Sodium butyrate inhibits the self-renewal capacity of endometrial tumor side-population cells by inducing a DNA damage response

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**Abbreviations list:** SP; side population, HDAC; histone deacetylase, NaB; sodium butyrate, ROS; reactive oxygen species

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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 $^{12}$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 (HDACs) 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 $\gamma$H2AX foci were increased by NaB treatment of both RK12V-SP cells and -NSP cells. The expression levels of $\gamma$H2AX, p21, p27 and phospho-p38 MAPK 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 -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 report of 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.
Introduction

Endometrial cancer (EC) is the most common gynecological malignancy in the industrialized world and can be classified into two different clinicopathological types, estrogen-related ECs (type I) and non-estrogen-related ECs (type II). The most frequent genetic alteration in type I ECs is PTEN inactivation, followed by microsatellite instability and mutations of the KRAS and β-catenin. In type II ECs, 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) (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, based on 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 et al. showed that SP cells derived from the endometrial cancer cell lines (An3CA) had features of cancer stem-like cells including low proliferative activity during nine days of cultivation, chemoresistance, and enhanced tumorigenicity (13). Hubbard et al. demonstrated that a small population of clonogenic cells from
endometrial cancer tissues showed self-renewing, differentiating and tumorigenic properties (14). Gotte et al. demonstrated that the adult stem cell marker, Musashi-1 was co-expressed 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 to secretary endometrium, suggesting the concept of a stem cell origin of endometriosis and endometrial carcinoma. Most recently, they demonstrated that siRNA 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 (HDACs) 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 demonstrated the antiproliferative or the proapoptotic effects of HDAC inhibitors on endometrial cancer cells (23-25). Thus, HDACs are important therapeutic targets for cancer and several HDAC inhibitors are in various stages of clinical development (26, 27). However, the effect of HDAC inhibitors 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 demonstrated 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
[\textsuperscript{12}Val] \textit{K}{\textsc{r}}{\textsc{a}}{\textsc{s}} (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 \([^{12}\text{Val}]\) human KRAS 4B cDNA was a gift from Dr. Channing Der (University of North Carolina) (30, 31). The pZeo-vector was purchased from Invitrogen, CA, USA. We cut the 1.1 kb fragment containing human \([^{12}\text{Val}]\) KRAS 4B cDNA from the pZIP-Neo SV(X)1 construct with Bam HI and ligated it to the Bam HI site of the pZeo vector.

Cell Culture

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

RENT4 cells harboring mutant \([^{12}\text{Val}]\) versions of KRA54B (RK12V cells) were established by transfecting RENT4 cells with pZeo constructs, containing cDNA sequences encoding \([^{12}\text{Val}]\) KRAS using lipofectoamine (Invitrogen, CA, USA). Stably transfected cells were selected and isolated in growth medium containing 400 μg/ml of Zeocin (Invitrogen, CA, USA) to establish cell lines expressing K-Ras 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, Japan) supplemented with 20 μg/mL Gly-His-Lys, 2 mM glutamine, 80 IU insulin (Sigma, St. Louis, USA) and 10% fetal bovine serum (FBS) (Hyclone, Utah, USA) (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 mL in DMEM containing 2% FBS. The cells were then labeled in the same medium at 37 °C for 90 min with 2.5μg/mL Hoechst 33342 dye (Molecular Probes, Eugene, ORE, USA), either alone or in combination with 50 μM verapamil (Sigma, St.Louis, USA). Finally, the cells were counterstained with 1 μg/mL propidium iodide (PI) to label dead cells. The cells were then analyzed in the EPICS ALTRA HyPerSort (Beckman Coulter, Fullerton, CA, USA) 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 FACS from the non-SP (NSP) cells and both fractions were seeded in a mesenchymal stem cell maintenance medium (MF-medium) (TOYOBO, Osaka, Japan) and 10% FBS on a collagen-coated 24-well plates (two cm²) (Iwaki, Funabashi, Japan). The cells were cultured for two to four 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, Japan). Cell viability was determined using trypan blue exclusion assay. Floating cells were washed away and the cells were detached from dishes by 0.25% trypsin. Collected cells were stained with 0.4% trypan blue and were counted 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 clonies 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, St. Louis, USA).

**Soft agar assays**

For the anchorage-independent growth assays in soft agar, 1x10^4 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 mM NaB. Cells were incubated for three weeks and the number of colonies per 4cm² was counted under a microscope.

**In vivo tumor formation assays**

We inoculated 1x10^4 cells in Matrigel (BD Matrigel Basement Membrane Matrix High Concentration, BD Bioscience, Bedford, MA, USA) into the subcutaneous connective tissue of five weeks old nude mice (Balb nu/nu). After six 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 -NSP cells were cultured for 96 hr in the presence or absence of 1μM cisplatin, 1μM doxorubicin, or 10nM paclitaxel. Viable cells determined by the trypan blue exclusion assay were counted using a hemocytometer.

**Cell cycle analysis**

The DNA content of cells was measured by flow cytometric analysis (EPICSXL, Beckman Coulter) using the propidium iodide (PI) staining method. Cells (2x10^5) in 6cm plates were treated with different concentrations of NaB for 24hr. After treatment, the attached cells were washed twice with ice cold phosphate buffered saline (PBS) and suspended in NP-40 lysis buffer (3.4 mM sodium citrate, 10 mM NaCl, 0.1%NP-40)
containing 0.5% PI. The proportion of cells in G1, S, G2/M phases was determined from DNA histograms using CellQuest software (Beckton Dickinson).

**Antibodies**

Primary antibodies used in this study were as follows: CD13 monoclonal antibody (3D8), vimentin monoclonal antibody (V9), p21 polyclonal antibody (C-19), p27 monoclonal antibody (F-8), β-actin antibody (C4), histone H3(FL-136), Ac-histone H3(Lys 9/14), all obtained from Santa Cruz Biotechnology, Inc.CA, USA. α-SMA monoclonal antibody (1A4) was purchased from MBL, Japan. Phospho-histone 2AX (Ser139), p38 MAPK antibody, and phospho-p38 MAPK (Thr180/Tyr182) were obtained from Cell Signaling Technology , Inc, MA,USA..

**Immunohistochemistry**

Formalin-fixed histological tumor sections from nude mice or cultured cells were used. Cultured cells were incubated on glass chamber slides (LAB-TEK; Nalge Nunc International Corp. Naperville, USA) and fixed by treatment with 10% formalin. Sections were rinsed twice in phosphate buffered saline (PBS, pH 7.4) for five min each. Samples were then incubated with 4% blocking horse serum (Vector Laboratories, Burlingame, USA) for one hr 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 performed overnight at 4 °C. Bound antibodies were detected with a biotinylated anti-rabbit IgG secondary antibody (1.5mg/ml) and an avidin-biotin complex linked to horseradish peroxidase (Vectastain, Vector Laboratories, Burlingame, USA), followed by incubation with diaminobenzidine tetrahydrochloride as the substrate. Osaka, Japan).
Analysis of the levels of intracellular reactive oxygen species (ROS)

The levels of intracellular ROS were evaluated by flow cytometry after staining RK12V-SP cells and -NSP cells with the CM-H$_2$DCFDA probe (Invitrogen). Both cells were cultured with MF medium in the presence or absence of 5 mM NaB for 72hr and incubated with phenol red-free Opti-MEM containing 1μM CM-H$_2$DCFDA for 30 min. Cells were washed in PBS and collected in 0.5mL PBS. Fluorescently stained cells were transferred to polystyrene tubes (Falcon, CA, USA) and were subjected to flow cytometric analysis (FACScan, Becton Dickinson) 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. Naperville, USA) in the presence or absence of 5 mM NaB for 24hr and cells were fixed by treatment with 10% formalin. γH2AX foci were analyzed by immunohistochemistry using phospho-histone 2AX (Ser139) antibody. γH2AX were counted visually by microscopy, examining ten cells in three different areas of the slide per each condition. Three independent experiments were performed.

Western blotting

To detect each protein expression, subconfluent cells were lysed with ice-cold lysis buffer (20mM Tris HCl, pH8.0, 1% Triton X-100, 10% glycerol, 137mM NaCl, 1.5mM MgCl$_2$, 1mM EGTA, 50mM NaF, 1mM Na$_3$VO$_4$) containing freshly added protease inhibitors (1mM phenyl methyl sulphonylfluoride, 1μg/mL leupeptin, and 10μg/mL aprotinin) (Sigma, St Louis, USA). After centrifugation at 13,000 X g for
ten min to remove debris, 10μg of the proteins were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane in a semi-dry transfer cell (Bio Rad Laboratories, Hercules, USA). The blots were incubated with diluted primary antibodies overnight at 4°C. Primary antibodies, including the anti-CD13 polyclonal antibody, anti-E-cadherin polyclonal antibody, and anti-vimentin polyclonal antibody were obtained from Santa Cruz Biotechnology, Inc., USA. After incubation with each primary antibody (1:1000 diluted), the blots were incubated with horseradish peroxidase-linked anti-rabbit antibodies and analysed with an ECL system (Amersham Bioscience, Buckinghamshire, UK). The levels of protein expression were quantitated using Image J software.

Microdissection and DNA extraction

Glass slides with an overlay of 4 mm of thin LM Film (PALM Microlaser Technologies GmbH, Bernried, Germany) were prepared. Formalin-fixed, paraffin-embedded tumor tissue was cut into seven μm sections and placed on the slides. The sections were then deparaffinized and stained with hematoxylin and eosin. Using a laser microdissection system (Leica Microsystems, Germany), 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, CA, USA) was added into the cap. The DNA was extracted by overnight incubation at 65 °C. The solution was then boiled for ten min to inactivate the proteinase K.

Polymerase Chain Reaction (PCR) of the KRAS gene

To amplify the KRAS gene (cDNA for RK12V-SP tumor), polymerase chain reaction (PCR) using a T3000 thermal cycler was performed (Biometra). The primers used for PCR were as follows: 5’-GACTGAATATAAACTT-3’ (sense); 5’-
CATAAATTACACACTTTGTCTT-3' for human KRAS cDNA. The PCR cycling conditions were as follows: 1) preheating for two min at 94 °C, 39 cycles of denaturation for one min at 94 °C, annealing for 30 sec at 59.3 °C, and extension for one min at 72 °C, 2) preheating for two minutes at 94 °C, 39 cycles of denaturation for one min at 94 °C, annealing for 30 sec at 59.3 °C, and extension for one min at 72 °C. After the last cycle, a final extension of five min 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 using the GFX PCR DNA gel band purification kit (GE Healthcare, UK). Direct sequencing of the PCR product was then performed 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, CA, USA). The primer used for sequencing was as follows: 5’-TTGAAACCCAAGGTACATTTCA-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 K12V-SP cells’ self-renewal capacity and potential to develop into stromal cells. When RK12V-SP cells were plated in collagen-coated dishes (10 cells/cm^2), they proliferated and formed large (>2mm) 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 -NSP cells were dissociated into single cells and then cultured these cells in 60mm collagen-coated plates (10 cells/cm^2). A single cell from the secondary colony of RK12V-SP cells generated tertiary colonies. RK12V-NSP 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 (ECM)-enriched stroma-like tissues in nude mice as previously shown (29). 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 [^{12}Val] KRAS 4B cDNA, these results clearly demonstrate 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 -NSP cells (Fig.3). Ninety-six hr 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 1μM cisplatin, 51% in 10nM paclitaxel and 56% in 1μM 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 demonstrated that 2mM NaB, a short-chain fatty acid HDAC inhibitor, induced senescence and apoptosis in several types of gynecologic cancer (34). Takai et al. showed that the effective dose of NaB that inhibited 50% clonal growth of the endometrial cancer cell lines ranged between 8.3 X 10^-4 M and 4.1 X 10^-3 M (23). Based on these previous data, we examined the effect of 2 or 5 mM NaB on cell proliferation of both RK12V-SP cells and -NSP cells.

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

Treatment with 2 or 5mM NaB for 96 hr 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 hr significantly inhibited the proportion of reproduced SP cells (control, 15%; 2 mM NaB, 1.5% p<0.02; 5 mM 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 mM NaB (Fig. 4C b).

Next, we analyzed the cell cycle alteration in response to NaB using flow cytometry with propidium iodide (PI) staining. Treatment with NaB for 24 hr resulted in a significant decrease in the fraction of RK12V-SP cells in S phase in a dose-dependent manner (control, 22.5%; 2 mM NaB, 14%; 5 mM NaB, 10%; p<0.05)(Fig.4D). Conversely, the percentages of cells in a sub-G1 population increased, but 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 -NSP cells by evaluating colony forming efficiency in soft agar cultures in the presence or absence of 2 or 5 mM NaB. After three 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 mM) 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 (DDR) signals and up-regulation of p21 (35). Thus, we examined the change of these signal levels in RK12V-SP cells and -NSP cells.
The levels of intracellular ROS were enhanced by 5 mM NaB treatment in both RK12V-SP cells and -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 mM NaB treatment in both RK12V-SP cells and -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 intracellular ROS and DNA damage in both RK12V-SP and -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 $^{[12]{\text{Val}}} KRAS 4B$ 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 -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, CD13), reduced long term proliferating capacity in culture, and enhanced tumorigenicity (29). We found in the present 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 well as do SP cells derived from a human endometrial cancer cell line, Hec1 cells.

It has been known that SP cells (but not NSP cells) express multi-drug resistance transporter proteins such as ABCG2/Brcp1 (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 performed the microarray assays to identify the over-expressed 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 (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.
HDAC inhibitors have multiple biological effects, including growth arrest, apoptosis, senescence, reactive oxygen-species facilitated cell death, mitotic cell death and anti-angiogenesis (17). HDAC inhibitors include short-chain fatty acids (e.g. butyrates and VPA), organic hydroxamic acids (TSA and suberoyl anilide bishydroxamine[SAHA]), cyclic tetra peptides (e.g. Trapoxin), and bezamides (e.g. MS-275). TSA, NaB, VPA and SAHA can inhibit malignant cells in vitro and in vivo (37-39). We have previously demonstrated 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 et al. demonstrated that SAHA treatment of cancer cells slow down replication forks, activates dormant origins and induces DNA damage (40). They also found γH2AX could be used as a convenient pharmacodynamic biomarker for HDAC inhibitors-induced DNA damage (41). We demonstrated 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 HDAC inhibitors. It is believed that stem cells generally proliferate slowly. Burgess et al. demonstrated that HDAC inhibitors can induce death of transformed cells in both proliferative and non-proliferative phases of the cell cycle (42).

Clinical trials of several HDAC inhibitors are currently underway, as mono therapies or in combination with other anti-cancer drugs and radiation. 140 studies for cancer are found in website of U.S. National Institute of Health (43). SAHA and
depsipeptide have been approved by the FDA for cancer therapy (26, 27). HDAC inhibitors emerge as promising drugs for targeting of cancer stem-like cells in clinical setting.
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References.


43. www.clinicaltrials.gov
Figure Legends

Figure 1

RK12V-SP cells possess self-renewal capacity.

A) RK12V-SP cells and -NSP cells were plated in collagen-coated dishes (10 cells/cm²) and cultured with mesenchymal stem cell maintenance medium (MF medium) for seven days. RK12V-SP cells proliferated and formed large (>2mm) colonies. In contrast, RK12V-NSP cells formed only small colonies (colony passage 1). The primary colonies of RK12V-SP cells or -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-NSP cells did not form the secondary colonies (colony passage two or three). The cloning plates were stained with crystal violet solution. Scale bar = 10mm

B) Colony numbers were counted in triplicate. Colony forming units (CFU) in each passage is shown. Error bar represents SEM from CFU in three independent experiments. CFU: the number of colonies (>2mm) per total plating cells

Figure 2

RK12V-SP cells differentiate to stromal-like cells.

A) CD13-positive stroma-like cells (a) and CD13-negative tumor cells (b) in tumor-derived RK12V-SP cells were microdissected, respectively. (magnification X50 in left panel, X100 in right panel)

B) DNA sequence of exon 1 (codons 17-39) in the KRAS gene is shown. The third base of codon 27 and codons 30-35 (underlined) differ between human and
mouse. An intron is inserted between codons 37 and 38. DNA sequences of microdissected tumor cells and stroma-like cells in the K12V-SP tumor were analyzed. Both the tumor cells (data not shown) and stroma-like cells contained human KRAS DNA. Arrowhead: third base of codon 27, codons 31-35

Three different regions containing stromal-like cells in RK12V-SP tumors were microdissected. The human K-ras gene sequences were detected in all of them (data not shown).

Figure 3
RK12V-SP cells display higher resistance to conventional chemotherapeutic drugs.

RK12V-SP cells and -NSP cells (2X10⁴) were plated and cultured with DMEM containing 10% FBS in the presence or absence of 1 μM cisplatin, 10 nM paclitaxel and 1 μM doxorubicin for 96 hr. The proportion of viable cells relative to control after incubation is shown. Inhibition (relative to control) was 61% in 1 μM cisplatin, 51% in 10 nM paclitaxel and 56% in 1 μM 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 growth of RK12V-SP cells. Error bar represents SEM in ten independent experiments.

Figure 4
Treatment of NaB inhibits self-renewal capacity of RK12V-SP cells.

A) RK12V-SP cells and -NSP cells were cultured with MF medium in the presence or absence of 2 mM NaB for 72 hr. The level of acetylated H3 or non-acetylated H3 was
analyzed by Western blot. The relative ratio of acetylated H3 levels by NaB treatment to control is shown. Representative data are shown. Similar results were obtained three times.

B) RK12V-SP cells and –NSP cells (2X10⁴) were plated and cultured with DMEM containing 10% FBS in the presence or absence of 2 mM or 5 mM NaB for 96 hr. The proportion of viable cells relative to control after incubation is shown. Treatment with 2 or 5 mM NaB for 96 hr significantly inhibited cell proliferation of RK12V-SP cells as well as RK12V-NSP cells (p < 0.01). Error bar represents SEM in three independent experiments.

C)(a) RK12V-SP cells were re-analyzed by flow cytometry two weeks after culture in the presence or absence of NaB. Treatment of NaB for 24 hr significantly inhibited the proportion of reproduced SP cells (control, 15%; 2 mM NaB, 1.5%, p<0.02; 5 mM NaB, 0.023%, p<0.01). Error bar represents SEM in four independent experiments.

(b) RK12V-SP cells were plated in collagen-coated dishes (10 cells/cm²) and cultured with mesenchymal stem cell maintenance medium (MF medium) in the presence or absence of 2 mM NaB for seven days. Treatment with 2 mM NaB completely suppressed primary colony-forming potential of RK12V-SP cells.

D) Flow cytometric analysis of cell cycle phase distribution following treatment with NaB. In RK12V-SP cells, treatment with 2 or 5 mM NaB for 24 hr resulted in a significant decrease in the fraction of cells in S phase in a dose-dependent manner (control 22.5%, 2 mM NaB, 14%; 5 mM NaB, 10%; p < 0.05). In RK12V-NSP cells, there were no significant changes in the cell cycle phase distribution. Error bar represents SEM in three independent experiments.
Figure 5

Treatment with NaB completely suppressed colony formation of RK12V-SP cells in soft agar cultures.

RK12V-SP cells and -NSP cells (1x10^4 cells) were seeded in 60 mm plates 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 mM NaB. After three weeks of culture, control cells in the absence of NaB formed many large RK12V-SP colonies. Treatment with NaB suppressed RK12V-SP colony formation. In contrast, no colony formed in soft agar cultures of RK12V-NSP cells. Error bar represents SEM in three independent experiments.

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 -NSP cells with the CM-H2DCFDA probe. Both types of cells were cultured with MF medium in the presence or absence of 5mM NaB for 72hr and incubated with phenol red free-Opti-MEM containing 1μM CM-H2DCFDA for 30 min. The levels of intracellular ROS are shown as mean fluorescence value. Error bar represents SEM in three independent experiments.

The ROS levels were enhanced by 5 mM NaB treatment in of RK12V-SP cells and -NSP cells.

B)Phosphorylated H2AX (γH2AX) foci were assessed by immunohistochemistry using phospho-histone 2AX (Ser139) antibody. γH2AX were counted visually by
microscopy, examining ten cells in three different areas of the slide per each condition. Error bar represents SEM in three independent experiments.

The number of γH2AX foci was increased by 5 mM NaB treatment of both RK12V-SP cells and -NSP cells.

C) RK12V-SP cells and -NSP cells were cultured with MF medium in the presence or absence of 5 mM NaB for six or 12 hr. 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 (two-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 three times.
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