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Efficient use of patient-derived organoids as a preclinical model for gynecologic tumors

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HIGHLIGHTS

- We established an efficient method for organoid culture of gynecologic tumors.
- Organoids retained many features of original tumors in histology and mutations.
- Different clones of organoids could be established from different sites within a single tumor.
- Drug response assay was feasible with organoid-derived spheroids.

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ABSTRACT

Objective. The relevance of patient-derived cancer cells has been recently increasing, particularly in terms of drug discovery and precision medicine. Whereas Matrigel-based organoid culture is a promising technique that enables infinite proliferation of cells from many types of organs in a physiological condition, its validity in gynecologic tumors remains to be established. To address this issue, we aimed at developing an efficient method for organoid culture of both ovarian and endometrial tumors.

Methods. We conducted 3D culture of 21 gynecologic tumors following our original and modified protocol for Matrigel bilayer organoid culture. We investigated whether propagated organoids retained various features of the original tumors by histopathological examination and targeted genome sequencing.

Results. We customized the protocol we previously optimized for murine normal and cancer tissues, so as to circumvent the digestion-resistant nature inherent to gynecologic tumors. Indeed, this modified protocol improved the success rate from 45 to 90%, for robust propagation of organoids from tumors with various stages and subtypes. Finally, 14 patient-derived organoids were established. The recovered organoids were enriched for cancer cells that retained many aspects of the original tumors, including histological features, mutation profiles, and intra-tumoral heterogeneity. A subset of the expanded organoids could develop xenografts in immunodeficient mice, potentially paving the way to drug screening *in vivo*. Drug response assay *in vitro* for paclitaxel and cisplatin was feasible using organoid-derived spheroids.

Conclusions. We showed that patient-derived organoids closely resembled the original gynecologic tumors, and thereby would serve as a promising resource for preclinical studies.

1. Introduction

Cell lines have long contributed to cancer research through functional studies in biochemistry, physiology and cell biology [1]. Although they originate from patient samples, it is generally difficult for primary cells to be established as immortalized adherent cells in a dish. Consequently, a subset of cell lines is subject to intensive use for a wide variety of studies. During many passages in culture with FBS, however, they can accumulate mutations, potentially leading to positive selection of specific clones without many features of the original tumors [2]. For example, many adenocarcinoma-derived cell lines no longer develop tumors with a glandular structure as xenografts in immunodeficient mice. Presumably for these reasons, the responses of these cell lines to therapeutic agents, either in culture or as xenografts, were not recapitulated in many clinical trials [3,4].

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In this regard, other platforms have been sought after as a resource for preclinical studies. One way to maintain patients' samples for a long duration is to directly inoculate tumor fragments into the dorsal skin of immunodeficient mice. Patient-derived xenografts (PDXs) thus generated not only retain the histological features of the original tumors, but also respond to drugs in a similar manner to those in clinical settings [5,6]. Another way is to generate spheroids, floating cell aggregates, in serum-free media. Spheroids are believed to mimic the physiological status of cancer cells more accurately than adherent cells, and to be enriched for stem-like cells responsible for drug resistance and metastasis [7]. Despite the potential usefulness of these two methods in cancer research, the success rates of both methods vary among cancer types or are not even mentioned [6,8,9], requiring alternative ways to maintain primary tumor cells in an efficient and robust manner.

Organoid culture is an emerging technique that enables infinite expansion of normal stem cells. With Matrigel as an extracellular matrix and defined factors that mimic the stem cell niche, tissue homeostasis can be reconstituted *in vitro* without any stromal cells [10], which has been applied to various research fields [11,12]. We also demonstrated that multistep carcinogenesis could be essentially recapitulated with gene transduction into murine organoids from the intestine [13,14], lung [15] and hepatobiliary system [16]. During the establishment of these models, we noted that a significantly high infection efficiency could be achieved by overnight incubation of single cells with lentiviral particles on solidified Matrigel, but not if cells were directly embedded in Matrigel [13,17]. The next day, viable cells exclusively attached onto Matrigel and readily propagated after being covered by Matrigel, strongly suggesting their stem or progenitor cells-like features. This culture method, which we referred to as Matrigel bilayer organoid culture (MBOC) [14], also proved efficient in eliminating dead or dying cells, as these could digest Matrigel or exert effects on viable cells if embedded together in Matrigel. Consequently, we extended the use of MBOC protocol to primary- and sub-cultures of organoids thereafter.

Whereas organoid culture has become common for patient-derived samples in diverse types of cancer [18–21], its validity in gynecologic tumors remains to be established, with only a small number of studies for gynecologic tumors [22,23]. In this study, we asked if the MBOC protocol could achieve robust propagation of organoids from gynecologic tumors. We eventually established a highly efficient method after introducing some modifications, and demonstrated that recovered organoids basically retained characteristics of the original tumors.

2. Methods

2.1. Patient information

This study was approved by the Chiba Cancer Center Ethics Committee (IRB approval number H28-J158 and H29-9). Tumor samples were obtained from patients with written informed consent. Twenty-two patients with gynecologic tumors (ovarian: 15 cases, endometrial: 6 cases) who underwent surgery in the Chiba Cancer Center between March 2017 and September 2018 were enrolled (Table 1). None of them had undergone chemotherapy or radiation therapy before surgery.

2.2. Isolation of gynecologic cancer cells

Tissue fragments of approximately 500–1000 mm^3 were obtained from ovarian or endometrial tumors immediately after tumor resection in the operating room. Non-necrotic lesions with solid or papillary growth were collected, and processed as previously described [13]. Tissue fragments were cut into 2–3 mm pieces, washed with cold PBS for several rounds and dissociated into small clusters or single cells, by digesting with 2 U/ml dispase II and 1 mg/ml collagenase P (Roche Diagnostics, Tokyo, Japan) for 45 min at 37 °C. Dissociated cells were washed with PBS and pelleted down. If a considerable amount of tissue pieces were left undigested, they were further digested with Accumax (Innovative Cell Technologies, San Diego, CA) for 5 min at 37 °C and washed with ice-cold PBS. Cancerous ascites were centrifuged at 4 °C for 10 min at 3000 rpm. Erythrocytes were ruptured by hypotonic buffer (168 mM NH_4Cl, 10 mM $KHCO_3$, 81.8 μM EDTA-4Na) treatment. In either case, the number of wells to seed tumor cells was empirically determined depending on the amount of cell pellets recovered. Typically, 2 to 4 wells in a 12-well plate were used to start the primary organoid culture.

Table 1
Summary of clinico-pathologic features and primary organoid culture of ovarian and endometrial tumors.

Case no.	FIGO staging	TNM staging	Histological diagnosis		NGS analysis	Organoid	MBOC	Tumorigenicity
Ovarian tumor								
Ov-1	IIB	pT2bN0M0	EMC			Failed	Standard	N.T.
Ov-2	IA	pT1aNxM0	BBT	(Borderline)		Propagated	Standard	N.T.
Ov-3	IIIA1	pT2bN1aM0	HGSC		Yes	Propagated	Standard	−
Ov-4	IIIA1	pT2bN1bM0	HGSC		Yes	Propagated	Standard	−
Ov-6	IA	pT1aN0M0	MB			Failed	Standard	N.T.
Ov-7	IC1	pT1c1N0M0	EMC			Failed	Standard	N.T.
Ov-8	IA	pT1aNxM0	MBT	(Borderline)		Failed	Standard	N.T.
Ov-9	IA	pT1aN0M0	HGSC		Yes	Propagated	Standard	−
Ov-10	IC2	pT1c2N0M0	MBT	(Borderline)		Failed	Standard	N.T.
Ov-11	IIIA1	pT2bN1bM0	HGSC			Failed	Modified	N.T.
Ov-12	IC1	pT1c1N0M0	EMC			propagated	Modified	−
Ov-13	IIB	pT2bNxM0	SBT	(Borderline)		Propagated	Modified	−
Ov-14	IA	pT1aNxM0	SBT	(Borderline)		Propagated	Modified	N.T.
Ov-15	IA	pT1aN0M0	MC			Propagated	Modified	N.T.
Ov-16	IC3	pT1c3N0M0	EMC			Propagated	Modified	+
Endometrial tumor								
Em-1	IB	pT1bN0M0	EMC G2		Yes	Propagated	Standard	+
Em-2	IB	pT1bN0M0	CCC			Failed	Standard	N.T.
Em-3	IA	pT1aN0M0	EMC G3			Propagated	Modified	N.T.
Em-4	IA	pT1aN0M0	EMC G1			Propagated	Modified	N.T.
Em-5	IA	pT1aN0M0	EMC G1			Propagated	Modified	−
Em-6	IB	pT1bN0M0	EMC G2			Propagated	Modified	N.T.

EMC, endometrioid carcinoma; BBT, borderline Brenner tumor: HGSC, high-grade serous carcinoma; MB, malignant Brenner tumor; MBT, mucinous cystadenoma; SBT, serous borderline tumor; MC, mucinous carcinoma; CCC, clear cell carcinoma; G, grade; N.T., not tested.

2.3. Organoid culture

The organoid culture media was advanced DMEM/F12 (Thermo Fisher Scientific, Watham, MA) supplemented with 50 ng/ml human EGF (Peprotech, Rocky Hill, NJ), 250 ng/ml R-spondin1 (R&D, Minneapolis, MN), 100 ng/ml Noggin (Peprotech), 10 μM Y27632 (Wako, Osaka, Japan), 1 μM Jagged-1 (AnaSpec, Fremont, CA), L-glutamine solution (Wako), penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO), and amphotericin B suspension (Wako). Organoid culture was conducted according to the modified MBOC protocol (Fig. 1A). Briefly, the cells resuspended in 800 μl/well of the media were plated on 65 μl of solidified Matrigel (BD Biosciences, Franklin Lakes, NJ) per well in a 12-well plate and incubated overnight at 37 °C (day 0). The following morning, floating non-viable cells were discarded along with the media. Viable cells attached onto Matrigel were covered with 70 μl of Matrigel and overlaid with the media (day 1). On many occasions where floating tissue fragments and cell aggregates appeared to contain many viable cells, they were collected and subjected to Accumax (Innovative Cell

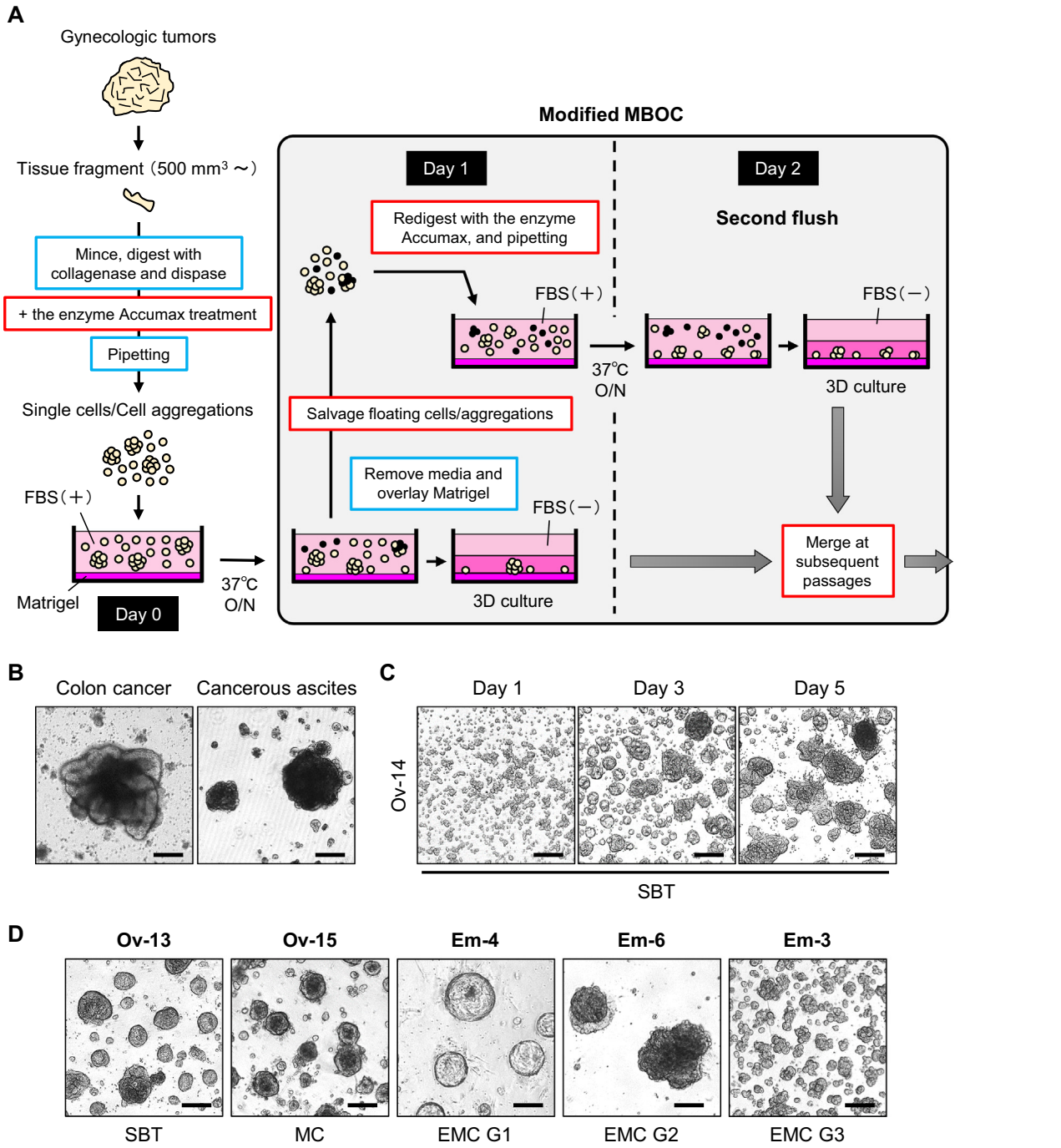

Fig. 1. Matrigel bilayer organoid culture (MBOC) for ovarian and endometrial tumors. (A) Schematic representation of the MBOC protocol. Procedures highlighted in red were added in modified MBOC. (B) Tumor-derived organoid propagated from clinical samples. (Left panel) Colon cancer tissue-derived organoids represented glandular-like budding structure. (Right panel) Cancerous ascites-derived organoids were propagated as dense organoids. Scale bar indicates 200 μm. (C) Representative time-lapse bright field images of primary tumor-derived organoids (Passage 0) at 1–5 days. Scale bar indicates 200 μm. (D) Representative bright field images of tumor-derived organoids. Morphology of organoids varied in each case. Scale bar indicates 200 μm. Ov, ovarian tumor; Em, endometrial tumor; SBT, serous borderline tumor; MC, mucinous carcinoma; EMC G, endometrioid carcinoma grade. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Technologies) treatment for 5 min at 37 °C and vigorous pipetting. Viable cells attached onto Matrigel were covered with 70 μl of Matrigel and overlaid with the media (day 2). These cells after two-rounds of dissociation were later integrated into the organoid culture, to increase the number of cancer cells. Passage was conducted at 70%–80% confluency, in which 5×10^5–2×10^6 cells were typically harvested. Depending on the proliferation speed, the cells were diluted at 1:1 to 1:4. In each passage, organoids, Matrigel, and the media were directly collected altogether with a cell scraper, washed with PBS, and dissociated into single cells, by Accutase treatment for 5 min at 37 °C and vigorous pipetting. Tumor-derived organoids were cryopreserved in Bambanker (GG Lymphotec, Tokyo, Japan) supplemented with 10 μM Y27632 and stored at −145 °C until used.

2.4. Pathological analysis

Organoids obtained by de-polymerization of Matrigel with Cell Recovery Solution (BD Biosciences, Franklin Lakes, NJ) were resuspended in iPGell (GenoStaff, Tokyo, Japan). The detailed method is shown in Supplementary methods.

2.5. Targeted next-generation sequencing (NGS) analysis

NGS analysis on tumor-derived organoids and formalin-fixed paraffin embedded (FFPE) samplese were conducted essentially as previously described [24], but with a slight modification. The detailed method is shown in Supplementary methods.

2.6. Cell proliferation and drug sensitivity assay

For *in vitro* assays, organoids were digested with Accumax. The dissociated cells were counted with TC20 Automated Cell Counter (Bio-Rad, Hercules, CA). The detailed method is shown in Supplementary methods.

2.7. Tumorigenicity assay

Immunodeficient nude mice BALB/cA$^{nu/nu}$ were purchased from CLEA Japan Inc. (Tokyo, Japan). Animal studies were carried out with the approval of the Chiba Cancer Center for Ethics in Animal Experimentation. Tumorigenicity assay was conducted as previously described [13]. Briefly, tumor-derived organoids were collected at 70–80% confluency and an aliquot of 1/10 volume was completely dissociated with Accumax for cell counting with TC20 Automated Cell Counter. Organoids corresponding to 5×10^5 cells were resuspended in 100 μl of advanced DMEM/F12, mixed with 100 μl of Matrigel at a 1:1 ratio, and inoculated into one side of the dorsal skin of nude mice. Tumor development was monitored for six months at the longest.

3. Results

3.1. Limitation of the MBOC protocol for human gynecologic tumors

To investigate whether the MBOC protocol (Fig. 1A) can be generally applicable to patient-derived samples, we first tested surgically resected colon cancer tissues and cancerous ascites, for which 3D culture has been widely conducted [9,25]. We successfully propagated organoids from 5 out of 6 colon cancer patients and from cancerous ascites and pleural effusion of one peritoneal cancer patient, if we define the successful cases as those achieving robust expansion over >2 passages (Fig. 1B). For gynecologic tumors, however, we frequently noted massive undigested tissue and cell aggregates floating in the supernatant even after regular enzymatic digestion of the tumors. These masses were supposed to be discarded according to the original protocol, leading to recovery of an insufficient number of cells attached to Matrigel. Consequently, we ended up with only a 45% of success rate (5/11 cases) (Table 1). To circumvent the digestion-resistant and aggregation-prone nature of gynecologic tumor tissues, we tried to increase the yield of single cells by passing them through cell strainers, which proved ineffective due to clogging by the cell aggregates. We also tried a standard “dome culture” with only partially digested tumors. However, this attempt resulted in rapid lysis of Matrigel and subsequent increase in cell death, presumably due to unknown deleterious factors from dead or dying cells, even in the presence of a proteolysis inhibitor FBS. Based on these observations, we reasoned that a more efficient release of single cancer cells from tumors would be required for substantial improvement of the success rate.

3.2. Robust propagation of organoids from gynecologic tumors by the modified MBOC protocol

To this end, tumor tissues could be subjected to overnight enzymatic digestion [18], but we declined this option to avoid any possible negative impact on the viability of primary cancer cells. Instead, we introduced a seven-minute digestion step with Accumax, another potent proteolytic and collagenolytic enzyme with DNase activity, following the routine digestion of tumors with dispase and collagenase. In addition, those cell aggregates that remained undissolved were collected on day 1, when the upper layer of Matrigel was supposed to be overlaid, to go through another round of Accumax treatment (Fig. 1A). Together, these modifications of the original MBOC protocol resulted in significant improvement in digestion of the tumor masses to release a sufficient number of single cancer cells, leading to robust expansion of organoids (Fig. 1C). With this modified protocol, we were able to propagate organoids from five ovarian (83%: 5/6 cases) and four endometrial (100%: 4/4 cases) tumors (Table 1). Reflecting inter-tumoral heterogeneity, recovered organoids varied in morphology ranging from solid, cystic structure to loosely cohesive clusters (Fig. 1D).

3.3. Recapitulation of original tumors' histological features in organoids

To examine to what extent propagated organoids retained the features of the original tumors, we first compared organoids and the original tumors for 4 representative cases. We confirmed that the organoids basically retained histological features of the original tumors (Figs. 2, S1A). Specifically, common features included cystic structure and cribriform for an ovarian serous borderline tumor (Ov-13), dense structure and/or cribriform for an endometrial endometrioid carcinoma (EMC) grade 2 (Em-1), loosely cohesive clusters of cancer cells with marked nuclear atypia and cannibalism for endometrial EMC grade 3 (Em-3), and a cystic structure of cancer cells with mucin production for mucinous carcinoma (Ov-15). In addition, immunostaining for estrogen receptor (ER), progesterone receptor (PgR), and p53 also resulted in concordant results (Fig. 2). We next inoculated tumor-derived organoids into the dorsal skin of nude mice to evaluate tumorigenic potentials. However, we obtained subcutaneous tumors in only two out of eight cases tested. In one case, organoids from two different parts of the same tumor were separately inoculated, but only one of which developed tumors with a more aggressive histology than the original tumor (Fig. S2). These results suggest that stochastic cooperation with the microenvironments and clonal selection might be necessary for tumor development. Considering the morphological similarity between organoids and cytology specimens, we conducted Papanicolaou staining, a routine procedure in cytological examination, on organoids. We observed atypical cells with anisokaryosis and marked nucleoli in most organoids, comparable to diagnosis with malignancy in cytology (Fig. S1B).

3.4. Retention and enrichment of tumor-derived somatic mutations in organoids

Accumulating evidence suggests that tumor-derived organoids retain the majority of somatic mutations and copy number variations of

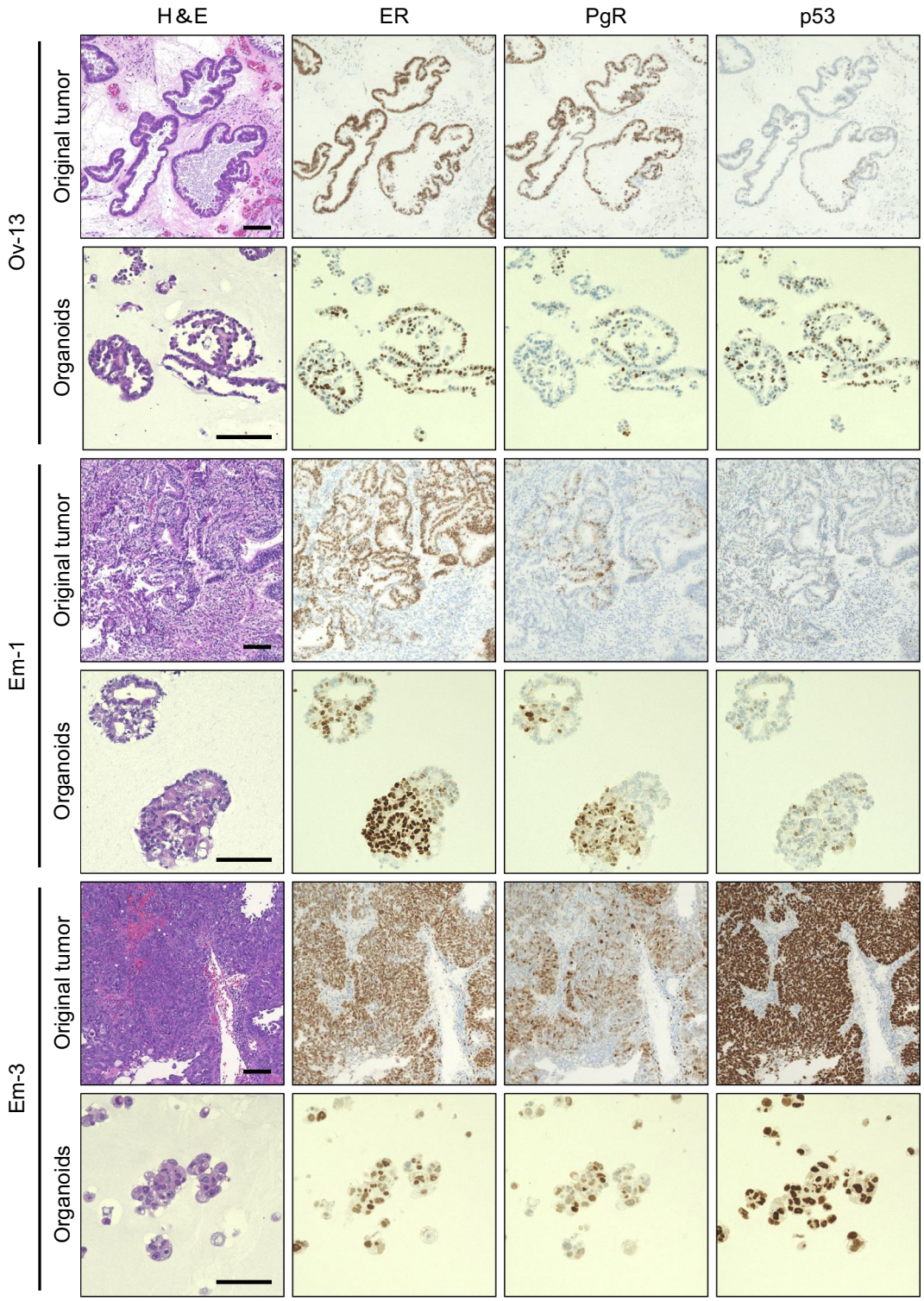

Fig. 2. Recapitulation of histological features of the original tumor in tumor-derived organoids. Histopathological examination of the original tumors and tumor-derived organoids by H&E and immunohistochemical staining against ER, PgR, p53. Tumor-derived organoids shared histopathological features with the original tumors. Scale bar indicates 100 μm. H&E, hematoxylin and eosin staining; ER, estrogen receptor; PgR, progesterone receptor.

the original tumors [25,26]. To verify this notion in gynecologic tumors, we first examined a high-grade serous carcinoma (HGSC) case (Ov-9), which was characterized by multilocular cysts with solid parts (Fig. 3A, B). With genomic DNA extracted from tumor-derived organoids and FFPE sample of the original tumor, we conducted targeted sequencing analysis of 409 cancer-related genes. For simplicity, somatic single nucleotide variants (S-SNVs) with variant allele frequency (VAF) higher than 10% were regarded as true mutations. A total of four S-SNVs were identified, three of which were shared by both organoids and the original tumor (Fig. 3C), confirming retention of somatic mutations in organoids.

Consistent with HGSC, a nonsense mutation in *TP53* was detected in this case, with the VAF at 70% and 98% in the tumor and organoids, respectively (Fig. 3D, Table S1). Generally speaking, VAF of a heterozygous

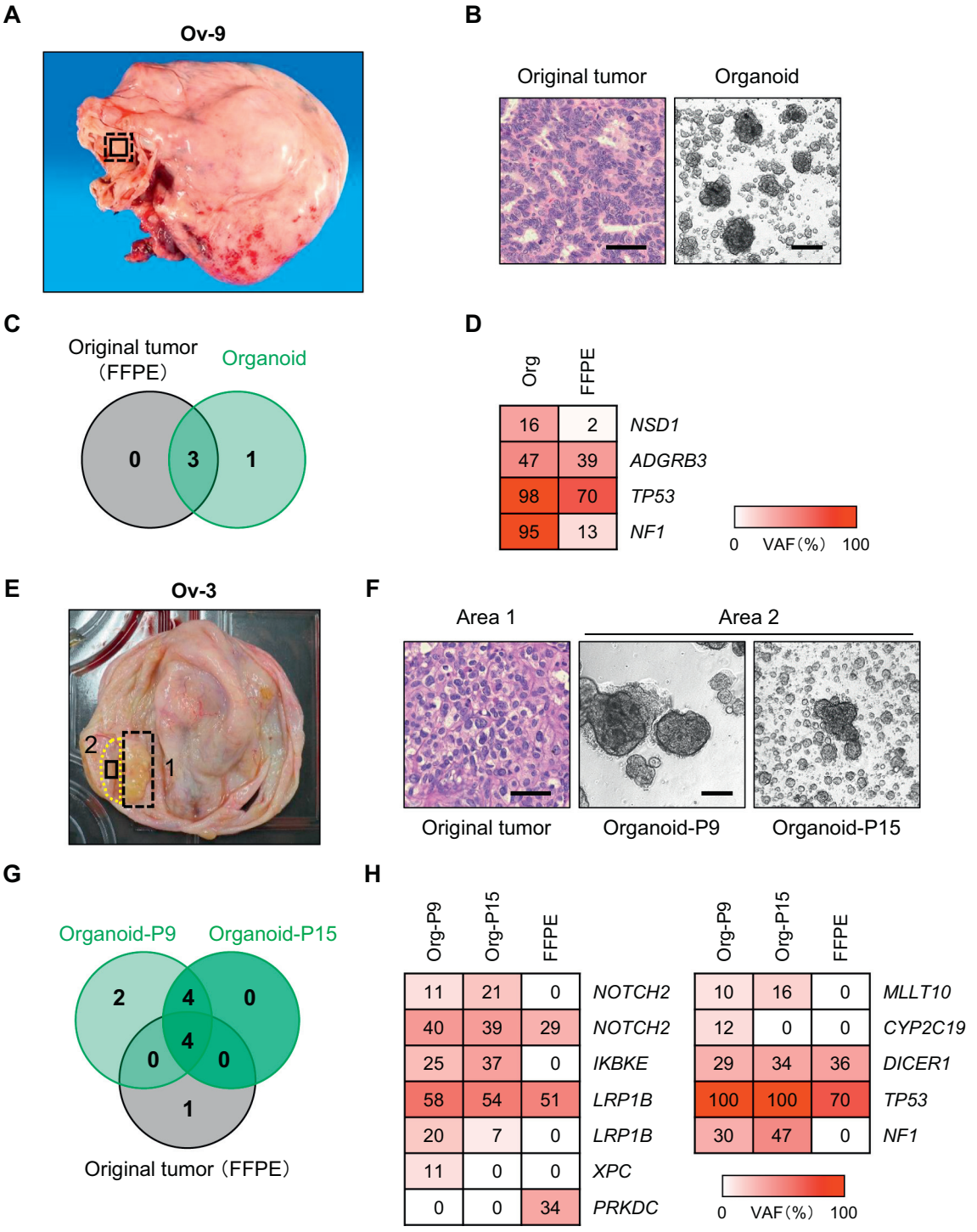

Fig. 3. Retention of somatic mutations of original tumors in tumor-derived organoids. (A–D) Organoid culture from ovarian multilocular cyst with solid parts (Ov-9). (A) Macroscopic image. Solid box indicates sampling site for organoid culture. Dashed box indicates sites where DNA was extracted from FFPE sample. (B) Bright field images of tumor-derived organoids together with H&E staining of the original tumor. Left and right scale bars indicate 50 μm and 200 μm, respectively. (C) Venn diagram of a total of 4 S-SNVs with VAF > 10% identified from each sample. (D) Detail of variant allele frequency (VAF) of 4 S-SNVs identified from tumor-derived organoids (Org) and the original tumor (FFPE). VAF is indicated according to the red color scale. (E–H) Organoids derived from an ovarian simple cyst with a thickened wall (Ov-3). (E) Macroscopic image after being opened and sampled to conduct rapid pathological diagnosis (yellow dash area). Solid box indicates sampling site for organoid culture. Dashed box indicates sites where DNA was extracted from FFPE sample. (F) Bright field images of tumor-derived organoids together with H&E staining of the original tumors. Left and middle scale bars indicate 50 μm and 200 μm, respectively. (G) Venn diagram of a total of 11 S-SNVs with VAF > 10% identified from each sample. (H) Detail of VAF of 11 S-SNVs identified from late passage tumor-derived organoids (Org-P9, Org-P15) and the original tumor (FFPE). VAF is indicated according to the red color scale. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mutation cannot be higher than 50%, even if the tumor purely consists of cancer cells. VAF of the mutant *TP53* at 70% therefore indicates that cancer cells with a point mutation and loss of heterozygosity (LOH) in *TP53* were already present in the tumor at the considerable level. Unexpectedly, the VAF of S-SNV in *NF1* also increased from 13% in the tumor to 95% in organoids (Fig. 3D, Table S1), although it was a synonymous SNV. In addition, high VAF value in organoids clearly indicated that the cancer cells in organoids had almost invariably undergone

LOH, raising the possibility that this S-SNV might have been positively selected for unknown reason. This notion prompted us to scrutinize the raw data in NGS, leading to identification of an additional 1 base frameshift deletion next to this S-SNV (Fig. S3). Together, these results suggest that the tumor was mainly composed of *TP53*-null cancer cells, but cancer cells nullizygous for both *TP53* and *NF1*, a possible minor fraction in the tumor, were significantly enriched during culture.

There is a general concern regarding prolonged culture for accumulation of genetic alterations and subsequent clonal selection [27]. To address this issue, we compared tumor-derived organoids after nine passages (P9) in eight months and 15 passages (P15) in twelve months of culture for another HGSC case (Ov-3) (Fig. 3E, F). A total of eleven S-SNVs were identified from these organoids, of which eight S-SNVs were shared by both P9 and P15 organoids (Fig. 3G). The other 3 S-SNVs specifically observed in P9 had low VAF and eventually disappeared in P15, ruling out the possibility of emergence and enrichment of novel mutations during culture. The VAF of S-SNV in *TP53* was enriched from 70% in the original tumor to 100% in organoids P9 and P15. There was mild enrichment during culture for a subset of mutations, including those in *NOTCH2*, *IKBKE*, *MLLT10*, and *NF1*, consistent with the potentially oncogenic roles of these mutations (Fig. 3H, Table S2).

3.5. Retention of intra-tumoral heterogeneity in organoids

With regard to ovarian HGSC (Ov-4), we were able to propagate organoids (Org-1, 2) derived from two macroscopically distinct lesions (Fig. 4A). Microscopic examination revealed that area 1 exhibited papillary growth with marked nucleoli, while area 2 showed solid growth (Fig. 4B). We previously showed that histologically distinct areas within the same tumor harbored distinct mutations in an ovarian cancer case [24]. Naturally, we asked if such intra-tumoral heterogeneity could be retained in organoids derived from the two distinct lesions. In addition to Org1 and Org2, FFPE sample of area 3, which is closer to and histologically resembles area 2 (Fig. 4A, B), was analyzed as a reference (FFPE-3) by NGS. Whereas Org-1 and Org-2 shared 3 S-SNVs in *TP53*, *PKHD1*, and *DST*, seven and six S-SNVs were exclusively detected in Org-1 and Org-2, respectively. On the other hand, Org-2 and FFPE-3 shared six S-SNVs, including *TP53*, *PKHD1*, and *DST*, while 3 and 2 S-SNVs were detected in Org-2 and FFPE-3, respectively. These results suggest that the mutations in *TP53*, *PKHD1*, and *DST* might be founder mutations of this tumor, and that mutational profiles reflecting intra-tumoral heterogeneity were indeed retained in organoids (Fig. 4C, D, Table S3).

To further verify intra-tumoral heterogeneity in organoids, we examined another case with exophytic endometrial cancer (Em-1).

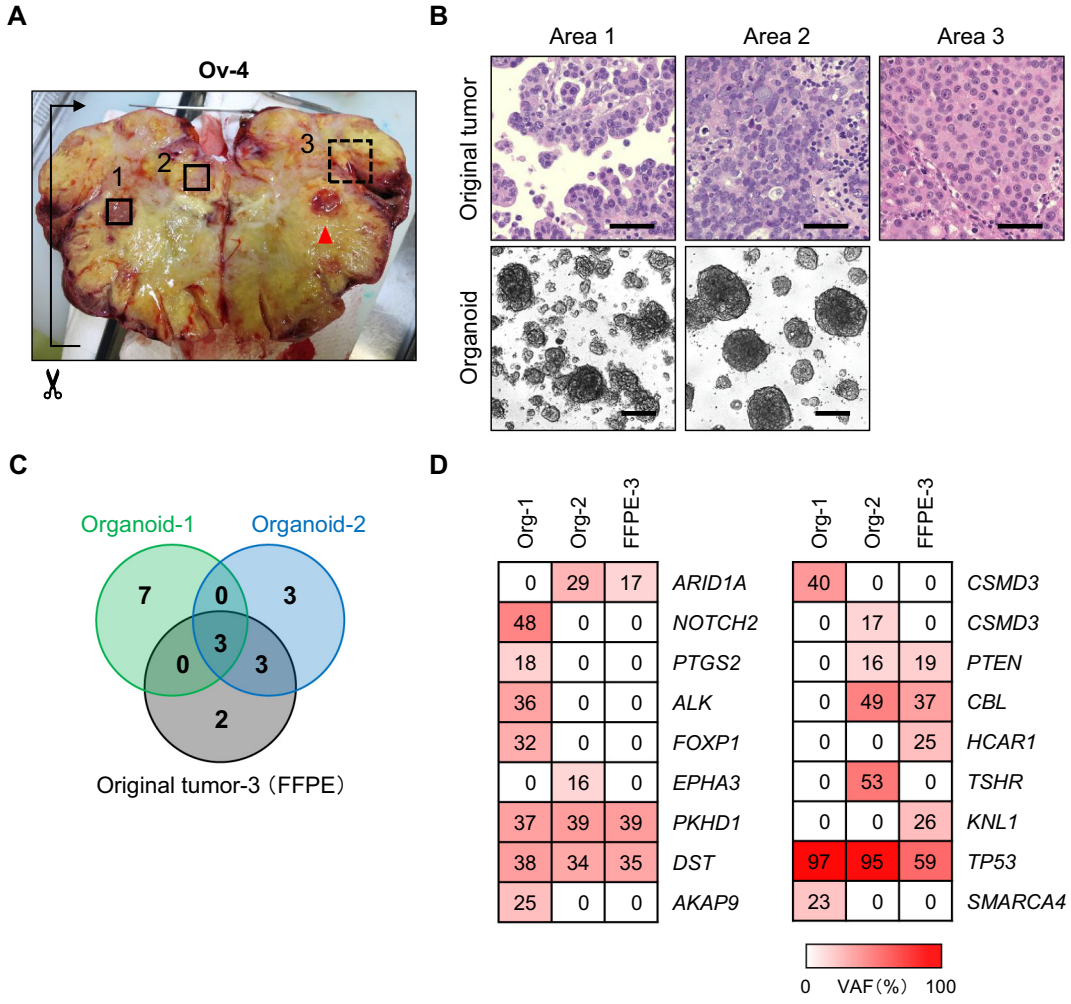

Fig. 4. Marked heterogeneity of tumor-derived organoids established from different sites within the solid ovarian tumor. (A) Macroscopic findings of a cut and opened section of an ovarian tumor (Ov-4). Solid boxes indicate sampling sites for organoid culture. Dashed box indicates sites where DNA was extracted from FFPE sample. Red arrow means opposite side of solid box 1 (B) Bright field images of tumor-derived organoids together with H&E staining of the original tumors. Upper and lower scale bars indicate 50 μm and 200 μm, respectively. (C) Venn diagram of a total of 19 S-SNVs with VAF > 10% identified from each sample. (D) Detail of VAF of 19 S-SNVs identified from 2 tumor-derived organoids (Org-1, Org-2) and the original tumor (FFPE-3). The VAF is indicated according to the red color scale. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Organoid culture was conducted from 2 lesions (Org-1, 2) without obvious macroscopic differences. Both organoids, in a cystic or dense structure with budding (Fig. S4A, B), were morphologically indistinguishable. A total of 34 S-SNVs were identified in the two organoids and the original tumor (FFPE-3) by NGS, in which 31 out of 34 S-SNVs were shared by all samples (Fig. S4C). These results suggest that this tumor might lack remarkable intra-tumoral heterogeneity. Somatic mutations identified included commonly mutated genes in endometrial cancer [28,29], such as *PTEN*, *PIK3CA*, and *ARID1A* (Fig. S4D, Table S4). Whereas the average VAF of S-SNVs identified in the tumor and both organoids was around 35% and 50%, respectively, suggesting uniform enrichment for epithelial cells in organoids. Similarly, the VAF of a *PTEN* mutation proportionally increased from 68% to 99%. These results suggested that cancer cells with a mutation and LOH in *PTEN* almost exclusively composed organoids, and that such oligopoly situation was already evident in the original tumor. In contrast, the VAF of mutant *ARID1A* was 77% in the tumor, but remained 81% in organoids, suggesting that cancer cells with mutant *ARID1A* might be rather excluded in organoid culture. Notably, S-SNVs of *CHEK2* were identified in this case (Fig. S4D, Table S4), strongly suggesting that this might account for the observed mutator phenotype. Taken together, organoids with distinct mutation profiles were established from macroscopically distinct sites, but not from similar lesions, of the same tumors. These observations suggest that organoids might robustly carry over intra-tumoral heterogeneity.

3.6. Feasibility of organoid-derived spheroids on drug sensitivity assay

Finally, we asked if drug sensitivity of the tumors could be evaluated with gynecologic tumor-derived organoids. As a Matrigel-based organoid culture could be bothersome in high-throughput screening, we determined to generate spheroids from organoids for ATP-based cell viability assay. We first compared the proliferative capacity of organoids in Matrigel and organoid-derived spheroids and found that organoids tended to grow faster than spheroids over time (Fig. 5A). As organoids and spheroids proliferated comparably, at least for a week from the beginning of the culture, we conducted drug sensitivity assay within a week. A total of nine patient-derived ovarian cancer cells

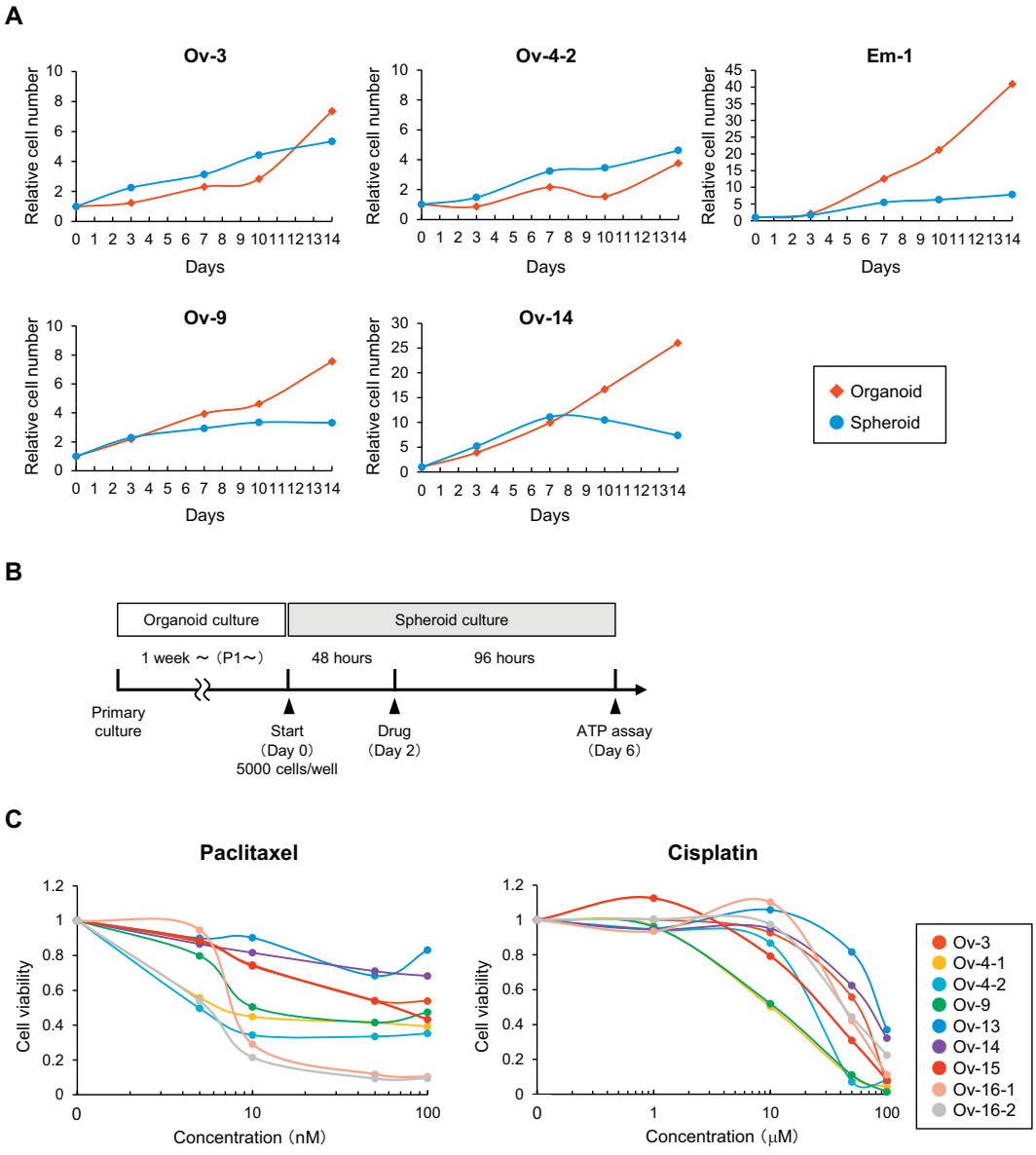

Fig. 5. Proliferative capacity and drug response of tumor-derived organoids and spheroids. (A) Comparison of proliferative capacity between Matrigel-based organoid culture and spheroid culture using established organoids. Red square and blue circle indicate organoid and spheroid, respectively. (B) Protocol of drug sensitivity assays from tumor-derived organoids. (C) Dose response curves for tumor-derived organoids treated with paclitaxel and cisplatin.

were treated by standard therapeutics for gynecologic cancer (Fig. 5B). Effective dose ranges for paclitaxel and cisplatin were 10 to 100 nM and 10 to 100 μM respectively (Fig. 5C), similar to earlier studies using patient-derived spheroids [30–32]. These results suggest that organoid-turned spheroids could be used for drug sensitivity assay of gynecologic tumors.

4. Discussion

In this study, we modified the original MBOC protocol [13,14] so as to cope with the digestion-resistant and aggregation-prone nature of resected human gynecologic tumors. These alterations resulted in a significant increase in the absolute number of recovered cells to initiate 3D culture, which in turn led to a remarkable improvement in the success rate of organoid culture. This finding is in line with the fact that past studies on spheroid culture of patient-derived ovarian cancers have been conducted predominantly with HGSC samples derived from ascites or peritoneal dissemination [9,32], which generally contain fewer stromal cells and are easily dissociated.

It was reported that the success rate of spheroid culture from ovarian HGSC tissues was only 13% [9]. More recently, a high success rate (80–90%) of organoid culture was reported for ovarian HGSC, but the tumors were mostly in advanced stages [33]. In contrast, we were able to conduct organoid culture not only with HGSC, but also mucinous and endometrioid carcinoma, and even three cases of borderline tumors. Similarly, we achieved a high success rate for endometrial cancer of all grades. The success rate of 62% was recently documented for endometrial cancer by the cancer tissue-originated spheroid (CTOS) technique [34], a modified method for primary culture from cell clusters that retained cell-cell contact [35]. One caveat with CTOS is that it does not grow beyond certain size. Accordingly, spheroid propagation was not mandatory for success in their criteria, and expansion of CTOS could be alternatively achieved by PDX development [35]. In contrast, in this study, endometrial cancer organoids robustly propagated *in vitro*, while organoid-turned spheroids could proliferate no faster than organoids, suggesting that organoids might have advantages in *in vitro* propagation over spheroid and CTOS.

On the other hand, xenograft take rate for organoids was 25% in this study, significantly lower than those in earlier PDX studies in endometrial [36] and ovarian cancer [37], achieving take rate at 60% and 53%, respectively. Due to the lack of direct comparison with the same tumor samples between the two methods, it is technically difficult to address which method is more efficient in establishing xenografts. One possible explanation for this discrepancy is that, whereas we mainly focused on low-grade tumor, the past PDX studies predominantly included high-grade tumor and used more severely immuno-compromised mice. Of course there is a possibility that organoid culture could select for cancer cells with lower tumorigenicity, which is further to be investigated.

Tumor-derived organoids retained both histological and genetic features and intra-tumoral heterogeneity of the original tumors in colon, breast, and bladder cancers [25,26,38,39]. In this study, we also confirmed that it was also the case with gynecologic tumors. Moreover, the mutation profile was not significantly affected by long-term organoid culture. Given a high value on both inter- and intra-tumor heterogeneity in preclinical studies of novel therapeutics, these features might highlight the relevance of gynecologic tumors organoids. Among mutations identified in this study, the VAFs of *TP53* and *PTEN* mutations were stereotypically enriched from 70% in tumors to nearly 100% in organoids, suggesting that LOH and a point mutation in each gene was a founder mutation shared by most cancer cells in the tumor, which were enriched simply as epithelial cells in organoids. In contrast, the VAFs of *NF1* and *ARID1A* mutations in organoids significantly increased from 13% to 95% and remained unaltered from 77% to 81%, suggestive of intra-tumoral heterogeneity and/or clonal selection during culture. Taken together, observed enrichment or exclusion of cancer cells with bi-allelic inactivation of tumor suppressor genes provided insights into the pathogenesis of gynecologic tumors. Although we conducted NGS analysis only a subset of samples for the purpose of genetic fingerprinting, it would be worth deep sequencing all the organoids obtained for the future application to drug discovery, which is currently ongoing.

In conclusion, we established a highly efficient and robust method for primary organoid culture of gynecologic tumors. Although further studies are required to examine many more tumors and cover all the histological subtypes, this approach will likely provide a foundation for using patient-derived tumors in many areas of gynecologic cancer research, including elucidation of its pathogenesis and development of novel therapies. Accordingly, future realization of precision medicine for gynecologic cancer will be also warranted.

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Conflict of interest statement

The authors declare no conflict of interest.

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Author contributions

Y.H. planned and supervised the study. Y.M. conducted the experiments. N.T. and M.I. analyzed the data. Y.M. and Y.H. wrote the manuscript.

References

[1] J. Greshock, K.E. Bachman, Y.Y. Degenhardt, J. Jing, Y.H. Wen, S. Eastman, et al., Molecular target class is predictive of in vitro response profile, Cancer Res. 70 (9) (2010) 3677–3686.
[2] J.P. Gillet, S. Varma, M.M. Gottesman, The clinical relevance of cancer cell lines, J. Natl. Cancer Inst. 105 (7) (2013) 452–458.
[3] J. Arrowsmith, P. Miller, Trial watch: phase II and phase III attrition rates 2011–2012, Nat. Rev. Drug Discov. 12 (8) (2013) 569.
[4] J. Arrowsmith, Trial watch: phase III and submission failures: 2007–2010, Nat. Rev. Drug Discov. 10 (2) (2011) 87.
[5] A. Bertotti, G. Migliardi, F. Galimi, F. Sassi, D. Torti, C. Isella, et al., A molecularly annotated platform of patient-derived xenografts ("xenopatients") identifies HER2 as an effective therapeutic target in cetuximab-resistant colorectal cancer, Cancer Discov. 1 (6) (2011) 508–523.
[6] A.T. Byrne, D.G. Alferez, F. Amant, D. Annibali, J. Arribas, A.V. Biankin, et al., Interrogating open issues in cancer precision medicine with patient-derived xenografts, Nat. Rev. Cancer 17 (4) (2017) 254–268.
[7] L.B. Weiswald, D. Bellet, V. Dangles-Marie, Spherical cancer models in tumor biology, Neoplasia 17 (1) (2015) 1–15.
[8] H. Ohata, T. Ishiguro, Y. Aihara, A. Sato, H. Sakai, S. Sekine, et al., Induction of the stem-like cell regulator CD44 by Rho kinase inhibition contributes to the maintenance of colon cancer-initiating cells, Cancer Res. 72 (19) (2012) 5101–5110.
[9] T. Ishiguro, A. Sato, H. Ohata, Y. Ikarashi, R.U. Takahashi, T. Ochiya, et al., Establishment and characterization of an in vitro model of ovarian cancer stem-like cells with an enhanced proliferative capacity, Cancer Res. 76 (1) (2016) 150–160.
[10] T. Sato, R.G. Vries, H.J. Snippert, M. van de Wetering, N. Barker, D.E. Stange, et al., Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche, Nature 459 (7244) (2009) 262–265.
[11] S. Bartfeld, T. Bayram, M. van de Wetering, M. Huch, H. Begthel, P. Kujala, et al., In vitro expansion of human gastric epithelial stem cells and their responses to bacterial infection, Gastroenterology 148 (1) (2015) 126–136 (e6).
[12] M. Matano, S. Date, M. Shimokawa, A. Takano, M. Fujii, Y. Ohta, et al., Modeling colorectal cancer using CRISPR-Cas9-mediated engineering of human intestinal organoids, Nat. Med. 21 (3) (2015) 256–262.

[13] K. Onuma, M. Ochiai, K. Orihashi, M. Takahashi, T. Imai, H. Nakagama, et al., Genetic reconstitution of tumorigenesis in primary intestinal cells, Proc. Natl. Acad. Sci. U. S. A. 110 (27) (2013) 11127–11132.
[14] Y. Maru, K. Onuma, M. Ochiai, T. Imai, Y. Hippo, Shortcuts to intestinal carcinogenesis by genetic engineering in organoids, Cancer Sci. 110 (3) (2019) 858–866.
[15] T. Sato, M. Morita, R. Tanaka, Y. Inoue, M. Nomura, Y. Sakamoto, et al., Ex vivo model of non-small cell lung cancer using mouse lung epithelial cells, Oncol. Lett. 14 (6) (2017) 6863–6868.
[16] M. Ochiai, Y. Yoshihara, Y. Maru, M. Tetsuya, M. Izumiya, T. Imai, et al., Kras-driven heterotopic tumor development from hepatobiliary organoids, Carcinogenesis (2019)https://doi.org/10.1093/carcin/bgz024 in press.
[17] Y. Maru, K. Orihashi, Y. Hippo, Lentivirus-based stable gene delivery into intestinal organoids, Methods Mol. Biol. 1422 (2016) 13–21.
[18] S.F. Boj, C.I. Hwang, L.A. Baker, I.I. Chio, D.D. Engle, V. Corbo, et al., Organoid models of human and mouse ductal pancreatic cancer, Cell 160 (1–2) (2015) 324–338.
[19] D. Gao, I. Vela, A. Sboner, P.J. Iaquinta, W.R. Karthaus, A. Gopalan, et al., Organoid cultures derived from patients with advanced prostate cancer, Cell 159 (1) (2014) 176–187.
[20] X. Li, H.E. Francies, M. Secrier, J. Perner, A. Miremadi, N. Galeano-Dalmau, et al., Organoid cultures recapitulate esophageal adenocarcinoma heterogeneity providing a model for clonality studies and precision therapeutics, Nat. Commun. 9 (1) (2018) 2983.
[21] H.H.N. Yan, H.C. Siu, S. Law, S.L. Ho, S.S.K. Yue, W.Y. Tsui, et al., A comprehensive human gastric cancer organoid biobank captures tumor subtype heterogeneity and enables therapeutic screening, Cell Stem Cell 23 (6) (2018) 882–897 (e11).
[22] M.Y. Turco, L. Gardner, J. Hughes, T. Cindrova-Davies, M.J. Gomez, L. Farrell, et al., Long-term, hormone-responsive organoid cultures of human endometrium in a chemically defined medium, Nat. Cell Biol. 19 (5) (2017) 568–577.
[23] E. Girda, E.C. Huang, G.S. Leiserowitz, L.H. Smith, The use of endometrial cancer patient-derived organoid culture for drug sensitivity testing is feasible, Int. J. Gynecol. Cancer 27 (8) (2017) 1701–1707.
[24] Y. Maru, N. Tanaka, M. Ohira, M. Itami, Y. Hippo, H. Nagase, Identification of novel mutations in Japanese ovarian clear cell carcinoma patients using optimized targeted NGS for clinical diagnosis, Gynecol. Oncol. 144 (2) (2017) 377–383.
[25] M. van de Wetering, H.E. Francies, J.M. Francis, G. Bounova, F. Iorio, A. Pronk, et al., Prospective derivation of a living organoid biobank of colorectal cancer patients, Cell 161 (4) (2015) 933–945.
[26] N. Sachs, J. de Ligt, O. Kopper, E. Gogola, G. Bounova, F. Weeber, et al., A living biobank of breast cancer organoids captures disease heterogeneity, Cell 172 (1–2) (2018) 373–386 (e10).
[27] UKCCCR guidelines for the use of cell lines in cancer research, Br. J. Cancer 82 (9) (2000) 1495–1509.
[28] C. Kandoth, N. Schultz, A.D. Cherniack, R. Akbani, Y. Liu, H. Shen, et al., Integrated genomic characterization of endometrial carcinoma, Nature 497 (7447) (2013) 67–73.
[29] M.P. Hayes, H. Wang, R. Espinal-Witter, W. Douglas, G.J. Solomon, S.J. Baker, et al., PIK3CA and PTEN mutations in uterine endometrioid carcinoma and complex atypical hyperplasia, Clin. Cancer Res. 12 (20 Pt 1) (2006) 5932–5935.
[30] H.G. Lee, S.J. Shin, H.W. Chung, Salinomycin Reduces Stemness and Induces Apoptosis on Human Ovarian Cancer Stem Cell, vol. 28(2), 2017 e14.
[31] J. Hirst, H.B. Pathak, S. Hyter, Z.Y. Pessetto, T. Ly, S. Graw, et al., Licofelone enhances the efficacy of paclitaxel in ovarian cancer by reversing drug resistance and tumor stem-like properties, Cancer Res. 78 (15) (2018) 4370–4385.
[32] S. Raghavan, P. Mehta, M.R. Ward, M.E. Bregenzer, E.M.A. Fleck, L. Tan, et al., Personalized medicine-based approach to model patterns of chemoresistance and tumor recurrence using ovarian cancer stem cell spheroids, Clin. Cancer Res. 23 (22) (2017) 6934–6945.
[33] S.J. Hill, B. Decker, Prediction of DNA repair inhibitor response in short term patient-derived ovarian cancer organoids, Cancer Discov. 8 (11) (2018) 1404–1421.
[34] Y. Kiyohara, K. Yoshino, S. Kubota, H. Okuyama, H. Endo, Y. Ueda, et al., Drug screening and grouping by sensitivity with a panel of primary cultured cancer spheroids derived from endometrial cancer, Cancer Sci. 107 (4) (2016) 452–460.
[35] J. Kondo, H. Endo, H. Okuyama, O. Ishikawa, H. Iishi, M. Tsujii, et al., Retaining cell-cell contact enables preparation and culture of spheroids composed of pure primary cancer cells from colorectal cancer, Proc. Natl. Acad. Sci. U. S. A. 108 (15) (2011) 6235–6240.
[36] J. Depreeuw, E. Hermans, S. Schrauwen, D. Annibali, L. Coenegrachts, D. Thomas, et al., Characterization of patient-derived tumor xenograft models of endometrial cancer for preclinical evaluation of targeted therapies, Gynecol. Oncol. 139 (1) (2015) 118–126.
[37] K.J. Eoh, Y.S. Chung, S.H. Lee, S.A. Park, H.J. Kim, W. Yang, et al., Comparison of clinical features and outcomes in epithelial ovarian cancer according to tumorigenicity in patient-derived xenograft models, Cancer Res. Treat. 50 (3) (2018) 956–963.
[38] S.H. Lee, W. Hu, J.T. Matulay, M.V. Silva, T.B. Owczarek, K. Kim, et al., Tumor evolution and drug response in patient-derived organoid models of bladder cancer, Cell 173 (2) (2018) 515–528 (e17).
[39] F. Weeber, M. van de Wetering, M. Hoogstraat, K.K. Dijkstra, O. Krijgsman, T. Kuilman, et al., Preserved genetic diversity in organoids cultured from biopsies of human colorectal cancer metastases, Proc. Natl. Acad. Sci. U. S. A. 112 (43) (2015) 13308–13311.