Title: SPARC was overexpressed in human endometrial cancer stem-like cells and promoted migratory activity

Article Type: Original Research Report

Keywords: SPARC, Endometrial cancer, EMT, CSCs, Cell migration

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Abstract: Goals: We previously demonstrated that side-population (SP) cells found in human endometrial cancer tissue have features of cancer stem cells (CSCs). Endometrial cancer SP cells are associated with the epithelial-mesenchymal transition (EMT). In this study, we analyzed the expression and function of a specific protein, SPARC (secreted protein acidic and rich in cysteine) which we found to be up-regulated in CSCs.

Methods: We performed microarray expression analysis to screen for up-regulated genes in CSCs using a set of SP cells and non-SP (NSP) cells. We used the MetaCore package to identify the Gene GO pathway MAPs associated with the up-regulated genes. Here, we investigated the expression and functions of SPARC, one of the genes up-regulated in endometrial CSCs. We established SPARC-overexpressing cells by transfecting endometrial cancer cells, (Ishikawa cells [IK-SPARC cells]). We characterized these cells' growth rate, tumorigenicity, migration and invasion activity. The levels and locations of SPARC protein expression in Hec-1 (an endometrial cancer cell line)-SP cells-derived tumors and endometrial cancer tissues were examined by immunohistochemistry.

Results: SPARC was detected by microarray expression analysis during screens for up-regulated genes in SP cells. SPARC enhanced fibronectin expression and promoted migration activity in IK cells. SPARC expression suppressed tumor growth but promoted formation of tumor stroma. SPARC was expressed in endometrial cancer tissues, in particular, poorly differentiated endometrioid adenocarcinoma, but not in normal endometrial tissue.

Conclusion: This is the first report of overexpression of SPARC in endometrial CSCs. SPARC expression is associated with cell migration and stroma formation.
Beth Y. Karlan, M.D.

Editor-in-Chief, *Gynecologic Oncology*

Dear Dr. Karlan:

Enclosed please find our revised manuscript entitled, "SPARC was overexpressed in human endometrial cancer stem-like cells and promoted migratory activity", by Nurismangul Yusuf and co-workers (Ms. No.: GYN-14-1). In accord with the comments of the Reviewer, we have added new data (Fig. 3, Fig. 4, Table 1 and Supplementary Tables 1, 2, and 3) and changed several sentences as described in the responses to the reviewers.

This work was supported by grants-in aid (23390392) and (24659736) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Each author took part in the design of the study, contributed to data collection, participated in writing the manuscript and all agreed to accept equal responsibility for the accuracy of the contents of this paper. We have no financial interests linked to this work. This paper is not under submission elsewhere and the data have not been published elsewhere in any form or language. Please consider this paper for publication in *Gynecologic Oncology*.

Your kind consideration would be greatly appreciated.

Sincerely,
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Replies to Reviewer 1
1) The authors provide an excellent synopsis comparing endometrial type I and type II cancers, but it is unclear both from the introduction and the body of the paper whether the experiments are conducted in one or both cancer types. If both, the similarities and differences need to be clearly highlighted. If only one, that should also be specified, and including the other type of endometrial cancer might be insightful as a control. This is especially important in the experiments conducted on patient samples, where it is unclear which grade of endometrial cancer was present. In this section, tumors are referred to as poorly differentiated vs. well differentiated, whereas in the introduction they were referred to as type I and type II, and the conclusion expands upon the differentiated phenotype as epithelial. This is confusing as it introduces many types of nomenclature for the different subtypes. Choosing one and using it consistently throughout the paper would be helpful to the reader.

Response
In accord with the Reviewer’s comment, we chose to refer to “poorly differentiated vs. well differentiated” as described below and we deleted the description of type II cancers (page 3 line 57 - 61 in the previous manuscript).

Page 3, line 52 - 59:
Endometrial cancer is classified into two clinicopathological types, i.e., estrogen-related type I and non-estrogen-related type II [1]. Endometrioid carcinoma is the most common histological type of endometrial cancer and it is classified into three types depending on differentiation grade, i.e., well differentiated- (G1), moderately differentiated- (G2) and poorly differentiated – (G3) adenocarcinoma. Clinically, poorly differentiated adenocarcinomas (G3) have poor prognoses compared with well- or moderately –differentiated adenocarcinoma.

2) (1) The Methods section lacks sufficient technical information, while other information is inconsistent with the rest of the manuscript. Specifically, the authors should state how they prepared the control "mock" cells and how they derived clones of the stably-transfected (with SPARC and mock) cells.
Along these lines, the authors should consider a more intuitive nomenclature for the two sets of clones used in the study (e.g clone A and B, or C1 and C2, etc.). The current names "pool" and "C3" are confusing and somewhat counter intuitive.

-Response

Based on the Reviewer’s comments, we added descriptions of the preparation of the control mock cells as described below.

Page 5, line 105 – 107:
IK cells harboring SPARC (IK-SPARC) and IK-mock cells were established by transfection of cells with the pcDNA3 vector carrying SPARC cDNA and the empty pcDNA3 vector, respectively.

As suggested by the Reviewer’s comment, we changed the nomenclature for the two sets of clones used in the study as described below and in other pages.

Page 6 line 114 – 117:
Stably transfected cells were selected and several colonies were isolated in growth medium containing 400 μg/mL G418 (Invitrogen). We analyzed two sets of clones (IK-SPARC C1 and C2, IK-mock C1 and C2).

3) The Methods section lists two different methods and cell numbers used to establish mouse xenografts (1x10^5 cells in Matrigel, and 300 uL of 10^6 cells/mL), while in the Results section contains yet another number (2x10^5 cells/mL).

-Response

We inoculated 1X10^5 cells of each cell type into the mouse to establish mouse xenografts. We corrected the description of cell number.
(page 10 line197, page 11 line 231)

4) The authors should provide evidence for increased fibronectin mRNA with a western blot showing a parallel increase in fibronectin protein expression.

-Response

In accord with the reviewer’s comment, we performed a Western blot and added the results showing an increase in fibronectin protein expression (Figure 3 A b).
Page 12 line 244-page 12 line 247 in the text
We investigated the level of fibronectin in two independent clones (C1 and C2) of IK-SPARC cells and mock cells by real-time PCR (Fig.3A a) and a Western blot (Fig.3A b). The levels of both SPARC mRNA and SPARC protein were enhanced in IK-SPARC cells compared with those in mock cells.
5) Why did the authors use conditioned media as an attractant in the invasion assay and an FBS gradient in the migration assay? In what medium were the cells suspended (upper chamber) in the invasion assay?

-Response
We performed each assay using BD BioCoat Matrigel Invasion chambers, either coated with Matrigel for the invasion assay or without Matrigel for the migration assay, in the same medium (the bottom chamber: conditioned medium contained 10%FBS, the upper chamber: serum-free medium). We corrected the sentences as described below.

Page 9 line 188-190:
*In vitro invasion assays were performed using BD BioCoat Matrigel Invasion Chambers (BD Biosciences, San Jose, CA) in 24-well plates in the same way of migration assay as described above.*

6) In Figure 4, the authors should provide IHC images of both Hec1-SP and Hec1-non-SP tumors. Only in this way can the readers truly appreciate any qualitative and quantitative differences in SPARC expression between tumors formed by the two cell populations.

-Response
As indicated in the Reviewer’s comment, we performed IHC on Hec1-NSP tumors. We provided IHC images of both Hec1-SP and Hec1-non-SP tumors (Figure 4A, right panel). We added text to explain this result as described below.

Page 13 line 257 – 259:
*We investigated SPARC expression in Hec1·SP and -NSP tumors by immunohistochemistry. SPARC was expressed in Hec1·SP tumors (Fig. 4A, left panel), but not in the Hec1·NSP tumor (Fig. 4A, right panel).*

7) Figure formatting is highly irregular, with figure 2B overlapping with the text. Text titles and labels range from excessively large (as in Fig 4a labels, Fig 4 Title, Fig 2 titles, etc), to very small (Fig 3C), and many labels are duplicated (Fig 3C). Consistency in formatting, fonts, clear labeling, and more logical axes (there is no reason why Fig 3C needs a negative component to its axis) as essential to improve the legibility of the figures.

-Response
As suggested by the Reviewer’s comment, we corrected figure format, fonts, and labels in
Figure 2, Figure 3 and Figure 4).
8) Table 1 - displaying the summary of SPARC expression in different stages of endometrial cancer would be much clearer if it contained information on the distribution of cancer subtypes within the population.

-Response
In response to Reviewer’s comment, we added information summarizing SPARC expression in different stages of each histological subtype.
(Supplementary Tables 2 and 3).

9) Both written and graphic parts of the manuscript contain grammatical and stylistic errors that should be corrected.

-Response
As suggested by the Reviewer’ we corrected grammatical and stylistic errors in the manuscript and figures.

Replies to Reviewer2

Reviewer #2: SPARC was overexpressed in human endometrial cancer stem-like cells and promoted migration activity.

(1)
1) Downfalls of the study are that there are no stage IV tumors and there is limited data (and no separate discussion) regarding the results for type II endometrial cancers.

-Response
We usually did not perform surgery on stage IV endometrial cancer patient. Therefore, we could not collect enough tissue samples to analyze and it was not possible to include stage IV samples. In accord with the Reviewer’s comment, we performed IHC on samples from two cases of clear cell adenocarcinoma, that were performed. We added the results (Supplementary Tables 1, 2, and 3) and added text to describe the results.
Page 13 line 275 – 278:

We summarized the expression levels in different stages of each histological type (Supplementary Table 2, 3). Notably, SPARC expression was weak in stroma of endometrioid G1, stage 1 (Supplementary Table 3).
2) I would suggest using samples from serous and clear cell tumors to see if staining patterns hold true in this setting.

-Response
In response to the Reviewer’s comment, we performed IHC on eleven cases of clear cell adenocarcinoma and four cases of serous adenocarcinoma and added the results of these samples (Figure 5, Table 1). The text below described the outcome.

Page 13 line 270 – 275:
We also investigated the level of SPARC in clear cell adenocarcinoma and serous adenocarcinoma. Interestingly, the levels were enhanced in both glandular and stromal tissues. That is, in clear cell adenocarcinoma, we observed strong staining in 64% of the glandular area and 73% of the stroma. In serous adenocarcinoma, we found strong staining in 50% of the glandular tissue and 50% of the stroma.

(2) Figures should be reviewed for accuracy. Spelling and labeling errors—for instance, "comtrol" in figure 3c.
-Response
We corrected grammatical and stylistic errors in the manuscript and figures.

(3) Line 303-304 states that SPARC is a new therapeutic target and predictive biomarker for response to nab paclitaxel. However, there is no data in the paper that would allow one to draw these conclusions. This portion of the discussion seems overstated for presented work. I would revise this a bit to make this point more of a hypothesis for future work.
-Response
As indicated the Reviewer’s comment, we deleted several sentences that described nab paclitaxel (page 15 line 310 - 316 in the previous manuscript).
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Article title: SPARC Was Overexpressed In Human Endometrial Cancer Stem-Like Cells And Promoted Migration Activity

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Please reprint and continue on a separate page for all authors.
SPARC was overexpressed in human endometrial cancer stem-like cells and promoted migration activity.

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Abstract

Goals: We previously demonstrated that side-population (SP) cells found in human endometrial cancer tissue have features of cancer stem cells (CSCs). Endometrial cancer SP cells show enhanced migration, the potential to differentiate into the mesenchymal cell lineage, and they are associated with the epithelial–mesenchymal transition (EMT).

In this study, we analyzed the expression and function of a specific protein, SPARC (secreted protein acidic and rich in cysteine) which we found to be up-regulated in endometrial cancer.

Methods: 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. We used the MetaCore package to identify the Gene GO pathway MAPs associated with the up-regulated genes.

Here, we investigated the expression and functions of SPARC, one of the genes up-regulated in endometrial CSCs.

We established SPARC-overexpressing cells by transfecting endometrial cancer cells, (Ishikawa cells ([IK-SPARC cells]). We characterized these cells’ growth rate, tumorigenicity, migration and invasion activity. The levels and locations of SPARC protein expression in Hec1SP cells-derived tumors and endometrial cancer tissues were examined by immunohistochemistry.

Results: SPARC was detected by microarray expression analysis during screens for up-regulated genes in SP and NSP CSC. The level of SPARC expression was enhanced in Hec1 SP cells compared with that in Hec1 non-SP cells. SPARC enhanced fibronectin
expression and promoted migration activity in IK cells. SPARC expression suppressed tumor growth but promoted formation of tumor stroma.

SPARC was expressed in endometrial cancer tissues, in particular, poorly differentiated endometrioid adenocarcinoma, but not in normal endometrial tissue.

Conclusion: This is the first report of overexpression of SPARC in endometrial cancer stem-like cells. SPARC expression is associated with cell migration and stroma formation.

Introduction
Endometrial cancer is a common gynecological malignancy in the industrialized world and its incidence is increasing. Endometrial cancer is classified into two clinicopathological types, i.e., types I and II [1]. The former is estrogen-related endometrial cancer, and occurs in both premenopausal and postmenopausal women. Type I endometrial cancer is histologically of the endometrioid type and of low cellular grade and is frequently preceded by endometrial hyperplasia. The prognosis of type I endometrial cancer is relatively good. Type II endometrial cancer is non-estrogen-related, primarily occurring in postmenopausal women. Histopathologically, Type II endometrial cancer is poorly differentiated endometrioid type, serous papillary or clear cell adenocarcinoma, and is related to atrophic endometrium but not hyperplastic endometrium. Type II endometrial cancer has a high cellular grade and a poor prognosis. The existence of cancer stem-like cells (CSCs) has been proposed and CSCs have been identified in leukemia and several solid tumors [2-4]. 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 [5]. 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) [6]. 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). SP cells
have features of CSCs, including the potential to differentiate into the mesenchymal cell
lineage [7].

The epithelial-mesenchymal transition (EMT) occurs during normal early
embryonic development. EMT is also a key developmental program that is often
activated during cancer cell 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. reported a direct link between EMT and
the gain of epithelial stem cell properties [8]. Most recently, we demonstrated EMT
processes in both RK12V-SP cells and Hec1-SP cells and the level of fibronectin
expression was enhanced in Hec1-SP cells [9]. We also performed microarray expression
analyses for to identify up-regulated genes in SP and NSP CSC. The gene coding for
secreted protein acidic and rich in cysteine (SPARC) was up-regulated and was associated
with EMT. The contributions of SPARC expression to the development of endometrial
cancer are unclear. In this study, we focused on SPARC and analyzed its expression and
functions in endometrial cancer.
Materials and Methods

Plasmids

The p3XFLAG CMV 7.1 vector and the pcDNA3- vector were purchased from Sigma Aldrich (St. Louis, MO) and Invitrogen (Carlsbad, CA), respectively. The human SPARC coding region was amplified by PCR and inserted into the Not1/Sal1 site of the p3XFLAG CMV 7.1 vector. We used this construct as the template to amplify the fragment containing FLAG-SPARC by PCR. We cut this fragment and ligated it to the BamHI/Xho1 site of the pcDNA3 vector. The human SPARC sequence was verified by sequencing.

Cell lines and cell culture

Two human endometrial cancer cell lines, Ishikawa (IK) and Hec-1 were used in this study. IK cells were purchased from Sigma. The Hec-1 cell line was established by Kuramoto et al. from human endometrial adenocarcinoma explants [10]. Both cell lines were authenticated by Takara Bio Inc. using short tandem repeat (STR) DNA profiling. The STR profiles of both 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.

IK cells harboring SPARC (IK-SPARC) were established by transfection of cells with the pcDNA3 vector carrying SPARC cDNA. Transfection was performed with the Amaxa® Cell Line Nucleofector® Kit L (Lonza, Basel, Switzerland) according to the manufacturer’s protocol. Briefly, cells were washed with PBS and detached with trypsin. Approximately
1 x 10^6 cells were transferred to a new tube, centrifuged at 200 g for ten min at room temperature and the pellet was suspended in 100 μL of transfection solution. In the electroporation cuvette, the cells were transfected with using program T-020. The sample was transferred to prepared six-well plates after the program was finished. Cells were incubated in a humidified incubator at 37°C with 5% CO₂. Stably transfected cells were selected and isolated in growth medium containing 400 μg/mL of G418 (Invitrogen). Cells were always used within the first two passages.

**Collection of Clinical Patient Specimens**

Endometrial cancer tissues and normal endometrium were obtained from patients who underwent surgical resection at the Department of Obstetrics and Gynecology, Juntendo University Tokyo, Japan from 1994 to 2001. Our study was approved by the Bioethics Committee of Juntendo University; written previous informed consents and approval were given by the patients.

**Immunohistochemistry**

The tissues were fixed in 10% formalin for histological sectioning. The tissue sections were deparaffinized and washed with distilled water, after proteinase K processing (ten-20/mL, 15 min) and were washed again with PBS. Next, we used the PAP method with primary antibody specific for osteonectin/SPARC (mouse IgG antibody 1:200; M125; TAKARA BIO INC) with 2% normal goat serum (Vector Laboratories, Inc.) with overnight incubation at 4 °C. Then, the second antibody (mouse IgG, 1:400, Dako) was added at room temperature for one h. HRP processing was carried out for 30 min (peroxidase-conjugated streptavidin, Dako P0397,1:400), followed by HE staining.
SPARC positive cells were examined by microscop. The intensity of SPARC staining was evaluated by a KS400 imaging analyzing system (Imaging Associate, Oxford, UK).

The optical density corresponded to the intensity of the transmitted light and was measured on a scale going from 0 (100% transmitted light) to 255 (0% transmitted light) for each pixel. In addition, the mean optical density of the immunoreactive including the cell bodies was also measured.

**RNA isolation**

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

**Real-Time Quantitative Reverse transcription (qRT)-PCR Assay**

Real-time quantitative PCR reactions were performed using a SYBR® Premix Ex Taq™ kit (Takara, Kyoto, Japan) according to the manufacture’s instruction. GAPDH was used as an internal standard. Primers used for SPARC mRNA analysis were as follows:

forward primer, 5’-CAACTGCTGAAACGGTAGCA-3’, and reverse primer, 5’-GAACTCTTGCCCTGTTCTGC-3’. Primers used for fibronectin mRNA analysis were as follows: forward primer, 5’-CAGTGGGAGACCTCGAGAAG-3’, and reverse primer, 5’-TCCCTCGGAACATCAGAAAC-3’. GAPDH was applied as an internal control: forward primer, 5’-ATCCTGACGGAGGAAGGCA-3’ and reverse primer, 5’-GCTTGATATGGCGTGCGAT-3’. The PCR protocol was as follows: 95°C for one min, then 40 cycles of 15 sec at 95°C and 31 sec at 60°C. Fold-expression was calculated using the ΔCt method. Reactions and analyses were performed using the ABI PRISM 7300 PCR and detection system (Applied Biosystems, Carlsbad, CA).
Western blot analysis

Twenty micrograms of total proteins were separated by SDS-PAGE and transferred onto a PVDF membrane. The membrane was then incubated overnight at 4°C with antibodies specific for SPARC (TAKARA; Osteonectin/SPARC 1:200 M125), and GAPDH (Sigma; 1:1,000). Bound antibodies were visualized using enhanced chemiluminescence. To confirm equal loading, membranes were stripped, washed three times with T-TBS and a second antibody was applied (anti-mouse IgG; 1:10000 Jackson 115-035-003).

Cell migration assay

The ability of cells to migrate through filters was measured using a BioCoat Matrigel invasion chamber (BD Biosciences, San Jose, CA) Cell culture inserts with eight μm pore sized PET membranes were used according to the protocol of the manufacturer. The bottom chamber included medium (0.75 mL) containing 10% FBS, whereas SPARC siRNA-transfected or control siRNA-transfected cells (1.0 × 10^6 suspended in 0.5 mL of medium containing 1% FBS) were seeded in the upper chamber and incubated overnight at 37°C in a humidified atmosphere containing 5% CO₂. Remaining cells on the upper surface were mechanically removed. Membranes were then washed, fixed, and stained by Diff-Quik (Medion Diagnostics). The number of cells that migrated to the lower surface of the filters was determined by counting stained cells under a light microscope in three independent fields (0.25 mm²/well).

Invasion assay
In vitro invasion assays were done using BD BioCoat Matrigel Invasion Chambers (BD Biosciences, San Jose, CA) in 24-well plates. Cancer cells (5 × 10^4/0.5 mL) were added to the upper chamber, and the lower chamber was filled with conditioned medium. After incubation for 48 h, the cells that migrated through the membrane to the lower surface were stained with Giemsa solution. Four randomly selected × 200 magnification fields were photographed, and the number of invading cells was counted. All experiments were repeated in duplicate.

**Transfection of SPARC-siRNA**

IK-SPARC cells were transfected with non-coding siRNA™ Duplex Oligoribonucleotides (SPARC–siRNA, Invitrogen stealth-RNAi SPARC CHSS110132) by electroporation (Nucleofector II) using program T-020 with transfection reagent V (Lonza, Amaxa, siRNA Test Kit, USA). Cells were collected 24 h later for Semi-Quantitative Reverse Transcription (RT) -PCR assay to validate transfection efficiency.

**Animal experiments**

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). Using two groups of ten nude mice, we injected 300 μL of 1.0 x10^6 cell/mL SPARC cells (SPARC over expressing cells) and mock1 cells (control) into the right and left sides of the first group of nude mice, and the same number of SPARC C3 cells and mock2 pool cells were injected into the second group of ten nude mice. The diameters of the tumors were measured regularly. All mouse
experiments were approved by the animal ethics committee of Juntendo University.

**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 the Student's t-test. For all analyses, two-sided tests of significance were used, with \( p < 0.05 \) considered significant.

**Results**

**SPARC was overexpressed in endometrial CSCs.**

We previously reported microarray expression analysis of RK12-SP and non-SP CSCs, focusing on up-regulated genes and Gene GO pathway MAPs [9]. The gene expression profile of endometrial CSCs was closely associated with the EMT pathway. The “cell adhesion – extracellular matrix (ECM) remodeling pathway” had the second highest P value (2.123X10^{-7}) and eight genes were included out of a total 52 (Fig.1). Among them, we investigated SPARC (3.4-fold up-regulated), an EMT-related gene.

The level of SPARC expression was enhanced in Hec1-SP cells compared with that in Hec1-non-SP cells (Fig. 2A, lanes -1, 2).

**SPARC reduced the growth of tumors initiated by endometrial cancer IK cells**

In order to investigate the effect of SPARC protein expression on the phenotype of endometrial cancer cells, we established IK cells that overexpressed SPARC. Enhanced
expression of SPARC protein and SPARC mRNA was confirmed by Western blotting and real-time PCR, respectively (Fig. 2 A, B).

We analyzed two independent clones, pooled clone and C3. Expression of SPARC did not have an effect on the growth rate of IK cells or the population of SP cells (data not shown). IK-SPARC cells and IK-mock cells were inoculated (2 × 10^5 cells/ mL) into subcutaneous tissues of nude mice. IK-mock cells and IK-SPARC cells formed palpable tumors after 45 days and 60 days of inoculation, respectively. Tumors generated from the IK-mock cells grew faster than those from IK-SPARC cells and IK-SPARC cells formed smaller-sized tumors in nude mice than did mock cells (Fig. 2C). These results demonstrated that SPARC expression inhibited the growth of IK tumor-forming cells.

The tumors generated from IK-SPARC cells contained enriched stroma compared with that derived from IK-mock cells (Fig. 2C).

**SPARC expression enhanced the level of fibronectin expression and migration activity.**

SPARC is an EMT-associated gene. We have previously demonstrated that the level of fibronectin was enhanced in Hec1-SP cells compared with that in Hec1-non SP cells [9]. We investigated the level of fibronectin in IK-SPARC cells and mock cells by real-time PCR. Its level was enhanced in IK-SPARC cells compared with that in mock cells (Fig. 3 A). Next, we examined the invasion and migration activities using Transwell chambers coated with or lacking Matrigel, respectively. Migration activity, but not invasion activity, was enhanced in IK-SPARC cells compared with that in mock cells (Fig. 3 B). We knocked down SPARC expression using SPARC-siRNA in IK-SPARC cells (Fig. 3 C).
Down-regulation of \textit{SPARC} in IK-SPARC cells led to a significant decrease in migration activity compared with that using control siRNA (Fig. 3 D).

\textbf{SPARC was overexpressed in intra- and peritumoral stroma in Hec1-SP tumors}

SPARC protein was overexpressed in Hec1-SP cells compared with Hec1-non SP cells (Fig.1). We investigated SPARC expression in Hec1-SP tumors by immunohistochemistry. SPARC was expressed strongly in stromal-like cells (Fig.4a, b). Cells expressing SPARC were also present at the edge of the tumor cell islands (Fig.4 c, d).

\textbf{SPARC was overexpressed in endometrial cancer}

The intensity of SPARC expression was measured with a KS400 image analyzing system. The expression of SPARC was below detection in normal endometrium. (Fig. 5) The level of SPARC was enhanced in the stroma of poorly differentiated endometrioid adenocarcinoma (G3: strong staining glandular 60%, stroma 60%) compared with well differentiated endometrioid adenocarcinoma (G1: strong staining glandular 27%, stroma 13%) (Table 1 ).

\textbf{Discussion}

In the present study, we investigated the effect of SPARC expression on the phenotype of endometrial cancer cells. SPARC was detected by microarray expression analysis screening for up-regulated genes in CSCs. The level of SPARC expression was enhanced in Hec1 SP cells compared with that in Hec1 non-SP cells. SPARC enhanced fibronectin expression and promoted migration activity in IK cells. SPARC expression suppressed tumor growth but promoted the formation of tumor stroma. SPARC was expressed in...
endometrial cancer tissue, in particular, in poorly differentiated endometrioid adenocarcinoma, but not in normal endometrial tissue. This represents the first report of overexpression of SPARC in endometrial cancer stem-like cells. SPARC expression was associated with cellular migration and stroma formation.

SPARC is a nonstructural component of the extracellular matrix that modulates cell-matrix interactions, particularly during tissue development remodeling and repair [11] [4]. SPARC represses E-cadherin and induces EMT during melanoma development [12]. The functions of SPARC in tumorigenesis are controversial. Overexpression of SPARC has been documented in several types of solid tumors, such as breast [13], melanoma [14], prostate [15], and glioblastomas [16]. In contrast, lower levels of SPARC expression have been found in other types of cancers such as ovarian [17], colorectal [18], pancreatic [19], and acute myelogenous leukemia [21]. These observations suggest that the tumorigenic effect of SPARC is cell-type specific and might depend on the tumor cells’ surrounding environment. SPARC has been reported to be either up-regulated or down-regulated depending on cancer type, suggesting that it acts both as an oncogene and a tumor suppressor gene [22].

There are few reports of SPARC expression in endometrial cancer. Rodriguez-Jimenez et al. [23] demonstrated down-regulation of the SPARC gene in endometrial epithelial tumor cells. In contrast, frequent overexpression of SPARC protein with strong immunoreactivity was detected in stromal cells. In our study, SPARC was overexpressed in stromal cells of Hec1-SP derived tumors and in both epithelial tumor cells and stromal cells of poorly differentiated endometrioid carcinoma. In addition,
overexpression of SPARC in cells enhanced fibronectin expression, a marker of EMT.

SPARC expression might be associated with an undifferentiated phenotype rather than a differentiated epithelial phenotype.

In addition, we demonstrated that SPARC overexpression suppressed the tumor growth and stimulated migration activity. These results are consistent with the phenotype of CSCs, which are slow-growing and are associated with EMT. Therefore, SPARC might be a marker of cancer stem-like cell-derived tumors.

In pancreatic cancer, it was observed that cancer cells were negative for SPARC expression whereas stromal fibroblasts were positive [24]. In the present study, SPARC was expressed in the peri-tumor stroma and stromal fibroblast of the tumor derived from Hec1-SP cells (Fig.4). This expression pattern might be responsible for the desmoplastic reaction and low vascularization of the stroma, which might cause a reduction in tumor size and an increase chemo-resistance.

Recently, nab-paclitaxel, a solvent-free, albumin-coupled form of paclitaxel, was developed. Several reports demonstrated that SPARC was a target for nab-paclitaxel in several cancers including breast, head, neck and pancreatic cancer [22]. SPARC plays an important role as a high-affinity receptor for albumin, leading to albumin-drug complex accumulation in SPARC-positive areas. In the present study, we showed that SPARC was overexpressed in endometrial cancer, in particular, poorly differentiated adenocarcinoma and stroma of tumors derived from Hec1-SP cells. This type of cancer is chemo-resistant, resulting in poor prognosis. SPARC is a new therapeutic target and a predictive biomarker for responsiveness to nab-paclitaxel. Nab-paclitaxel might be considered a
chemotherapeutic drug for endometrial cancers, that express SPARC, such as poorly
differentiated carcinoma and CSCs.
Acknowledgment

We thank Laboratory of Molecular and Biochemical Research, Research Support Center, Juntendo University Graduate School of Medicine, Tokyo, Japan, for technical assistance. This work was supported by grants-in-aid (23390392) and (24659736) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Conflict of interest statement

Nurismangul Yusuf, Tetsunori Inagaki, Soshi Kusunoki, Hitomi Okabe, Izumi Yamada, Akemi Matsumoto, Yasuhisa Terao, Satoru Takeda, Kiyoko Kato

The authors declare that there are no conflicts of interest.
References


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**Figure legends**

Figure 1.  
*SPARC* was overexpressed in endometrial CSCs  
The Gene GO pathway MAPs involved in up-regulated genes in CSCs determined from RK12V-SP cells and –non-SP cells [9]. The “cell adhesion –extracellular matrix (ECM)” remodeling pathway ” had the second highest P value (2.123X10^-7) and eight genes were included out of a total of 52 genes.

Figure 2.  
*SPARC* reduced the growth rate of tumors initiated by endometrial cancer IK cells.  
We established IK cells overexpressing *SPARC* (IK-SPARC cells) as described in Materials and Methods.  
A), B) Elevated SPARC expression in IK-SPARC cells was confirmed by Western blotting (A) and real-time PCR (B).  
C) Two independent clones of IK-SPARC cells (pool, C3) and IK-mock cells (pool 1, pool 2) were inoculated (2 x 10^5 cells/mL) into the subcutaneous tissues of nude mice.  
Tumors generated from the IK-SPARC cells formed smaller tumors in the nude mice than did mock cells. The tumor generated from IK-SPARC cells contained enriched stroma compared with that derived from IK-mock cells. (Magnification X 20, Scale bar : 100 μm)

Figure 3
SPARC expression enhanced the level of fibronectin and migration activity.

A) The level of fibronectin gene expression in IK-SPARC cells and mock cells was assessed by real-time PCR. Its level was enhanced in IK-SPARC cells compared with that in mock cells.

B) We examined the invasion activity and migration activity using Transwell chambers coated with or lacking Matrigel, respectively. Migration activity, but not invasion activity, was enhanced in IK-SPARC cells compared with that in mock cells.

C) SPARC gene expression was suppressed using SPARC-siRNA in IK-SPARC cells.

D) Down-regulation of SPARC in IK-SPARC cells led to a significant decrease in migration activity compared with that using control siRNA.

Figure 4.

SPARC was overexpressed in intra- and peritumoral stroma in Hec1-SP tumors

We investigated SPARC expression in Hec1-SP tumors by immunohistochemistry.

SPARC was expressed strongly in stroma-like cells (a, b). Cells expressing SPARC were also present at the edge of tumor cell islands (c, d). (Magnification X50, scale bar, 100 μm, Magnification X100, scale bar, 50 μm, Magnification X200; 20μm)

Figure 5

SPARC was overexpressed in endometrial cancer.

The level of SPARC protein expression in normal endometrium and endometrial cancer tissue (total 43 cases; G1 29 cases, G2 9 cases, G3 5 cases) was investigated by immunohistochemistry. The expression of SPARC was not detected in normal endometrium. The intensity of SPARC expression was measured by a KS400 image
analyzing system. (scale bar, 100 μm)
SPARC was overexpressed in human endometrial cancer stem-like cells and promoted migratory activity.

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Abstract

Goals: We previously demonstrated that side-population (SP) cells found in human endometrial cancer tissue have features of cancer stem cells (CSCs). Endometrial cancer SP cells show enhanced migration and the potential to differentiate into the mesenchymal cell lineage, and they are associated with the epithelial–mesenchymal transition (EMT).

In this study, we analyzed the expression and function of a specific protein, SPARC (secreted protein acidic and rich in cysteine), which we found to be up-regulated in endometrial cancer.

Methods: 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. We used the MetaCore package to identify the Gene GO pathway MAPs associated with the up-regulated genes. Here, we investigated the expression and functions of SPARC, one of the genes up-regulated in endometrial CSCs.

We established SPARC-overexpressing cells by transfecting endometrial cancer cells (Ishikawa cells ([IK-SPARC cells]). We characterized these cells’ growth rate, tumorigenicity, migration and invasion activity. The levels and locations of SPARC protein expression in Hec1SP and NSP cells-derived tumors and endometrial cancer tissues were examined by immunohistochemistry.

Results: SPARC was detected by microarray expression analysis during screens for up-regulated genes in SP and NSP CSC. The level of SPARC expression was enhanced in Hec1 SP cells compared with that in Hec1 non-SP cells. SPARC enhanced fibronectin
expression and promoted migration activity in IK cells. SPARC expression suppressed
tumor growth but promoted formation of tumor stroma.

SPARC was expressed in endometrial cancer tissues, in particular, poorly differentiated
endometrioid, clear and serous adenocarcinoma, but not in normal endometrial tissue.

Conclusion: This is the first report of overexpression of SPARC in endometrial cancer
stem-like cells. SPARC expression is associated with cell migration and stroma
formation.

Introduction

Endometrial cancer is a common gynecological malignancy in the industrialized world
and its incidence is increasing. Endometrial cancer is classified into two
clinicopathological types, estrogen-related type I and non-estrogen-related type II [1].
Endometrioid carcinoma is the most common histological type of endometrial cancer and
is classified into three types depending on differentiation grade, well differentiated-(G1),
moderately differentiated-(G2) and poorly differentiated-(G3) adenocarcinoma.
Clinically, poorly differentiated adenocarcinomas (G3) have poor prognoses compared
with well- or moderately-differentiated adenocarcinomas.

The existence of cancer stem-like cells (CSCs) has been proposed and CSCs have been
identified in leukemia and several solid tumors [2 - 4]. 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 [5]. 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) [6]. 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). SP cells have features of CSCs, including the potential to differentiate into the mesenchymal cell lineage [7].

The epithelial-mesenchymal transition (EMT) occurs during normal early embryonic development. EMT is also a key developmental program that is often activated during cancer cell 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. reported a direct link between EMT and the gain of epithelial stem cell properties [8]. Most recently, we demonstrated EMT processes in both RK12V-SP cells and Hec1-SP cells and the level of fibronectin expression was enhanced in Hec1-SP cells [9]. We also performed microarray expression analyses to identify up-regulated genes in SP and NSP CSC. The gene coding for secreted protein acidic and rich in cysteine (SPARC) was up-regulated and was associated with EMT. The contributions of SPARC expression to the development of endometrial cancer are unclear. In this study, we focused on SPARC and analyzed its expression and functions in endometrial cancer.

Materials and Methods
Plasmids

The p3XFLAG CMV 7.1 vector and the pcDNA3- vector were purchased from Sigma Aldrich (St. Louis, MO) and Invitrogen (Carlsbad, CA), respectively. The human SPARC coding region was amplified by PCR and inserted into the NotI/SalI site of the p3XFLAG CMV 7.1 vector. We used this construct as the template to amplify the fragment containing FLAG-SPARC by PCR. We cut this fragment and ligated it to the BamHI/XhoI site of the pcDNA3 vector. The human SPARC sequence was verified by sequencing.

Cell lines and cell culture

Two human endometrial cancer cell lines, Ishikawa (IK) and Hec-1 were used in this study. IK cells were purchased from Sigma. The Hec-1 cell line was established by Kuramoto et al. from human endometrial adenocarcinoma explants [10]. Both cell lines were authenticated by Takara Bio Inc. using short tandem repeat (STR) DNA profiling. The STR profiles of both 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. IK cells harboring SPARC (IK-SPARC) and IK-mock cells were established by transfection of cells with the pcDNA3 vector carrying SPARC cDNA or the empty pcDNA3 vector, respectively. Transfection was performed with the Amaxa® Cell Line Nucleofector® Kit L (Lonza, Basel, Switzerland) according to the manufacturer’s protocol. Briefly, cells were washed with PBS and detached with trypsin. Approximately
109 1 x 10^6 cells were transferred to a new tube, centrifuged at 200 X g for ten min at room temperature and the pellet was suspended in 100 μL of transfection solution. In the electroporation cuvette, the cells were transfected with program T-020. The sample was transferred to prepared six-well plates after the program was finished. Cells were incubated in a humidified incubator at 37ºC with 5% CO2. Stably transfected cells were selected and several colonies were isolated in growth medium containing 400 μg/mL G418 (Invitrogen).

115 We analyzed two sets of clones (IK-SPARC C1 and C2 and IK-mock C1 and C2). Cells were always used within the first two passages.

118 **Collection of clinical patient specimens**

119 Endometrial cancer tissues and normal endometrium were obtained from patients who underwent surgical resection at the Department of Obstetrics and Gynecology, Juntendo University Tokyo, Japan from 1994 to 2001. Our study was approved by the Bioethics Committee of Juntendo University. Written previous informed consents and approval were given by the patients.

125 **Immunohistochemistry**

126 The tissues were fixed in 10% formalin for histological sectioning. The tissue sections were deparaffinized and washed with distilled water, after proteinase K processing (ten-20/ mL, 15 min) and were washed again with PBS. Next, we used the PAP method with primary antibody specific for osteonectin/ SPARC (mouse IgG antibody 1:200; M125; TAKARA BIO Inc.) with 2% normal goat serum (Vector Laboratories, Inc.) with
overnight incubation at 4 ℃. Then, the second antibody (mouse IgG, 1:400, Dako) was added at room temperature for one h. HRP processing was carried out for 30 min (peroxidase-conjugated streptavidin, Dako P0397, 1:400), followed by HE staining. SPARC positive cells were examined by microscopy. The intensity of SPARC staining was evaluated by a KS400 imaging analyzing system (Imaging Associate, Oxford, UK). The optical density corresponded to the intensity of the transmitted light and was measured on a scale from 0 (100% transmitted light) to 255 (0% transmitted light) for each pixel. In addition, the mean optical density of the immunoreactive including the cell bodies was measured.

RNA isolation

RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Five μg of total RNA was reverse-transcribed to cDNA using oligo dT primers and Superscript II reverse-transcriptase (Invitrogen, Carlsbad USA).

Real-Time Quantitative Reverse transcription (qRT)-PCR Assay

Real-time quantitative PCR reactions were performed using a SYBR® Premix Ex Taq™ kit (Takara, Kyoto, Japan) according to the manufacturer’s instruction. GAPDH was used as an internal standard. Primers used for SPARC mRNA analysis were as follows: forward primer, 5'-CAACTGCTGAAACGGTAGCA-3', and reverse primer, 5'-GAACTCTTTGCCCTGTTCTGC-3'. Primers used for fibronectin mRNA analysis
were as follows: forward primer, 5'-CAGTGGGAGACCTCGAGAAG-3', and reverse primer, 5'-TCCCTCGGAACATCAGAAAC-3'. GAPDH was applied as an internal control: forward primer, 5'-ATCCTGACGGAGGAAGGCA-3' and reverse primer, 5'-GCTTGATATGGCGTGCGAT-3'. The PCR protocol was as follows: 95°C for one min, then 40 cycles of 15 sec at 95°C and 31 sec at 60°C. Fold-expression was calculated using the ΔCt method. Reactions and analyses were performed using the ABI PRISM 7300 PCR and detection system (Applied Biosystems, Carlsbad, CA).

**Western blot analysis**

Twenty micrograms of total proteins were separated by SDS-PAGE and transferred onto a PVDF membrane. The membrane was then incubated overnight at 4°C with antibodies specific for SPARC (TAKARA; Osteonectin/SPARC 1:200 M125), and GAPDH (Sigma; 1:1,000). Bound antibodies were visualized using enhanced chemiluminescence. To confirm equal loading, membranes were stripped, washed three times with T-TBS and a second antibody was applied (anti-mouse IgG; 1:10000 Jackson 115-035-003).

**Transfection of SPARC-siRNA**

IK-SPARC cells were transfected with non-coding siRNA™ Duplex Oligoribonucleotides (SPARC–siRNA, Invitrogen stealth-RNAi SPARC CHSS110132) by electroporation (Nucleofector II) using program T-020 with transfection reagent V (Lonza, Amaxa, siRNA Test Kit, USA). Cells were collected 24 h later for Semi-Quantitative Reverse Transcription (RT)-PCR assay to validate transfection efficiency.
Cell migration assay

The ability of cells to migrate through filters was measured using a BioCoat Matrigel control chamber without matrigel (BD Biosciences, San Jose, CA). Cell culture inserts with eight μm pore sized PET membranes were used according to the protocol of the manufacturer. The bottom chamber included conditioned medium (0.75 mL) containing 10% FBS. Cells (2.5 × 10^5 suspended in 0.5 mL of serum-free medium DMEM) were seeded in the upper chamber and incubated at 37°C for 48 h in a humidified atmosphere containing 5% CO₂. Remaining cells on the upper surface were mechanically removed. Membranes were then washed, fixed, and stained by Diff-Quik (Medion Diagnostics). The number of cells that migrated to the lower surface of the filter was determined by counting stained cells under a light microscope in three independent fields (0.25 mm²/well).

Invasion assay

In vitro invasion assays were performed using BD BioCoat Matrigel Invasion Chambers (BD Biosciences, San Jose, CA) in 24-well plates in the same way as the migration assay described above. After incubation for 48 h, the cells that migrated through the membrane to the lower surface were stained with Giemsa solution. Four randomly selected × 200 magnification fields were photographed, and the number of invading cells was counted. The number of cells on the lower side of the filter was normalized to cells using the control chamber without matrigel. All experiments were repeated in duplicate.
Animal experiments
We inoculated $1 \times 10^5$ 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). Using two groups of ten nude mice, we injected IK-SPARC C1 cells and IK-mock C1 cells into the right and left sides of the first group of nude mice, and IK-SPARC C2 cells and IK-mock C2 cells into the right and left sides of the second group of ten nude mice. The diameters of the tumors were measured regularly. All mouse experiments were approved by the animal ethics committee of Juntendo University.

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
SPARC was overexpressed in endometrial CSCs.
We previously reported microarray expression analyses of RK12-SP and -NSP CSCs, focusing on up-regulated genes and Gene GO pathway MAPs [9]. The gene expression profile of endometrial CSCs was closely associated with the EMT pathway. The “cell adhesion – extracellular matrix (ECM) remodeling pathway” had the second highest P value ($2.123 \times 10^{-7}$) and eight genes were included out of a total 52 (Fig.1). Among them,
we investigated SPARC (3.4-fold up-regulated), an EMT-related gene.

The level of SPARC expression was enhanced in Hec1-SP cells compared with that in Hec1-NSP cells (Fig. 2 A, lanes -1, 2).

SPARC reduced the growth of tumors initiated by endometrial cancer IK cells

In order to investigate the effect of SPARC protein expression on the phenotype of endometrial cancer cells, we established IK cells that overexpressed SPARC. Enhanced expression of SPARC protein and SPARC mRNA was confirmed by Western blotting and real-time PCR, respectively (Fig. 2 A, B).

We analyzed two independent clones. Expression of SPARC did not have an effect on the growth rate of IK cells or the population of SP cells (data not shown). IK-SPARC cells and IK-mock cells were inoculated (1 × 10^5 cells) into subcutaneous tissues of nude mice. IK-mock cells and IK-SPARC cells formed palpable tumors after 45 days and 60 days of inoculation, respectively. Tumors generated from the IK-mock cells grew faster than those from IK-SPARC cells and IK-SPARC cells formed smaller-sized tumors in nude mice than did mock cells (Fig. 2C). These results demonstrated that SPARC expression inhibited the growth of IK tumor-forming cells. The tumors generated from IK-SPARC cells contained enriched stroma compared with that derived from IK-mock cells (Fig. 2D).

SPARC expression enhanced the level of fibronectin expression and migratory activity.
SPARC is an EMT-associated gene. We have previously demonstrated that the level of fibronectin was enhanced in Hec1-SP cells compared with that in Hec1-NSP cells [9]. We investigated the level of fibronectin in two independent clones (C1 and C2) of IK-SPARC cells and mock cells by real-time PCR (Fig. 3A a) and by Western blotting (Fig. 3A b). The levels of both SPARC mRNA and SPARC protein were enhanced in IK-SPARC cells compared with those in mock cells. Next, we examined the invasion and migration activities using Transwell chambers coated with or lacking Matrigel, respectively. Migration activity, but not invasion activity, was enhanced in IK-SPARC cells compared with that in mock cells (Fig.3 B). We knocked down SPARC expression using SPARC-siRNA in IK-SPARC cells (Fig.3 C). Down-regulation of SPARC in IK-SPARC cells led to a significant decrease in fibronectin expression and migratory activity compared with those using control siRNA (Fig. 3 C, D).

**SPARC was overexpressed in intra- and peritumoral stroma in Hec1-SP tumors**

SPARC protein was overexpressed in Hec1-SP cells compared with Hec1-NSP cells (Fig.1). We investigated SPARC expression in Hec1-SP and -NSP tumors by immunohistochemistry. SPARC was expressed in the Hec1-SP tumors (Fig. 4A, left panel), but not in the Hec1-NSP tumor (Fig. 4 A, right panel). SPARC was expressed strongly in stroma-like cells of Hec1-SP tumors (Fig.4B left panel). Cells expressing SPARC were also present at the edge of the tumor cell islands (Fig.4 B right panel).

**SPARC was overexpressed in endometrial cancer tissues**
The intensity of SPARC expression of endometrial cancer tissue was measured with a KS400 image analyzing system (Patient characteristics are shown in Supplementary Table 1). The expression of SPARC was below detection in normal endometrium (Fig. 5). The level of SPARC was enhanced in the stroma of poorly differentiated endometrioid adenocarcinoma (G3: strong staining glandular 60%, stroma 60%) compared with well differentiated endometrioid adenocarcinoma (G1: strong staining glandular 28%, stroma 13%) (Table 1). We also investigated the level of SPARC in clear cell adenocarcinoma and serous adenocarcinoma. Interestingly, the levels were enhanced in both glandular and stromal tissues. That is, in (clear cell adenocarcinoma, we observed strong staining in 64% of the glandular area and 73% of the stroma. In serous adenocarcinoma, we found strong staining in 50% of the glandular tissue and stroma 50%) of the stroma. We summarized the expression levels in different stages of each histological type (Supplementary Table 2-the glandular, 3-the stroma). Notably, SPARC expression was weak in stroma of endometrioid G1, stage I (Supplementary Table 3).

Discussion

In the present study, we investigated the effect of SPARC expression on the phenotype of endometrial cancer cells. SPARC was detected by microarray expression analysis screening for up-regulated genes in CSCs. The level of SPARC expression was enhanced in Hec1-SP cells compared with that in Hec1-NSP cells. SPARC enhanced fibronectin expression and promoted migration activity in IK cells. SPARC expression suppressed tumor growth but promoted the formation of tumor stroma. SPARC was expressed in endometrial cancer tissue, in particular, in poorly differentiated adenocarcinoma, but not
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might be associated with an undifferentiated phenotype rather than a differentiated epithelial phenotype.

In addition, we demonstrated that SPARC over-expression suppressed tumor growth and stimulated migration activity. These results are consistent with the phenotype of CSCs, which are slow-growing and are associated with EMT. Therefore, SPARC might be a marker of cancer stem-like cell-derived tumors.

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Recently, nab-paclitaxel, a solvent-free, albumin-coupled form of paclitaxel, was developed. Several reports demonstrated that SPARC was a target for nab-paclitaxel in several cancers including breast, head, neck and pancreatic cancer [22]. Nab-paclitaxel might be considered a chemotherapeutic drug for endometrial cancers that express SPARC.

In summary, we demonstrated that SPARC was overexpressed in cancer stem-like cells and its expression was associated with cellular migration and stroma formation. This represents the first report of overexpression of SPARC in endometrial cancer stem-like cells and cancer tissues, in particular, poorly differentiated adenocarcinoma.
Acknowledgment

We thank Laboratory of Molecular and Biochemical Research, Research Support Center, Juntendo University Graduate School of Medicine, Tokyo, Japan, for Technical assistance. This work was supported by grants-in aid (23390392) and (24659736) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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Figure Legends

Figure 1.

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Figure 2.

*SPARC* reduced the growth rate of tumors initiated by endometrial cancer IK cells.

We established IK cells overexpressing *SPARC* (IK-SPARC cells) as described in Materials and Methods.

A), B) Elevated *SPARC* expression in IK-SPARC cells was confirmed by Western blotting (A) and real-time PCR (B).

C) Two independent clones of IK-SPARC cells (C1, C2) and IK-mock cells (C1, C2) were inoculated (1 x 10^5 cells) into the subcutaneous tissues of nude mice. Tumors generated from the IK-SPARC cells formed smaller tumors in the nude mice than did mock cells. The tumor generated from IK-SPARC cells contained enriched stroma compared with that derived from IK-mock cells. (Magnification X 20, Scale bar: 100 \(\mu\)m)

Figure 3

*SPARC* expression enhanced the level of fibronectin and migration activity.
A) The levels of fibronectin gene expression in IK-SPARC cells and mock cells were assessed by real-time PCR (a) and a Western blot (relative ratio of protein expression is shown) (b). Its level was enhanced in IK-SPARC cells compared with that in mock cells.

B) We examined the invasion activity and migration activity using Transwell chambers coated with or lacking Matrigel, respectively. Migration activity, but not invasion activity, was enhanced in IK-SPARC cells compared with that in mock cells.

C) (a) SPARC gene expression was suppressed using SPARC-siRNA in IK-SPARC cells. (b) Down-regulation of SPARC in IK-SPARC cells led to a significant decrease in fibronectin expression compared with that using control siRNA. \( p < 0.003 \)

D) Down-regulation of SPARC in IK-SPARC cells led to a significant decrease in migration activity compared with that using control siRNA. \( p < 0.005 \)

Figure 4.

SPARC was over-expressed in intra- and peritumoral stroma in Hec1-SP tumors

We investigated SPARC expression in Hec1-SP and -NSP tumors by immunohistochemistry.

A) SPARC was expressed in Hec1-SP tumors (left panel), but not in Hec1-NSP tumors (right panel).

B) SPARC was expressed strongly in stromal-like cells of Hec1-SP tumors (left panel) and cells expressing SPARC were also present at the edge of the tumor cell islands (right panel).

(Magnification X 50, scale bar, 100 μm, Magnification X 100, scale bar, 50 μm,
SPARC was overexpressed in endometrial cancer tissues. The level of SPARC protein expression in normal endometrium and endometrial cancer tissues was investigated by immunohistochemistry. The study population include 43 cases of endometrioid carcinoma (G1, 29 cases; G2, 9 cases; G3, 5 cases), 11 cases of clear adenocarcinoma, 4 cases of serous adenocarcinoma. The expression of SPARC was not detected in normal endometrium. The intensity of SPARC expression was measured by a KS400 image analyzing system and was evaluated by scoring as described in Materials and Methods (strong expression: 130-150, middle expression: 120-129, weak expression 100-119). Representative pictures of each histological type are shown. (Scale bar, 100 μm)
### Table 1: Summary of SPARC expression

<table>
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<th></th>
<th>Score*</th>
<th>100-119 weak+</th>
<th>120-129 middle+</th>
<th>130-150 strong++</th>
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<td><strong>G1</strong></td>
<td>glandular</td>
<td>10(34%)</td>
<td>11(38%)</td>
<td>8(28%)</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>stroma</td>
<td>19(66%)</td>
<td>6(21%)</td>
<td>4(13%)</td>
<td>29</td>
</tr>
<tr>
<td><strong>G2</strong></td>
<td>glandular</td>
<td>0 (0%)</td>
<td>3(33%)</td>
<td>6(67%)</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>stroma</td>
<td>2(22%)</td>
<td>2(22%)</td>
<td>5(55%)</td>
<td>9</td>
</tr>
<tr>
<td><strong>G3</strong></td>
<td>glandular</td>
<td>1(20%)</td>
<td>1(20%)</td>
<td>3(60%)</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>stroma</td>
<td>0(0%)</td>
<td>2(40%)</td>
<td>3(60%)</td>
<td>5</td>
</tr>
<tr>
<td>Clear cell</td>
<td>glandular</td>
<td>-</td>
<td>4(36%)</td>
<td>7(64%)</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>stroma</td>
<td>-</td>
<td>3(27%)</td>
<td>8(73%)</td>
<td>11</td>
</tr>
<tr>
<td>Serous</td>
<td>glandular</td>
<td>-</td>
<td>2(50%)</td>
<td>2(50%)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>stroma</td>
<td>-</td>
<td>2(50%)</td>
<td>2(50%)</td>
<td>4</td>
</tr>
</tbody>
</table>

* Score of SPARC expression by KS400
Fig. 1: Cell adhesion ECM remodeling

IBM4

5. Figure
Click here to download 5. Figure: Figures.pptx
Fig. 2

A

<table>
<thead>
<tr>
<th>Positive control</th>
<th>Hec1</th>
<th>IK-SPARC</th>
<th>Mock</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSP</td>
<td>SP</td>
<td>C1</td>
<td>C2</td>
</tr>
</tbody>
</table>

43KD

B

GAPDH

Relative ratio of expression

C

Relative ratio of expression

D

Mock C1 | Mock C2 | SPARC C1 | SPARC C2

Mock | C2 X20

Mock | C1 X20
Fig. 4

A) Hec1-SP (X50)

B) Hec1-SP (X100)

Hec1-NSP (X50)
Fig. 5

Normal endometrium

Endometrioid adenocarcinoma

G1

Glandular strong++
Stroma weak+-

G3

Glandular middle+
Stroma strong++

Clear cell adenocarcinoma

Glandular strong++
Stroma middle+

Clear cell adenocarcinoma

Glandular strong++
Stroma middle+

Serous adenocarcinoma

Glandular strong++
Stroma strong++
<table>
<thead>
<tr>
<th>Histology</th>
<th>Endometrioid</th>
<th>Serous</th>
<th>Clear cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>G2</td>
<td>G3</td>
<td></td>
</tr>
<tr>
<td>age</td>
<td>median</td>
<td></td>
<td>(range)</td>
</tr>
<tr>
<td></td>
<td>(32-82)</td>
<td>(27-75)</td>
<td>(44-74)</td>
</tr>
<tr>
<td>I</td>
<td>25</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>II</td>
<td>2</td>
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<tr>
<td>III</td>
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<td>3</td>
</tr>
<tr>
<td>IV</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>Total patients</td>
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<td>9</td>
<td>5</td>
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</table>
### Supplementary Table 2

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<tr>
<th>Stage</th>
<th>Histology expression</th>
<th>G1 S M W</th>
<th>G2 S M W</th>
<th>G3 S M W</th>
<th>Clear S M W</th>
<th>Serous S M W</th>
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<td>5</td>
<td>0 1 1</td>
<td>0 1 0</td>
<td>0 1 0</td>
<td>1 0 0</td>
<td>0 0 0</td>
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<tr>
<td>III</td>
<td>11</td>
<td>0 1 1</td>
<td>1 0 0</td>
<td>2 1 0</td>
<td>2 1 0</td>
<td>1 1 0</td>
</tr>
<tr>
<td>IV</td>
<td>2</td>
<td>0 0 0</td>
<td>0 0 0</td>
<td>0 0 0</td>
<td>1 1 0</td>
<td>0 0 0</td>
</tr>
<tr>
<td>Total</td>
<td>58</td>
<td>6 15 8</td>
<td>3 4 2</td>
<td>3 2 0</td>
<td>7 4 0</td>
<td>2 2 0</td>
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</tbody>
</table>

The level of expression in the glandular was evaluated by KS400 scoring.

S: strong expression (130-150)  M: middle expression (120-129)  W: weak expression (100-119)
<table>
<thead>
<tr>
<th>stage</th>
<th>Histology expression</th>
<th>Endometrioid</th>
<th>Clear</th>
<th>Serous</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>G1</td>
<td>G2</td>
<td>G3</td>
</tr>
<tr>
<td>I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
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<td>1</td>
</tr>
<tr>
<td>III</td>
<td></td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>IV</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>total</td>
<td></td>
<td>4</td>
<td>6</td>
<td>19</td>
</tr>
</tbody>
</table>

The level of expression in the stroma was evaluated by KS400 scoring.

S:strong expression (130-150)  M:middle expression (120-129)  W:weak expression (100-119)
7. Highlights (for review)

Highlights

We found SPARC was up-regulated in endometrial cancer stem-like cells.

SPARC suppressed the tumor growth and stimulated migratory activity.

SPARC was overexpressed in poorly differentiated endometrioid adenocarcinoma.