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ORIGINAL ARTICLE

Prostate Disease

Potential therapeutic effect of epigenetic therapy on treatment-induced neuroendocrine prostate cancer

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Although adenocarcinomas of the prostate are relatively indolent, some patients with advanced adenocarcinomas show recurrence of treatment-induced neuroendocrine prostate cancer, which is highly aggressive and lethal. Detailed biological features of treatment-induced neuroendocrine prostate cancer have not been characterized owing to limited biopsies/resections and the lack of a cellular model. In this study, we used a unique cellular model (LNCaP/NE1.8) to investigate the potential role of cancer stem cells in treatment-induced neuroendocrine prostate cancer with acquired resistance to hormonal therapy and chemotherapy. We also studied the role of cancer stem cells in enhancing invasion in treatment-induced neuroendocrine prostate cancer cells that recurred after long-term androgen-ablation treatment. Using an *in vitro* system mimicking clinical androgen-ablation, our results showed that the neuroendocrine-like subclone NE1.8 cells were enriched with cancer stem cells. Compared to parental prostate adenocarcinoma LNCaP cells, NE1.8 cells are more resistant to androgen deprivation therapy and chemotherapeutic agents and show increased cancer cell invasiveness. Results from this study also suggest a potential epigenetic therapeutic strategy using suberoylanilide hydroxamic acid, a histone deacetylase inhibitor, as a chemotherapeutic agent for therapy-resistant treatment-induced neuroendocrine prostate cancer cells to minimize the risk of prostate cancer recurrence and metastasis.

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INTRODUCTION

Prostate cancer (PCa) is the most commonly diagnosed nonskin malignancy in men and the second leading cause of cancer death in the United States.[1] The vast majority of PCa are prostatic acinar type adenocarcinomas (AdenoCa) consisting mostly (>90%) of tumor cells showing luminal features and requiring androgen for growth. Low-grade, organ-confined PCa is curable by surgery or radiation therapy. For patients with advanced or metastatic PCa where local therapies can no longer be used, the treatment of choice is hormonal therapy, which inhibits androgen production and/or blocks androgen receptor (AR) function. However, this therapy is not curative and cancer recurs after an initial response period averaging 18 months. This recurrent tumor is known as castration-resistant prostate cancer (CRPC). To treat CRPC, newer agents have been approved, including enzalutamide (ENZA) and abiraterone, which better block AR signaling and inhibit intratumoral androgen synthesis, respectively. Unfortunately, these effects are generally short-lived, and resistance to therapy develops quickly.

Detailed histologic and molecular studies of CRPC are lacking because biopsy or resection occurs very rarely in a clinical setting. However, studies have revealed that a significant number of metastatic tumors after hormonal therapy belong to a histological variant form of carcinoma known as neuroendocrine prostate cancer (NEPC), or treatment-induced NEPC (tNEPC), a hormone-refractory PCa subtype.[2] While prostate AdenoCa is relatively slow growing and indolent, tNEPC is highly aggressive and rapidly lethal in addition to being refractory to currently available therapies.[3]

tNEPC is composed solely of neuroendocrine (NE) cells of the small-cell or large-cell type.[4] In a normal mature prostate, approximately 1% of the cell population is NE cells, which are distributed along the epithelial compartment in the prostate gland. These NE cells contain dense core granules in the cytoplasm and secrete several neuronal markers as well as growth-stimulatory factors, but they lack AR expression.[5] Studies have shown that NE cells in the normal prostate, originating from endodermal epithelial cells, are terminally differentiated[6] and may play a functional role in regulating the growth and differentiation of epithelial cells in an androgen-independent manner.[7] For prostate carcinogenesis, NE differentiation is a common feature[8] and is often associated with tumor reprogression after hormonal therapy failure, leading to a poor prognosis.[8–11] In an *in vitro* system mimicking the clinical androgen-ablation condition, Zhang *et al.*[12] have successfully established NE-like subclone cells (designated as NE1.3 and NE1.8) from the human prostate AdenoCa LNCaP cell line with long-term androgen deprivation therapy (ADT). Characterization of these cells showed not only that ADT can induce human prostate

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epithelial NE differentiation in androgen-sensitive AdenoCa (LNCaP cells) but also that developed NE cells have suppressed AR expression and prostate-specific antigen (PSA) level.[5,12] This indicates a potential limit for current therapeutic agents targeting AR signaling, such as ENZA and abiraterone, in clinical management of patients with recurrent tumors after hormonal therapy.

In this study, we used the LNCaP/NE1.8 cellular model to investigate the role of cancer stem cells (CSCs) in both acquiring resistance to hormonal therapy and chemotherapy, and enhancing invasion of tNEPC cells. Suberoylanilide hydroxamic acid (SAHA) has been used as an epigenetic treatment in the management of certain solid tumors.[13] In this study, we examined the potential therapeutic effects of SAHA on the differentiation of enriched CSCs, sensitization to chemotherapeutic agents, and inhibition of cancer cell invasiveness in NE1.8 cells. The results presented here indicate that SAHA is a promising candidate to target tNEPC enriched with CSCs.

MATERIALS AND METHODS

Cell culture

Prostate AdenoCa LNCaP cells and subcloned NE1.8 cells, derived from LNCaP cells, were maintained in a steroid-reduced (SR) environment mimicking the androgen-ablation condition in clinical setting. These cells were provided by Dr. Ming-Fong Lin (University of Nebraska Medical Center, Omaha, NE, USA). LNCaP cells were authenticated by DDC Medical. LNCaP cells were maintained in ATCC-formulated RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (Denville Scientific, Metuchen, NJ, USA). NE cells were maintained in SR-conditioned medium (phenol red-free RPMI-1640 medium supplemented with 10% charcoal-dextran-stripped FBS [Gemini Gemini Bio-Products, Woodland, CA, USA], 5% sodium pyruvate, and 5% nonessential amino acid). Cells were incubated in a humidified incubator at 37°C and 5% CO_2.

Clonogenic assay

Clonogenic assays were performed to evaluate the ability of cells to form colonies in response to treatments ADT, ENZA (Selleckchem, Houston, TX, USA), or docetaxel (DTX; Sigma-Aldrich, St. Louis, MO, USA). For ADT, LNCaP and NE1.8 cells were detached using StemPro Accutase (Life Technologies, Carlsbad, CA, USA) and washed twice with PBS. Cells (1×10^3) were then plated in 60 mm dishes and grown in phenol red-free RMPI 1640 medium containing 10% FBS (as control), or in SR-conditioned medium (as ADT) for 21 days. The medium was changed every 3 days. For ENZA treatment, plated cells were maintained in RPMI-1640 medium containing 10% FBS and 10 μmol l^{-1} ENZA, or dimethyl sulfoxide (DMSO; American Type Culture Collection, Manassas, VA, USA) as control, for 14 days, and the medium was changed every 3 days. For treatment with DTX, plated cells were treated with 1 nmol l^{-1} DTX for 2 days, and then cells were maintained in fresh medium for 20 days, changing medium every 3 days. After staining with crystal violet, colonies consisting of >50 cells were considered as survival colonies and were directly scored using an inverted microscope. The average number of survival colonies was plotted versus that in control to determine the 50% lethal dose (LD_{50}) for each treatment, or survival fractions.

When SAHA (Sigma-Aldrich, St. Louis, MO, USA) pretreatment was applied, NE1.8 cells were treated with 1 μmol l^{-1} SAHA, or DMSO as control, for 72 hours. Cells were then replated and treated with ADT or indicated chemo-drugs as described above.

Invasion assay

LNCaP or NE1.8 cells (5×10^4) in growth medium containing 1% FBS were seeded in 1× BME-coated cell culture inserts (for 24-well plate; Millipore, Billerica, MA, USA). Complete growth medium containing 10% FBS was placed outside the chambers, and cells were allowed to invade toward the attractant of full-serum medium. After 24 hours, processing of chamber filter and visualization/quantification of invasion was performed, as previously described.[14]

Flow cytometric analysis

LNCaP and NE1.8 cells were detached by StemPro® Accutase (Life Technologies) and washed twice with PBS. Cells were then stained with PE-conjugated anti-Sox2, anti-Oct3/4, and anti-Nanog antibodies (BD Biosciences, San Jose, CA, USA), or stained with PE-conjugated anti-CD133 (Miltenyi Biotec, San Diego, CA, USA), or co-stained with PE-conjugated anti-CD24 (BD Biosciences, San Jose, CA, USA) and fluorescein isothiocyanate (FITC)-conjugated anti-CD44 antibodies (BD Biosciences, San Jose, CA, USA). For neuron-specific enolase (NSE; DAKO, Carpinteria, CA, USA) staining, cells were first fixed and permeabilized with BD Perm/WashTM buffer (BD Biosciences, San Jose, CA, USA) as per the manufacturer's instructions and were then incubated with primary anti-NSE antibody followed by incubation with secondary Alexa Fluor @ 568 goat anti-rabbit IgG (Life Technologies, Carlsbad, CA, USA). In the staining process for Sox2, Oct3/4, and Nanog, BD Perm/WashTM buffer was also used. PE- or FITC-positive cells were quantified by flow cytometric analysis on Flow Cytometer LSRII (BD Biosciences, San Jose, CA, USA), with up to 5×10^4 cells counted per run.

For bromodeoxyuridine (BrdU) incorporation assay, 10 μmol l^{-1} BrdU (BD Biosciences, San Jose, CA, USA) was added into the cell suspension 2 hours before collection. Cells were then fixed with cold 70% ethanol and labeled with FITC-conjugated anti-BrdU monoclonal antibody (BD Biosciences, San Jose, CA, USA) as per the manufacturer's instructions. Propidium iodide (Sigma-Aldrich, St. Louis, MO, USA) was added before the flow cytometric analysis. Detection of BrdU incorporation in DNA synthesizing cells was conducted by flow cytometry.

For cell cycle analysis, cells were collected and fixed with 75% ethanol, stained with propidium iodide and analyzed by flow cytometry with 5×10^4 events counted per run, as described previously.[15] The percentages of cells in the G1, S, and G2/M phases of the cell cycle were determined using FlowJo software (FlowJo, Ashland, OR, USA).

Western blot analysis

Cell lysates were prepared in RIPA buffer with mild sonication and subjected to SDS-PAGE for immunoblot assays.

Tumor-initiating test

The tumor-initiating test was conducted following the described methods.[16] The protocol was reviewed and approved by the Chancellor's Animal Research Committee (ARC) at the University of California at Los Angeles, Los Angeles, California, USA (ARC #2009-063-13). Freshly prepared cells were resuspended in serum-free PBS/Matrigel mixture (1:1 *v*/*v*), and 500 LNCaP and NE1.8 cells were inoculated subcutaneously into the bilateral flanks of the same animal (8-week-old male nonobese diabetic (NOD)/severe combined immunodeficiency disease (SCID) mice; Jackson Laboratory, Bar Harbor, ME, USA). The mice were euthanized 5 weeks later, and the tumors were excised. Tumor volumes were determined from caliper measurements of tumor length (L) and width (W) according to the formula $(L \times W^2)/2$. Hematoxylin and eosin staining was performed for validation of tumors formed.

For SAHA treatments, cells were treated with 1 μmol l^{-1} SAHA, or DMSO as control, for 3 days before inoculation. SAHA

(20 mg kg^{-1} body weight day^{-1}) was administered for 5 days intraperitoneally after inoculation of the cancer cells.

Statistical analysis

Statistical analyses were performed using the paired Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Resistance of NE1.8 cells to ADT, ENZA, and DTX treatments

To investigate the biological features of prostate NE cells derived from AdenoCa with long-term treatment of androgen deprivation, we first performed clonogenic survival assays in NE1.8 cells and their parental LNCaP cells with ADT, ENZA, and DTX treatments. Our results showed that as compared to parental LNCaP cells, NE1.8 cells are more resistant to these treatments, showing decreased survival fractions ($P < 0.05$; **Figure 1a**). Invasion assays also showed that cancer cell invasiveness was dramatically enhanced in NE1.8 cells versus LNCaP cells (**Figure 1b**). In NE1.8 cells, we validated the reduced protein levels of PSA and AR, increased expression of NSE, and elevated ERK1/2 activation (without changes of ERK1/2 protein levels; **Figure 1c**), as reported previously. We also detected higher levels of phosphorylated Akt in NE1.8 cells. Interestingly, we found that NE1.8 cells showed increased basal levels of Akt protein (**Figure 1d**). The observed changes of Akt protein level and Akt activation suggest that NE1.8 cells have intrinsic properties of enhanced cell survival.[17] In addition, we detected increased protein levels of AURKA in NE1.8 cells versus LNCaP cells. AURKA is a kinase protein, which is overexpressed in the majority of tNEPC cases and plays a role in tNEPC development (**Figure 1d**).[18,19]

CSC Enrichment in NE1.8 cells

CSCs represent a subpopulation of tumor cells endowed with self-renewal and multi-lineage differentiation capacity. These cells have an innate resistance to cytotoxic agents. This resistance provides major clinical challenges toward the complete eradication of residual disease in cancer patients.[20]

In this study, we examined the potential enrichment of CSCs in NE1.8 cells. To determine the putative CSCs, we used prostatic stem cell marker CD133,[21] embryonic stem cell markers Oct3/4,[22] Sox2,[23] and Nanog,[24] and an early PCa progenitor/stem cell marker CD44$^+$/CD24$^{-/low}$.[25] Flow cytometric analyses showed significant increase in CD133-positive-stained populations in NE1.8 cells (0.74 ± 0.05 for LNCaP *vs* 14.31 ± 1.97 for NE1.8, **Figure 2a**), Oct3/4 (2.32 ± 0.33 for LNCaP *vs* 42.71 ± 4.67 for NE1.8, **Figure 2b**), and CD44$^+$/CD24$^{-/low}$ (2.60 ± 0.30 for LNCaP *vs* 9.53 ± 1.63 for NE1.8, **Figure 2c**). Although no differences were detected for the percentages of cells with positive staining for Sox2 and Nanog between the LNCaP and NE1.8 cells, we observed a notable shift in the cell populations toward positive staining for Sox2 in NE1.8 cells (**Figure 2d** and **2e**).

In NOD/SCID mice, we found that all the sites inoculated with NE1.8 cells (500 cells injection^{-1}) developed tumors (3/3) with an average volume of 57 mm^3 at day 31 whereas only one of the three sites inoculated with LNCaP cells showed detectable tumors with a volume of 28 mm^3 (**Figure 3a**). We also detected a lower BrdU incorporation rate in NE1.8 cells (3.78% ± 0.28%) than in LNCaP cells (10.58% ± 1.82%) (**Figure 3b**), indicating that NE1.8 cells are quiescent or slow cycling, which are the characteristics of CSCs.[20] In addition, we detected increased NSE expression in NE1.8 cells using both flow cytometric analysis and immunochemistry of NE1.8-derived xenograft tumors (**Figure 3c** and **3d**).

We further tested whether the expression changes of these putative CSC markers in NE1.8 cells were driven by ADT treatment. For this, we examined parental LNCaP cells with ADT treatment. Our results showed that ADT treatment for 2 weeks resulted in increased cell fractions with positive staining of Oct3/4 (from 1.06 ± 0.16 to 3.44 ± 0.51, $P = 0.0015$) and CD133 (from 0.64 ± 0.04 to 1.99 ± 0.24, $P = 0.0007$) (**Figure 4a**). Although Sox2 and Nanog can be detected in almost all LNCaP cells, ADT treatment led to dramatic shifts of the cell populations toward positive staining for Sox2 and Nanog (**Supplementary Figure 1a and 1b**). In addition, ADT treatment did not cause any obvious change in fractions stained for CD44$^+$/CD24$^{-/low}$ in LNCaP cells (**Supplementary Figure 1c**). However, we did notice an increased percentage of cells with CD44 expression (**Figure 4b**). CD44 is a cell surface protein that plays a role in tumor growth and metastasis. Interestingly, CD44 alone has also been suggested to be a valuable cell surface marker for CSCs.[26,27]

SAHA treatment induces differentiation of CSCs in NE1.8 cells

Our above results showing the differences in expression of multiple proteins in NE1.8 cells (*vs* parental LNCaP cells) suggest the potential of epigenetic changes during the development of NE1.8 cells. This raises the possibility of using epigenetic therapy for NE1.8 cells, or cancerous prostate NE cells. We therefore investigated the effects of SAHA, an histone deacetylase (HDAC) inhibitor that has shown promising results in clinical trials as an epigenetic therapy for human malignancies,[13] on NE1.8 cells.

We first examined the effects of SAHA on enriched CSCs in NE1.8 cells. As shown in **Figure 5a**, we found that treatment with 1 μmol l^{-1} SAHA for 72 hours reduced the fraction of NE1.8 cells with positive staining of CD44$^+$/CD24$^{-/low}$ (from 8.73 ± 1.04 to 1.83 ± 0.89, $P = 0.0003$), Oct3/4 (from 39.2 ± 0.78 to 3.61 ± 0.18, $P = 0.0002$), and CD133 (from 14.12 ± 1.01 to 8.62 ± 0.66, $P = 0.0013$). SAHA treatment did not cause obvious changes in the percentage of NE1.8 cells stained positive for Sox2. However, we noticed a subpopulation showing weak Sox2 staining in both NE1.8 cells treated with or without SAHA, and the percentage of this subpopulation increased significantly in SAHA-treated cells (1.53 ± 0.33 for DMSO and 2.42 ± 0.16 for SAHA, $P = 0.0139$) (**Figure 5b**). Exposure to SAHA also resulted in the presence of a subpopulation of NE1.8 cells that did not show NSE staining. Statistical analysis indicated a significant difference in the percentages of this subpopulation in SAHA-treated cells versus control cells (0.81 ± 0.04 for control *vs* 1.52 ± 0.11 for SAHA, $P = 0.00004$) (**Figure 5c**). This observation indicates the potential of SAHA to induce transdifferentiation of the NE phenotype in NE1.8 cells.

Our Western blot results further showed that in NE1.8 cells, SAHA exposure increased protein expression of the differentiation markers of involucrin and syndecan-3,[28,29] as well as p21Waf1/Cip1, which served as a positive control for SAHA treatment (**Figure 5d**).[30]

In the tumor-initiating assay, we found that all three sites inoculated with NE1.8 cells without SAHA treatment developed tumors by day 31, with an average tumor volume of 98.9 ± 14.2 mm^3. Two of the three sites inoculated with NE1.8 cells that were treated with SAHA developed tumors with an average tumor volume of 53.1 ± 37.8 mm^3 (**Figure 5e**). SAHA treatment also led to longer latency for tumor formation when compared to control cells (17 ± 4 days *vs* 24 ± 3 days). IHC staining showed reduced NSE expression in tumors formed with SAHA-treated NE1.8 cells (**Figure 5f**).

In addition, we observed that SAHA treatment reduced total Akt levels and Akt phosphorylation. Exposure to SAHA also slightly decreased protein expression of AURKA in NE1.8 cells (**Figure 5g**).

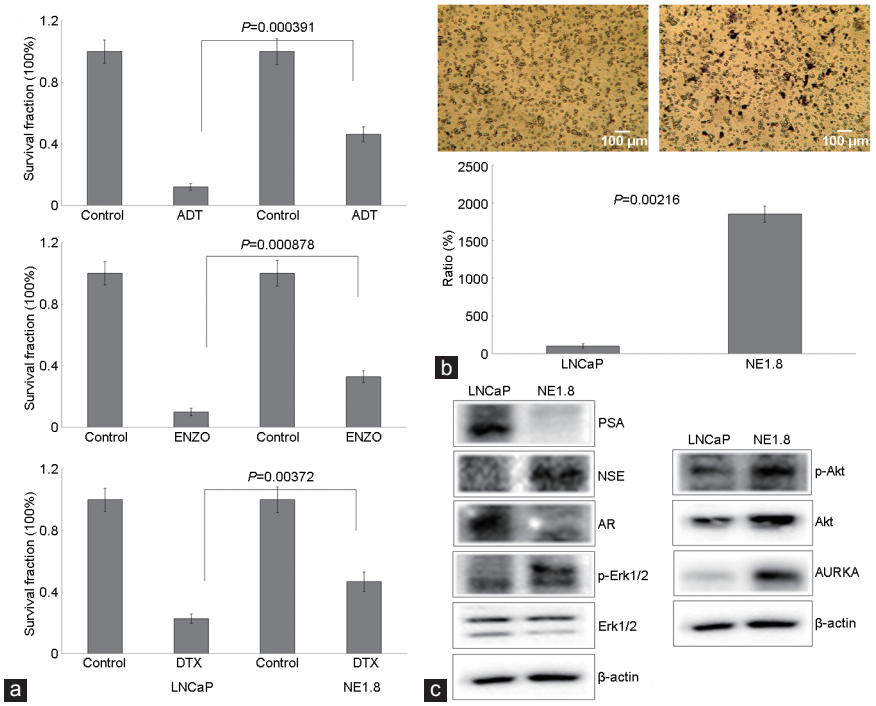

Figure 1: NE1.8 cells are more resistant to treatments of ADT, ENZA, and DTX, and also show elevated invasiveness. (**a**) Clonogenic survival analysis showing the resistance of NE1.8 cells to treatments of ADT, ENZA (10 μmol l^{-1}), and DTX (1 nmol l^{-1}). (**b**) Invasion assay showing NE1.8 cells are more invasive compared to LNCaP cells; top: representative images for transwell invasion assay; bottom: relative quantification of cellular invasiveness. (**c**) Western blot analysis. *P* values were determined from at least three independent experiments. Error bars indicate standard deviation. ADT: androgen deprivation treatment; ENZA: enzalutamide; DTX: docetaxel; PSA: prostate-specific antigen; NSE: neuron-specific enolase; AR: androgen receptor.

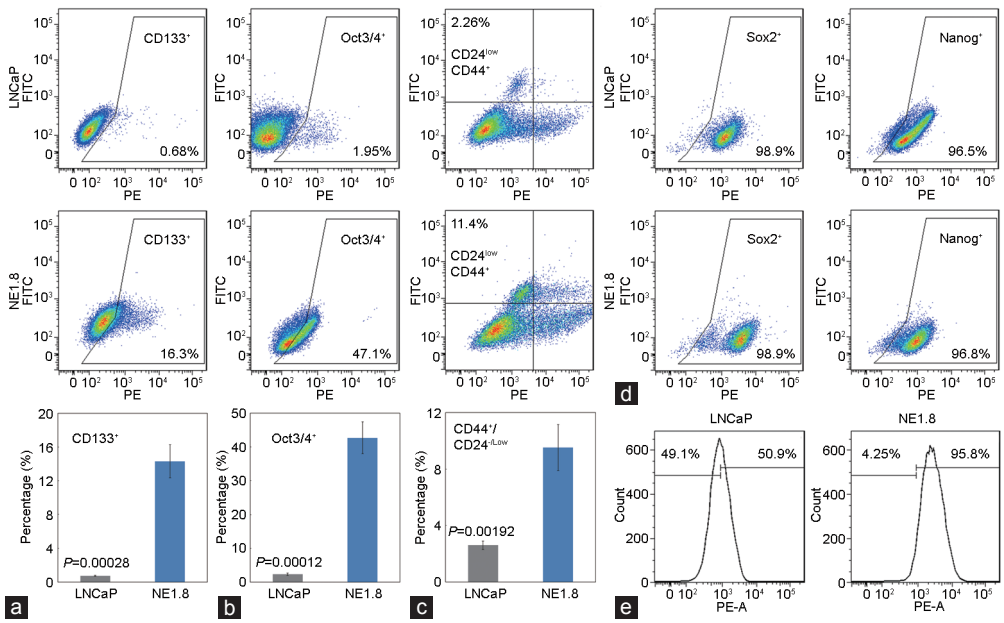

Figure 2: Flow cytometric analysis for stem cell surface markers. Increased cell fractions with positive staining of putative stem cell markers CD133 (**a**), Oct3/4 (**b**), and $CD44^{+}/CD24^{-/low}$ (**c**) in NE1.8 cells compared to parental LNCaP cells. Top: flow cytometric analysis; bottom: diagram showing the percentages of cell populations with positive staining. *P* values were determined from at least three independent experiments. Error bars indicate standard deviation. (**d**) Flow cytometric analysis for putative stem-cell surface markers Sox2 (left) and Nanog (right) in NE1.8 and LNCaP cells. (**e**) Flow cytometric analysis showing the shift of cell population toward positive staining for Sox2 in NE1.8 cells when compared to LNCaP cells. FITC: fluorescein isothiocyanate.

Therapeutic potential of SAHA on NE1.8 cells

We next evaluated the potential effects of SAHA on the sensitivity of NE1.8 cells to treatments of ADT or chemotherapeutic agents. The results showed that pretreatment with SAHA significantly reduced the clonogenic survival ($P < 0.05$) of NE1.8 cells in response to ADT, ENZA (10 μmol l^{-1}), and DTX (1 nmol l^{-1}) treatments (**Figure 6a**). We further noticed that SAHA treatment reduced the invasiveness of NE1.8 cells ($P = 0.0043$) (**Figure 6b** and **6c**).

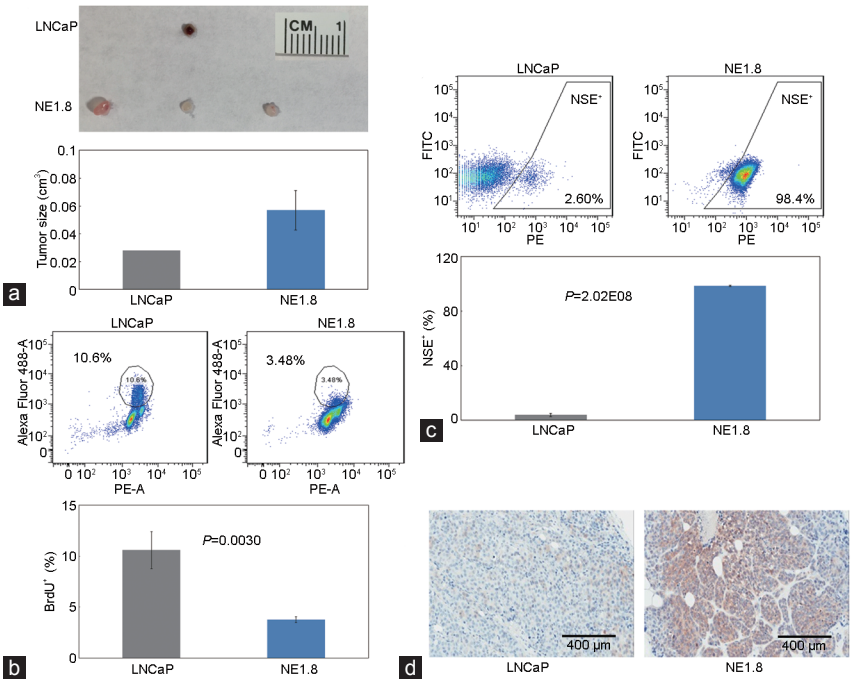

Figure 3: Tumorigenicity. (**a**) Tumorigenesis of NE1.8 cells versus LNCaP cells; top: images for collected tumors from tumor initiating test; bottom: diagram showing the average of tumor volumes. (**b**) BrdU incorporation assay; numbers indicate the percentage of BrdU-positive cells. (**c**) Flow cytometric analysis for NSE staining; diagram showing the percentages of cell populations with positive staining (bottom). (**d**) IHC staining of NSE in tumor specimen collected from tumor initiating test. *P* values were determined from at least three independent experiments. Error bars indicate standard deviation. BrdU: bromodeoxyuridine; NSE: neuron-specific enolase; IHC: immunohistochemistry; FITC: fluorescein isothiocyanate.

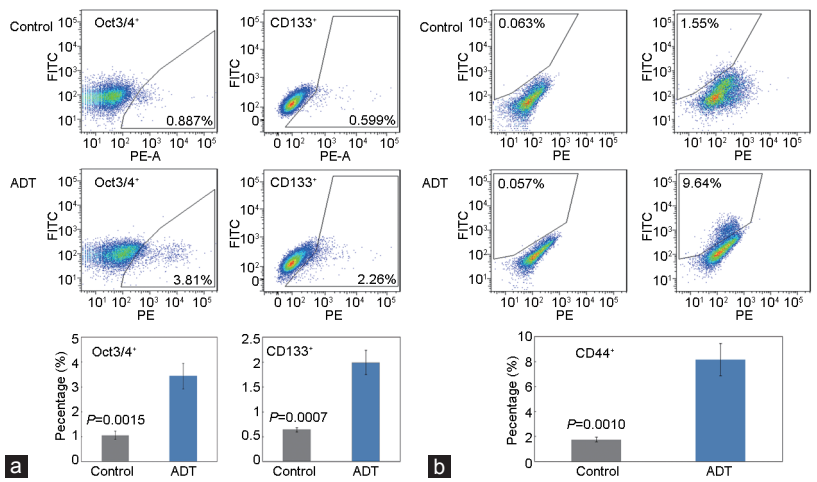

Figure 4: Effects of ADT treatment on CSC enrichment in LNCaP cells. (**a**) Effects of ADT treatment on expression of putative stem-cell surface markers of Oct3/4 and CD133 in LNCaP cells; top: representative results of flow cytometric analysis; bottom: diagram showing the percentages of cell populations with positive staining for indicated marker. (**b**) Effects of ADT treatment on CD44 expression in LNCaP cells; top left: flow cytometric analysis of gating control for LNCaP cells without ADT treatment; top right: flow cytometric analysis of CD44 for LNCaP cells without ADT treatment; middle left: flow cytometric analysis of gating control for LNCaP cells with ADT treatment; middle right: flow cytometric analysis of CD44 for LNCaP cells with ADT treatment; bottom: diagram showing the percentages of cell populations with positive CD44 staining. *P* values were determined from at least three independent experiments. Error bars indicate standard deviation. ADT: androgen deprivation treatment; CSC: cancer stem cell; FITC: fluorescein isothiocyanate.

Taken together, our results show that SAHA treatment can cause epigenetic changes, induce differentiation of enriched CSCs, and reduce the invasiveness of hormone-resistant prostate NE cancer cells. SAHA treatment also restores the therapeutic effect of ADT treatment and increases the cytotoxic effects of ENZA and DTX in NE cancer cells.

DISCUSSION

Evidence has shown that the transition of PCa from an androgen-sensitive state to an androgen-independent state is correlated with transdifferentiation of luminal-type cancer cells into NE-like cells. Histologically, focal NE differentiation ranges from 10% to 100% in prostate adenocarcinomas treated by ADT. However, it is

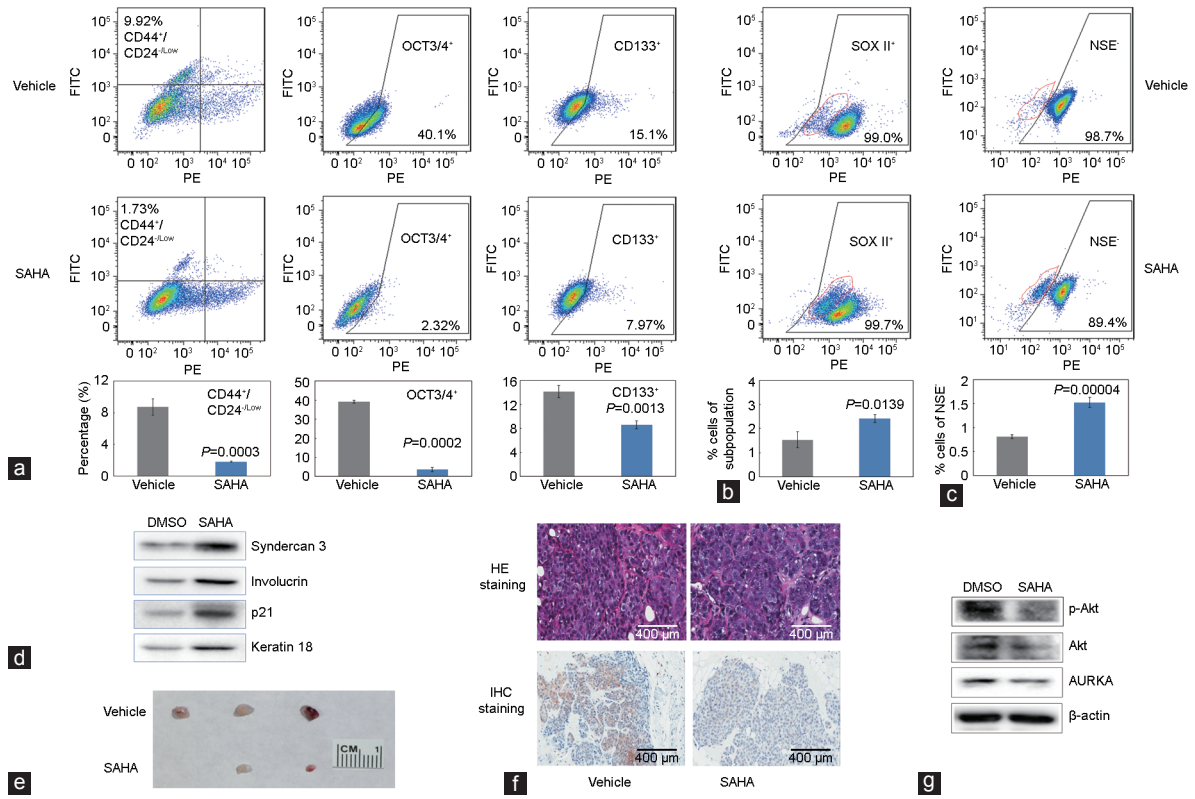

Figure 5: SAHA induces differentiation of enriched CSCs in NE1.8 cells. (**a**) Effects of SAHA treatment on cell populations of $CD44^{+}/CD24^{-/low}$, Oct3/4, and CD133 in NE1.8 cells; top: representative results of flow cytometric analysis; bottom: diagram showing the percentages of cell populations with positive staining of indicated marker. (**b**) Effect of SAHA treatment on Sox2 expression in NE1.8 cells; top: representative results of flow cytometric analysis; bottom: diagram showing the percentages of the cell subpopulation with weak expression of Sox2 as circulated. (**c**) Effect of SAHA treatment on NSE expression in NE1.8 cells; top: representative results of flow cytometric analysis; bottom: diagram showing the percentages of cell populations with negative NSE staining as circulated. (**d**) Western blots showing that SAHA treatment induces expression of differentiation markers of involucrin and Syndecan 3 proteins in NE1.8 cells. β-actin was included for equivalent protein loading. (**e**) Images of collected tumors from tumor initiating test with SAHA-treated NE1.8 cells. (**f**) HE and IHC staining of NSE in a tumor specimen collected from the tumor initiating test with SAHA-treated NE1.8 cells. (**g**) Western blots showing that the effects of SAHA treatment on activation and expressions of Akt, and on AUKRA protein level in NE1.8 cells. *P* values were determined from at least three independent experiments. Error bars indicate standard deviation. SAHA: suberoylanilide hydroxamic acid; CSC: cancer stem cell; NSE: neuron-specific enolase; HE: hematoxylin and eosin stain; IHC: immunohistochemistry; FITC: fluorescein isothiocyanate.

also possible that the original population of NE cells (approximate 1%) acts as CSCs, which can survive hormonal therapy due to their AR-independent nature and then expand its population leading to CRPC.[31–33] Nevertheless, patients with prostate NE cancers often face poor outcomes, and few therapeutic strategies can be applied to these patients in the clinic.[4] Thus, a clearer understanding of biological features and appropriate handling of cancerous prostate NE cells in PCa will benefit clinical practice.

Accumulating experimental and clinical evidence supports the notion that a rare subset of a cancer cell population exists with stem cell properties. These cells could give rise to a hierarchy of proliferative and differentiated bulk tumor cells, leading to tumor initiation, progression, recurrence, and metastasis to distant organs. This subset of cells, or CSCs, has been identified in many types of cancer, including PCa.[34,35] The discovery of the ability of CSCs to form early micrometastases and survive after chemotherapy suggests the importance of CSCs for cancer recurrence.[36] CSCs also show an innate resistance to cytotoxic agents which is a major clinical challenge toward the complete eradication of residual disease in cancer patients.[37] Therefore, targeting CSCs has been suggested to be one of the most promising strategies for the improvement of clinical management for cancer patients.[38]

Previous studies indicate that NE cells show several features of CSCs, including quiescence, expression of PCa CSC marker CD44, and androgen independence for growth.[20,33,39–41] Our data demonstrate that NE1.8 cells, cancerous prostate NE cells derived from AdenoCa (LNCaP) with long-term treatment of androgen-ablation (ADT), are potentially enriched with CSCs. Compared to parental AdenoCa cells, these cancerous NE cells are more resistant to AR signaling-dependent treatments (ADT and ENZA) and chemotherapeutic agent (DTX). We also observed that NE1.8 cells have an elevated capability of invasiveness.

Our results also show that exposure to SAHA could restore the sensitivity of NE1.8 cells to treatment with ADT and ENZA and increase the cytotoxic effect of DTX. SAHA treatment also induces differentiation of enriched CSCs and reduces cancer cell invasiveness of NE1.8 cells. These observations suggest the therapeutic potential of epigenetic treatment on developing cancerous prostate NE cells. Epigenetic therapy has been recently implemented in clinical cancer management as an effective and well-tolerated treatment.[42,43] Histone proteins are the therapeutic target of a cancer epigenome. Posttranslational modifications that occur on certain amino acid

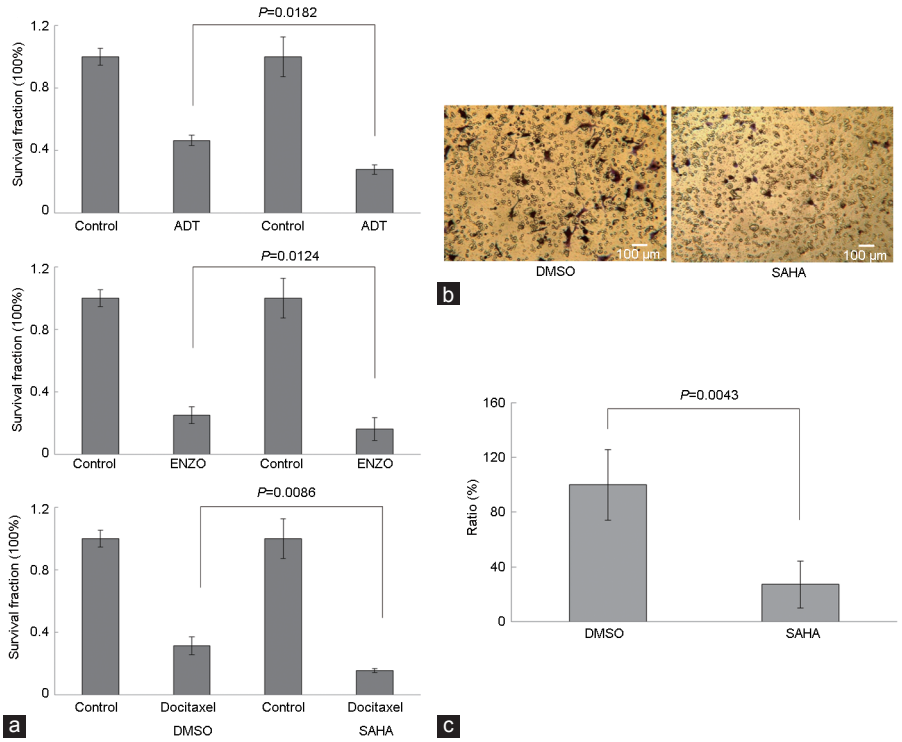

Figure 6: Exposure to SAHA increases the sensitivity of NE1.8 cells to treatments of ADT, ENZA, and DTX. (**a**) SAHA treatment reduced clonogenic survival of NE1.8 cells in response to treatments of ADT, ENZA (10 μmol l^{-1}), and DTX (1 nmol l^{-1}). (**b**) Representative images of transwell invasion assay for NE1.8 cells with or without SAHA treatment. (**c**) Relative quantification of cellular invasiveness. *P* values were determined from at least three independent experiments. Error bars indicate standard deviation. DMSO: dimethyl sulfoxide; SAHA: suberoylanilide hydroxamic acid; ADT: androgen deprivation treatment; ENZA: enzalutamide; DTX: docetaxel.

residues of histone protein tails modify the chromatin structure, and thereby alter gene expression. Among the covalent histone modifications, histone acetylation is the best understood. The HDAC enzymes, together with their counterparts, the histone acetyl-transferases (HATs), control the level of histone acetylation. Studies have demonstrated that HDAC inhibitors (HDACis) can induce tumor cell apoptosis, growth arrest, senescence, differentiation, immunogenicity, and inhibit angiogenesis. These HDACis are therefore of great interest for cancer epigenetic therapy. One of the known HDACis, SAHA (vorinostat), has been recently approved for clinical trials for the treatment of solid tumors, including CRPC (also available on www.clinicaltrials.gov).[44]

CONCLUSION

Our results reveal the biological features of CSC enrichment, which causes resistance to ADT and chemotherapeutic agents along with increased invasiveness of NE1.8 cells. The observed effects of SAHA on NE1.8 cells not only indicate the potential clinical impacts of SAHA as a single agent for epigenetic therapy in therapy-resistant cancerous prostate NE cells, but also suggest approaches for the combination of SAHA and standard anti-PCa therapy as novel therapeutic strategies for clinical management aiming to minimize the risk of recurrent/metastatic tNEPC. However, future studies are needed to evaluate the clinical applicability of SAHA as part of a therapeutic regimen, as well as to uncover the overall role of epigenetic alterations for this potential regimen.

AUTHOR CONTRIBUTIONS

XX, YHH, XFC, and MZ designed the study, analyzed the data, and drafted the manuscript. YJL, AC, ZL, JS, and ZW analyzed the data. JTH supervised the study. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declared no competing interests.

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