Commentary

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Side-population Cells Derived from Non-tumorigenic Rat Endometrial Cells are a Candidate Cell of Origin for Malignant Endometrial Tumors

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Abstract

We have previously demonstrated that side population (SP) cells derived from endometrial cancer cells have cancer stem-like cell features. However, the role of stem cell-enriched subpopulations, SP cells in normal endometrium for carcinogenesis is still poorly understood. In the present study, to modeled early carcinogenesis in normal endometrium, we established two cell lines by introducing the oncogenic KRAS gene into SP (RSP) cells and non-SP (RNSP) cells from a rat non-tumorigenic endometrial cell line. Tumorigenicity was enhanced in SP cells harboring mutant KRAS (RSP-K12V cells) compared with that in NSP harboring mutant KRAS gene (RNSP-K12V cells). The primary cultured tumor cells derived from RSP-K12V cells exhibited long-term proliferating capacity in culture and had the capacity to form serial tumors in vivo. In contrast, the primary cultured tumor cells derived from RNSP-K12V cells failed to grow and became senescent. The proportion of SP cells was higher in RSP-K12V cells than in RSP cells and was highest in the RSP-K12V tumor cells and it was correlated with tumorigenicity. The levels of c-Myc and Oct4, and the transcriptional activity of the estrogen receptor were enhanced in RSP-K12V cells and their tumor cells compared with those in RNSP-K12V cells and their tumor cells, respectively. Tumor cells derived RSP-K12V acquired the potential for estrogen-independent proliferation. This is the first report which demonstrates that the occurrence of KRAS gene mutations in SP cells rather than NSP cells derived from non-tumorigenic endometrial cells, contributes to the development of malignant endometrial tumors.

Introduction

Endometrial cancer (EC) is the most common gynecological malignancy in the industrialized world. It is classified into one of two clinicopathological types. The first, type I, is estrogen-related ECs, develops in pre-and postmenopausal women, has endometrioid histology, and is frequently preceded by endometrial hyperplasia. The second, type II, is non-estrogen-related ECs, occurs in postmenopausal women, encompasses histologies other than endometrioid type (mainly papillary serous or clear cell carcinomas), and is not associated with hyperplasia. Type I EC is positive for the estrogen receptor (ER, especially ERα), has a low cellular grade, and carries a good prognosis. In contrast, type II EC is negative for the ER and progesterone receptor (PR), has a high cellular grade, and is associated with a poor prognosis. The most frequent genetic alteration in type I endometrioid carcinoma is *PTEN* inactivation, followed by microsatellite instability, and mutations of the *KRAS* and *β-catenin*. In type II cancer, *TP53* mutation is the most frequent genetic alteration, followed by amplification of *ERBB2* [1].

The estrogen signal, which is potently mitogenic, is transmitted to the nucleus by estrogen binding to the Estrogen Receptor (ER). The dimerized E2-ER complex tightly binds to the estrogen response element (ERE) where it regulates E2-dependent gene expression by acting as a transcription factor. ER has two transcriptional activation domains, AF1 and AF2, which are located in the N-terminal A/B region and in the ligand-binding region E, respectively [2]. Signaling through the membrane-associated receptor tyrosine kinase/Ras/Raf/MAPK pathway enhances the activity of ER-AF1 by stimulating the phosphorylation of the serine residue at 118 [3]. Thus, two independently transmitted signals, E2-ER binding and ER-AF1 activation, are necessary for the dominant activation of the ER.

Previously, we have shown that activated K-ras 4B significantly enhances the ERα expression level and its transcriptional activity. The enhanced transcriptional activity of ER contributes to oncogenic K-ras-mediated NIH3T3 cell transformation [4]. Functional inactivation of the ER by a dominant negative mutant of ERα in the presence of activated K-ras 4B induces cell senescence and abrogates tumorigenic potential [5]. In addition, we have demonstrated that blockage of the MAPK/Estrogen receptor signal pathway by treatment with the ER antagonist (ICI 182, 780) and a MEK inhibitor, suppresses cell proliferation of endometrial cancer cells [6]. Though the molecular events involved in endometrial cancer tumorigenesis are now being elucidated, the precursor cells in which these alterations occur have not yet been analyzed in endometrial cancer.

Two models of cancer development have been proposed [7]. One is the stochastic model, which states that the cell population within a tumor is heterogeneous, but all cells have an equal possibility of acquiring mutations and initiating tumors. The other is the hierarchical

model, which proposes that only a distinct and small subset of cells within a tumor are highly efficient at initiating tumors, whereas the majority of tumor cells are differentiated cells with limited replicative potential. The cell of origin of most cancers, including endometrial cancer, however, remains unclear.

The human endometrium is a highly dynamic tissue undergoing cycles of growth, differentiation, shedding and regeneration throughout the reproductive life of a woman. Endometrial adult stem/progenitor cells are likely responsible for endometrial regeneration [8]. Recently, stem cell-enriched 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 [9]. We have isolated SP cells in the human endometrium for the first time and demonstrated that they have long-term repopulating properties and produce gland and stromal-like cells [10]. Tsuji et al. [11] have demonstrated that BCRP/ABCG2, a marker of SP cells, is strongly expressed in the vascular endometrium. Several groups have also shown that endometrial SP cells have multi-lineage developmental potential [12-14].

We have also isolated SP cells from a human endometrial cancer cell-line, Hec1, and characterized their properties [15]. Hec1-SP cells have cancer stem-like cell features. Although it is clear that endometrial cancer harbors cancer stem-like cells, the role of stem cells in the normal endometrium in carcinogenesis or generation of cancer stem-like cells is still poorly understood. Recently, Gotte et al. [16] demonstrated that the adult stem cell marker, Musashi-1 expressing cells were significantly increased in proliferative endometrium, and endometrial carcinoma tissue, compared to secretary endometrium. This alludes to the concept of a stem cell origin of endometrial carcinoma. Although it is well-known that occurrence of KRAS gene mutations is an early event of endometrial carcinogenesis [17], the contribution of KRAS mutations in stem cells of normal endometrium to carcinogenesis has not been studied.

In the present study, we modeled early carcinogenesis in the endometrium by introducing the activated KRAS gene into SP cells and non-SP cells from a non-tumorigenic rat endometrial cell line (RENT4 cells).

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) [18,19]. The pZeo-vector was purchased from Invitrogen, CA, USA. A 1.1 kb fragment containing human [12Val] *KRAS* 4B cDNA from the pZIP-Neo SV (X) 1 construct was excised with Bam HI and ligated into the Bam HI site of the pZeo vector. An estrogen-responsive reporter plasmid, pERE-luciferase, was obtained from Dr.Shinichi Hayashi (Tohoku University).

Cell-line

A non-tumorigenic immortalized rat endometrial cell line (RENT4) was used in the present study. RENT4 cells were established from primary rat endometrial cells by introducing the immortalizing oncogene, SV40 large T antigen by Wiehle et al. [20]. We obtained them from the European Collection of Cell Cultures (ECACC). No authentication of the cell line was done by the authors. Cells were cultured with growth medium, Dulbecco's Modified Eagle's medium (DMEM) (Nacalai Tesque, Kyoto, 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) [20]. Cells used were always less than 20 passages.

Isolation of SP cells

To identify and isolate RENT4-SP cells, the RENT4 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 2.5 µg/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 using the Vantage FACS (Becton Dickinson, Bedford, USA) or 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 weeks. The cells were then transferred to collagen-coated 60 mm plates.

Establishment of RSP-K12V cells and RNSP-K12V cells

RENT4-SP (RSP) cells or RENT4–NSP (RNSP) cells harboring mutant [12Val] versions of *KRAS*4B (RSP-K12V cells or RNSP-K12V cells) were established by transfecting RENT4 cells with pZeo constructs, containing cDNA sequences encoding [12Val] *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. Expression of *KRAS* mRNA was confirmed by RT-PCR using the primer compatible human *K-RAS* cDNA described below. Pooled populations were used for the assay. Cells were cultured with Dulbecco's Modified Eagle's medium (DMEM) supplemented with 20 µg/mL Gly-His-Lys, 2 mM glutamine, 80 IU insulin and 10% FBS. All cells were used prior to the completion of 20 passages.

Isolation of primary tumor cells

Tumors were excised from nude mice and finely diced, and dissociated in HBSS containing HEPES (25 mmol), penicillin (200 U/ml), streptomycin (200 µg/ml), and collagenase (1 mg/ml, 15U/mg) (Sigma, St. Louis, USA) for 30 minutes at 37°C with agitation. The dispersed tumor cells were separated by filtration through a wire sieve. Primary tumor cells were then cultured with growth medium.

Reverse transcription-Polymerase Chain Reaction (RT-PCR) of the KRAS gene

To detect expression of *KRAS* mRNA, reverse transcription-polymerase chain reaction (RT-PCR) was performed using a T3000 thermal cycler was performed (Biometra). RNA was extracted using Isogen (Nippon gene, Toyama, Japan) according to the manufacturer's instructions. Total RNA (1 µg) was converted to first-stranded cDNA using an Oligo (dT) primer and SuperScript™ II Reverse Transcriptase (Invitrogen, Carlsbad, CA). The primers used for the PCR reactions were as follows: 5'-GACTGAATATAAACTT-3'(sense); 5'-CATAATTACACACTTTGTCTT-3' for human K-ras cDNA. The PCR cycling conditions were as follows: 1) preheating for 2 min at 94C,

39 cycles of denaturation for 1 min at 94°C, annealing for 30 sec at 59.3°C, and extension for 1 min at 72°C, 2) preheating for 2 minutes at 94°C, 39 cycles of denaturation for 1 min at 94°C, annealing for 30 sec at 59.3°C, and extension for 1 min at 72°C. After the last cycle, a final extension of 5 min at 72°C was added.

PCR products were analyzed on a 2% agarose gel containing ethidium bromide.

Growth rate assay

2×10^4 cells were plated with MF medium or phenol red free DMEM containing 10% ordinary FBS (E2 present) or charcoal treated FBS (E2 deprived condition) for three weeks. Cell viability was determined using trypan blue exclusion assay. Floating cells were washed off and the adherent cells detached from the dishes with 0.25% trypsin. Collected cells were stained with 0.4% trypan blue and were counted using hematocytometer.

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. Cells were incubated for three weeks and the number of colonies per 4 cm^2 was counted under a microscope.

In vivo tumor formation assays

We inoculated 1×10^4 cells in Matrigel (BD Matrigel Basement Membrane Matrix High Concentration, BD Bioscience, Bedford, MA, USA) into the subcutaneous connective tissue of five week old nude mice (Balb nu/nu). After thirty weeks, the mice were killed and the tumors were excised. All mouse experiments were approved by the animal ethics committee of Kyushu University.

Senescence-associated β-Galactosidase Staining

Cells were cultured in DMEM containing 10% FBS. Senescence-associated β-Galactosidase Staining was performed by Senescence β-Galactosidase Staining Kit (Cell Signaling Technology Inc., MA, USA) according to the manufacturer's instructions.

Luciferase assay

Transient transfection of 2.5 μg of a luciferase reporter plasmid containing an ERE-sequence in the promoter region [21] was performed using LipofectAMINE (Invitrogen) according to the manufacturer's instructions. The cells were incubated with phenol red-free DMEM containing 10% charcoal-dextran-treated fetal calf serum for 72 hr after transfection. The medium was replaced with phenol red-free DMEM containing 1% charcoal-dextran-treated fetal calf serum in the presence or absence of 10^{-7} M 17β-estradiol (Sigma) or 10% fetal calf serum for 16 hr. Luciferase assays were performed using a luciferase assay kit (Stratagene, La Jolla, CA, USA). Transfection efficiency was normalized by β-galactosidase assays using β-galactosidase staining kits (Invitrogen).

Antibodies

Primary antibodies used in this study were as follows: Oct3/4 polyclonal antibody (H-134), c-Myc monoclonal antibody (C-8), ERα polyclonal antibody (MC-20), ERβ polyclonal antibody (H-150), and a GAPDH polyclonal antibody (FL-335), all were obtained from Santa Cruz Biotechnology, Inc., CA, USA. The MAPK polyoclonal antibody was purchased from Millipore Billirica MA, USA.

Western blotting

To measure protein expression, subconfluent cells were lysed with ice-cold lysis buffer (CelLytic M Cell Lysis Reagent; Sigma, St Louis, MO, USA) containing freshly added protease inhibitors (Protease inhibitor Cocktail; Sigma, St Louis, MO, USA). After centrifugation at 13,000 X g for ten min 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, Hercules, USA). The blots were incubated with diluted primary antibodies overnight at 4°C. After incubation with each primary antibody (1:1000 dilution), the blots were incubated with horseradish peroxidase-linked anti-rabbit antibodies and analyzed with an ECL system (Amersham Bioscience, Buckinghamshire, UK). The levels of protein expression were quantitated using Image J software.

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

Tumorigenicity was enhanced in SP cells harboring mutant *KRAS* compared with that in NSP harboring mutant *KRAS* gene

We used a non-tumorigenic immortalized rat endometrial cell-line (RENT4). SP cells (RSP cells) and NSP cells (RNSP cells) were isolated from RENT4 cells and incubated with MF medium (Figure 1A). Initially, both RSP cells and RNSP cells grew at the same rate and RSP cells grew faster than RNSP cells after seven weeks of culturing (Figure 1B). RSP cells and RNSP cells harboring mutant [12Val] versions of K-ras4B (RSP-K12V cells and RNSP-K12V cells) were established
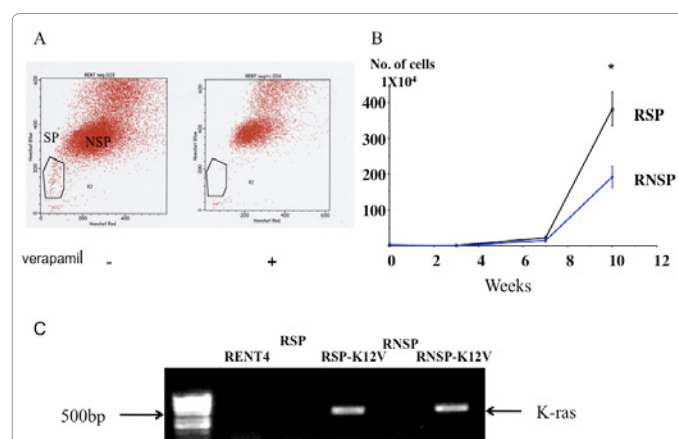

Figure 1: RSP and RNSP cell lines harboring mutant [12Val] versions of *KRAS* 4B (RSP-K12V cells and RNSP-K12V cells) were established.

A) SP cells and NSP cells were isolated from RENT4 cells. Verapamil treatment blocked the dye efflux and rendered the SP cells undetectable.

B) SP cells and NSP cells were incubated with MF medium for ten weeks. The growth rate was investigated. RSP cells grew faster than RNSP cells after seven weeks of culturing. Cell numbers are represented as the means ± SEM from three independent experiments. * $p < 0.05$

RSP cells and RNSP cells harboring mutant [12Val] versions of *KRAS* 4B (RSP-K12V cells and RNSP-K12V cells) were established as described in Materials and Methods. Expression of [12Val] *KRAS* gene was confirmed by RT-PCR using the human KRAS cDNA specific primers.

by transfecting RENT4 cells with pZeo constructs, containing cDNA sequences encoding [12Val] human *KRAS*. Expression of the [12Val] *KRAS* mRNA was confirmed by RT-PCR using human KRAS cDNA specific primers (Figure 1C). Cells were cultured on collagen-coated plates in DMEM containing 10% FBS. The growth rate was analyzed for ten weeks. The growth rate of the RSP-K12V cells increased after seven weeks of culture. In contrast, RNSP-K12V cells continued to grow slowly (Figure 2A).

Next, anchorage-independent cell growth was investigated. Neither RSP nor RNSP cells formed colonies in soft agar (data not shown). Both RSP-K12V cells and RNSP-K12V cells generated colonies in soft agar. The number of colonies in RSP-K12V cells was 1.7-fold greater compared with that in RNSP-K12V cells (Figure 2B).

Cells were inoculated into the subcutaneous tissue of nude mice (n=8 per group). Both RSP and RNSP cells did not form tumors in nude mice (data not shown). RSP-K12V cells formed palpable tumors at 20 weeks following injection in three mice. In contrast, the RNSP-K12V cells only formed a tumor in one mouse 30 weeks following inoculation. The RSP-K12V tumors grew faster and were larger than the RNSP-K12V tumor (Figure 2C).

SP cells, but not NSP cells harboring mutant *KRAS* had the capacity for serial tumor formation *in vivo*

The tumors were excised from the mice after 32 weeks of inoculation. We isolated cells from each tumor (tumor cells derived from RSP-K12V cells or RNSP-K12V cells) and cultured these cells on collagen-plates with DMEM containing 10% FBS. Although growth rate in these primary cultured cells is slow, tumor cells derived from RSP-K12V cells exhibited long-term proliferating capacity in culture. In contrast, the primary cultured tumor cells derived from RNSP-K12V cells failed to grow and died within four weeks (Figure 3A). Tumor cells derived from RNSP-K12V cells exhibit increased size and flattened morphology, suggesting that senescence was induced in these cells. We examined whether these cells expressed a senescence-associated β-gal (SA-β-gal) activity. SA-β-gal activity was detected in tumor cells derived from RNSP-K12V cells, but not in tumor cells derived from RSP-K12V cells (Figure 3B).

We transplanted these tumor cells derived from RSP-K12V cells or RNSP-K12V cells into the subcutaneous tissue of nude mice (n=4 per group). Tumor cells derived from RSP-K12V cells produced large tumors in all mice, indicating that RSP-K12V cells had the capacity to form serial tumors in vivo. In contrast, tumor cells derived from RNSP-K12V cells did not form tumors (Figure 3C). We confirmed mRNA expression of introduced *KRAS* gene in each type of primary culture cells by RT-PCR (Figure 3D).

SP cells harboring mutant *KRAS* and the tumor cells derived from them exhibited a stemness phenotype

We investigated the stemness phenotype in each type of cells. First, we analyzed the proportion of SP cells for each of the cell types. The proportion of SP cells was higher in RSP-K12V cells than in RSP cells and was highest in the RSP-K12V tumor cells (Figure 4). Interestingly, the proportion of SP cells correlated with tumorigenicity (Table 1).

It has previously been shown that c-Myc and Oct4, which are associated with stemness phenotype, are expressed in endometrium [22,23]. Therefore, we investigated the levels of c-Myc and Oct4 in each
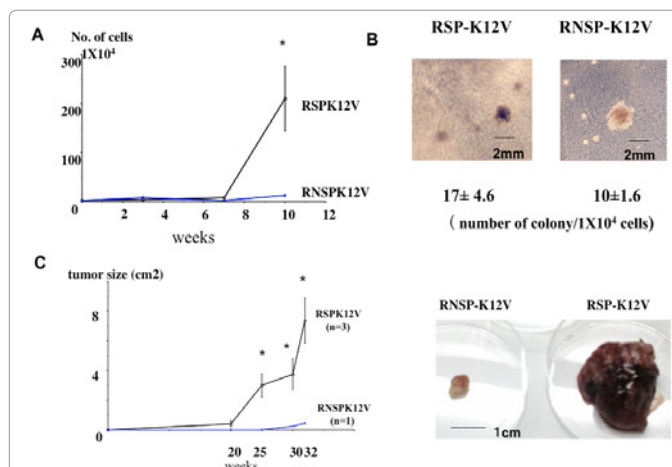

Figure 2: Tumorigenicity was enhanced in RSP-K12V cells compared with that in RNSP-K12V cells.

A) RSPK12V cells and RNSPK12V cells were cultured in DMEM containing 10% FBS. The cell growth rate was then analyzed for ten weeks. The growth rate of RSP-K12V cells was increased after seven weeks of culturing. In contrast, RNSP-K12V cells continue to grow slowly. Cell numbers are represented as the means ±SEM from three independent experiments. * *p* < 0.05

B) Both RSP-K12V cells and RNSP-K12V cells generated colonies in soft agar. Colony numbers are represented as the means ± SEM from three independent experiments. Scale bar; 2mm

C) RSP-K12V cells and RNSP-K12Vcells were inoculated into the subcutaneous tissue of nude mice (n=8 per group). RSP-K12V cells formed palpable tumors after twenty weeks of inoculation in three mice. In contrast, RNSP-K12V cells formed a tumor in only one mouse after thirty weeks of inoculation. The tumors generated from RSP-K12V cells grew faster and larger than the single RNSP-K12V tumor. Scale bar; 1cm
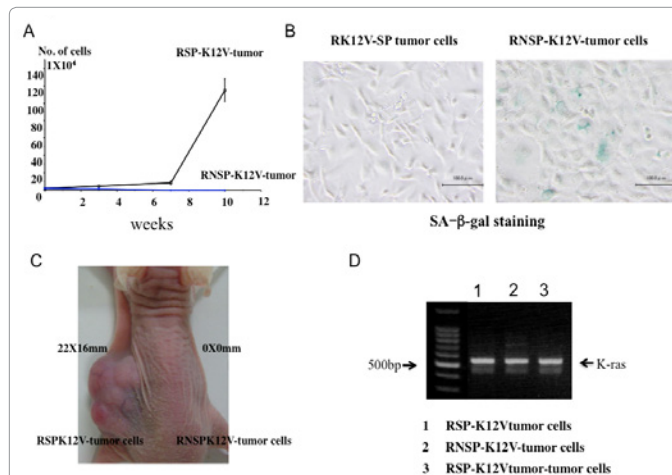

Figure 3: Primary cultured cells from RSP-K12V-tumors are capable of serial tumor formation *in vivo*.

A) Cells were isolated from RSP-K12V-tumors and RNSP-K12V-tumors and cultured on collagen-plates with DMEM containing 10% FBS. The primary cultured RSP-K12V-tumor cells exhibited long-term proliferating capacity in vitro. In contrast, the primary cultured RNSP-K12V-tumor cells failed to grow and died within two weeks.

B) A senescence associated β-gal (SA-β-gal) staining was performed. SA-β-gal activity was detected in RNSP-K12V tumor cells, but not in RSP-K12V-tumor cells.

C) RSP-K12V-tumor cells and RNSP-K12V-tumor cells were transplanted into the subcutaneous tissue of nude mice. RSP-K12V-tumor cells produced tumors. In contrast, RNSP-K12V-tumor cells did not form tumors.

D) Expression of K-ras in each type of primary cultured cells was confirmed by RT-PCR.

cell type. The levels of both proteins were increased in RSP-K12V cells and tumor cells derived from RSP-K12V cells compared with those in RNSP-K12V cells and tumor cells derived from RNSP-K12V cells, respectively. Enhancement of c-Myc and Oct4 expression levels was more pronounced in tumor cells derived from RSP-K12V cells than in RNSP-K12V cells.

ER transcriptional activity was enhanced in SP cells harboring the mutant *KRAS* gene in comparison to NSP cells harboring the mutant *KRAS* gene

We previously have demonstrated that activated K-Ras enhanced transcriptional activity of ER in NIH 3T3 cells and functional inactivation of ER induced senescence [4,5]. We investigated whether this was the case in cells used in the present study. We first analyzed the levels of ERα and β expression, which are critical for estrogen-dependent function (Figure 5A). The level of ERα protein was increased in RSP and RSP-K12V cells compared with the levels in NSP and NSP-K12V cells, respectively. The level of ERβ was constant across all cell types. Although the total MAPK level, which is shown as an internal control, was similar in all of the cells, activation of MAPK (upper band denoted by the arrow) was observed in RSP-K12V cells and RNSP-K12V cells, but not in RSP cells and RNSP cells. This indicates that the introduced [^{12}Val] human K-Ras functionally activates MAPK in both RSP-K12V cells and RNSP-K12V cells (Figure 5A).

Next, we employed a luciferase reporter assay to measure ER transcriptional activity. A luciferase reporter plasmid containing an ERE-sequence in the promoter region was transfected into each of cell types in the presence or absence of 10^{-7}M E2 and/or 10% serum. We calculated the ratio of luciferase activity in each of cell types to the activity in RSP-mock cells in the absence of both 10^{-7}M E2 and 10% serum. ER activity in all conditions was significantly greater in RSP-K12V cells compared with that in RSP-mock cells, RNSP-mock cells and RNSP-K12V cells (Figure 5B) ($p < 0.05$).

Tumor cells derived RSP-K12V cells demonstrated enhanced transcriptional activity of ER and acquired the potential for estrogen-independent proliferation

We analyzed the levels and transcriptional activity of ER in primary cultured tumor cells derived from RSP-K12V cells and RNSP-K12V cells by a luciferase assay. The ERα expression level was greater in tumor cells derived from RSP-K12V cells than in tumor cells derived from RNSP-K12V cells (Figure 6A). The ERE-luciferase activity of tumor cells derived from RSP-K12V cells was enhanced by stimulation with E2 (3.8-fold) or serum (8-fold) over the levels achieved following stimulation of tumor cells derived from RNSP-K12V cells ($p < 0.01$). ER activity was not enhanced by stimulation of E2 or serum in tumor cells derived from RNSP-K12V cells (Figure 6B).

Growth rates of RSP cells, RSP-K12V cells and primary cultured tumor cells derived from RSP-K12V cells in the presence or deprivation of E2 were investigated. Cells were cultured with phenol red free DMEM containing ordinary FBS (E2 present condition in serum) or charcoal treated FBS (E2 deprived condition in serum) for 3 weeks. Cell growth of RSP cells and RSP-K12V cells was suppressed in the deprivation of E2 in comparison to growth in the presence of E2 ($p < 0.05$). In contrast, the growth rate of tumor cells derived from RSP-K12V cells was not influenced by the presence of E2 (Figure 6C). These results indicate that primary cultured tumor cells derived from serial transplanted tumors of RSP-K12V cells acquired the potential for estrogen-independent proliferation.

Discussion

In this study, we investigated the role of SP cells in non-tumorigenic endometrial cells for development of endometrial cancer. We demonstrated that oncogenic K-Ras produced different biologic effects in SP cells and NSP cells using a rat endometrial cell line, i.e., i) tumorigenicity and the ER transcriptional activity were enhanced in SP cells harboring mutant *KRAS* compared with that in NSP harboring mutant *KRAS* gene, ii) SP cells harboring mutant K-ras and the tumor cells derived from them exhibited a stemness phenotype and the capacity for serial tumor formation *in vivo*.

Ras signaling is involved in the development of endometrial cancer. K-ras mutations occur in 20% of endometrial cancer [24] and are also found in endometrial hyperplasia [17]. Endometrial cancer is also estrogen-dependent. We have previously demonstrated that activated K-Ras 4B significantly enhances the transcriptional activity of the ER [4]. ER function contributes to oncogenic K-Ras mediated NIH3T3 cell transformation with escape from senescence by modulating the p53 pathway [5]. In this study, we demonstrated that the transcriptional activity of the ER was increased following stimulation with E$_2$ or serum to a greater extent in RSP-K12V cells than in RNSP-K12V cells. This suggests that oncogenic K-ras is associated with ER-dependent features in SP cells rather than in NSP cells of endometrium. RSP-K12V cells themselves were more tumorigenic than RNSP-K12V cells. RSP-K12V cells formed larger tumors in nude mice than did RNSP-K12V cells. These results suggest that the occurrence of *KRAS* mutations in SP cells rather than NSP cells is critical for development of malignant endometrial tumors.

RSP-K12V cells, unlike the RNSP-K12V cells, also retained their tumorigenic properties in both primary cultures as well as in secondary xerograft experiments. Tumor cells derived from RNSP-K12V cells
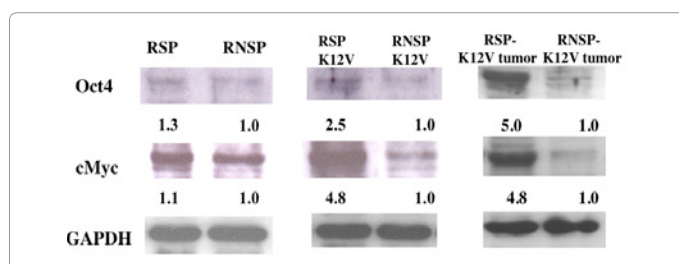

Figure 4: The levels of c-Myc and Oct4 were enhanced in RSP-K12V cells and RSP-K12V tumor cells compared with those in RNSP-K12V cells and RNSP-K12V tumor cells

The levels of c-Myc and Oct4 in each of cell types were investigated by Western blot. The levels of both proteins were increased in RSP-K12V cells and RSP-K12V tumor cells compared with that in RNSP-K12V cells and RNSP-K12V tumor cells, respectively. The level of GAPDH was used as an internal control.

Cell	Proportion of SP cells (% :mean+SEM) (n=3)	Tumorigenicity No.of mouse formed tumor INo. of mouse inoculated cells
RSP	0.92 ± 0.10	0/8 (0 %)
RSP-K12V	2.30 ± 0.20	3/8 (38%)
RNSP-K12V	0.54 ± 0.24	1/8 (13%)
RSP-K12V-tumor	13.03 ± 3.20	4/4 (100%)
RNSP-K12V-tumor	0.41 ± 0.22	0/4 (0%)

Table 1: The proportion of SP cells correlated with tumorigenicity.

have the low level of ERα expression and activity and become senescent. These results are consistent with our previous report that functional inactivation of the ER by a dominant negative mutant of ERα in the presence of activated K-ras 4B in NIH 3T3 cells induces cell senescence and abrogates tumorigenic potential [5].

The levels of the stem cell markers, c-Myc and Oct4, were increased in RSP-K12V cells and tumor cells derived from RSP-K12V cells compared with their NSP counterparts. Jung et al. demonstrated that there are 4 ERE target sequence with minor sequence variation at *OCT4* promoter region and E2 induced Oct4 expression in MCF7 mammospheres by the binding ER at *OCT4* promoter region [25]. c-*MYC* is a well-known estrogen-induced gene, too. Wang et al. [26] examined c-*MYC* gene structure in this global genomic context and found a distal ER-binding region located at about 67 kb upstream of the transcriptional start site of the c-*MYC* gene. They demonstrated that E2 induced c-*MYC* gene expression via an upstream enhancer activated by the ER and the AP-1 transcription factor. Consistent with these previous evidences, increases of c-Myc and Oct4 levels were correlated with enhancement of ER activity in the present study. As expected based on these observations, the proportion of SP cells gradually increased by serial transplantation (RSP-K12V cells < primary cultured tumor cells derived from RSP-K12V cells), suggesting an increase in the population of cancer stem-like cells.
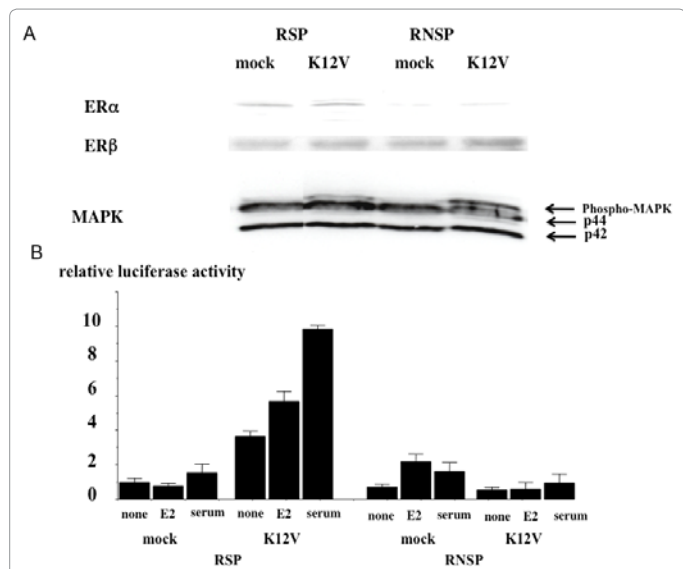

Figure 5: The transcriptional activity of the ER was enhanced in SP cells harboring the mutant *KRAS* gene compared with the activity in NSP cells harboring the mutant *KRAS* gene.

A) The expression levels of ERα and β were analyzed by Western blot. The level of ER β protein was enhanced in RSP and RSP-K12V cells compared with the levels in NSP and NSP-K12V cells. The level of ERβ was constant across all cell types. The total MAPK level is shown as an internal control. Although the levels of total MAPK were similar in all of the cell types, activation of MAPK (upper band denoted by the arrow) was observed in RSP-K12V cells and RNSP-K12V cells, but not in RSP cells or RNSP cells. Representative data are shown. Similar results were obtained in three independent experiments.

The ER transcriptional activity in each of cells type by a luciferase reporter assay using luciferase reporter plasmid containing ERE-sequence in the promoter region in the presence or absence of 10^{-7}M E2 and/or 10% serum. The ratio of luciferase activity to RSP-mock cells in the absence of both 10^{-7}M E2 and 10% serum was calculated. ER activity in all conditions was significantly enhanced in RSP-K12V cells compared with that in RSP-mock cells, RNSP-mock cells and RNSP-K12V cells ($p < 0.05$). Data are represented as the means ±SEM from three independent experiments.
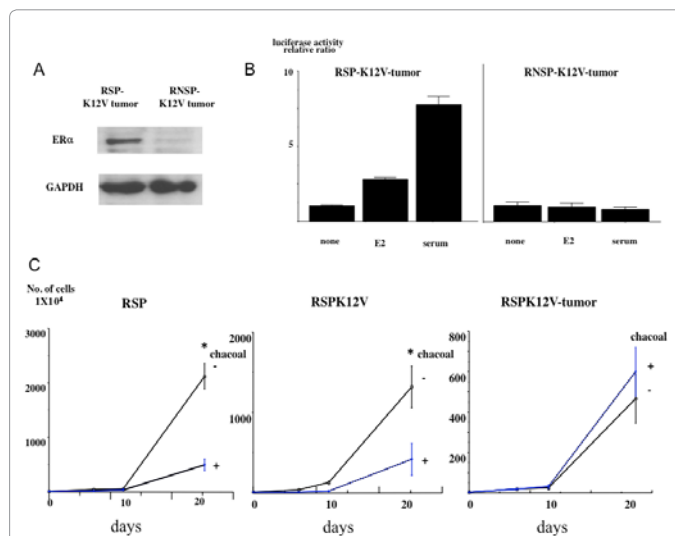

Figure 6: Tumor cells derived from RSP-K12V cells had enhanced transcriptional activity of the ER and the capacity for estrogen-independent proliferation.

A) The level of ERα expression was enhanced in RSP-K12V-tumor cells compared with that in RNSP-K12V-tumor cells. GAPDH served as the internal control. Representative data are shown. Similar results were obtained in three independent experiments.

B) The levels and transcriptional activity of the ER in RSP-K12V-tumor cells and RNSP-K12V-tumor cells were analyzed by a luciferase reporter assay. The luciferase activity of RSP-K12V-tumor cells was enhanced by stimulation of E2 (3.8-fold) or serum (8-fold) over the level in RNSP-K12V-tumor cells ($p < 0.01$).

C) The cell growth rate of RSP-K12V cells and RSP-K12V tumor cells was investigated in the E2 present or E2 deprived condition. Cells were cultured with phenol red free DMEM containing 10% ordinary FBS (E2 present condition) or 10% charcoal treated FBS (E2 deprived condition) for three weeks. Cell growth of RSP cells and RSP-K12V cells was suppressed in the E2 deprived condition compared with that in the E2 present condition. In contrast, growth of RSP-K12V tumor cells was not influenced by estrogen. Data of cell numbers were represented as the means ±SEM from three independent experiments. * $p < 0.05$

The growth rates of RSP cells and RSP-K12V cells were suppressed in E2-deprived medium. In contrast, the growth rate of RSP-K12V-tumor cells was not influenced by E2, suggesting that RSP-K12-tumor cells progress to a hormone-refractory state. Although the precise mechanism of this phenomenon is still unclear, one possibility is alteration of property of tumor cells during serial tumor formation. A second possibility is the effect of non-tumor cells, which are component of the tumors (stroma cells, fibroblast, endothelial cells etc.), because these cells are primary cultured cells derived from tumors. The precise mechanism is under investigation.

The results of the present study suggest that the occurrence of genetic alterations (e.g. *KRAS* gene mutations) in endometrial SP cells contributes to the development of malignant endometrial tumors. Introduction of an activating *KRAS* mutation into SP cells promoted the acquisition of cancer stem cell properties, escape from senescence and induced malignant progression into a hormone-refractory state. In contrast, the same genetic alteration in NSP cells was insufficient for the development of malignant endometrial tumors.

Vitale-Cross et al. has shown that the conditional expression of activated K-*ras* in the basal layer of stratified epithelium, which includes the epithelial stem cells is sufficient to induce the rapid formation of malignant squamous cell carcinoma [27]. Wang et al. have demonstrated that rare luminal cells of the prostate that express

Nkx3-1 in the absence of testicular androgen display stem/progenitor properties during prostate regeneration. Targeted deletion of the *PTEN* tumor suppressor gene in these cells results in rapid carcinoma formation [28]. Barker et al. [29] have shown that when *APC* is deleted in short-lived transit-amplifying cells, the growth of induced microadenomas is rapidly stalled. In contrast, Lgr5-EGFP+ intestinal stem cells persistent are transformed within days and form β-catenin high microadenomas after loss of *APC*.

In summary, we demonstrated that SP cells rather than NSP cells in endometrium contributed to carcinogenesis for the first time. Our present observations suggest that endometrial cancer likely arises from a stem cell containing a genetic alteration, which is critical for cancer development.

Acknowledgment

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