA	NM_016200
β-Actin	GACAGGATGCAGAAGGAGAT	TGCTTGCTGATCCACATC TG	X00351