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The inhibitory effect of salinomycin on the proliferation, migration and invasion of human endometrial cancer stem-like cells

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HIGHLIGHTS

- Salinomycin reduced the level of fibronectin expression, which was enhanced in endometrial cancer stem cells.
- Salinomycin induced apoptosis and inhibited Wnt signaling.
- Salinomycin inhibited the proliferation, migration, invasiveness and tumorigenicity of endometrial cancer stem cells.

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ABSTRACT

Goals. We previously demonstrated that side-population (SP) cells in human endometrial cancer cells (Hec1 cells) and in rat endometrial cells expressing oncogenic human K-Ras protein (RK12V cells) have features of cancer stem cells (CSCs). Hec1-SP cells showed enhanced migration and the potential to differentiate into the mesenchymal cell lineage. In this study, we analyzed the association of the epithelial–mesenchymal transition (EMT) with the properties of these endometrial CSCs. We also assessed the effects of salinomycin (a compound with EMT-specific toxicity) on the proliferative capacity, migration and invasiveness of these endometrial CSCs using Hec1-SP cells.

Method. We performed microarray expression analysis to screen for up-regulated genes in CSCs using a set of RK12V-SP cells and -non-SP(NSP) cells and used the Metacore package to identify the Gene GO pathway MAPs involved in the up-regulated genes. To analyze their association with EMT, the expression of several EMT associated genes in Hec1-SP cells was investigated by real time PCR and compared with that in Hec1-NSP cells. We assessed the expression of *BAX*, *BCL2*, *LEF1*, *cyclinD* and *fibronectin* by real time PCR. We also evaluated the viabilities, migration and invasive activities, and tumorigenicities of these SP cells and NSP cells in the presence or absence of salinomycin.

Results. We demonstrated that i) EMT processes were observed in both RK12V-SP cells and Hec1-SP cells, ii) the level of *fibronectin* was enhanced in Hec1-SP cells and salinomycin reduced the level of *fibronectin* expression, iii) salinomycin induced apoptosis and inhibited Wnt signaling, and iv) salinomycin inhibited the proliferation, migration, invasiveness and tumorigenicity of these SP cells.

Conclusion. This is the first report of an inhibitory effect of salinomycin on the properties of endometrial CSCs.

Introduction

Stem cells have a marked self-renewal capacity and are pluripotent. They can be identified in embryonic tissues as well as in normal adult tissues. The existence of cancer stem-like cells (CSCs) has been proposed and CSCs have been identified in leukemia and several solid tumors [1–3]. The properties of CSCs are as follows: i) they possess self-renewal capacity, ii) they can produce progeny cells, iii) they constitute a small minority of neoplastic cells within a tumor, and iv) they possess the developmental potential for expression of multiple specific markers [4]. CSCs are resistant to current cancer treatment, resulting in an increased risk of recurrence.

Side-population (SP) cells are enriched in stem cells and have been isolated and characterized, using fluorescence-activated cell sorting (FACS). The methodology is based on the cells' ability to reduce the intracellular concentration of the fluorescent dye Hoechst 33342 [5]. The identification of SP cells is associated with a high

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expression level of the ATP-binding cassette transporter protein ABCG2/BCRP1. The ATP-binding-cassette (ABC) transporters represent the largest family of transmembrane proteins capable of exporting a wide variety of molecules and structurally unrelated chemotherapeutic drugs from the cytosol. These proteins confer multidrug resistance to CSCs [6,7].

We isolated and characterized SP cells present in human endometrial cancer cells (Hec1 cells) and in rat endometrial cells expressing oncogenic human K-Ras protein (RK12V cells). The SP cells showed reduced expression levels of differentiation markers, long-term proliferative capacity of the cell cultures, self-renewal capacity, enhanced tumorigenicity, and enhanced migration. These findings demonstrate that SP cells have features of CSCs, including the potential to differentiate into the mesenchymal cell lineage [8].

The epithelial–mesenchymal transition (EMT) occurs during normal early embryonic development. This program allows a polarized epithelial cell, which normally interacts with the basement membrane via its basal surface, to undergo multiple biochemical changes that enable it to assume a mesenchymal cell phenotype, which includes enhanced migratory capacity and invasiveness [9]. EMT is also a key developmental program that is often activated during cancer invasion and metastasis. The EMT program enables cancer cells to disseminate from a primary tumor by losing epithelial characteristics and acquiring a mesenchymal phenotype. Mani et al. have reported a direct link between EMT and the gain of epithelial stem cell properties [10]. Human breast cancer stem cells express Snail and Twist, EMT-inducing transcription factors. When their expression is elevated, the size of the stem cell population is increased and expression levels of mesenchymal markers fibronectin and vimentin are enhanced [10]. Thus, EMT promotes the generation of cancer stem cells.

Salinomycin is a selective inhibitor of CSCs [11]. Salinomycin is an antibacterial and coccidiostatic therapeutic drug. It has been shown to kill breast cancer stem cells in mice at least 100-times more effectively than the commonly used anti-cancer drug paclitaxel. Although several studies demonstrate that salinomycin has an inhibitory effect on proliferation of CSCs, the effects of salinomycin on the properties of endometrial CSCs are unclear.

In this study, we used Hec1-SP cells and RK12V-SP cells to analyze the association of EMT with the properties of endometrial CSCs and the effects of salinomycin on the proliferative capacity, migration and invasiveness of endometrial CSCs.

Materials and methods

Cell lines and cell culture

An endometrial cancer cell line (Hec-1) and a rat endometrial cell line (RENT4) were used in the present study. The Hec-1 cell line was established by Kuramoto et al. from explants of adenocarcinoma of human endometrium [12] and it was a gift from Dr. Kuramoto. RENT4 cells were established by Wiehle et al. [13] and obtained from the European Collection of Cell Cultures (ECACC). Both cell lines were authenticated by Takara Bio Inc. using the short tandem repeat (STR) DNA profiling. The STR profiles of Hec-1 cells were matched to their original profiles. Interspecies contamination was ruled out by the STR profiles in both cell lines. Both cell lines were cultured at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM) + 10% fetal bovine serum (FBS) + 100 U/mL penicillin, and 100 μg/mL streptomycin.

Isolation of SP cells

To identify and isolate Hec1 SP cells, the cells were dislodged from the culture dishes with trypsin and EDTA, washed, and suspended at a concentration of 1×10^6 cells/mL in DMEM containing 10% 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, OR), either alone or in combination with 50 μmol/L verapamil (Sigma-Aldrich). Finally, the cells were counterstained with 1 μg/mL propidium iodide (PI) to label dead cells. The cells were then analyzed in a FACS Vantage fluorescence-activated cell sorter (BD Biosciences, San Jose, CA) using dual wavelength analysis (blue, 424–444 nm; red, 675 nm) after excitation with 350 nm UV light. PI-positive dead 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) containing 10% FBS on collagen-coated 24-well plates (2 cm^2) (Iwaki, Funabashi, Japan). The cells were cultured for two to four weeks. The cells were then transferred to collagen-coated plates (60 mm).

RNA isolation

RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Real time quantitative PCR analysis

Real time PCR was carried out using a 7300 Real Time PCR System with SDS RQ Study software (Applied Biosystems). cDNA templates were combined with SYBER Green premix with Rox (Takara Bio) to perform quantitative-PCR reactions. Primers used in this study are shown in Supplementary Table 1. Reactions were carried out for 1 cycle at 94 °C for 5 min; 40 cycles at 94 °C for 30 s, 59 °C for 30 s and 72 °C for 30 s; 1 cycle at 94 °C for 1 min; and 1 cycle at 55 °C for 30 s. The transcript level of each specific gene was normalized to GAPDH amplification.

In vitro scratch assay

The SP and NSP cells were cultured on 24-well plates in complete medium. Upon reaching confluence, the medium was replaced with conditioned medium DMEM containing 1% FBS in the presence or absence of salinomycin (1 μM) for an additional 24 h, and the cell layer was wound with 200 μL tips. After 24 h of incubation, the cells were photographed with a BZ-8100 microscope (Keyence, Japan).

Invasion assays

Cell invasiveness was assessed with a BioCoat Matrigel Invasion Chamber kit according to the protocol of the manufacturer (Becton Dickinson Labware, Bedford, MA). We seeded 5×10^4 Hec1-SP or -NSP cells treated with or without salinomycin (1 μM) into the Transwell insert chamber with a filter coated with Matrigel and placed the inserts in the lower chambers filled with 750 μL of DMEM containing 10% FBS. Chambers were incubated at 37 °C under a 5% CO_2 atmosphere for 24 h. Thereafter, we removed the inserts and scraped off the non-invading cancer cells remaining on the upper side of the filter. The cells that had invaded the lower side of the filter were viewed under a Nikon phase-contrast microscope and counted in >10 fields of view at ×200 magnification. The number of cells on the lower side of the filter was normalized to cells using the control chamber without matrigel. The assay was done in triplicate.

In vivo tumor formation assay

We inoculated 1×10^5 cells in Matrigel (BD Matrigel Basement Membrane Matrix High Concentration; BD Biosciences, Bedford, MA) into the subcutaneous connective tissue of five-week-old nude mice (Balb nu/nu). Four to six months after injection of Hec1-SP cells, mice were sacrificed and the tumors excised. Eight mice were injected subcutaneously with 0.26 μM salinomycin and another eight mice were injected with 100 1/4 L DMSO twice a week. The size of the tumor Q3

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was measured every week. The weight of each mouse was recorded once a week. Blood was taken from the mice with a syringe, which was flushed with heparin before blood withdrawal. All mouse experiments were approved by the animal ethics committee of Juntendo University.

TUNEL labeling

The Hec1-SP and NSP cells were cultured on chamber slide (BD Falcon™), and they were fixed in 1% paraformaldehyde in PBS, PH 7.4, for 10 min at room temperature. The cells were post-fixed in pre-cooled ethanol: acetic acid (2:1) for 5 min at −20 °C. The sections were immersed in 3% hydrogen peroxide for 10 min, and DNA 3-end labeling of apoptotic cells was performed by the Apop-Tag Plus Peroxidase Kit (Oncor, Inc. Gaithersburg, MD). After washing several times in PBS and the equilibration buffer, the sections were incubated with terminal deoxynucleotidyl transferase (TdT) for 1 h in a humidified chamber at 37 °C. Subsequently, the sections were immersed in stop wash buffer for 15 min, washed in PBS and incubated in anti-digoxigenin peroxidase for 30 min, at 37 °C. After several washes in

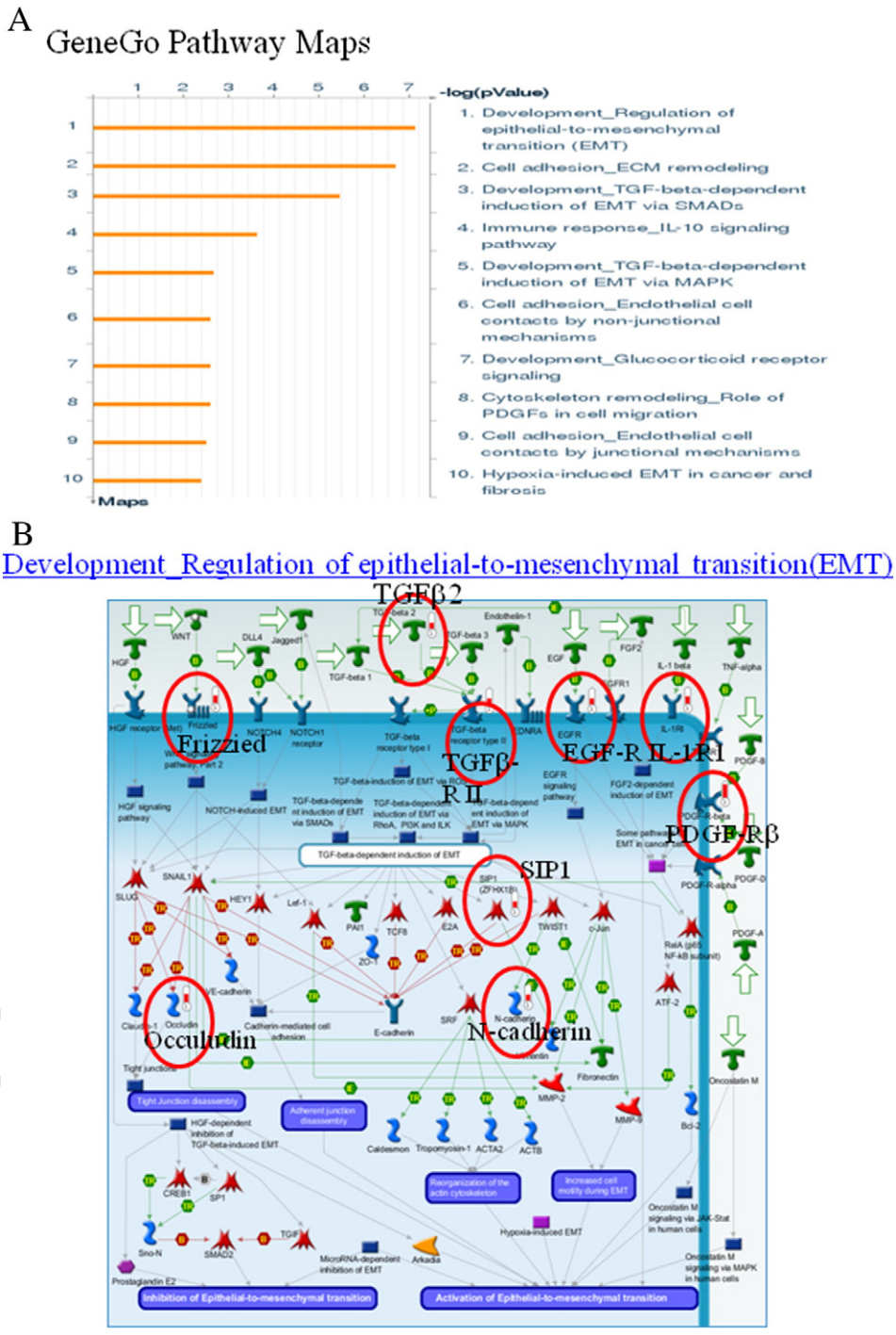

Fig. 1. EMT and the phenotype of endometrial CSCs. We used the Metacore package to identify the Gene GO pathway MAPs involved in upregulated genes in the microarray data on a set of K12V-SP cells and -NSP cells. A) Four Gene GO pathways of EMT were involved in the top ten scoring pathways. B) Nine genes were included in a total of 64 genes involved in the development regulation of EMT.

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PBS, the reaction was revealed by DAB (3.3′-diaminobenzidine tetrahydrochloride) and finally, the sections were counterstained with Mayer's hematoxylin. Negative controls were processed in the same manner, except that TdT was replaced with distilled water. Moreover, involuting mammary gland sections (provided by Apop-Tag Kit/Oncor, Inc. Gaithersburg) were used as positive controls for the TUNEL method. The sections were examined and photographed with an OLYMPUS BX-50.

Microarray

Total RNA was extracted using RNeasy Mini Kits according to the manufacturer's instructions (QIAGEN). Microarray analysis was performed by Takara Bio customer service. Briefly, cDNAs were synthesized by GeneChip T7-Oligo (dT) Promoter Primer Kit (Affymetrix) and TaKaRa cDNA Synthesis Kit (TaKaRa Bio) from 5 μg total RNA. Biotinylated cDNAs were synthesized with the IVT Labeling Kit (Affymetrix). Following fragmentation, 10 μg of cDNA were hybridized for 16 h at 45 °C on GeneChip Rat Genome 230 2.0 Array. GeneChips were scanned using a GeneChip Scanner 3000. Single Array Analysis was calculated by Microarray Suite version 5.0 (MAS5.0) with Affymetrix default settings.

Pathway analysis

Genetic pathways were evaluated using the MetaCore Analytical Suite (Gene Go Inc.). Enrichment analysis consisted of mapping gene IDs of the dataset onto IDs in entities of built-in functional ontologies represented in MetaCore by pathway maps and networks.

Data analysis

Data are represented as the means ± SEM and were analyzed with Student's *t*-test. For all analyses, two-sided tests of significance were used, with $p < 0.05$ considered significant.

Results

EMT: an important characteristic of endometrial CSCs

We previously demonstrated that both Hec1-SP cells and RK12V-SP cells have features of CSCs [8,14]. We found that the quality of mRNA derived from RK12V-SP cells isolated by flow cytometry was better than that from Hec1-SP cells. Thus, we performed microarray expression analysis to screen for up-regulated genes in CSCs on a set of RK12V-SP cells and -non-SP (NSP) cells. We identified 450 genes that were up-regulated more than two-fold in RK12V-SP cells compared with those in RK12V-NSP cells. The top 50 genes are shown in Supplementary Table 2. We used the Metacore package to identify the Gene GO pathway MAPs involved in the upregulated genes in RK12V-SP cells. The gene map with the highest P value (7.97×10^{-8}) was "Developmental Regulation of EMT" and nine genes were included out of a total of 64 genes of this pathway (Fig. 1A, B). Four Gene GO pathways of EMT were involved in the top ten scoring pathways (Fig. 1A). In agreement with the previous study by Mani et al., the microarray data on a set of RK12V-SP cells and -NSP cells showed that the pathways associated with EMT were important characteristics of endometrial CSCs [10].

The levels of EMT-associated genes were enhanced in Hec1-SP cells

We subjected Hec1-SP cells to real time PCR to analyze the levels of several types of EMT-associated genes that were enhanced in breast CSCs as shown by Mani et al. [10] and compared the results with those in Hec1-NSP cells. As expected, the expression levels of *CD9*, which is a glandular marker, in Hec1-SP cells were reduced ($p < 0.01$). In contrast, the levels of EMT-associated genes were increased. In particular, the level of *fibronectin* expression was significantly enhanced in Hec1-SP cells compared with that in Hec1-NSP cells (Fig. 2). These results demonstrated that EMT processes are occurring in Hec1-SP cells as well as in RK12V-SP cells.

Salinomycin inhibited the proliferation of Hec1-SP cells

Next, we examined the effect of salinomycin (a compound with EMT-specific toxicity) on the proliferation of Hec1-SP cells and Hec1-NSP cells. We assessed the viability of Hec1-SP cells and Hec1-NSP cells in the presence or absence of salinomycin (0.1, 1.0 and 5.0 μM) for 72 h. Proliferation of Hec1-NSP cells was inhibited at all of these concentrations. The viability of Hec1-SP cells was suppressed by treatment with concentrations of salinomycin more than 1 μM (Fig. 3A). Chromatin fragmentation was observed in Hec1-SP cells treated with 1 μM salinomycin, suggesting that apoptosis was induced (Fig. 3B). We confirmed apoptotic cells by TUNEL staining and SP cells treated with 1 μM salinomycin contained more apoptotic cells than non-treated SP cells (Fig. 3C). Salinomycin inhibited he proliferation of RK12V-SP cells as well as Hec1-SP cells (Fig. 3A).

Salinomycin induced apoptosis and inhibited Wnt signaling

To examine the induction of apoptosis by salinomycin in Hec1-SP cells and Hec1-NSP cells, we evaluated the expression of *BAX* and *BCL2* by real time PCR. The expression of *BAX*, a pro-apoptotic protein, was increased although it was not significantly different, and that of *BCL2*, an anti-apoptotic protein, was suppressed by treatment with 1 μM salinomycin significantly (Fig. 4A).

A recent study demonstrated that salinomycin downregulated the expression of Wnt target genes such as *LEF1*, *cyclin D1*, and *fibronectin* [15]. Thus, we assessed the expression of *LEF1* and *cyclin D1* by real time PCR. A gradual decrease of *LEF1* and *cyclin D1* expression was observed during treatment with salinomycin (Fig. 4B).

Salinomycin suppressed fibronectin expression and inhibited migration and invasion of Hec1-SP cells

We evaluated the level of *fibronectin* expression in Hec1-SP cells and Hec1-NSP cells in the presence or absence of salinomycin. The

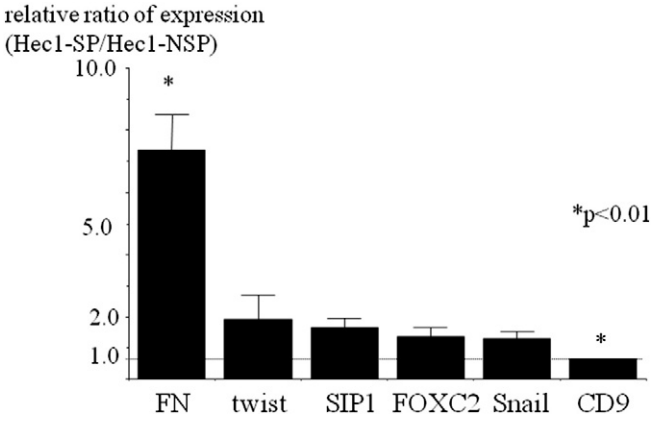

Fig. 2. The levels of EMT-associated genes were enhanced in Hec1-SP cells. We subjected Hec1-SP cells to real time PCR to analyze the levels of several types of EMT-associated genes and compared the results with those in Hec1-NSP cells. The expression levels of *CD9*, which is a glandular marker, in Hec1-SP cells were reduced ($p < 0.01$). In contrast, the levels of EMT-associated genes were increased. In particular, the level of *fibronectin* expression was significantly enhanced in Hec1-SP cells compared with that in Hec1-NSP cells.

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level of *fibronectin* was reduced in Hec1-SP cells treated with 1 μM salinomycin (Fig. 5A).

We previously demonstrated that Hec1-SP cells showed prominent migration activity. Thus, we investigated the effect of salinomycin on migration of Hec1-SP cells by time-lapse videoscopy. Migration of Hec1-SP cells was gradually inhibited by treatment with 1 μM salinomycin and completely suppressed after 16 h of treatment (Supplementary Fig. 1).

Next, we analyzed cell migration activity using the in vitro scratch assay. Migration of Hec1 and RK12V-SP cells was enhanced compared with that of Hec1 and RK12V-NSP cells. Enhanced migration of these SP cells was completely suppressed by treatment with 1 μM salinomycin for 24 h (Fig. 5B). We also examined cell invasiveness using the Boyden chamber assay with a filter coated with Matrigel. The invasiveness of Hec1 and RK12V-SP cells was enhanced compared with that of Hec1and RK12V-NSP cells. Enhanced migration of these SP cells was significantly suppressed by treatment with 1 μM salinomycin for 24 h (Fig. 5C).

Salinomycin inhibited the tumorigenicity of Hec1-SP cells

Finally, we investigated the effect of salinomycin on the tumorigenicity of Hec1-SP cells in vivo. Hec1-SP cells (1×10^5) were inoculated into the subcutaneous tissue of nude mice. After palpable tumors ($1\ cm^3$) had developed, we injected 0.26 μM of salinomycin or DMSO directly into the tumors. Tumors in DMSO-treated mice continued to grow. In contrast, tumors in salinomycin-treated mice stopped growing. In fact, tumor size in salinomycin-treated mice was reduced compared with tumors in DMSO-treated mice (Fig. 6). Primary cultured cells derived from tumors of salinomycin-treated mice did not form colonies. Finally, we evaluated the toxicity of salinomycin. No hematologic toxicity was observed except for elevation of K^+.

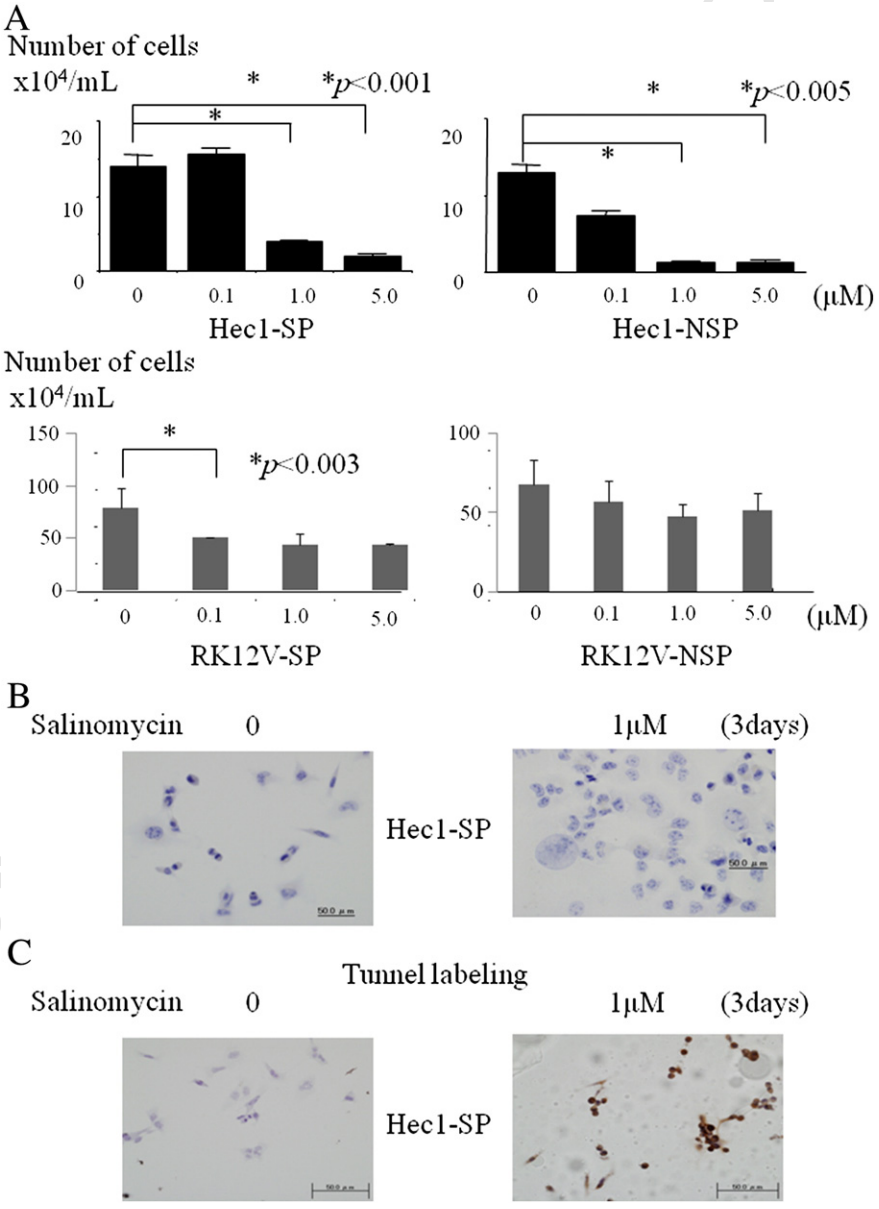

Fig. 3. Salinomycin inhibited proliferation of Hec1-SP cells. A) We assessed the viability of Hec1 and RK12V-SP cells and Hec1 and RK12V-NSP cells in the presence or absence of salinomycin (0.1, 1.0, and 5.0 μM) for 72 h. Viability of these-SP cells was suppressed by treatment with more than 1.0 μM salinomycin. B) Chromatin fragmentation was observed in Hec1-SP cells treated with 1.0 μM salinomycin, suggesting that apoptosis was induced. C) Tunnel labeling of apoptotic cells was performed by the Apop-Tag Plus Peroxidase Kit. SP cells treated with one μM salinomycin contained more apoptotic cells than non-treated SP cells.

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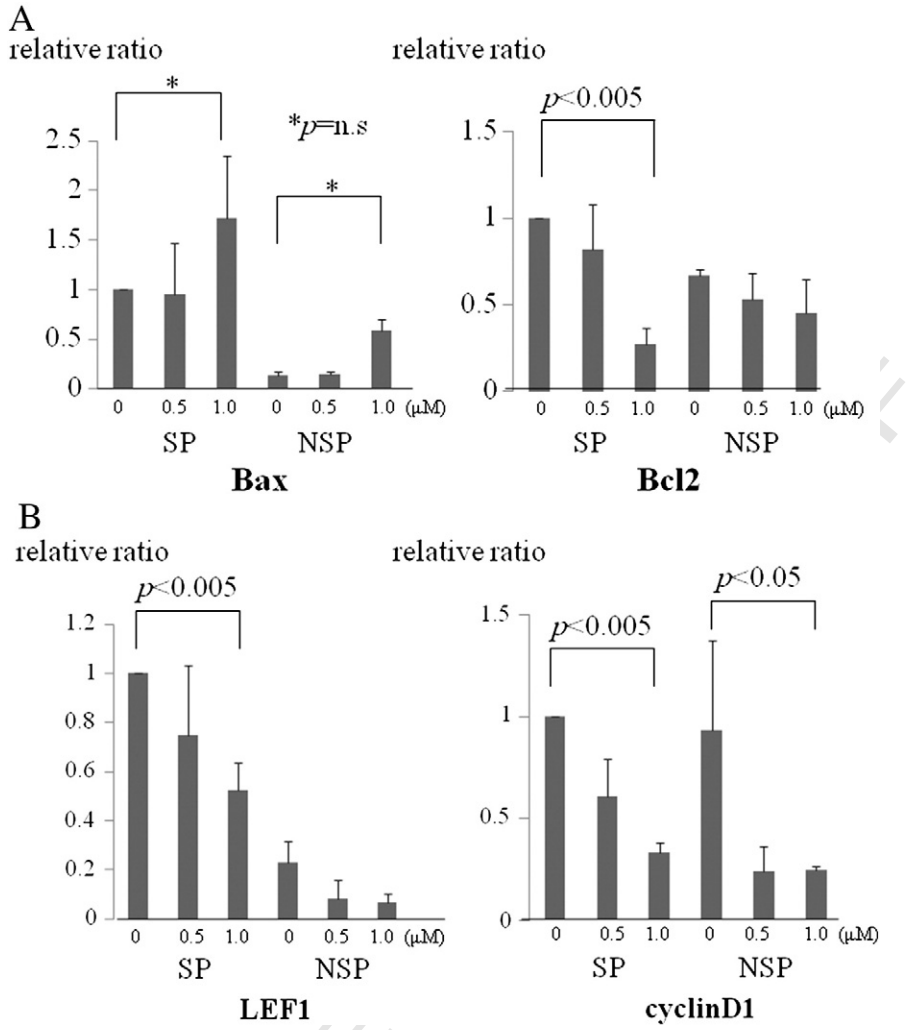

Fig. 4. Salinomycin induced apoptosis and inhibited Wnt signaling. A) The expressions of *BAX* and *BCL2* were investigated by real-time PCR. The expression of *BAX*, a pro-apoptotic protein was increased although it was not significantly different, and the expression of *BCL2*, an anti-apoptotic protein was suppressed by treatment with 1 μM salinomycin significantly. B) The expression of *LEF1* and *cyclin D1* was investigated by real-time PCR. A gradual decrease of *LEF1* and *cyclin D1* expression was caused by treatment with salinomycin.

Elevated K^+ was also seen in DMSO-treated mice and control mice (non-treated mice). Therefore, we suspect that the change in K^+ was not associated with salinomycin (Supplementary Table 3).

Discussion

Previous studies have shown that CSCs are resistant to chemo- or radio-therapy [7,16–18]. Current cancer treatment may fail to eliminate CSCs and surviving CSCs can regenerate new tumors, leading to relapse and metastasis. Mani et al. showed that EMT in normal or neoplastic mammary epithelial cell populations results in the enrichment of stem-like cells [10]. Gupta et al. demonstrated that normal and cancer cell populations engaged in the EMT process exhibit increased resistance to chemotherapy. These data suggests that EMT is associated with the resistance to cancer therapy, and may be linked to relapse or metastasis [11].

We showed in the present study that fibronectin is overexpressed in Hec1-SP cells compared with Hec1-NSP cells. Fibronectin is an extracellular matrix (ECM) ligand. Cell adhesion to the ECM initiates signaling cascades regulated by cell surface integrin receptors, affecting the proliferation and invasiveness of cells. The interaction of fibronectin with α5β1 integrin leads to integrin activation that is critical for cell survival and proliferation [19]. Recent evidence has shown that fibronectin is also associated with resistance to cancer therapy in breast cancer cells, leukemic cells and prostate cancer cells [20–22]. Amudson et al. have reported that fibronectin 1 is a potent predictor of radiation resistance in head and neck cancer [23]. It is likely that enhancement of fibronectin levels in Hec1-SP cells contributes to the properties of CSC, including EMT or chemo-resistance in endometrial cancer.

In 2009, Gupta et al. developed a new, high throughput screening method to search for compounds that attack CSCs [11]. Of 16,000 different chemicals that were tested, only a small number of substances (salinomycin, etoposide, abamectin and nigercin) showed selective toxicity for breast cancer stem cells. Among them, salinomycin was the most selective inhibitor of CSCs. Since then, several studies have shown that salinomycin has an inhibitory effect on cell proliferation in human cancer cells [24–26].

Salinomycin is a 751 Da monocarboxylic polyether antibiotic that consists of a large pentacyclic molecule with a unique tricyclic spiroketal ring system and an unsaturated six membered ring. Salinomycin acts in cytoplasmic and mitochondrial membranes as an ionophore with strict selectivity for alkali ions and a strong preference for potassium, thereby promoting mitochondrial and cellular potassium efflux and inhibiting mitochondrial oxidative phosphorylation. Salinomycin has been used as an anti-coccidial drug in poultry and is fed to cattle to increase their size [27]. Recent studies demonstrated that salinomycin induces apoptosis

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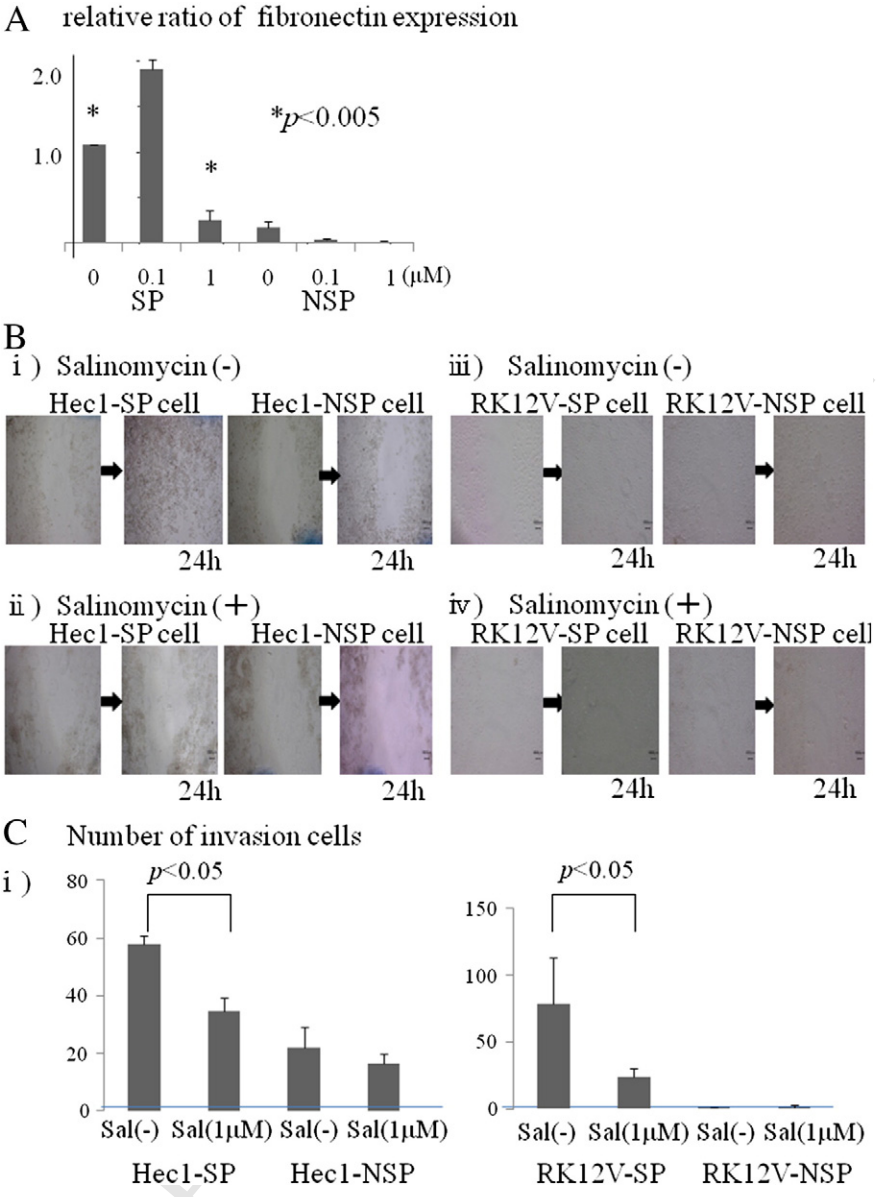

Fig. 5. Salinomycin suppressed *fibronectin* expression and inhibited migration and invasion of Hec1-SP cells. A) The level of *fibronectin* expression was investigated in Hec1-SP cells and Hec1-NSP cells in the presence or absence of salinomycin. The level of *fibronectin* was reduced in Hec1-SP cells treated with 1.0 μM salinomycin. B) Cell migration activity was investigated with the in vitro scratch assay. Migration of Hec1 and RK12V-SP cells was enhanced compared with that of Hec1 and RK12V-NSP cells. Enhanced migration of Hec1 and RK12V-SP cells was completely suppressed by the treatment with 1 μM salinomycin for 24 h. C) Cell invasion activity was examined with the Boyden chamber assay with a filter coated with Matrigel. Invasion of Hec1 and RK12V-SP cells was enhanced compared with that of Hec1 and RK12V-NSP cells. Enhanced migration of Hec1-SP and RK12V-NSP cells was significantly suppressed by treatment with 1 μM salinomycin for 24 h.

and overcomes apoptosis resistance by overexpression of Bcl-2, P-glycoprotein or 26S proteasomes with deregulated proteolytic activity in human cancer cells of different origins [25,26,28]. Furthermore, salinomycin overcomes multidrug resistance in CSCs that express ATP-binding cassette (ABC) transporters capable of removing chemotherapeutic drugs from the cytosol. Salinomycin sensitizes cancer cells to the effects of chemotherapy or radiation treatment by increasing DNA damage [26]. Fuchs et al. demonstrated that salinomycin induced massive apoptosis in CD4 + T cell leukemia and failed to induce apoptosis in normal CD4+T cell. These results suggest that salinomycin kills apoptosis-resistant cancer cells, but not normal cells [24].

Salinomycin effectively kills not only cancer cells but also cancer stem cells from different origins displaying ABC transporter-mediated multidrug resistance [24,29]. Dong et al. reported that salinomycin reduces the proportion of $CD133^+$cell subpopulations in human colorectal cancers cell lines (CRC) and down-regulates the expression of vimentin and induces E-cadherin expression in CRC cell lines [30].

Lu et al. reported that salinomycin downregulated the expression of Wnt target genes such as *LEF1*, *cyclin D1*, and *fibronectin*. Moreover, normal human peripheral blood lymphocytes resisted salinomycin toxicity [15]. We also demonstrated in the present study that salinomycin suppressed the level of fibronectin and inhibited proliferation, migration and invasion of endometrial CSCs (Hec1 and RK12V-SP cells). Salinomycin would likely be an effective anticancer agent for endometrial CSCs.

Salinomycin is widely used as an antibiotic for domestic animals, however, use of salinomycin has not reached the human trial stage. In China, a group accidentally ingested high levels of salinomycin during a banquet due to the mistaken substitution of salinomycin powder for starch. Symptoms included dizziness, nausea, vomiting, stomach ache, diarrhea, limb anesthesia, weakness, and red to brown urine [31]. It is necessary to examine salinomycin dosage carefully to determine human toxicity and to develop new derivatives of salinomycin with reduced toxicity for human clinical applications.

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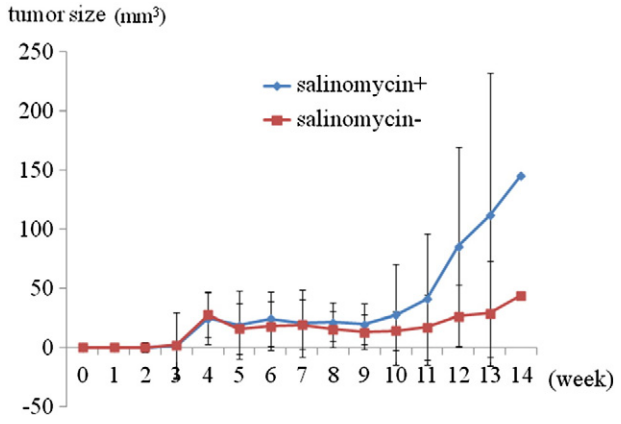

Fig. 6. Salinomycin inhibited tumorigenicity of Hec1-SP cells. The effect of salinomycin on tumorigenicity of Hec1-SP cells in vivo. Hec1-SP cells (1×10^5) were inoculated into the subcutaneous tissue of nude mice. When palpable tumors ($1\ cm^3$) developed, we injected 0.26 μM or DMSO directly into tumors. Tumor size in salinomycin-treated mice ($n = 5$) was reduced compared with tumors in DMSO-treated mice ($n = 6$).

In this study, we demonstrated that i) EMT processes were underway in both RK12V-SP cells and Hec1-SP cells, ii) the level of fibronectin was enhanced in Hec1-SP cells and salinomycin reduced the level of fibronectin expression, and iii) salinomycin induced apoptosis and inhibited proliferation, migration, invasion and tumorigenicity in these SP cells. This is the first report of an inhibitory effect of salinomycin on the properties of endometrial CSCs.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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