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Clonal analysis revealed functional heterogeneity in cancer stem-like cell phenotypes in uterine endometrioid adenocarcinoma

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ABSTRACT

Uterine endometrial carcinoma is one of the common cancers in females. Cancer stem-like cells (CSCs)/cancer-initiating cells (CICs) are a small subpopulation of cancer cells that are tumorigenic and are resistant to treatments, thus they are focused as treatment targets. However, the heterogeneity of CSCs/CICs is still elusive, and we therefore analyzed CSCs/CICs at the clonal level. We previously established sphere-cultured CSCs/CICs from primary human uterine endometrial carcinoma, and we isolated several clones from CSCs/CICs in this study. Interestingly, we established two types of clones based on the growth pattern. The clones were termed sphere clones (S clones) and leukemia-like clones (LL clones). Functional analysis revealed that S clones are resistant to chemotherapy, whereas LL clones are sensitive to chemotherapy. On the other hand, S clones are less tumorigenic, while LL clones are highly tumorigenic. Transcriptome analysis using serial analysis of gene expression sequencing (SAGE-Seq) revealed distinctive gene expression profiles in S clone cells and LL clone cells. The results indicate that CSCs/CICs are composed of functionally heterogenic subpopulations including highly tumorigenic clones and treatment-resistant clones and that the characteristics of CSCs/CICs might be determined by the characteristics of different clones that compose CSCs/CICs.

1. Introduction

Uterine corpus carcinoma is one of the common female cancers. It was estimated that there would be about 61,380 new cases and 10,920 deaths in the United States in 2017 (Siegel et al., 2017). Since the major symptom of endometrial cancer is bleeding after menopause, it is often diagnosed in an early stage, with about 83% of the patients being in stage I or II (Creasman et al., 2006). Endometrial cancer in early stages can be controlled by operation in early stages. However, it cannot be controlled in an advanced stage or after recurrence, and the survival rate is very low (< 20%) (Brasseur et al., 2016). Thus, a novel approach for treatment of resistant endometrial cancer in an advanced stage is needed to improve the outcome.

Cancer stem-like cells(CSCs)/cancer-initiating cells(CICs) are defined as a small population of heterogeneous cancer cells that are endowed with high levels of tumor-initiating ability, self-renewal ability and differentiation ability (Hirohashi et al., 2010; Visvader and Lindeman, 2008). Recent studies have revealed that CSCs/CICs are resistant to chemotherapy and radiotherapy, and that they are responsible for recurrence (Murase et al., 2009; Park et al., 2009). Furthermore, cancer cells that have stem cell like phenotype were shown to have epithelial-mesenchymal transition (EMT) indicating that CSCs/CICs are metastatic cancer cells (Mani et al., 2008). Thus, CSCs/CICs would be appropriate targets for treatment strategies. CSCs/CICs from solid cancers can be isolated as CD44-positive cells, CD133-positive cells, side population (SP) cells, aldehyde dehydrogenase-positive ($ALDH^+$) cells using the ALDEFLUOR assay and sphere-forming cells (Al-Hajj et al., 2003; Asano et al., 2016; Ginestier et al., 2007; Inoda et al., 2011; Kondo et al., 2004; O'Brien et al., 2007). CSCs/CICs have also been isolated from endometrial cancers as $ALDH^{high}$ cells, SP cells and

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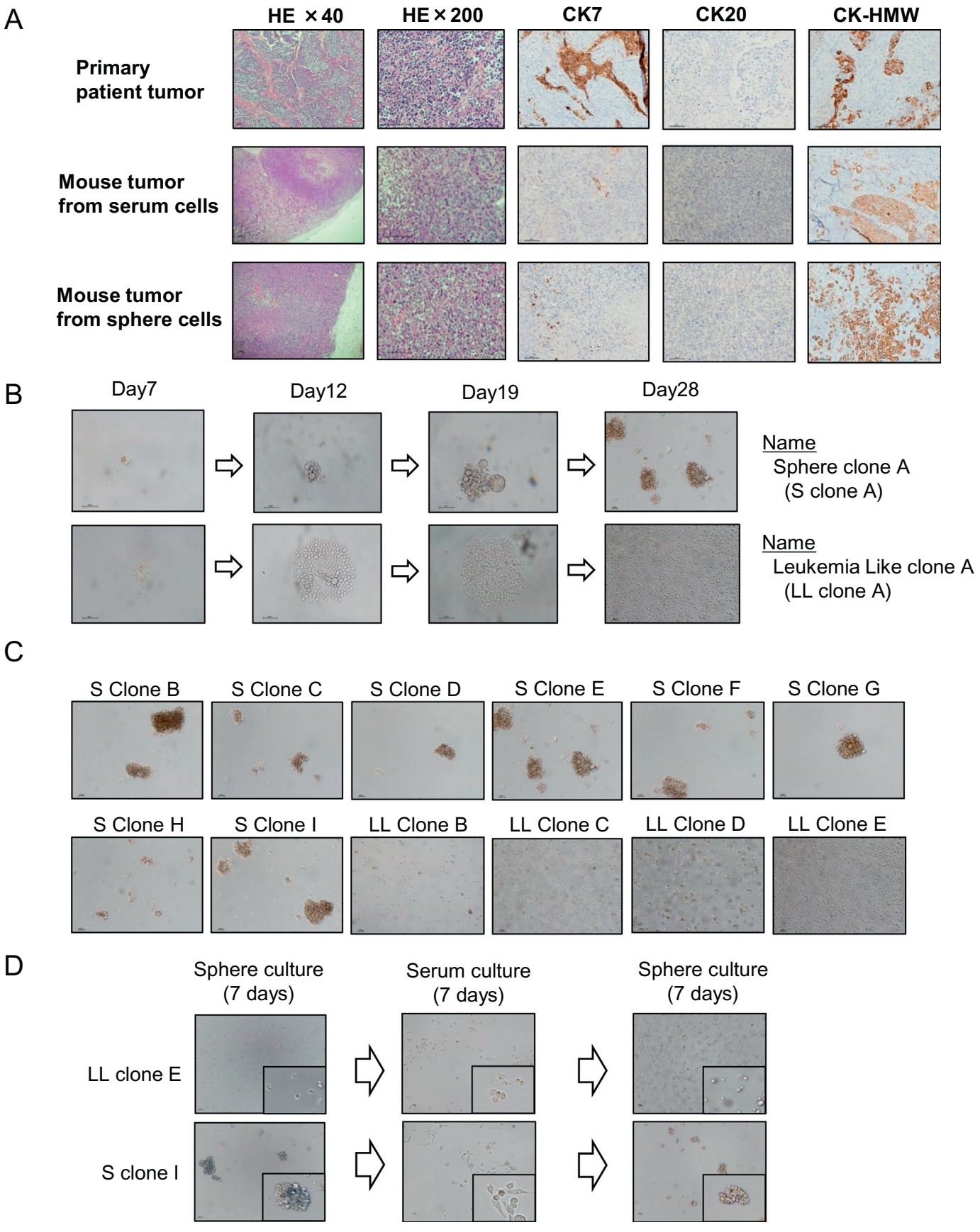

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Fig. 1. Pathological findings of primary patient tumor and mouse tumors derived from serum-cultured cells and sphere-cultured cells, and two types of clones from sphere-cultured cells.
(A) Hematoxylin and eosin staining and immunohistochemical staining. Black bar size is 100 μm.
(B) Clones established by single cell cloning. Representative S clone cells and LL clone cells are shown according to time course. Black bar size is 100 μm.
(C) Clones established by single cell cloning. Pictures were taken 28 days after cloning. Black bar size is 100 μm.
(D) S clone cells and LL clone cells were stable in serum culture. S clone cells and LL clone cells were cultured at in a sphere culture medium using a non-attachment plate for 7 days, all of the cells were collected and seeded in an attachment plate with serum culture medium and cultured for 7 days. The cells were collected and seeded again in a sphere culture medium and cultured for 7 days.

CD133-positive cells using cancer cell lines (Kato et al., 2010; Nakamura et al., 2010; Rahadiani et al., 2011; Rutella et al., 2009; Yasuda et al., 2016). Although CSCs/CICs isolated from cancer cell lines are reasonable sources for analyzing CSCs/CICs, a recent study has suggested that some cell lines show different genetic properties from those of primary cancer cells (Domcke et al., 2013). Therefore, analysis of endometrial CSCs/CICs from primary cancer cells is critical for understanding primary human cancers.

Previously, we successfully isolated established CSCs/CICs from human endometrial carcinoma as sphere-cultured cells (Hashimoto et al., 2017). The sphere-cultured cells showed higher tumor-initiating ability than that of serum-cultured cells, indicating that sphere-cultured cells are enriched with CSCs/CICs. Moreover, single cell transcriptome analysis revealed heterogeneity in serum-cultured cells (Hashimoto et al., 2017). We thus analyzed primary sphere-cultured cells at single cell levels and found functional heterogeneity in the cells. These findings indicate that CSCs/CICs isolated as sphere-forming cells are composed of heterogenic cells including higher tumorigenic clones and treatment-resistant cells. This report is the first report on heterogeneity of CSCs/CICs, and our model is a reasonable model for analyzing the heterogeneity of CSCs/CICs.

2. Materials and methods

2.1. Ethics statement

Mice were maintained and experimented on in accordance with the guidelines of and after approval by the Committee of Sapporo Medical University School of Medicine, Animal Experimentation Center under permit number 08-006. Any animal found unhealthy or sick was promptly euthanized. All the studies were approved by the Institutional Review Board (IRB) of Sapporo Medical University Hospital. Written informed consent was obtained from all patients according to the guidelines of the Declaration of Helsinki.

2.2. Isolation of primary cancer cells from clinical tumors

This study was approved by the IRB of Sapporo Medical University Hospital. Written informed consent was obtained from all patients according to the guidelines of the Declaration of Helsinki. Solid tumors were cut into fragments, washed in phosphate buffered saline (PBS), and centrifuged at 2000 rpm for 10 min. Then cell aggregates were incubated at 37 °C for about 30 min with 2 mg Liberase™ research grade (Roche, Basel, Switzerland) in DMEM/F12 (ThermoFisher Scientific, MA, USA) until they had been separated into single cells. The cells obtained by these procedures were cultured as described below.

2.3. Cell culture conditions and establishment of S clone cells and LL clone cells

We cultured cells from a primary tumor in two conditions. One condition was culture using an attachment plate in DMEM/F12 medium containing 10% FBS and 1% penicillin and streptomycin (serum culture). The other condition was a culture using an ultra-low attachment flask (Corning Inc., Corning, NY, USA) in serum-free DMEM/F12 medium supplemented with N-2 supplement (Wako, Osaka, Japan), 20 ng/ml recombinant human epithelial growth factor (ThermoFisher Scientific), 10 ng/ml human basic fibroblast growth factor (Sigma-Aldrich, MO, USA), 4 μg/ml heparin (AY pharma, Tokyo, Japan) and 1% penicillin and streptomycin (sphere culture).

Single cell cloning was performed to establish clone cells from sphere cultured cells by single cell sorting using FACS Aria II™ (Becton, Dickinson and Campany, NJ, USA) or limiting dilution. Clone cells were classified into sphere clone (S clone) cells and leukemia-like clone (LL clone) cells.

2.4. Reverse transcription polymerase chain reaction analysis (RT-PCR)

RT-PCR was performed to check the expression of candidate genes acquired by SAGE-Seq. The thermal cycling conditions were 94 °C for 2 min followed by 40 cycles of 15 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C. Primers for GAPDH (glyceraldehyde-3-phosphate dehydrogenase), used as an internal control, were 5'-ACCACAGTCCATGCCATCAC-3′ and 5'-TCCACCACCCTGTTGCTGTA-3′ with an expected PCR product size of 452 bp. Other primer information is summarized in Supplementary Table S1.

2.5. Quantitative real-time PCR analysis (qRT-PCR)

Quantitative real-time PCR (qRT-PCR) was performed using an ABI PRISM 7000 Sequence Detection System (ThermoFisher Scientific) according to the manufacture's protocol. Primers and probes were designed by the manufacturer (ThermoFisher Scientific). Thermal cycling was performed using 40 cycles of 95 °C for 15 s followed by 60 °C for 1 min. Each experiment was done in triplicate and normalized to the GAPDH gene as an internal control.

2.6. Resistance to chemotherapeutic reagents

Cells were counted, and 5000 cells were cultured in a medium with 10% FBS overnight. After overnight incubation, the medium was changed to a medium containing CBDCA and PTX at several concentrations. CBDCA was used at 10, 33, 100 and 200 μM, and PTX was used ad 0.1, 1 and 2 μM. Cells cultured in an agent-free medium were prepared as control cells. After incubation for 48 h in the medium with reagents, all of the cells are collected gently using a cell scraper and stained by trypan blue. Cells that were not stained were counted as surviving cells, and cell survival rates were calculated from the ratio of treated/control cells.

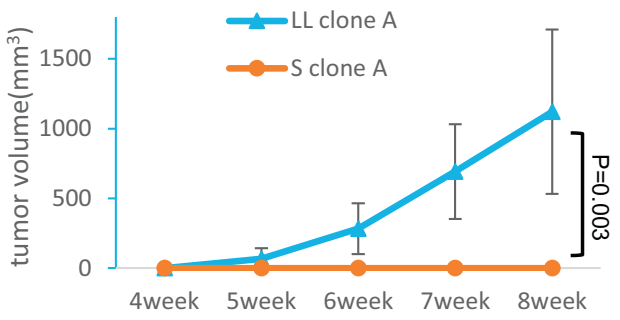
2.7. ALDEFLUOR assay and cell cycle assay

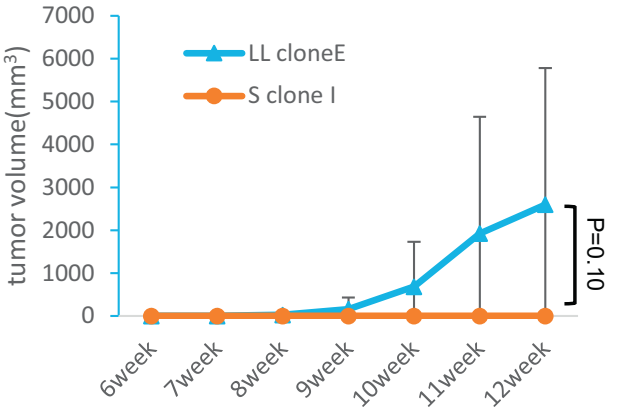
The ALDEFLUOR assay and cell cycle assay were performed as described previously (Kuroda et al., 2013; Nakatsugawa et al., 2011).

2.8. Cell proliferation analysis

Cells were seeded into a 6-well plate at 1×10^5 cells per well. After incubation for 24, 48 and 96 h, the cells were removed by trypsin and viable cell numbers were determined using Countess® (ThermoFisher Scientific).

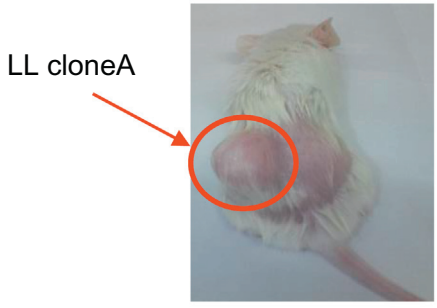
A

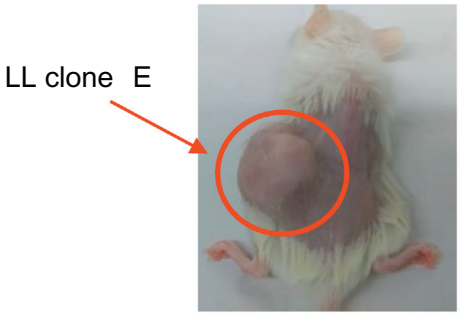




Number of cells transplanted	LL clone A	S clone A
1.0×10^2	6/6	0/6

Number of cells transplanted	LL clone E	S clone I
1.0×10^2	4/5	0/5





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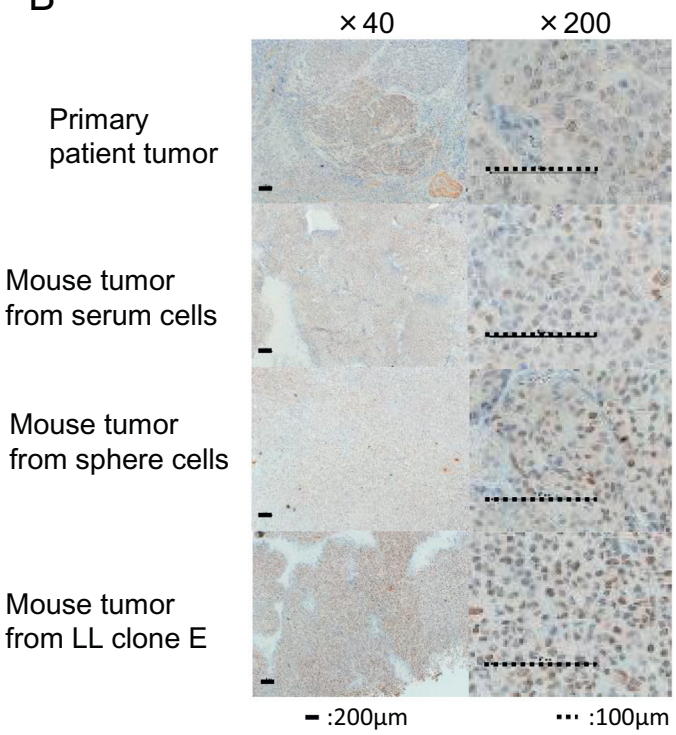

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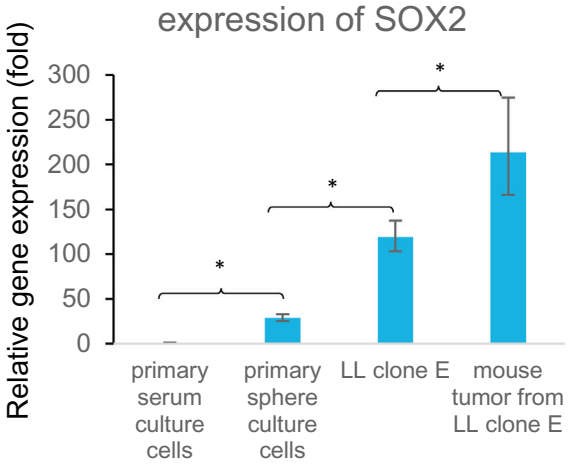

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Fig. 2. Tumorigenicity of LL clones and expression of SOX2.
(A) Growth curves of tumors derived from LL clone A and S clone A cells (left panel), and from LL clone E and S clone I cells (right panel). 1×10^2 cells were injected into NSG mice, and tumors were measured weekly. The X-axis is the number of days and the Y-axis is tumor volume (mm^3).
(B) Expression of SOX2 determined by immunohistochemical staining in primary patient tumor, mouse tumors derived from serum-cultured cells, sphere-cultured cells and LL clone E cells.
(B) Expression of SOX2 in primary serum-cultured cells, primary sphere-cultured cells, LL clone E cells and sphere-cultured cells from the mouse xenograft tumor of LL clone E cells was evaluated by quantitative RT-PCR. Data are shown as means ± SD. An asterisk indicates a statistically dignificant difference ($P < .05$).

2.9. Mouse xenograft assay

Xenograft transplantation experiments using animals were performed in accordance with the institutional guidelines for the use of laboratory animals. LL clone cells and sphere clone cells were suspended at 100 cells in 100 µl PBS mixed with Matrigel (BD) at a 1:1 volume and injected subcutaneously into the backs of 4–6-week-old female NSG mice. Tumor size was assessed weekly using a caliper and calculated using following the formula: tumor size (mm^3) = (longest diameter × shortest diameter^2)/2.

2.10. Immunohistochemical staining (IHC)

Immunohistochemical staining of CK7, CK20, CK-HMW and SOX2 was performed with formalin-fixed paraffin-embedded sections as described previously (Kitamura et al., 2013; Michifuri et al., 2012). Anti-CK7 (N1626, DAKO), anti-CK20 (N1627, DAKO), and anti-CK-HMW (N1553, DAKO) were used. For SOX2 staining, a rabbit anti-SOX2 polyclonal antibody (48-1400, ThermoFisher Scientific) was used at 2 µg/ml.

2.11. Serial analysis of gene expression-sequencing (SAGE-Seq) and pathway analysis

SAGE-Seq was performed as described previously (Hashimoto et al., 1999a; Hashimoto et al., 1999b; Hashimoto et al., 2000). More than 20,000,000 tags per sample were analyzed and the tag sequence was analyzed by SAGE software (Velculescu et al., 1995), CGAP SAGE database (http://www.ncbi.nlm.nih.gov/SAGE/), and NCBI's sequence search tool (Advanced BLAST search, http://www.ncbi.nlm.nih.gov/BLAST/). Enriched pathways of genes with high expression levels in LL clone, S clone and sphere-cultured cells were estimated by Metascape (http://metascape.org). Genes with high expression levels in LL clone were extracted by the following conditions: FPKM of LL clone cells > 5000 and FPKM of serum-cultured cells < 1000. Genes with high expression levels in S clone cells were extracted by the following condition: FPKM of S clone cells > 4000 and FPKM of serum-cultured cells < 500. Genes with high expression levels in sphere-cultured cells were extracted by the following condition: FPKM of sphere-cultured cells > 2000 and FPKM of serum-cultured cells < 100.

Data set of normal endometrial tissue was obtained from GEO database (GDS3975) and Spearman's rank correlation coefficient was calculated using SAGE-seq data of serum culture cells, sphere culture cells, S clone I cells and LL clone E cells.

2.12. Statistical analysis

Student's *t*-test was used to compare two groups. $P < 0.05$ was considered significant.

3. Results

3.1. Xenograft analysis revealed phenotypical similarity of serum-cultured cells and sphere-cultured cells from primary endometrioid adenocarcinoma

We previously reported that sphere-cultured cells showed higher tumor-initiating ability compared with that of serum-cultured cells derived from human primary endometrioid adenocarcinoma cells, suggesting that sphere-cultured cells are enriched with CSCs/CICs (Hashimoto et al., 2017). In this study, we performed further analysis using the primary cell culture sample. We examined the histological features of the primary tumor and xenografted tumors. The primary tumor sample was from a 52-year-old woman in post menopause who was newly diagnosed as having endometrioid adenocarcinoma stage IVB, and the tumor sample was obtained after chemotherapy (PTX + CBDCA). Analysis of the primary tumor tissue, the xenograft tumor derived from serum-cultured cells and the xenograft tumor derived from sphere-cultured cells revealed poorly differentiated adenocarcinomas that were morphologically similar (Fig. 1A). Immunohistochemical staining (IHC) revealed that all the samples were partially positive for cytokeratin (CK)7, negative for CK20 and partially positive for high-molecular weight cytokeratin (CK-HMW) (Fig. 1A). These results indicate that the primary tumor tissue and tissues derived from xenograft tumors are histologically similar and that serum-cultured cells and sphere-cultured cells have characteristics of the primary tumor.

3.2. Establishment of distinctive clones from sphere-cultured cells

The sphere-cultured cells showed higher tumor-initiating ability, indicating that the sphere-cultured cells are enriched with CSCs/CICs. Thus, sphere-cultured cells are a reasonable model for CSCs/CICs. The sphere-cultured cells were established with only a short period of *in vitro* culture and and the sphere-cultured cells might contain heterogenous subpopulation cells. We therefore performed single cell cloning to determine the characteristics of sphere-cultured cells at single cell levels. Single cell clones were established by the limiting dilution method and single cell sorting using a flow cytometer. Isolated single cells were cultured under a non-adhesive culture condition using ultra-low attachment plates, and several clone cells were successfully established. Interestingly, the cell growth pattern could be classified into two types (Fig. 1B). We termed the two clone types clones as sphere (S) clones (cells that grow to form spheres) and leukemia-like (LL) clones (cells that grow separately from each other like leukemia cells). All of the established clone cells could be classified into S clones (8 S clones: A, B, C, D, E, F, G, H and I) and LL clones (5 LL clones: A, B, C, D and E) (Fig. 1C).

The cell growth pattern was stable and each of the S clone cells and LL clone cells sustained the morphological pattern for several passages. Since sphere-cultured cells can be differentiate into non-CSCs/CICs under a serum-culture condition, we examined the plasticity of S clone cells and LL clone cells. After culture under a serum-culture condition for 7 days, both S clone cells and LL clone cells showed an attached growth pattern (Fig. 1D). Interestingly, re-sphere culture of serum-cultured S clone cells and LL clone cells showed a growth pattern similar to that of parental clone cells (Fig. 1D). These results indicate that S clone cells and LL clone cells can differentiate into non-CSCs/CICs and that the phenotypes are plastic. However, the growth patterns are stable even in a serum-culture condition.

3.3. Functional variations of LL clones and S clones

Since CSCs/CICs are defined by their higher tumor-initiating ability, we compared the tumor-initiating abilities of LL clone cells (A and E)

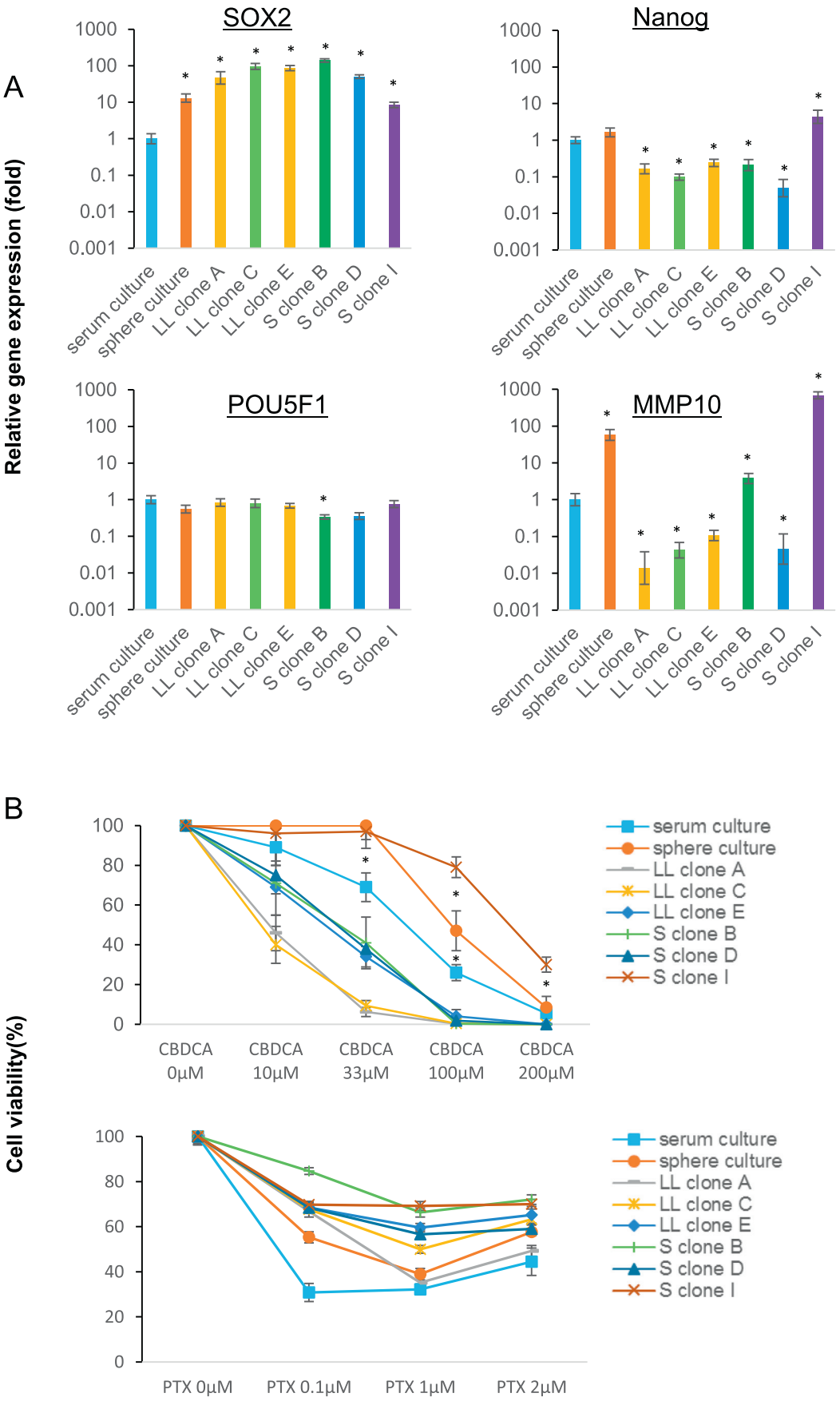

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Fig. 3. Gene expression of clones and resistance to chemotherapeutic agents.
(A) Expression of stemness genes in serum-cultured cells, sphere-cultured cells, LL clone cells and S clone cells was evaluated by quantitative RT-PCR. Data are shown as means ± SD. An asterisk indicates a statistically dignificant difference ($P < .05$).
(B) Resistance of serum-cultured cells, sphere-cultured cells, LL clone cells and S clone cells to chemotherapeutic agents. Calculated cell viability after 48 h of treatment with CBDCA (carboplatin) and PTX (paclitaxel) is shown. The X-axis is dose intensity of the chemotherapeutic agent. The Y-axis is calculated cell viability (%). Data are shown as means ± SD. An Asterisk indicates statistically significant difference ($P < 0.05$).

and S clone cells (A and I). LL clone cells (A and E) showed tumor initiation 8 weeks after infection of 10^2 cells. On the other hand, S clone cells (A and I) did not show tumor initiation (Fig. 2A). Histological analysis showed histological similarities in samples obtained from a primary patient tumor, a mouse tumor derived from serum-cultured cells, a mouse tumor derived from sphere-cultured cells and a mouse tumor derived from LL clone E cells (Fig. 2B). In previous studies, the stem cell-related gene SOX2 was shown to be highly expressed in CSCs/CICs (Nakatsugawa et al., 2011; Yasuda et al., 2013). We thus examined the expression of SOX2 in samples of the primary tumor and tumors derived from cultured cells. Immunohistochemical (IHC) staining revealed that tumors derived from LL clone E cells had a higher rate of SOX2 than did the primary tumor and tumors derived from serum-cultured cells and sphere-cultured cells (Fig. 2B). Sphere-cultured cells were established from a mouse xenograft tumor-derived from LL clone E cells, and the expression levels of SOX2 were examined by quantitative (q)RT-PCR. LL clone E cells showed a higher expression level of SOX2 than that in serum-cultured cells and sphere-cultured cells (Fig. 2C). And the expression level of SOX2 was increased by xenograft transplantation (Fig. 2C).

Further analysis of several LL clone cells and S clone cells was performed. It was shown by qRT-PCR analysis that sphere-cultured cells, LL clone cells (clones A, C and E) and S clone cells (clones B, D and I) had higher expression levels of SOX2 than serum culture cells (Fig. 3A). In our previous study, we found that MMP10 was over-expressed in sphere-cultured ovarian CSCs/CICs (Mariya et al., 2016). We thus examined the expression of MMP10 and found that the expression level of MMP10 was highest in S clone I cells; however, other S clone cells (B and D) and LL clone cells (A, C and E) showed lower expression levels than the level in parental sphere-cultured cells. There were no significant tendencies in the expression of POU5F1 and NANOG (Fig. 3A). CSCs/CICs show resistance to treatments including chemotherapy by several molecular mechanisms (Park et al., 2009), and Calboplatin (CBDCA) and pacritaxel (PTX) are key drugs for uterine endometrioid adnoecarcinoma. We therefore examined the sensitivities of serum-cultured cells, sphere-cultured cells, LL clone cells and S clone cells to these drugs. Sphere-cultured cells showed greater resistance than serum-cultured cells to CBDCA and PTX. Interestingly, LL clone cells (clones A, C, E) were more sensitive than parental serum-cultured cells and sphere-cultured cells to CBDCA. S clone I cells were resistant to CBDCA and PTX, whereas LL clone E cells were sensitive to PTX (Fig. 3B). These findings suggest that S clone cells are resistant to CBDCA and PTX, whereas LL clone cells are resistant to PTX.

Previous studies showed by the ALDEFLUOR assay that CSCs/CICs are enriched in aldehyde dehydrogenase-high ($ALDH^{high}$) cells (Ginestier et al., 2007; Kuroda et al., 2013; Yasuda et al., 2013). In the present study, serum-cultured cells showed a low percentage of $ALDH^{high}$ cells (2.3%), whereas sphere-cultured cells, LL clone A, C and E cells and S clone B, D and I cells showed relatively higher percentages of $ALDH^{high}$ cells (Fig. 4A). A cell growth assay revealed that serum cultured cells and LL clones A and E cells are rapidly growing cells under active cell cycle, whereas sphere-cultured cells and S clones B, D and I cells are slowly growing cells (Fig. 4B).

3.4. Distinctive gene expression landscape of LL clone cells and S clone cells shown by SAGE-seq (serial analysis of gene expression sequencing)

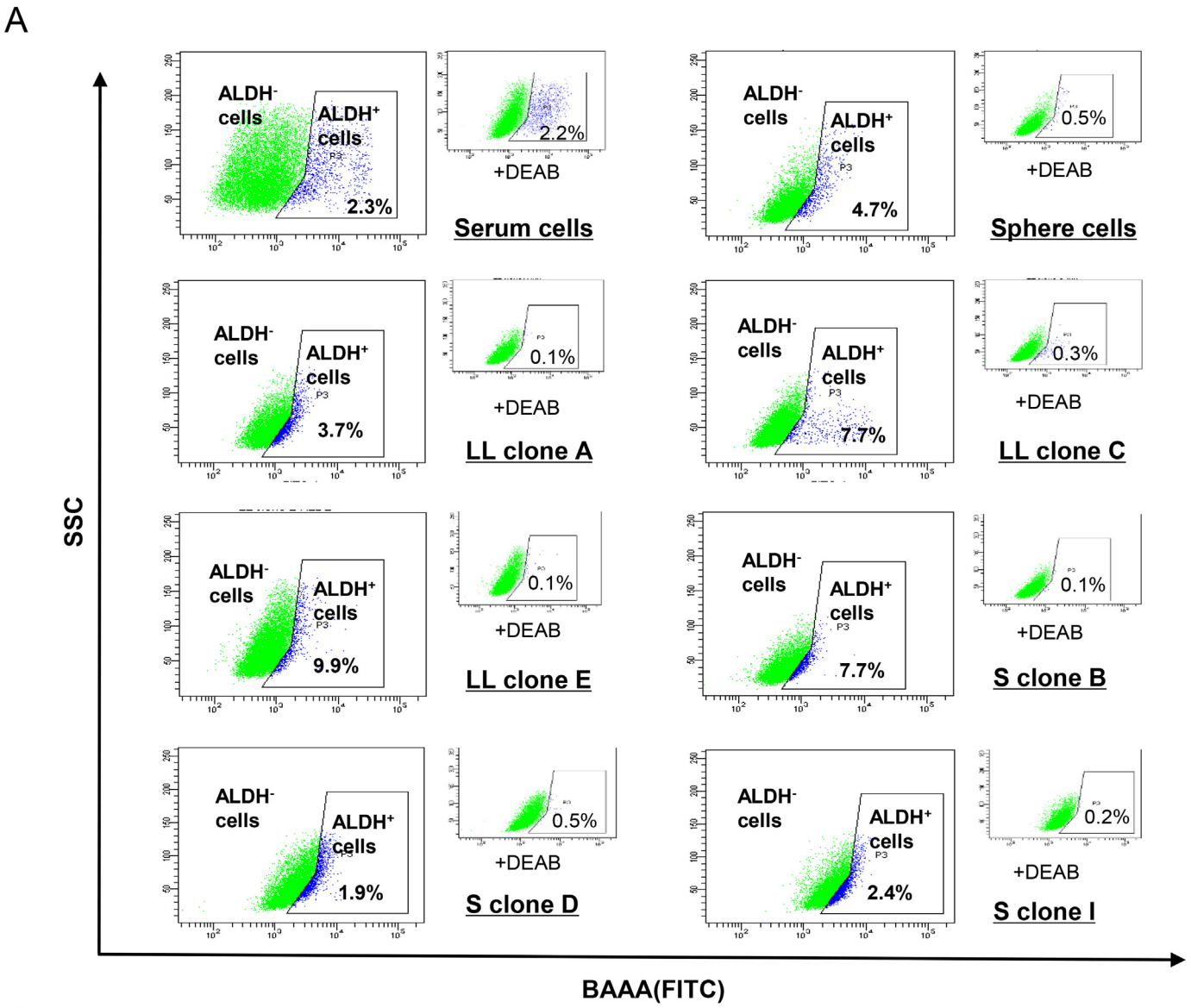
To analyze the transcriptome of serum-cultured cells, sphere-cultured cells, LL clone cells and S clone cells, we performed Serial analysis of gene expression sequencing (SAGE-Seq). More than 20,000,000 tags were read per sample, and a total of 18,587 genes were detected in the analysis. Since the primary cancer cells were obtained from uterine endometrioid adenocarcinoma, we analyzed the similarity of gene expression profile to normal gene expression of the endometrium. The serum-cultured cells showed gene expression profile close to that of the normal endometrium, and LL clone A cells showed a gene expression profile with less similarity to that of the normal endometrium (Fig. 5A).

Characteristic genes expressed in LL clone cells are shown in Table 1 and those expressed in S clone cells and sphere-cultured cells are shown in Table 2. We performed RT-PCR to confirm the gene expression. Several genes including *ADAMTS9, ALOX5AP, BHLHE41, C10orf90, CCR1, CHRNA1, KCNH1 NRP2, UGT2B7* and *OTX2* were specifically expressed in sphere-cultured cells and S clone cells. Other genes including *ZMAT4*, *RFX4*, *F13A1* and *PDGFB* were specifically expressed in LL clone cells (supplemental Fig. S1). To analyze the characteristics of gene expression, pathway analysis was performed using Metascape (http://metascape.org) (Supplementary Fig. S2). LL clone cells showed a significant gene expression signature of the MAPK cascade, whereas S clone cells showed a significant gene expression signature of stem cell differentiation (supplemental Fig. S2).

4. Discussion

Endometroid adenocarcinoma cells with stem cell features have been isolated by ALDH1 (Rahadiani et al., 2011), CD44 and CD133 (Nakamura et al., 2010; Rutella et al., 2009). Recently, novel candidate markers for endometrioid adenocarcinoma CSCs/CICs have been reported (Chen et al., 2015; Li et al., 2015; Yusuf et al., 2014). However, previous analysis was performed using long cultured cell lines. In this study, we successfully established distinctive CSC/CIC clones from primary patient tumor sample. The important point of this study is the cell samples had the similar features of the primary patient tumor. The results of histological studies including IHC staining revealed similarity of the primary tumor tissue and xenograft tumor tissues derived from established CSC/CIC clones, suggesting that the CSC/CIC clones established in this study are reasonable models for analyzing primary endometrioid CSCs/CICs. We previously reported that sphere-cultured cells have the characteristics of CSCs/CICs in cervical cancer (Asano et al., 2016) and ovarian cancer (Mariya et al., 2016), and we therefore we tried to analyze sphere-cultured cells. We established two cell clone types from sphere-cultured cells. Previous studies showed that highly tumorigenic and chemo-resistant cells are enriched in sphere-cultured cells compared with parental serum-cultured cells (Asano et al., 2016; Mariya et al., 2016). However, S clone cells (clones A and I) showed less tumorigenicity than that of LL clone cells (clones A and E). On the other hand, S clone I cells showed greater chemo-resistance than that of LL clone cells. These results indicate that different phenotypes of CSCs/CICs might be due to the heterogeneity of CSCs/CICs and that different CSC/CIC clone cells might have different phenotypes.

LL clone cells showed (1) higher tumorigenicity, (2) high expression level of SOX2 (3) high ratio of $ALDH^{high}$ cells, and (4) high proliferation rate *in vitro*. On the other hand, S clone cells showed (1) less tumorigenicity, (2) high expression level of MMP10, (3) lower ratio of $ALDH^{high}$ cells and (4) low proliferation rate *in vitro*. The most remarkable phenotype of LL clone cells is higher tumorigenicity. Previous



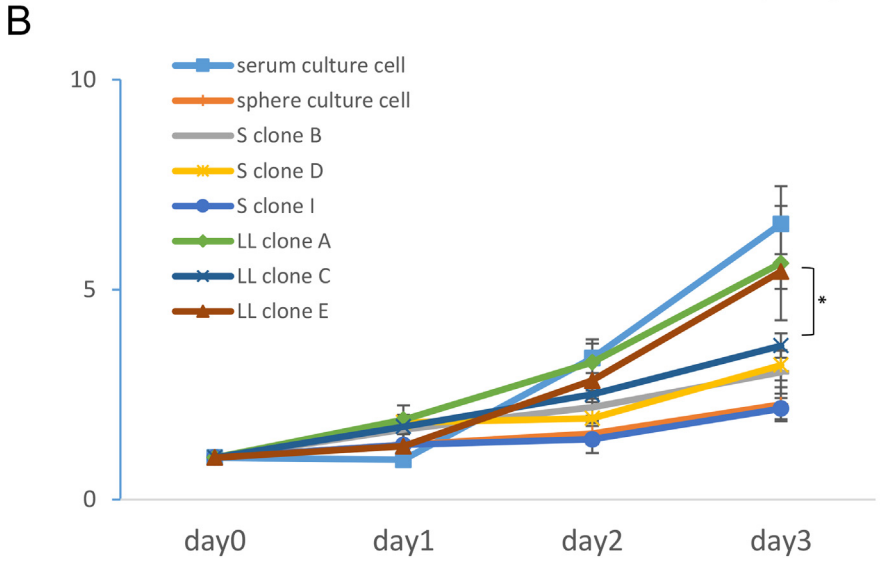


Fig. 4. Functional analysis of serum-cultured cells, sphere-cultured cells, LL clone cells and S clone cells.
(A) ALDEFLUOR assays of serum-cultured cells, sphere-cultured cells, LL clone cells and S clone cells.
(B) Cell proliferation assay of serum-cultured cells, sphere-cultured cells, LL clone cells and S clone cells.

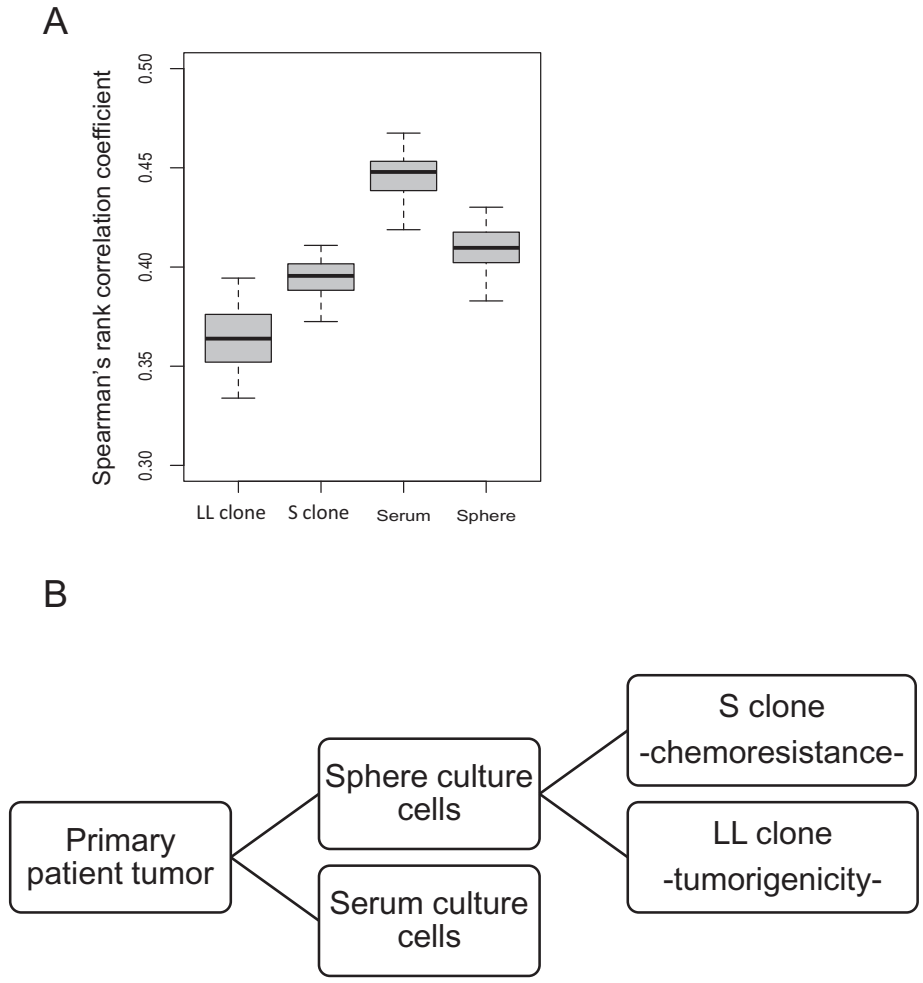


Fig. 5. Genetic similarity to normal endometrial tissue and schematic image of this study.
(A) Spearman's rank correlation coefficients for gene expression profiles of LL clone cells, S clone cells, serum-cultured cells and sphere-cultured cells compared to normal endometrial tissues (Reference ID of the NCBI GEO is GSE 23339).
(B) Schematic summary of CSC/CIC heterogeneity.

Table 1
Genes with high expression levels in LL clone cells compared to the levels in sphere clone, serum-cultured cells and sphere-culture cells.

Gene name	Locus	LL clone FPKM	S clone FPKM	Serum cultured FPKM	Sphere cultured FPKM	reference sequence
CHST15	chr10:124044399-124044427	3,453	0	0	0	NM_015892
F13A1	chr6:6144599-6144651	7,108	0	0	60	NM_000129
FBXL7	chr5:15939426-15939478	5,737	0	219	0	NM_012304
FBXL7	chr5:15708128-15708157	4,519	33	44	0	NM_012304
LINC00473	chr6:165939268-165939295	5,585	0	0	60	NR_026861
LOC400867	chr21:38912971-38912998	3,097	66	0	0	NR_120405
LRRN4	chr20:6040864-6040891	4,976	99	656	0	NM_152611
MIR421	chrX:74205900r-74219869	4,214	0	0	0	NR_030398
NOS2	chr17:27793643-27793727	17,009	0	0	0	NM_000625
PDGFB	chr22:39224087-39224115	4,620	0	44	0	NM_033016
PSMG1	chr21:39174718-39174817	4,417	33	219	0	NR_049728
RBM24	chr6:17292232-17292260	5,433	66	87	60	NM_153020
RFX4	chr12:106761765-106761792	5,433	0	0	0	NM_213594
VWA3A	chr16:22156598-22156632	5,280	0	0	0	NM_173615
ZMAT4	chr8:40531033-40531137	15,993	33	0	60	NM_024645

studies showed that SOX2 (Hubbard et al., 2009; Pitynski et al., 2015; Weina and Utikal, 2014) and ALDH^{high} are involved in CSCs/CICs and are related to tumorigenicity (Ginestier et al., 2007; Kuroda et al., 2013; Mariya et al., 2016). Our results showed that LL clone cells express higher expression level of SOX2 and higher ratio of ALDH^{high} cells. In contrast, S clone cells showed no of tumorigenicity despite having a higher expression level of SOX2 and higher ratio of ALDH^{high} cells than those of parental serum-cultured cells. These results indicate that SOX2 and ALDH^{high} are not only factors that define tumorigenicity. The cells were isolated from patient primary tumor who received chemotherapy before surgical resection. The tumor showed partial response (PR) to chemotherapy, thus remaining cancer cells should be resistant to chemotherapy. Therefore, chemo-resistant but low tumorigenic S clone cells might be isolated in this analysis.

Table 2
Genes with high expression levels in S clone and sphere-cultures cells compared to LL clone and serum-cultured cells.

Gene name	Locus	LL clone FPKM	S clone FPKM	Serum culture FPKM	Sphere culture FPKM	Reference sequence
ADAMTS9	chr3:64515826-64515929	51	14,242	0	13,009	NM_182920
ALOX5AP	chr13:30735579-30735632	0	7,483	0	5,875	NM_001629
BHLHE41	chr12:26120554-26120581	51	3,659	0	13,848	NM_030762
C10orf90	chr10:126468979-126469006	0	2,670	0	2,997	NM_001004298
C10orf90	chr10:126425349-126425401	51	11,835	44	12,290	NM_001004298
CCR1	chr3:46201999-46202027	0	3,264	0	2,518	NM_001295
CHRNA1	chr2:174748101-174748219	51	159,988	44	52,515	NM_001039523
KCNH1	chr1:210678507-210678557	0	2,374	0	1,739	NM_172362
LOC101929199	chr4:27277191-27277256	0	3,165	0	11,151	NR_125921
NOS3	chr7:151014563-151014615	0	5,703	0	2,338	NM_001160111
NRP2	chr2:205698081-205698109	0	2,143	0	2,398	NM_201279
OTX2	chr14:56801276-56801328	0	2,341	0	17,805	NR_073036
PRR16	chr5:120687204-120687254	0	2,769	0	1,019	NM_016644
PURG	chr8:30995899-30995927	609	2,110	0	1,199	NM_013357
SCML4	chr6:107702198-107702225	102	41,373	0	14,927	NM_198081
SERTAD4	chr1:210243448-210243475	0	2,505	87	1,559	NM_019605
SHC3	chr9:89177628-89177680	406	2,275	0	1,079	NM_016848
SOBP	chr6:107629463-107629586	51	4,220	0	5,395	NM_018013
SSPN	chr12:26229829-26229857	0	2,967	0	5,935	NM_005086
UGT2B7	chr4:69112493-69112597	102	29,505	44	92,321	NM_001074

FPKM (Fragments Per Kilobase of exon per Million fragments mapped) indicates the number of fragments per sample which were read by the reference sequence.

Carboplatin (CBDCA) inhibits DNA synthesis and Paclitaxel (PTX) inhibits the microtubule assembly, and suppress cell proliferation (Jiang et al., 2015). Therefore, these drugs target fast growing cancer cells well, and LL clones, which have thigh proliferation ability, are very sensitive to chemotherapeutic reagents. Since S clone I cells are very slow-growing cells in a dormant state, this is one factor that might explain the chemo-resistance of S clone I cells. However, there was no significant difference between the cell proliferation ability of S clone I cells and that of sphere-cultured cells, whereas S clone I cells showed greater resistance than sphere-cultured cells to CBDCA and PTX. We previously reported that MMP10 is involved in CSCs/CICs and is related to chemo-resistance (Mariya et al., 2016). Therefore, a high expression level of MMP10 in S clone I cells might the reason for chemo-resistance. Interestingly, a high expression level of SOX2 is not related to chemo-resistance in this study.

Estimated enriched pathway analysis revealed the differences between features of genes with high expression levels in S clone cells and LL clone cells. Molecules involved in tissue development pathways (such as embryo, skeletal, sensory organ and mesoderm development) are enriched in S clone cells, and S clone cells have a tendency to have stem cell features. On the other hand, pathways related to cell proliferation are enriched in LL clone cells. Second-messenger signaling, which is related to cell proliferation and differentiation is enriched in LL clone cells. Recently, a strategy for cancer therapy targeting calcium signaling which is second-messenger signaling has been reported (Cui et al., 2017). MAPK signaling has been reported to be related to tumor progression and it can be a target of cancer therapy (Cheng et al., 2013). Genes related to the MAPK pathway are enriched in LL clone cells. These pathways might have a role in the higher tumorigenicity of LL clone cells.

CSCs/CICs have been reported to have high levels of tumorigenicity and chemo-resistance (Hirohashi et al., 2010; Murase et al., 2009; Park et al., 2009; Visvader and Lindeman, 2008). However, we could not explain these features of CSCs/CICs by single gene or one clone cells, even by analysis of the sample from the patient. We think that the degree of malignancy and the prognosis for a patient are determined not by one gene or one clone cells but by multiple genes or multiple phenotypical CSCs/CICs. In this study, the phenotypes as carcinoma were shared by multiple clones, including LL clone cells as tumor-initiating clones and S clones as chemo-resistant cells (Fig. 5B), and the gene expression profiles were quite different among the clones. Therefore, analysis of a sample from one patient at clonal level, might be nucessary to elucidate the complexity of CSC/CIC phenotypes.

In summary, we established several clone cells from a primary endometrial adenocarcinoma sample and analyzed their functions, and we found that heterogenous CSC/CIC includes tumorigenic clone cells and chemo-resistant clone cells.

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Disclosure statement

The authors have no conflict of interest.

Author contribution statement

YT, YH, TS and TTorigoe wrote the main manuscript. YT, SH, TM, TA, KI, TKuroda, AM, SU and NK performed experiments. YT, YH, SH, KI, TKubo, MN, TKanaseki, TTsukahara, TS and TTorigoe analyzed data. All authors reviewed the manuscript.

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