Sodium Butyrate Inhibits the Self-Renewal Capacity of Endometrial Tumor Side-Population Cells by Inducing a DNA Damage Response

Kiyoko Kato1, Aya Kuhara2, Tomoko Yoneda2, Takafumi Inoue2, Tomoka Taka2, Tatsuhiro Ohgami2, Li Dan2, Ayumi Kuboyama2, Soshi Kusunoki1, Satoru Takeda1, and Norio Wake2

Abstract

We previously isolated side-population (SP) cells from a human endometrial cancer cell line, Hec1, and determined that Hec1-SP cells have cancer stem–like cell features. In this study, we isolated SP cells and non-SP (NSP) cells derived from a rat endometrial cell line expressing human [Val] KRAS (RK12V cells) and determined the SP phenotype. RK12V-SP cells showed self-renewal capacity, the potential to develop into stromal cells, reduced expression levels of differentiation markers, long-term proliferating capacity in cultures, and enhanced tumorigenicity, indicating that RK12V-SP cells have cancer stem–like cell features. RK12V-SP cells also display higher resistance to conventional chemotherapeutic drugs. In contrast, treatment with a histone deacetylases (HDAC) inhibitor, sodium butyrate (NaB), reduced self-renewal capacity and completely suppressed colony formation of RK12V-SP cells in a soft agar. The levels of intracellular reactive oxygen species (ROS) and the number of γH2AX foci were increased by NaB treatment of both RK12V-SP cells and RK12V-NSP cells. The expression levels of γH2AX, p21, p27, and phospho-p38 mitogen-activated protein kinase were enhanced in RK12V-SP cells compared with RK12V-NSP cells. These results imply that treatment with NaB induced production of intracellular ROS and DNA damage in both RK12V-SP and RK12V-NSP cells. Following NaB treatment, DNA damage response signals were enhanced more in RK12V-SP cells than in RK12V-NSP cells. This is the first article on an inhibitory effect of NaB on proliferation of endometrial cancer stem–like cells. HDAC inhibitors may represent an attractive antitumor therapy based upon their inhibitory effects on cancer stem–like cells. Mol Cancer Ther; 10(8): 1430–9. ©2011 AACR.

Introduction

Endometrial cancer is the most common gynecologic malignancy in the industrialized world and can be classified into 2 different clinicopathologic types, estrogen-related endometrial cancers (type I) and non-estrogen-related endometrial cancers (type II). The most frequent genetic alteration in type I endometrial cancers is PTEN inactivation, followed by microsatellite instability and mutations of the KRAS and β-catenin. In type II endometrial cancers, TP53 mutation is the most frequent genetic alteration, followed by amplification of ERBB2 (1). Some of these pathways are important determinants of stem cell activity (Wnt-, β-catenin, and PTEN ref. 2–4). These findings suggest a stem cell contribution to endometrial carcinoma development.

Recent evidence suggests that cancer stem–like cells exist in several malignant tumors, such as leukemia (5, 6), breast cancer (7), and brain tumors (8), and that these stem cells express surface markers similar to those expressed by normal stem cells in each tissue (5, 9). Stem cell subpopulations [side-population (SP) cells] have been identified in many mammals, including humans, on the basis of the ability of these cells to efflux the fluorescent dye Hoechst 33342 (10). The SP phenotype is associated with a high expression level of the ATP-binding cassette transporter protein ABCG2/BCRP1 (11). Established malignant cell lines, which have been maintained for many years in culture, have also been shown to contain SP cells as a minor subpopulation (12). Friel and colleagues showed that SP cells derived from the endometrial cancer cell lines (An3CA) had features of cancer stem–like cells including low proliferative activity during 9 days of cultivation, chemoresistance, and enhanced tumorigenicity (13). Hubbard and colleagues showed that a small population of clonogenic cells from endometrial cancer tissues showed self-renewing, differentiating, and tumorigenic properties (14). Gotte and colleagues showed that the adult stem cell marker
Musashi-1 was coexpressed with Notch-1 in a subpopulation of endometrial cells (15). Furthermore, they showed that telomerase and Musashi-1 expressing cells were significantly increased in proliferative endometrium, endometriosis, and endometrial carcinoma tissue, compared with secretory endometrium, suggesting the concept of a stem cell origin of endometriosis and endometrial carcinoma. Most recently, they showed that short interfering RNA depletion of Musashi-1, an adult stem cell marker enriched in the SP, in the endometrial carcinoma cell line Ishikawa leads to interference with the Notch signaling pathway and p21 expression, resulting in an antiproliferative effect and induction of apoptosis (16).

The regulation of histone acetylation is a major mechanism controlling cellular differentiation and the biological phenotype of cancer cells (17). Histone deacetylases (HDAC) and histone acetyl transferases are enzymes that ensure the homeostatic levels of histone acetylation. Deregulated HDAC activity has been found in certain human cancers (18–22). Several studies have shown the antiproliferative or the proapoptotic effects of HDAC inhibitors (HDACi) on endometrial cancer cells (23–25). Thus, HDACs are important therapeutic targets for cancer and several HDACi’s are in various stages of clinical development (26, 27). However, the effect of HDACi’s on proliferation of cancer stem cells is unknown.

We have isolated SP cells from normal human endometrium and from a human endometrial cancer cell line, Hec1, and characterized their properties (28, 29). We have shown that Hec1-SP cells have cancer stem-like cell features. In this study, SP cells and non-SP (NSP) cells derived from a rat endometrial cell line expressing human [12Val] KRAS (RK12V cells) were isolated. We analyzed the biological characteristics and assessed the antiproliferative effect of a HDAC inhibitor (NaB).

Materials and Methods

Plasmid

pZIP-Neo SV(X)1 containing [12Val] human KRAS 4B cDNA was a gift from Dr. Channing Der (University of North Carolina; refs. 30, 31). The pZeo-vector was purchased from Invitrogen. We cut the 1.1-kb fragment containing human [12Val] KRAS 4B cDNA from the pZIP-Neo SV(X)1 construct with BamHI and legated it to the BamHI site of the pZeo vector.

Cell culture

A rat endometrial cell line (RENT4) was used in this study. RENT4 cells were established by Wiehle and colleagues (32) and obtained from the European Collection of Cell Cultures. No authentication for the cell line was done by the authors.

RENT4 cells harboring mutant [12Val] versions of KRAS4B (RK12V cells) were established by transfecting RENT4 cells with pZeo constructs, containing cDNA sequences encoding [12Val] KRAS by using Lipofectamine (Invitrogen). Stably transfected cells were selected and isolated in growth medium containing 400 μg/mL of Zeocin (Invitrogen) to establish cell lines expressing KRAS protein, previously described elsewhere (33). Pooled populations were used for the assay. Cells were cultured with Dulbecco’s Modified Eagle’s Medium (DMEM; Nissui Seika) supplemented with 20 μg/mL Gly-His-Lys, 2 mmol/L glutamine, 80 IU insulin (Sigma), and 10% FBS (Hyclone; ref. 32). Cells used were always less than 20 passages.

Isolation of SP cells

To identify and isolate Hec1 and RK12 V SP cells, the cells were dislodged from the culture dishes with trypsin and EDTA, washed, and suspended at a concentration of 10^6 cells per milliliter in DMEM containing 2% FBS. The cells were then labeled in the same medium at 37°C for 90 minutes with 2.5 μg/mL Hoechst 33342 dye (Molecular Probes), either alone or in combination with 50 μmol/L verapamil (Sigma). Finally, the cells were counterstained with 1 μg/mL propidium iodide (P’I) to label dead cells. The cells were then analyzed in the EPICS ALTRA HyPerSort (Beckman Coulter) by using dual-wavelength analysis (blue, 424–444 nm; red, 675 nm) after excitation with 350 nm UV light. PI-positive cells were excluded from the analysis.

The SP cells were separated by flow cytometry (the EPICS ALTRA HyPerSort) from the NSP cells and both fractions were seeded in a mesenchymal stem cell maintenance medium (MF medium; TOYOBO) at 10% FBS on a collagen-coated 24-well plates (2 cm²; Iwaki). The cells were cultured for 2 to 4 weeks. The cells were then transferred to collagen-coated 60-mm plates.

Growth rate assay

Cells were plated in a MF medium in the presence or absence of NaB (Sigma-Aldrich). Cell viability was determined by using trypan blue exclusion assay. Floating cells were washed away and the cells were detached from dishes with 0.25% trypsin. Collected cells were stained with 0.4% trypsin blue and were counted by using hematocytometer.

Self-renewal assays

SP cells or NSP cells were plated in 24-well collagen-coated dishes (10 cells/cm²). SP cells, but not NSP cells, formed colonies. Cells from individual colonies of SP cells were reseeded at 10 cells/cm² in triplicate in 60-mm collagen-coated plates to generate colonies. Colonies were monitored to ensure they were derived from single cells. The secondary colonies were reseeded in a similar manner to generate tertiary colonies. The cloning plates were stained with the crystal violet solution (Sigma).

Soft agar assays

For the anchorage-independent growth assays in soft agar, 1 × 10^4 cells were seeded in 60-mm dishes containing growth medium, supplemented with 10% FBS and 0.3% Bactoagar over a hardened 0.5% agar base layer in
the presence or absence of 2 or 5 mmol/L NaB. Cells were incubated for 3 weeks and the number of colonies per 4 cm² was counted under a microscope.

**In vivo tumor formation assays**

We inoculated 1 × 10⁶ cells in Matrigel (BD Matrigel Basement Membrane Matrix High Concentration; BD Bioscience) into the subcutaneous connective tissue of 5-weeks-old nude mice (Balb nu/nu). After 6 weeks, mice were killed and the tumors excised. All mouse experiments were approved by the animal ethics committee of Kyushu University.

**In vitro sensitivity to chemotherapeutic agents**

RK12V-SP cells and RK12V-NSP cells were cultured for 96 hours in the presence or absence of 1 μmol/L cisplatin, 1 μmol/L doxorubicin, or 10 nmol/L paclitaxel. Viable cells determined by the trypsin blue exclusion assay were counted by using a hemocytometer.

**Cell-cycle analysis**

The DNA content of cells was measured by flow cytometric analysis (EPICSXL; Beckman Coulter) by using the PI staining method. Cells (2 × 10⁶) in 6-cm plates were treated with different concentrations of NaB for 24 hours. After treatment, the attached cells were washed twice with ice-cold PBS and suspended in NP-40 lysis buffer (3.4 mmol/L sodium citrate, 10 mmol/L NaCl, and 0.1% NP-40) containing 0.5% PI. The proportion of cells in G1, S, and G2-M phases was determined from DNA histograms by using CellQuest software (Beckton Dickinson).

**Antibodies**

Primary antibodies used in this study were as follows: CD13 monoclonal antibody (3DB), vimentin monoclonal antibody (V9), p21 polyclonal antibody (C-19), p27 monoclonal antibody (F-8), β-actin antibody (C4), histone H3(FL-136), and Ac-histone H3(Lys 9/14), all obtained from Santa Cruz Biotechnology, Inc.; α-Smooth muscle actin monoclonal antibody (1A4) was purchased from MBL. Phospho-histone 2AX (Ser139), p38 mitogen-activated protein kinase (MAPK) antibody, and phospho-p38 MAPK (Thr180/Tyr182) were obtained from Cell Signaling Technology, Inc.

**Immunohistochemistry**

Formalin-fixed histologic tumor sections from nude mice or cultured cells were used. Cultured cells were incubated on glass chamber slides (LAB-TEK; Nalge Nunc International Corp.) and fixed by treatment with 10% formalin. Sections were rinsed twice in PBS (pH 7.4) for 5 minutes each. Samples were then incubated with 4% blocking horse serum (Vector Laboratories) for 1 hour at room temperature in a humidified chamber, followed by incubation with the primary antibody (200 μg/mL, 1:100 diluted). We also used nonimmune mouse or rabbit IgG as a control for the primary antibody. Staining with the primary or control antibody was conducted overnight at 4°C. Bound antibodies were detected with a biotinylated anti-rabbit IgG secondary antibody (1.5 mg/mL) and an avidin-biotin complex linked to horseradish peroxidase (Vectastain, Vector Laboratories), followed by incubation with diaminobenzidine tetrahydrochloride as the substrate.

**Analysis of the levels of intracellular reactive oxygen species**

The levels of intracellular reactive oxygen species (ROS) were evaluated by flow cytometry after staining RK12V-SP cells and RK12V-NSP cells with the CM-H₂DCFDA probe (Invitrogen). Both cells were cultured with MF medium in the presence or absence of 5 mmol/L NaB for 72 hours and incubated with phenol red-free Opti-MEM containing 1 μmol/L CM-H₂DCFDA for 30 minutes. Cells were washed in PBS and collected in 0.5 mL PBS. Fluorescently stained cells were transferred to polystyrene tubes (Falcon) and were subjected to flow cytometric analysis (FACScan, Becton Dickinson) by using CellQuest software for data acquisition and analysis. The levels of intracellular ROS were shown as mean fluorescence values.

**Analysis of γH2AX foci**

Cells were incubated on glass chamber slides (LAB-TEK; Nalge Nunc International Corp.) in the presence or absence of 5 mmol/L NaB for 24 hours and cells were fixed by treatment with 10% formalin. γH2AX foci were analyzed by immunohistochemistry by using phospho-histone 2AX (Ser139) antibody. γH2AX were counted visually by microscopy, examining 10 cells in 3 different areas of the slide per each condition. Three independent experiments were conducted.

**Western blotting**

To detect each protein expression, subconfluent cells were lysed with ice-cold lysis buffer (20 mmol/L Tris-HCl, pH 8.0, 1% Triton X-100, 10% glycerol, 137 mmol/L NaCl, 1.5 mmol/L MgCl₂, 1 mmol/L EGTA, 50 mmol/L NaF, and 1 mmol/L Na₃VO₄) containing freshly added protease inhibitors (1 mmol/L phenyl methyl sulphonylfluoride, 1 μg/mL leupeptin, and 10 μg/mL aprotinin; Sigma). After centrifugation at 13,000 × g for 10 minutes to remove debris, 10 μg of the proteins were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane in a semi-dry transfer cell (Bio Rad Laboratories). The blots were incubated with diluted primary antibodies overnight at 4°C. After incubation with each primary antibody (1:1,000 diluted), the blots were incubated with horseradish peroxidase-linked anti-rabbit antibodies and analyzed with an ECL system (Amersham Bioscience). The levels of protein expression were quantitated by using ImageJ software.

**Microdissection and DNA extraction**

Glass slides with an overlay of 4 mm of thin LM Film (PALM Microlaser Technologies GmbH) were prepared. Formalin-fixed, paraffin-embedded tumor tissue was cut...
into 7-μm sections and placed on the slides. The sections were then deparaffinized and stained with hematoxylin and eosin. By using a laser microdissection system (Leica Microsystems), tumor cells or stromal cells were isolated into the cap of a 0.5-mL microtube. After retrieval of the cells, 50 μL proteinase K solution (Pico Pure DNA Extraction kit; ARCTURUS) was added into the cap. The DNA was extracted by overnight incubation at 65°C. The solution was then boiled for 10 minutes to inactivate the proteinase K.

**PCR of the KRAS gene**

To amplify the KRAS gene (cDNA for RK12V-SP tumor), PCR by using a T3000 thermal cycler was performed (Biometra). The primers used for PCR were as follows: 5'-GACTGAATATAAACTT-3' (sense); 5'-CAT-AATTACACACTTTGTCTT-3' for human KRAS cDNA. The PCR cycling conditions were as follows: (i) preheating for 2 minutes at 94°C, 39 cycles of denaturation for 1 minute at 94°C, annealing for 30 seconds at 59.3°C, and extension for 1 minute at 72°C, (ii) preheating for 2 minutes at 94°C, 39 cycles of denaturation for 1 minute at 94°C, annealing for 30 seconds at 59.3°C, and extension for 1 minute at 72°C. After the last cycle, a final extension of 5 minutes at 72°C was added.

**Determination of the KRAS sequence**

PCR products were electrophoresed on a 2% agarose gel and the band corresponding to the desired target cut from the gel. DNA was extracted by using the GFX PCR DNA Gel Band Purification Kit (GE Healthcare). Direct sequencing of the PCR product was then done by using an ABI PRISM Big Dye Termination Ver3.1 Cycle Sequencing Kit according to the manufacturer’s instructions and the ABI PRISM 3100 (Applied Biosystems). The primer used for sequencing was 5'-TTGGA-ACCCAAGGTACATTTC-3' (antisense) for KRAS DNA.

**Data analysis**

Data are represented with the means ± SEM and were analyzed with Student’s t test. A P value of less than 0.05 was considered statistically significant.

**Results**

RK12V-SP cells show features of cancer stem-like cells

First of all, we analyzed self-renewal capacity of K12V-SP cells and potential to develop into stromal cells. When RK12V-SP cells were plated in collagen-coated dishes (10 cells/cm²), they proliferated and formed large (>2 mm) colonies. In contrast, RK12V-NSP cells formed only small colonies (Fig. 1A). We tested their self-renewal capacity by evaluating serial colony forming potential. Primary colonies of RK12V-SP cells or RK12V-NSP cells were dissociated into single cells and then cultured these cells in 60-mm collagen-coated plates (10 cells/cm²). A single cell from the secondary colony of RK12V-SP cells or RK12V-NSP cells were dissociated into single cells and then cultured these cells in 60-mm collagen-coated plates (10 cells/cm²). A single cell from the secondary colony of RK12V-SP cells generated tertiary colonies. RK12V-SP cells did not form the secondary colonies (Fig. 1B).

Next, we investigated whether RK12V-SP cells had the ability to develop into stromal cells. RK12V-SP cells formed large, invasive tumors with extracellular matrix–enriched stroma-like tissues in nude mice as previously shown (29).

![Figure 1](https://www.aacrjournals.org)
We microdissected CD13-positive stroma-like cells and CD13-negative tumor cells, respectively, and sequenced exon 1 (codons 27–35) in the KRAS gene (Fig. 2A). Several bases in this region differ between the human and mouse, enabling the origin of the cells to be determined. As expected, both tumor cells (data not shown) and the surrounding CD13-positive stroma-like cells contained the human KRAS gene sequences (Fig. 2B). Three different regions containing stroma-like cells in RK12V-SP tumors were microdissected. Human KRAS gene sequences were detected in all of them. As RK12V cells were transfected with human mutant [12Val] KRAS 4B cDNA, these results clearly show that the surrounding stroma-like cells, at least in part, originated from the inoculated RK12V-SP cells.

**RK12V-SP cells display higher resistance to conventional chemotherapeutic drugs**

Next, we investigated the effect of cisplatin, paclitaxel, and doxorubicin (clinically used for endometrial cancer chemotherapy) on the proliferation of RK12V-SP cells and RK12V-NSP cells (Fig. 3). Ninety-six hours of incubation of RK12V-NSP cells with medium containing these chemotherapeutic drugs inhibited proliferation compared with control. Relative to control, the extent of inhibition was 61% in 1 μmol/L cisplatin, 51% in 10 nmol/L paclitaxel, and 56% in 1 μmol/L doxorubicin. All of these drugs inhibited the proliferation of RK12V-NSP cells significantly compared with control ($P < 0.001$). In contrast, none of these drugs had an inhibitory effect on the growth of RK12V-SP cells.

**Treatment of NaB inhibits self-renewal capacity of RK12V-SP cells**

We previously showed that 2 mmol/L NaB, a short-chain fatty acid HDAC inhibitor, induced senescence and apoptosis in several types of gynecologic cancer (34). Takai and colleagues showed that the effective dose of NaB that inhibited 50% clonal growth of the endometrial...
cancer cell lines ranged between $8.3 \times 10^{-4}$ and $4.1 \times 10^{-3}$ mol/L (23). On the basis of these previous data, we examined the effect of 2 or 5 mmol/L NaB on cell proliferation of both RK12V-SP cells and RK12V-NSP cells.

We first confirmed by Western blot that the levels of acetylated H3 were enhanced in both RK12V-SP cells and RK12V-NSP cells treated with 2 mmol/L NaB for 72 hours compared with that in untreated cells (Fig. 4A).

Treatment with 2 or 5 mmol/L NaB for 96 hours significantly inhibited cell proliferation of RK12V-SP cells as well as RK12V-NSP cells (Fig. 4B; P < 0.01). RK12V-SP cells have a potential to regenerate SP cells after incubation, which is an important characteristics of stem-like cells. Treatment with NaB for 24 hours significantly inhibited the proportion of reproduced SP cells (control, 15%; 2 mmol/L NaB, 1.5% P < 0.02; 5 mmol/L NaB, 0.023%; P < 0.01; n = 4, Fig. 4C a). The primary colony-forming potential of RK12V-SP cells was completely suppressed by treatment by 2 mmol/L NaB (Fig. 4C b).

Next, we analyzed the cell-cycle alteration in response to NaB by using flow cytometry with PI staining. Treatment with NaB for 24 hours resulted in a significant decrease in the fraction of RK12V-SP cells in S phase in a dose-dependent manner (control, 22.5%; 2 mmol/L NaB, 14%; 5 mmol/L NaB, 10%; P < 0.05; Fig. 4D). Conversely, the percentages of cells in a sub-G1 population increased; however, it was not statistically significant. In RK12V-NSP cells, there was no significant change in the cell-cycle phase distribution.

**NaB treatment suppresses colony formation of RK12V-SP cells in soft agar cultures**

Next, we investigated the effect of NaB treatment on tumorigenicity of RK12V-SP cells and RK12V-NSP cells by evaluating colony forming efficiency in soft agar cultures in the presence or absence of 2 or 5 mmol/L NaB. After 3 weeks of culture, RK12V-SP cells yielded many large colonies in the control culture lacking NaB (Fig. 5). In contrast, no colony formed in soft agar cultures in RK12V-NSP cells. Treatment with NaB (both 2 and 5 mmol/L) suppressed colony formation of RK12V-SP cells in soft agar cultures. As anticipated, RK12V-NSP cells did not form colonies in soft agar cultures.

**RK12V-SP cells show higher susceptibility to NaB-induced DNA damage**

Finally, we investigated the molecular mechanism associated with the inhibitory effect of NaB treatment of RK12V-SP cells. Recently, we reported that treatment with NaB induced cell death in several cancer cell lines mediated by enhanced ROS levels, DNA damage response signals, and upregulation of p21 (35). Thus, we examined the change of these signal levels in RK12V-SP cells and RK12V-NSP cells.

The levels of intracellular ROS were enhanced by 5 mmol/L NaB treatment in both RK12V-SP cells and RK12V-NSP cells (Fig. 6A). Phosphorylated H2AX (γH2AX) foci, which are indicators of DNA damage, were assessed by immunohistochemistry. The numbers of γH2AX foci were increased by 5 mmol/L NaB treatment in both RK12V-SP cells and RK12V-NSP cells (Fig. 6B). The levels of γH2AX proteins were markedly increased in RK12V-SP cells (44-fold) compared with that in RK12V-NSP cells (2-fold; Fig. 6C). The p21, p27, and phospho-p38 MAPK expression levels were also enhanced more in RK12V-SP cells than in RK12V-NSP cells. These results imply that treatment with NaB induced production of

![Figure 3](https://www.aacrjournals.org/MolCancerTher/10(8)/1435 Figure 3.png)
intracellular ROS and DNA damage in both RK12V-SP and RK12V-NSP cells.

Signals involved in DNA damage responses were enhanced more in RK12V-SP cells (cancer stem–like cells) than in RK12V-NSP cells by NaB treatment.

Discussion

In this study, we analyzed the biological characteristics of the SP fraction derived from rat endometrial cells harboring human mutant [Val] KRAS 4B genes (RK12V cells). We determined that the phenotype of RK12V-SP cells resembled that of cancer stem–like cells. We found that the HDAC inhibitor, NaB inhibited proliferation of RK12V-SP and RK12V-NSP cells. Although these findings may not be representative of all endometrial carcinomas, this is the first report of an inhibitory effect of NaB on proliferation of endometrial cancer stem–like cells.

As previously shown, RK12V-SP cells have reduced expression levels of certain differentiation markers (CD9 and CD13), reduced long-term proliferating capacity in
culture, and enhanced tumorigenicity (29). We found in this study that RK12V-SP cells possessed self-renewal capacity and the potential to develop into stromal cells. These results indicate that RK12V-SP cells have cancer stem–like cell features as that of SP cells derived from a human endometrial cancer cell line, HeC1 cells.

It has been known that SP cells (but not NSP cells) express MDR transporter proteins, such as ABCG2/Bcrp1 (11, 36). As expected, RK12V-SP cells displayed higher resistance to conventional chemotherapy (Fig. 3), indicating a requirement for new targets for the treatment of cancer stem–like cells. To develop new approaches to molecular cancer therapy, we did the microarray assays to identify the overexpressed genes in RK12V-SP cells compared with those in RK12V-NSP cells (data not shown). The expression level of a number of genes, including cytokines and growth factors, was enhanced in RK12V-SP cells (Kato and colleagues, manuscript in preparation), suggesting that multiple signal pathways exist to maintain the phenotype of SP cells. It would be difficult to identify a single, selective molecular target to SP cells.

HDACi's have multiple biological effects, including growth arrest, apoptosis, senescence, ROS facilitated cell death, mitotic cell death, and antiangiogenesis (17). HDACi’s include short-chain fatty acids (e.g., butyrates and valporic acid), organic hydroxamic acids [trichostatin A and suberoyl anilide bishydroxamine (SAHA)], cyclic tetrapeptides (e.g., trapoxin), and bezamides (e.g., MS-275). Trichostatin A, NaB, valporic acid, and SAHA can inhibit malignant cells in vitro and in vivo (37–39). We have previously shown that NaB induced p21 expression, resulting in growth arrest and cell death (34). Recently, we have also shown that DNA damage signals were involved in NaB-induced cell death (35). In this study, NaB inhibited primary colony-forming potential and regeneration of SP cells, indicating suppression of self-renewal capacity. Most recently, Conti and colleagues showed that SAHA treatment of cancer cells slows down replication forks, activates dormant origins, and induces DNA damage (40). They also found γH2AX could be used as a convenient pharmacodynamic biomarker for HDACi-induced DNA damage (41). We showed that γH2AX was markedly enhanced in RK12V-SP cells compared with that in RK12V-NSP cells, implying that RK12V-SP cells were highly sensitive to NaB-induced DNA damage (Fig. 6). These results indicated that cancer stem–like cells, which are resistant to conventional chemotherapy, were sensitive to treatment with HDACi’s. It is believed that stem cells generally proliferate slowly. Burgess and colleagues showed that HDACi’s can induce death of transformed cells in both proliferative and nonproliferative phases of the cell cycle (42).

Clinical trials of several HDACi’s are currently underway, as monotherapies or in combination with other anticancer drugs and radiation. A total of 140 studies for cancer are found in website of U.S. NIH (www.clinicaltrials.gov). SAHA and depsipeptide have been approved by the FDA for cancer therapy (26, 27). HDACi’s emerge as promising drugs for targeting of cancer stem–like cells in clinical setting.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank Dr. Channing Der (University of North Carolina) for generously donating pZIP neoSV (X)-K-Ras4B(12V) and Miwako Ando for technical assistance. The authors also thank Drs. Ryota Souzaki, Tatsuro Tajiri, and Tomoaki Taguchi (Department of Pediatric Surgery, Kyushu University) for technical advice on a laser microdissection.

Grant Support

This work was supported by grants-in-aid 20659259, 22659302, and 22591869 from the Ministry of Education, Culture, Sports, Science and Technology, Japan and the Environment Technology Development Fund of the Ministry of the Environment, Japan for K. Kato.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received November 29, 2010; revised April 26, 2011; accepted May 24, 2011; published OnlineFirst June 1, 2011.

References


Figure 6. RK12V-SP cells show high responsiveness to NaB-induced DNA damage. A, the levels of intracellular ROS were evaluated by flow cytometric analysis after staining RK12V-SP cells and RK12V-NSP cells with the CM-H2DCFDA probe. Both types of cells were cultured with MF medium in the presence or absence of 5 mmol/L NaB for 72 hours and incubated with phenol red free-Opti-MEM containing 1 μmol/L CM-H2DCFDA for 30 minutes. The levels of intracellular ROS are shown as mean fluorescence value. Error bar represents SEM in 3 independent experiments. The ROS levels were enhanced by 5 mmol/L NaB treatment of RK12V-SP and RK12V-NSP cells. B, phosphorylated H2AX (γH2AX) foci were assessed by immunohistochemistry using phospho-histone 2AX (Ser139) antibody. γH2AX were counted visually by microscopy, examining 10 cells in 3 different areas of the slide per each condition. Error bar represents SEM in 3 independent experiments. The number of γH2AX foci was increased by 5 mmol/L NaB in the treatment of both RK12V-SP and RK12V-NSP cells. DMSO, dimethyl sulfoxide. C, RK12V-SP and RK12V-NSP cells were cultured with MF medium in the presence or absence of 5 mmol/L NaB for 6 or 12 hours. The level of each protein was analyzed by Western blot and the relative ratio to control is shown. The levels of γH2AX proteins were markedly increased in RK12V-SP cells (44-fold) compared with that in RK12V-NSP cells (2-fold). Expression levels of p21, p27, and phospho-p38 MAPK were also enhanced more in RK12V-SP cells than in RK12V-NSP cells. β-Actin was the internal control. Representative data are shown. Similar results were obtained 3 times.
An Effect of HDAC Inhibitor on Cancer SP Cell Proliferation


Molecular Cancer Therapeutics

Sodium Butyrate Inhibits the Self-Renewal Capacity of Endometrial Tumor Side-Population Cells by Inducing a DNA Damage Response


Mol Cancer Ther 2011;10:1430-1439. Published OnlineFirst June 1, 2011.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-10-1062

Cited articles
This article cites 41 articles, 14 of which you can access for free at:
http://mct.aacrjournals.org/content/10/8/1430.full#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
http://mct.aacrjournals.org/content/10/8/1430.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.