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Optimization of endometrial cancer organoids establishment by cancer-associated fibroblasts

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Most endometrial cancers (EC) are diagnosed at an early stage with a favorable prognosis. However, for patients with advanced or recurrent disease, the chemotherapy response rate and overall survival remain poor. A novel *in vitro* model, tumor organoids, has important value in providing a more individualized treatment plan for tumor patients. However, the slow growth of the established EC organoid seriously hinders the application of EC organoids. Cancer-associated fibroblasts (CAFs), the main component of tumor stroma, have been reported to promote the proliferation of endometrial cancer cell lines and primary endometrial cancer cells *in vivo* and *in vitro*. Therefore, we optimized the current endometrial cancer organoid by introducing CAFs isolated from EC lesions. Here we developed long-term expandable organoids from endometrial cancer lesions, which show disease-associated traits and cancer-linked mutations. Based on the co-culture of CAFs and endometrial cancer organoids, we found that CAFs could promote the growth of endometrial cancer organoids, might by secreting factors according to the result that CAFs could also promote the growth. Our research provided a more promising model for the basic and preclinical study of endometrial cancer.

Key words: endometrial cancer, organoids, cancer-associated fibroblasts, optimization

Endometrial cancer (EC) is the sixth most common female cancer worldwide and the fourth most common in high-income countries [1]. The incidence of EC has risen by 57% since the early 1990s in some populations [1]. There are two types of endometrial cancer: type I EC is estrogen-dependent with a favorable prognosis (85% 5-years survival rate); type II EC is estrogen-independent and frequently high grade, with a poorer prognosis (25–60% 5-years survival rate) and a high risk of metastasis [2].

Most endometrial cancer patients are diagnosed at an early stage with a favorable prognosis. However, for patients with advanced or recurrent disease, the chemotherapy response rate and overall survival remain poor [3]. Precision therapies, including chemotherapy and targeted therapies after drug sensitization, are emerging as an individualized treatment for advanced and recurrent endometrial cancer patients [3]. A model that more closely resembles *in vivo* tumors needs to be developed and applied in individualized treatment.

Organoids, as an excellent preclinical model, have important value in providing a more individualized treatment plan for tumor patients and improving the prognosis of tumor patients [4–8]. However, the slow growth of the established EC organoids seriously limits their application [9]. Thus, a more efficient culture system needs to be established. It is well known that the tumor microenvironment contributes to the development and progression of tumors [10]. As the main component of the tumor microenvironment, cancer-associated fibroblasts (CAFs) constitute a major portion of the reactive tumor stroma and play a crucial role in tumor progression [11–13]. Current studies have found that CAFs could promote the proliferation of both EC cell lines and primary tumor cells by secreting various factors including EGF and FGF [14, 15]. Thus, optimizing the EC organoid culture system with CAFs to promote EC organoid proliferation would be feasible and remarkable.

Here, we successfully established endometrial cancer cell line-originated spheroid (CCLOS) and patient-derived

endometrial cancer organoids. The patient-derived organoids showed long-term expandability, genomic, and replicate disease diversity. We found that the proliferation of EC CCLOS/organoids was promoted after co-culturing with CAFs. Furthermore, we also found that the culture supernatant of CAFs promoted the proliferation of EC CCLOS/organoids. In summary, we successfully established an EC organoid culture system with higher proliferation efficiency, which provided a more effective model for the investigation and preclinical study of EC.

Patients and methods

Human tissues and cell lines. EC tissues were obtained from Shanghai Tenth People's Hospital Affiliated with Tongji University. Only "leftover" tissues were utilized from hysterectomy specimens from EC patients who had not been treated with preoperative chemotherapy or radiotherapy. The study was approved by the Ethics committee of Shanghai Tenth People's Hospital of Tongji University (20K158). The demographic and tumor characteristics of the enrolled subjects are shown in Supplementary Table S1. The specimens and associated clinical information were anonymized.

EC cell lines HEC-1A and RL95-2 were purchased from Shanghai EK-Bioscience Biotechnology Co., Ltd with short tandem repeat (STR) identification reports.

Patient-derived organoids/endometrial cancer cell line-originated spheroid establishment. Endometrial cancer cell line-originated spheroids (CCLOS) were established with RL95-2 and HEC-1A cells. RL95-2 and HEC-1A cells were cultured in a complete medium (DMEM/F12 and RPMI1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin, respectively) unless specifically stated. The cells were washed with PBS and digested with 0.25% trypsin for 3 min at 37°C. After digestion, the cells were neutralized in the complete medium. Then, cells were centrifuged at 1,300×g for 3 min. After centrifugation, the cells were recovered, resuspended in the complete medium, and counted in a hemocytometer. The cell pellets were resuspended in the cold medium in preparation for bulk cultivation. For bulk spheroid culture, dissociated endometrial cancer cells, suspended in 75% Matrigel (Corning, USA)/25% complete medium at 2×10^6/ml, were deposited in droplets of 15 µl into prewarmed 24-well plates (30 µl/well in total). The inverted plates were placed at 37°C with 5% CO_2, and the droplets were allowed to solidify for 10 min. Then, the droplets were dissociated from the plate and suspended in the 1 ml cell line complete medium (Supplementary Figure S1). Cultures were maintained by medium change every 72 h.

As for patient-derived organoids, biopsies were obtained from patients with EC (Supplementary Table S1). The fresh EC tissues were obtained and transported to the laboratory in HBSS (Thermo Fisher, USA). The tissues were minced to the size of 1 mm^3 and then were digested with collagenase I (1 mg/ml) in DMEM/F12 supplemented with 10 µM Rock inhibitor (Y-27632) for 1–3 h at 37°C. Digestion was stopped by medium dilution (without serum) and the cell suspension was filtered with a 100 µm filter. After digestion, the tissues were treated with erythrocyte lysis solution and washed twice with PBS. Part of the pellets was used for the establishment of primary fibroblast cells; the rest of the pellets was used to establish organoids with the same method as cell lines organoids except for medium. The basic medium for patient-derived organoids is described in Supplementary Table S2. Organoids were cultured with the basic medium and the medium was changed every two to three days. For passaging (performed every 10–20 days), organoids were recovered by digesting the Matrigel drop with TrypLE Express (Thermo Fisher) and mechanical pipetting to ensure the maximum collection of organoids. The mixture was centrifuged at 1,300×g for 3 min and the fragments and cells obtained were resuspended in 75% Matrigel/25% organoid basic medium and droplets were deposited in 24-well plates. Established organoids were amplified, cryopreserved for biobanking, and subjected to downstream analyses. Unless otherwise stated, organoids of low passage number (P3–P6) were used for the experiments described (Supplementary Figure S1).

Immunohistochemistry. The sections of tumor tissue and organoid were fixed overnight in 4% PFA prior to paraffin wax professing and embedding. Tissue sections were cut to 4 µm thickness. For immunohistochemical analysis, endogenous peroxidase was blocked with 0.3% hydrogen peroxide for 30 min. Antigen was retrieved using a sodium citrate buffer method by heating at 100°C for 30 min. Slides were then incubated with the following antibodies for 1 h, diluted according to the manufacturer protocol: ER-α (1:100 dilutions, Abcam), PGR (1:100 dilutions, Affinity), Ki-67 (1:200 dilutions, BD Biosciences), KRT7 (1:200 dilutions, Abclonal), TP53 (1:200 dilutions, Abcam) (Supplementary Table S2). A labeled streptavidin-biotin system with a horse-radish peroxidase label was used to detect the primary antibodies and visualized by incubation with 3,3'-diaminobenzidine chromogen and hydrogen peroxide substrate for 10 min. The slides were then counterstained with hematoxylin and mounted in dibutyl phthalate xylene.

Whole-exome sequencing. Whole-exome gene sequencing was performed using tumor tissues and paired organoids derived from the patients. The clustering of the index coded samples was performed on a cBot Cluster Generation System using Hiseq PE Cluster Kit (Illumina) according to the manufacturer's instructions. After cluster generation, the DNA libraries were sequenced with the Illumina Hiseq platform and 150 bp paired-end reads were generated. Quality control statistics including total reads number, raw data, raw depth, sequencing error rate, percentage of reads with Q30 (the percent of bases with Phred-scaled quality scores greater than 30), and GC content distribution were calculated and summarized. Same tools mpileup and bcftools were used to do the variant calling and identify SNP, InDels, as previously described [16]. ANNOVAR was performed

to do annotation for Variant Call Format obtained in the previous effort [17]. dbSNP, 1000 Genome, and other related existing databases were applied to characterize the detected variants. The somatic SNV was detected by muTect [18] and the somatic InDel by Strelka [19]. Control FREEC was used to detect somatic CNV [20].

Establishment of primary fibroblast cells. The digested and filtered tissues mentioned above were also used for primary fibroblast cells establishment. The pellets were cultured in DMEM/F12 medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C. The medium was changed every 72 h, and subculture was performed after the cultures reached confluence. After two to three passages, a unique homogeneity of stromal fibroblasts was formed (Supplementary Figure S1). All the stromal fibroblasts used in the experiments were at less than 10 passages, to maintain the closest phenotype to the primary tissues.

Immunofluorescence. CAFs were harvested on slides and fixed in 4% PFA. Then, the slides were permeated with 0.5% Triton X-100/PBS for 5 min and blocked with 3% bovine serum albumin. Slides were incubated with anti-α-SMA (1:200 dilutions, Abcam, USA), anti-Vimentin (1:200 dilutions, Abcam) overnight at 4°C (Supplementary Table S2). Slides were washed with PBS and incubated with secondary antibody (1:1000 dilutions) for 2 h at RT. Nuclei were counterstained with DAPI (1:1000 dilutions) for 30 min.

Proliferation evaluation of endometrial cancer organoids/CCLOS. 1×10^5 CAFs were seeded at the bottom of a 12-well plate with CCLOS (1×10^3 cells) cultured in the Transwell insert (0.4 μm) in the cell line complete medium. After 24 h, the medium was changed to DMEM/F12 or RPMI 1640 medium supplemented with 2% FBS and 1% penicillin/streptomycin, respectively. Then, cell growth was analyzed at 0 h, 24 h, 48 h, and 72 h with the Alamar Blue reagent after 12 h incubation following the manufacturer's directions. The medium was changed every two days.

CAFs were seeded and cultured in DMEM/F12 medium supplemented with 10% FBS for 24 h, before being cultured in the medium containing 2% FBS for the following 24 h. The culture supernatant was collected using a 15 ml centrifuge tube by centrifugation at 200×g for 3 min. Spheroids containing 1×10^3 HEC-1A cells or RL95-2 cells respectively were seeded in 1 ml complete medium in 24-well plates. At 24 h post-seeding, 500 μl fibroblast culture supernatant and 500 μl DMEM/F12 containing 2% FBS were added to the test group and 1 ml DMEM/F12 containing 2% FBS was added to the control group. As for patient-derived organoids, organoids of low passage (P3–P6) were used for the CAFs culture supernatant treatment experiment. 500 cells/15 μl Matrigel drop were plated into 24 well plates (1000 cells/well in total). At post-seeding, 500 μl CAFs culture supernatant and 500 μl organoid basic medium were added to the test group. 500 μl CAFs complete medium and 500 μl organoid basic medium were added to control wells. Cell growth was analyzed at 0 h, 24 h, 48 h, and 72 h after treatment of CAFs culture supernatant with the Alamar Blue reagent after 12 h incubation following the manufacturer's directions.

Statistics. Data are presented as the mean ± SD unless otherwise noted. The significance of differences was evaluated with Student's t-test. All analyses were performed with GraphPad Prism. When the p-value was <0.05, the results were considered to be statistically significant.

Result

Establishment of patient-derived endometrial cancer organoids. We first established a culture system for patient-derived cancer organoids (PDOs). Biopsies obtained from endometrial cancer patients were minced, digested, and seeded in Matrigel to grow as organoids (Supplementary Figure S1). Three patient-derived organoid lines (Supplementary Table S1) were used in the study, and the brightfield pictures showed their proliferation (Figure 1A).

Then, we verified the consistency between the organoid and the derived tissue by hematoxylin-eosin (HE) staining and immunohistochemistry (IHC). Estrogen receptor (ER) and progesterone receptor (PR) are thought to be molecular markers for endometrial cancer classification [21, 22]. Ki-67, tumor protein 53 (TP53), and keratin 7 (KRT7) are used for the verification of epithelial cancer [23]. HE staining showed that the organoids had a similar morphology to original tumor tissues (Figure 1B). IHC analysis showed that the organoids exhibited a similar expression pattern of ER, PR, Ki-67, KRT7, and TP53 with the corresponding tumor tissues (Figure 1B). We also noticed that, as the result in the brightfield pictures, the faster proliferating PDO had a higher Ki-67 ratio, indicating the different proliferative rates among the organoids derived from different patients and organoids derived from the same patient (Figure 1C).

Next, whole-exome sequencing (WES) was applied to compare the differences in genetic alteration between tumor tissues (EC-Primary) and their corresponding patient-derived organoids (EC-O). The results showed that the single nucleotide mutation type of EC-Primary and corresponding EC-O remained highly consistent (Figure 2A). Then we analyzed the gene mutations of EC-Primary and EC-O. The gene mutations were mostly the same in EC-Primary and corresponding EC-O (Figure 2B). The results showed that the gene mutation status in the EC-Primary was well reflected in the EC-O. 25 genes that were frequently mutated in EC were screened for further analysis. In cancer organoids, two of them (EC-O_1 and EC-O_3) were highly consistent in the number and type of mutant genes (Figure 2C). In particular, we found conservative PTEN mutations in EC-O_1 and EC-O_3 (Figure 2C). As a tumor suppressor gene, PTEN mutations are closely related to the occurrence of endometrial cancer (the probability of PTEN mutation in endometrioid adenocarcinoma is as high as 77%). In addition, the mutations of the oncogene PIK3CA and the tumor suppressor gene TP53 in EC-Primary_1 were also observed in the corre-

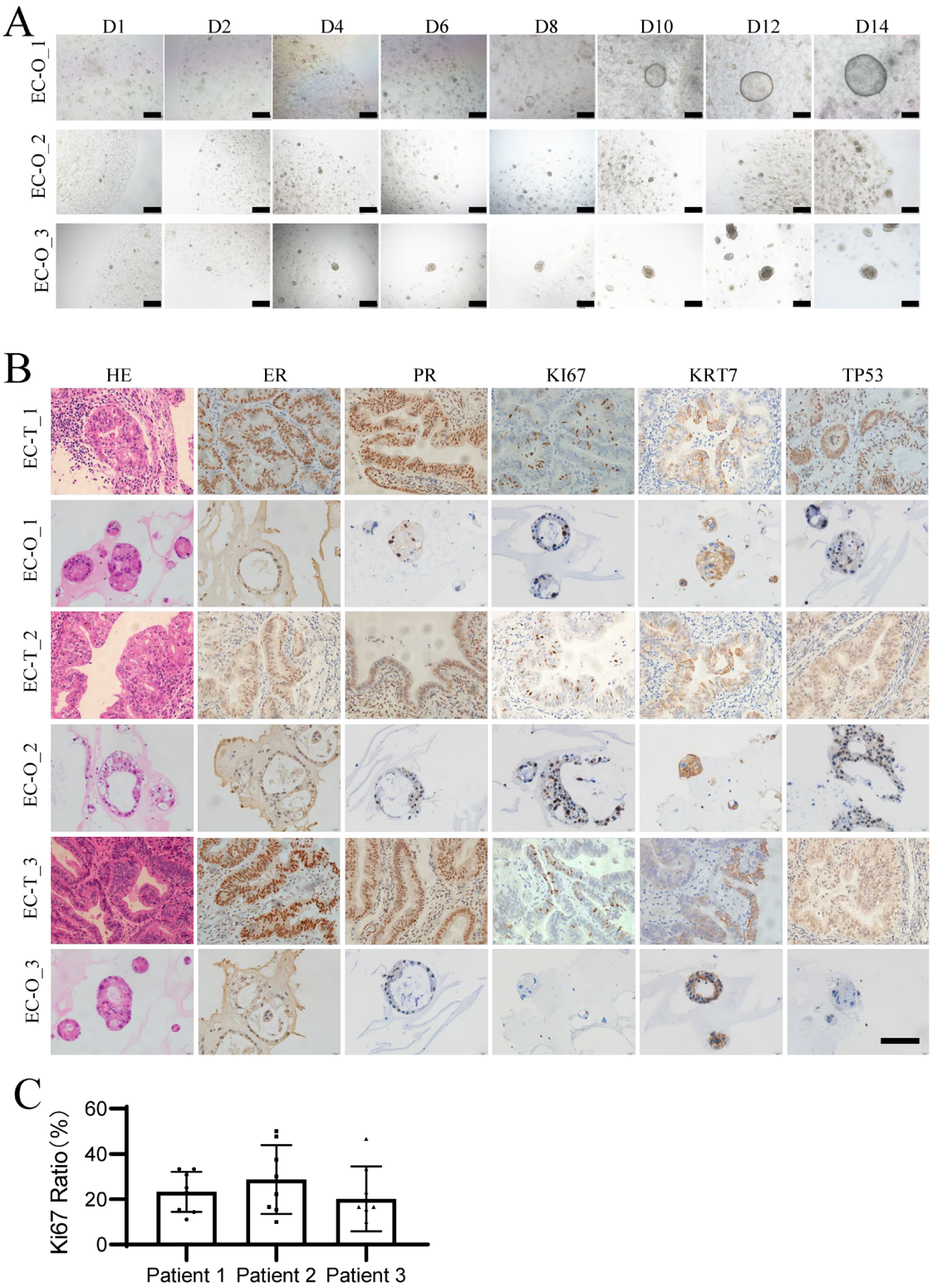

Figure 1. Patient-derived endometrial cancer organoids displayed a disease-associated phenotype. A) Representative bright-field images of EC organoids. The morphology of organoids varied in each case (Scale bar, 200 μm). B) Representative hematoxylin-eosin and IHC results for PDO cultures and the corresponding tumors (Scale bar, 100 μm). C) The Ki-67 ratio in different patient-derived organoids. For each patient, 8 organoids were included for counting respectively, and each dot represented an organoid. Ki-67 ratio = Number of Ki-67$^+$ cells/total number of cells. The data are shown as the means ± SD.

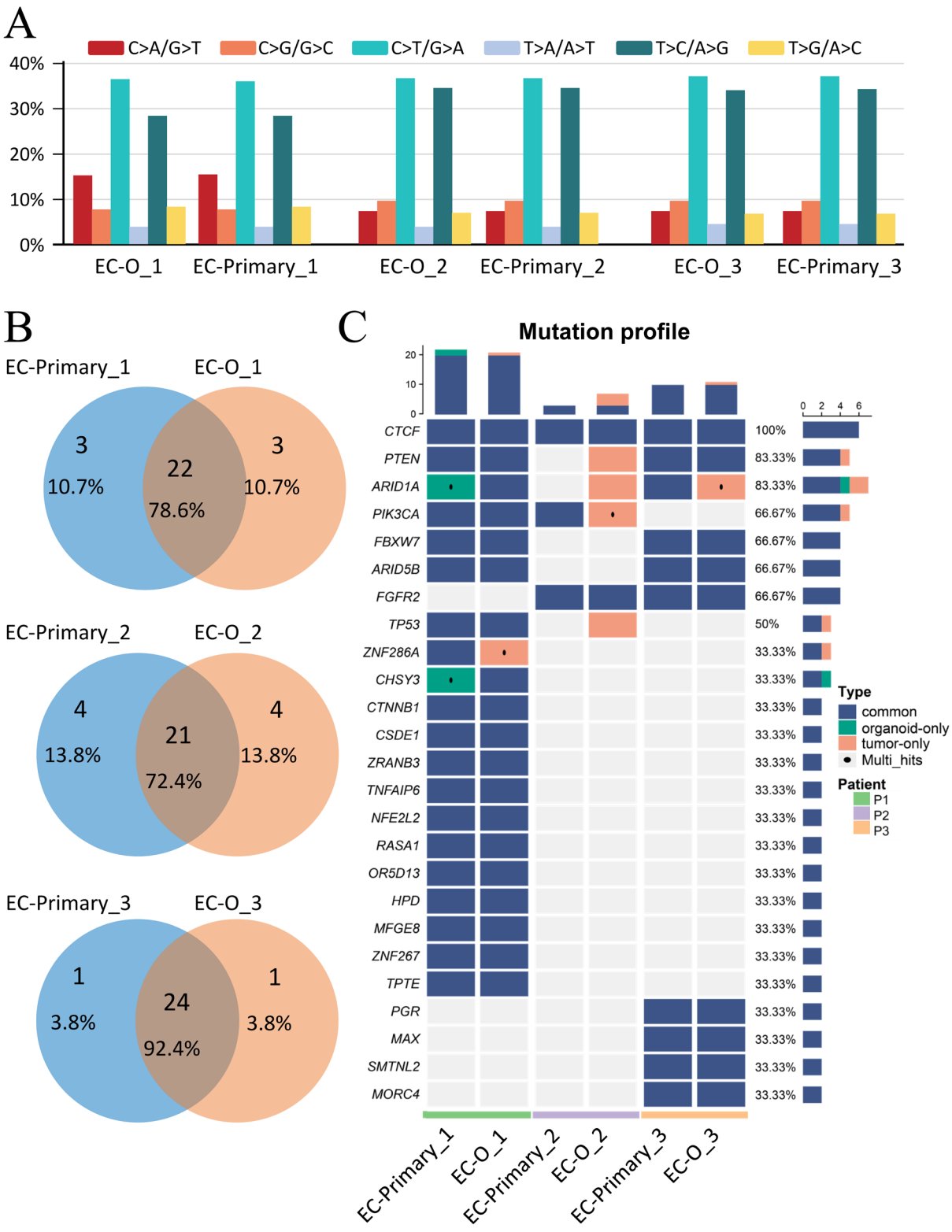

Figure 2. Patient-derived endometrial cancer organoids displayed consistent gene mutations to corresponding tissues. A) Diagram of single nucleotide mutation types of endometrial cancer tissues and corresponding organoids. B) Overlap of gene mutations in organoids and corresponding tissues. C) Landscape map of EC tissues and corresponding organoid gene mutations.

sponding organoid EC-O_1 (Figure 2C). We also noticed that EC-O_1, EC-O_2, and EC-O_3 all had new mutations compared to tumor tissue mutations, such as the ZNF286A gene in EC-O_1, PTEN in EC-O_2, ARAID1A, PIK3CA, FGFR2, and ARAID1A in EC-O_3.

CAFs promote the proliferation of organoids from EC cell lines. After the establishment of patient-derived endometrial cancer organoid (PDO), we intended to optimize the culture system based on CAFs. To establish primary fibroblast cells, EC tissues were digested and cultured in the FBS-contained medium to obtain pure CAFs. The primary fibroblast cells isolated from tumor tissues displayed cruciform or stellate-shaped features as typical CAFs (Figure 3A). The fibroblast cells were labeled with anti-α-SMA and anti-Vimentin antibodies to verify the purity. The results showed that CAFs isolated from EC tissues were highly positive for α-SMA and Vimentin expression (Figure 3B) and exhibited high purity.

Cancer cell line-originated spheroids (CCLOS) derived from EC cell lines, HEC-1A and RL95-2, were co-cultured with CAFs respectively to investigate whether CAFs promote the proliferation of CCLOS. The Alamar Blue assay analysis showed that both HEC-1A and RL95-2 CCLOS co-cultured with CAFs demonstrated stronger proliferation ability compared to the control ones (Figures 3C, 3D).

The culture supernatant of CAFs promoted the proliferation of EC organoids. The effect of the culture supernatant of CAFs on organoid proliferation was also accessed because it has been reported that CAFs culture supernatant promoted tumor cell proliferation and self-renewal of cancer stem cells [24, 25]. In detail, The CCLOS were treated with CAFs culture supernatant for 0 h, 24 h, 48 h, and 72 h. It showed that the CAFs culture supernatant promoted proliferation in both HEC-1A and RL95-2 CCLOS in a time-dependent manner (Figures 4A, 4B), which was consistent with the co-culture experiment (Figures 3C, 3D).

We further verified the promotional role of CAFs based on the PDO proliferation. EC organoids were treated by CAFs culture supernatant at 0 h, 24 h, 48 h, 72 h, and 96 h and sent for the Alamar Blue assay analysis. Similar to CCLOS, PDO treated with CAFs culture supernatant showed enhanced proliferation capacity in a time-dependent manner as CCLOS did (Figure 4C).

Taken together, the results showed that CAFs derived from EC tissues promoted the growth of spheroids/organoids derived from both EC cell lines and patient-derived EC tissues.

Discussion

The two-dimensional monolayer of cancer cells has been the most widely used *in vitro* model for tumors for the easy establishment and maintenance and long-term viability. However, it is often not as reflective of the biological properties of tumors *in vivo* due to the lack of 3D structural architecture [26]. Organoids are cultured in 3D

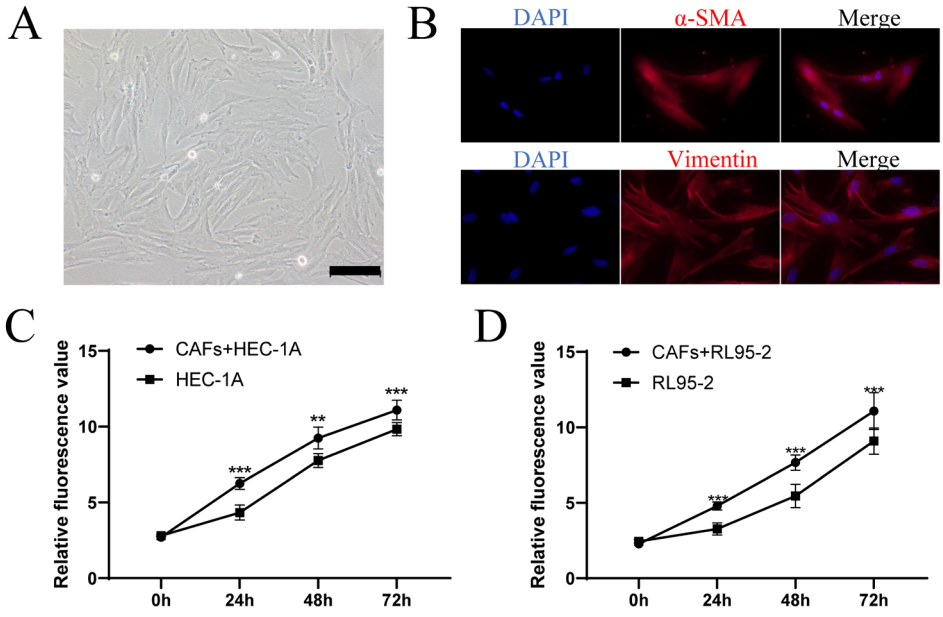

Figure 3. Identification of stromal fibroblasts and CAFs promoting the proliferation of endometrial cancer cell line-originated spheroids (CCLOS). A) Bright field of CAFs (Scale bar, 1000 μm). B) The immunofluorescence of DAPI, α-SMA, Vimentin, and merge of CAFs (Scale bar, 200 μm). C, D) CCLOS from HEC-1A (C) and RL95-2 (D) cell lines were co-cultured with CAFs. At 0 h, 24 h, 48 h, and 72 h after co-culture, Alamar Blue was used to measure cell proliferation (excitation wavelength is 530 nm, the emission wavelength is 590 nm), and standardized with the standard group, the experimental group and the control group have significant differences at 24 h, 48 h, and 72 h (mean ± SD, **p<0.01, ***p<0.001).

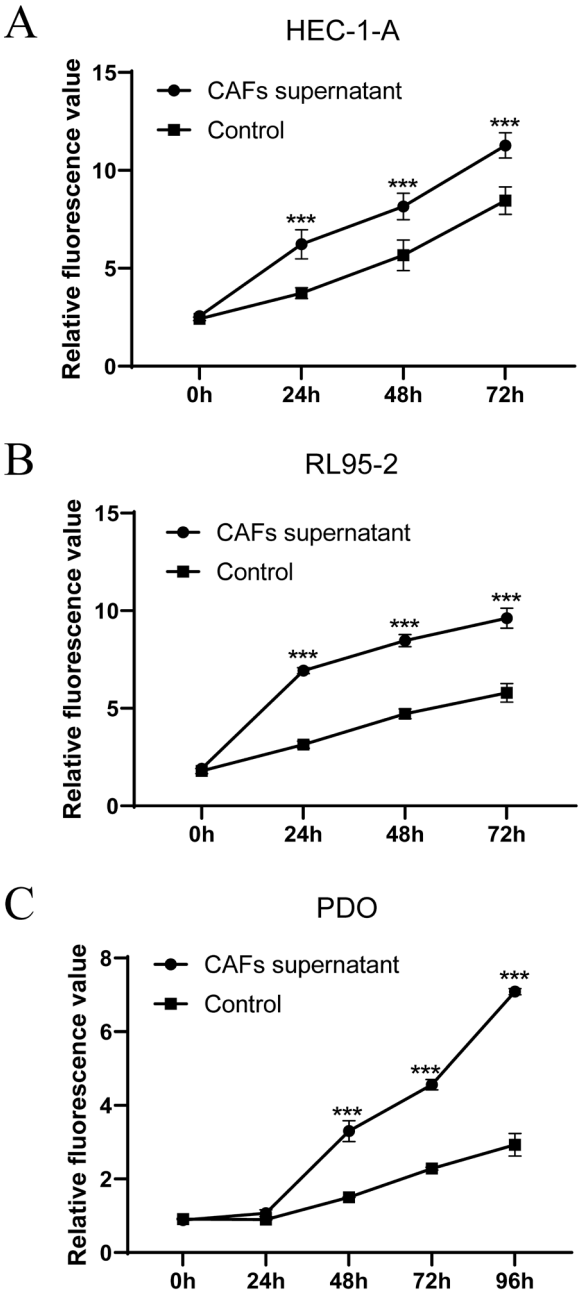

Figure 4. CAFs culture supernatant promoted the proliferation of EC CCLOS/PDO. A, B) CCLOS of HEC-1A (A) and RL95-2 (B) cell lines were treated by CAFs culture supernatant. At 0 h, 24 h, 48 h, 72 h after co-culture, Alamar Blue was used to measure cell proliferation, and standardized with the standard group, the experimental group (CAFs supernatant) and the control group (Control) had significant differences at 24 h, 48 h, and 72 h. C) Primary EC organoids were co-cultured with CAFs culture supernatant for 0 h, 24 h, 48 h, 72 h, 96 h. The cell proliferation was measured using Alamar Blue, and standardized with a standard set. The results showed that the experimental group (CAFs supernatant) and the control group (Control) had significant differences at 48 h, 72 h, and 96 h. Measured with Alamar Blue Cell proliferation, and standardize the data with the label set. The results showed that the experimental group and the control group had significant differences at 48 h, 72 h, and 96 h (mean ± SD, **p<0.05, **p<0.01, ***p<0.001).

and can provide a good platform for basic and preclinical research on tumors and have huge application prospects [27–33]. Our study established endometrial cancer patient-derived organoids and verified tissue consistency with tumor markers and WES. PTEN, PIK3CA, TP53, FBXW7, and CTCF mutations were observed in EC tissues and PDO, which were frequently mutated in EC as known. PTEN, PIK3CA, and FBXW7 mutations are usually thought to be good prognosis predictors [3]. WES results showed that gene mutations were mostly the same in EC tissues and corresponding PDO with some new mutations occurred in PDO, indicating that PDO inherited genetic traits in general but still had some differences in the culture. It reminded us that PDO should be examined and selected when experimenting with specific targets.

In the present study, we first confirmed that the proliferation of EC spheroids/organoids was promoted by CAFs co-culturing. Then, we found that CAFs culture supernatant also promoted the growth of EC organoids. It would provide a better platform for basic and preclinical research on EC.

In our study, we observed that CAFs could promote the proliferation of EC spheroids/organoids and can optimize the culture system based on co-culturing. However, we did not further explore the specific mechanism. It is well known that tumor stromal cells play an important role in the formation and maintenance of tumors [10]. As the main part of tumor stroma, CAFs have been proven to promote the growth of EC *in vivo* and *in vitro* [34, 35]. CAFs are reported to EC growth via activation of the interleukin-6/STAT-3/c-Myc pathway by secreting high levels of interleukin-6 [34]. SDF-1α secreted by CAFs also contributed to promoting proliferation, migration, and invasion as well as *in vivo* tumorigenesis of EC cells [35]. It is possible that these secreted factors are involved in the enhancement of organoid proliferation.

More studies are needed to focus on the interaction between tumor stroma and the tumor on the organoid platform. The specific effect factors secreted by CAFs, which contribute to promoting EC cell proliferation, need to be further clarified. And these secreted factors should make it easier to establish PDO for further application such as the establishment of tumor progression and tumor microenvironment models and preclinical trials in precision medicine and so on.

Taken together, our study demonstrates that CAFs derived from EC tissues promoted the growth of endometrial cancer cell line-originated spheroids and patient-derived endometrial cancer organoids. An optimized culture medium based on the result has been established for EC organoids.

Supplementary information is available in the online version of the paper.

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Optimization of endometrial cancer organoids establishment by cancer-associated fibroblasts

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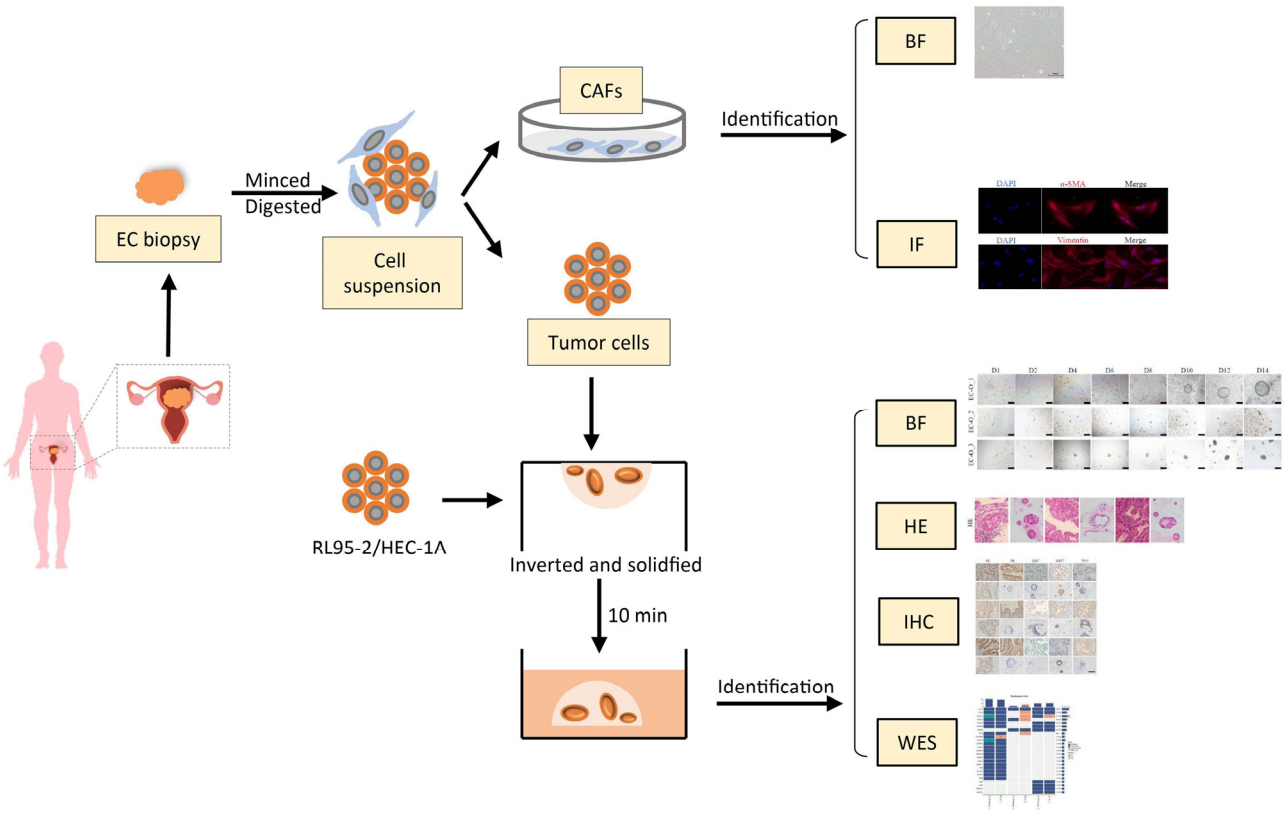
Supplementary Information



Supplementary Figure S1. The flow chart for the establishment and identification of organoids derived from tumor cell line and tumor tissues and CAFs derived from tumor tissues. Abbreviations: EC-endometrial cancer; BF-bright field; IF-immunofluorescence; HE-hematoxylin-eosin staining; IHC-immunohistochemistry; WES-whole exome sequencing

Supplementary Table S1. Characteristics of endometrial cancer patient in the study.

Id	Age	BMI	Pathology	Grade
EC-1	47	20	Endometrioid adenocarcinoma with partial clear differentiation	3
EC-2	44	25	Endometrioid adenocarcinoma	1–2
EC-3	50	22.3	Endometrioid adenocarcinoma	2

Supplementary Table S2. List of medium composition and antibodies.

Usage	Reagent	Company	Cat. No	Dilution /Concentration
PDO Basic medium	Advanced DMEM/F12	ThermoFisher	12634028	/
	Glutamax	ThermoFisher	35050061	1/100
	HEPES	ThermoFisher	15630080	1/100
	Nicotinamide	Sigma Aldrich	N0636	10 mM
	N-acetyl L-cysteine	Sigma Aldrich	A9165	1.25 mM
	SB202190	Sigma Aldrich	S7076	10 μM
	Prostaglandin E2	R&D Systems	2296	1 μM
	Noggin	Novoprotein	CB89	100 ng/ml
	A8301	Sigma Aldrich	SML0788	500 nM
	R-spondin-1	Novoprotein	CX83	100 ng/ml
	Y27632 2HCl	Selleck	S1049	5 mM
	β-Estradiol	Sigma Aldrich	E2758	100 nM
	B27	Gibco	17504044	1/50
	Matrigel	Corning	354230	/
IHC	ER-α	Abcam	ab108398	1/100
	PGR	Affinity	DF6829	1/100
	Ki67	BD Biosciences	556003	1/200
	KRT7	Abclonal	A2574	1/200
	TP53	Abcam	ab1101	1/200
ICC	Anti-α-SMA	Abcam	ab5694	1/200
	Anti-Vimentin	Abcam	Ab16700	1/200

Abbreviations: PDO-patient-derived endometrial cancer organoid; ICC-immunocytochemistry; IHC-immunohistochemistry