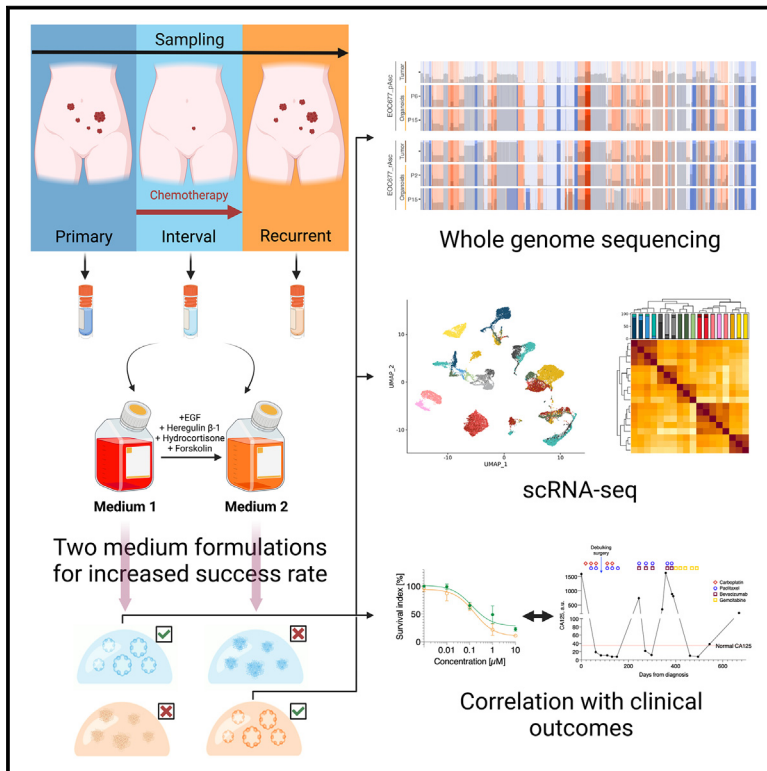


Developmental Cell

A platform for efficient establishment and drug-response profiling of high-grade serous ovarian cancer organoids

Graphical abstract



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In brief

Establishment of robust high-grade serous ovarian cancer (HGSC) organoid cultures has proven difficult. Senkowski et al. present a protocol for long-term culture of HGSC organoids from cryopreserved tissue at improved success rates, providing deep molecular model characterization and insights into correlating organoid drug responses with clinical outcomes.

Highlights

- Protocol for establishment and long-term culture of HGSC organoids
- Two culture medium formulations improve organoid establishment success rate
- HGSC organoids retain genomic and phenotypic characteristics of original tumors
- Culture medium impacts correlation of organoid drug responses with clinical outcomes



Technology

A platform for efficient establishment and drug-response profiling of high-grade serous ovarian cancer organoids

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SUMMARY

The broad research use of organoids from high-grade serous ovarian cancer (HGSC) has been hampered by low culture success rates and limited availability of fresh tumor material. Here, we describe a method for generation and long-term expansion of HGSC organoids with efficacy markedly improved over previous reports (53% vs. 23%–38%). We established organoids from cryopreserved material, demonstrating the feasibility of using viably biobanked tissue for HGSC organoid derivation. Genomic, histologic, and single-cell transcriptomic analyses revealed that organoids recapitulated genetic and phenotypic features of original tumors. Organoid drug responses correlated with clinical treatment outcomes, although in a culture conditions-dependent manner and only in organoids maintained in human plasma-like medium (HPLM). Organoids from consenting patients are available to the research community through a public biobank and organoid genomic data are explorable through an interactive online tool. Taken together, this resource facilitates the application of HGSC organoids in basic and translational ovarian cancer research.

INTRODUCTION

High-grade serous ovarian cancer (HGSC) is the most prevalent and lethal type of ovarian cancer (OC), accounting for 70%–80% of OC mortality.¹ HGSC is characterized by high molecular heterogeneity and shows only a few recurrent genetic abnormalities, including an almost universal loss of functional *TP53* (91%–96% of patients) or mutations in *BRCA1/2* genes (20%).^{2,3} HGSC patient survival rate has seen little improvement over the last few decades.¹ Cytoreductive surgery combined with platinum- and taxane-based chemotherapy remains the first-line treatment and despite the favorable initial response, most patients eventually relapse.¹ Introduction of poly(ADP-ribose) polymerase (PARP) inhibitors has increased the overall

survival in patients with *BRCA1/2* mutations or other defects in DNA double-stranded break repair, highlighting that identification of predictive biomarkers for patient stratification can yield clinical benefits in HGSC.⁴ Thus, capturing the enormous molecular complexity of HGSC in preclinical model systems has been deemed crucial to facilitate the discovery of new biomarkers and matched treatment strategies.^{1,5,6}

Cancer organoids—patient-derived, self-renewing three-dimensional cell cultures—retain the genetic heterogeneity and recapitulate morphological characteristics of original tumors more closely than standard cell lines.^{7,8} They are also less costly, more scalable and easier to maintain than patient-derived xenografts (PDXs). In recent years, organoids from OC and other solid tumors have been generated and utilized in molecular biology



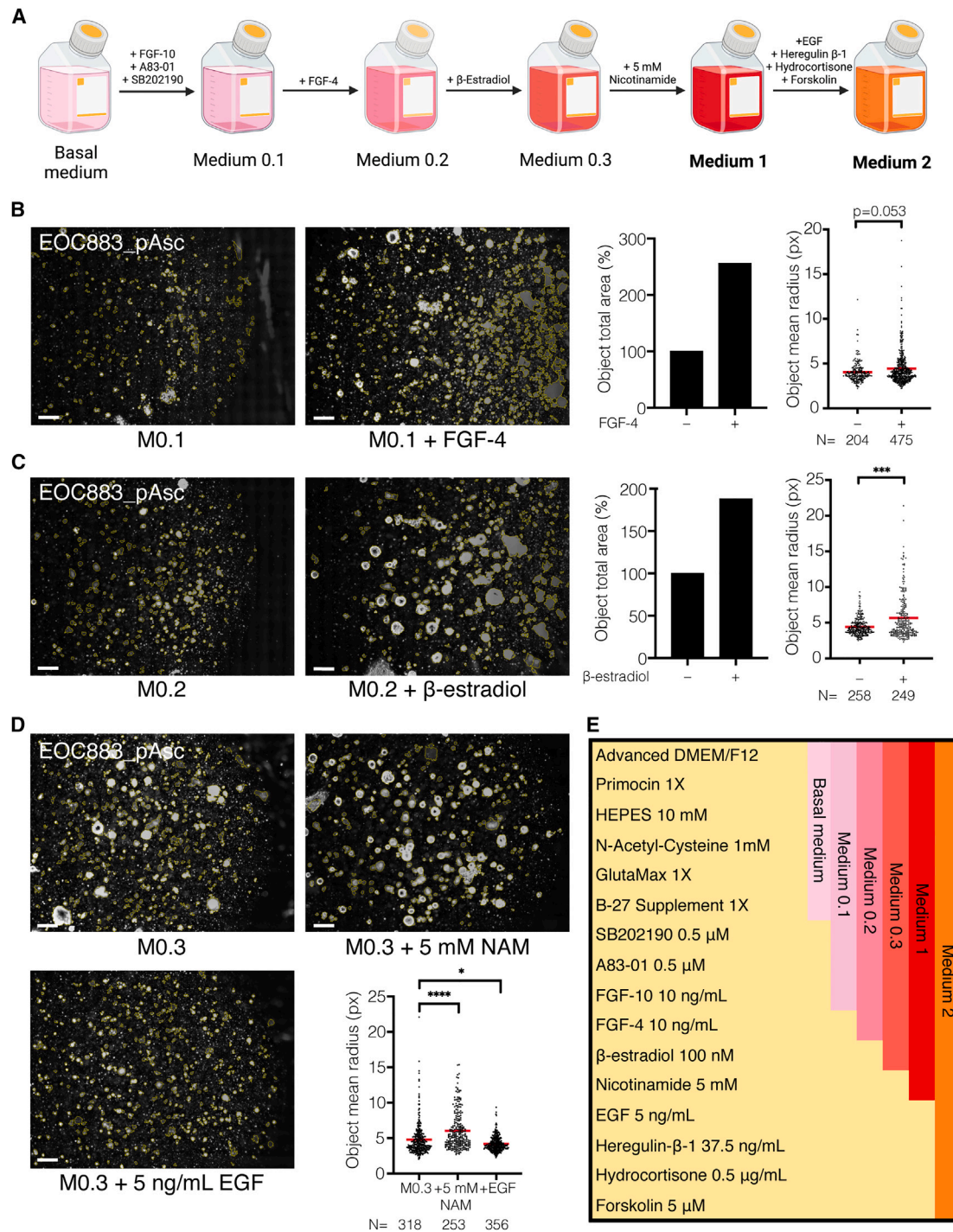


Figure 1. Establishment of HGSC organoid media formulations

(A) Summary of the medium composition establishment process.

(B) Left: phase-contrast images of basement membrane extract (BME) droplets with objects (outlined in yellow) identified with CellProfiler. EOC883_pAsc cells were cultured in M0.1 or M0.1 supplemented with FGF-4 (10 ng/mL) for 38 days (passaged once on day 17). Scale bars, 200 μm. Right: total area of objects and mean (marked with a line) object radius in the particular picture, estimated using CellProfiler.

(C) Left: phase-contrast images of BME droplets with objects identified as above. EOC883_pAsc cells were cultured in M0.2 or M0.2 supplemented with β-estradiol (100 nM) for 39 days (passaged once on day 20). Scale bars, 200 μm. Right: total object area and mean object radius in the particular picture, as above.

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research and drug screening.^{9–14} However, organoid establishment success rates vary across tumor types, limiting their broad usability.^{15,16} Several studies in recent years have described short-term culture of primary HGSC cells (often, somewhat inaccurately, referred to as organoid cultures) with very high success rates.^{17,18} While establishment of organoids from many OC types has been successful, reported success rates of derivation of self-renewing, robust organoid cultures from HGSC have, in fact, remained relatively low, from 23% to 38%.^{11,19,20} Furthermore, HGSC organoids have mainly been developed from fresh surgical specimens, which are viable only for a limited time and require geographical closeness and costly infrastructures between hospitals and research institutions. This poses major limitations for the establishment and broad availability of HGSC organoids for OC research.

Design

Cancer organoid culture media compositions vary across tumor types. Historically, most cancer organoid media were designed by altering the composition of pre-existing formulations used for maintenance of organoids from matching healthy tissue types. This method has been successful in the culture of cancer organoids from a number of tumor types, including low-grade serous, mucinous, or mucinous borderline OC.^{11,21} However, in case of HGSC, this approach is sub-optimal, as cancer organoid media based on fallopian tube or endometrial organoid media were not sufficient to maintain the majority of attempted samples.^{11,19} Medium components promoting survival of healthy epithelium might also result in persisting contamination of organoid culture with normal cells, which has been observed in HGSC organoids.^{11,19} Furthermore, the physiological relevance of standard organoid media in functional assays has also been questioned. Organoid media are nutrient-rich and supplemented with a number of growth-promoting molecules, which could result in exaggerated growth rates and distorted drug responses.^{6,22,23} Thus, there is a need to design medium conditions for efficient, long-term culture of HGSC organoids, but also to evaluate their relevance in organoid-based functional assays.

Here, we developed and optimized two medium formulations for long-term culture and expansion of HGSC organoids. With the method, we generated a collection of 17 expandable HGSC organoid cultures from 10 patients, encompassing samples from different tissue sites and disease progression stages. We established all organoid cultures from cryopreserved samples, from 53% of attempted patients, a success rate markedly improved over previous reports. We validated the organoids using whole-genome sequencing (WGS), immunohistochemistry (IHC) and single-cell RNA sequencing (scRNA-seq) showing that they are genetically and phenotypically representative of the original patient samples over long-term culture. Based on patient consents, we deposited two organoid cultures in a publicly accessible biobank. We also observed that organoid drug re-

sponses in physiologic human plasma-like medium (HPLM) were markedly different from those in nutrient- and growth factor-rich expansion medium and were more closely correlated to clinical patient outcomes.

RESULTS

Establishment of an HGSC organoid culture medium

For HGSC organoid medium optimization and subsequent organoid derivation, we used cancer patient material from debulking surgery, laparoscopic biopsies or ascites drainage (Table S1). Following the surgical procedure, the tissue was immediately processed and frozen. Cryopreserved cell suspensions were shipped to the laboratory, thawed and seeded for organoid growth. Sample/organoid names indicate the patient number, clinical course phase at sampling and tumor location (for instance, EOC989_iOme indicates sample from patient EOC989, taken during interval debulking surgery, from tumor located in omentum; full explanation of abbreviations is available in Table S1).

As a starting point for medium optimization, we used Advanced DMEM/F12 medium, supplemented with Glutamax, Primocin, N-acetyl-cysteine and B27 supplement (“basal medium”) (Figures 1A and 1E). We then assessed the influence of individual medium additives, reported by Hill et al., on the short-term organoid formation and growth of HGSC primary cells.¹⁷ Addition of fibroblast growth factor (FGF)-10, p38 inhibitor SB202190 or TGF- β receptor inhibitor A83-01 resulted in improved organoid formation (Figure S1). On the other hand, addition of EGF, FGF-2, or Noggin resulted in increased cellular attachment and decreased organoid formation. R-Spondin 1, nicotinamide (1 mM) or prostaglandin E did not cause any observable effect. Thus, we proceeded with the basal medium, supplemented with FGF-10, SB202190 and A83-10 (“Medium 0.1; M0.1”) to further optimization (Figures 1A and 1E).

Despite promoting growth after seeding, Medium 0.1 did not sustain growth over passaging. Thus, we set out to explore molecules that support the establishment of long-term, self-renewing organoid culture. We tested medium additives that have been reported to support cancer organoid growth as well as additives that have not been used as cancer organoid media components, but are important signaling molecules for cancer stem-like cells (CSCs) in HGSC. The full list of tested additives and their effects on HGSC organoid growth is available in Table S2. Of these, addition of FGF-4 resulted in increased organoid formation over passaging (“Medium 0.2; M0.2”; Figure 1B). Notably, FGF-4 has been previously reported to promote the tumorigenicity of OC CSCs,²⁴ but has not been included in cancer organoid media before. Further, we observed that addition of Wnt-pathway activating R-spondin-1 and Wnt-conditioned media, alone or in combination, caused decreased HGSC organoid formation (Figure S2A).

(D) Top and bottom-left: phase-contrast images of BME droplets with objects identified as above. EOC883_pAsc cells were cultured in M0.3 or M0.3 supplemented with nicotinamide (NAM, 5 mM) or EGF (5 ng/mL) for 38 days (passaged once on day 19). Scale bars, 200 μ m. Bottom-right: mean object radius in the particular picture, as above.

(E) Overview of particular HGSC organoid media formulations.

n, number of analyzed objects * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$, unpaired two-tailed Mann-Whitney test. See also Figures S1 and S2 and Tables S1 and S2.

In the following experiment, supplementation of M0.2 with β -estradiol increased the HGSC organoid formation and growth over passaging (“Medium 0.3; M0.3”; Figure 1C), in concordance with previous reports.^{11,19,20} Addition of nicotinamide further improved the organoid formation (Figure 1D), but the effect was concentration-dependent (Figure S2B). Addition of 5 or 10 mM nicotinamide was most advantageous, in agreement with previous reports,^{11,17,19} whereas 1 mM nicotinamide, reported by others²⁰ yielded sub-optimal organoid growth. Thus, we included 5 mM nicotinamide in the final HGSC organoid medium formulation (“Medium 1; M1”; Figures 1A and 1E).

Two medium formulations improve the success rate of HGSC organoid culture

Interestingly, when M0.3 was supplemented with EGF, a component of all previously published HGSC organoid media, we observed markedly reduced growth in the EOC883_pAsc sample and eventual collapse of the culture (Figure 1D). In contrast, EGF promoted growth of several other samples (Figure S2C; Table S2). Combining EGF with additives reported previously by others,^{11,19} including heregulin β -1, hydrocortisone, and adenylyl cyclase activator forskolin, further improved growth and expansion of these samples (Figure S2D; Table S2). On the contrary, EOC883_pAsc growth and organoid formation were harmed by these supplements (Figure S2E; Table S2). These experiments demonstrated that the addition of EGF, heregulin β -1, hydrocortisone, and forskolin can either promote or restrict organoid growth, depending on the sample. Thus, we concluded that, to maximize the likelihood of successful organoid growth, every HGSC sample should be cultured in parallel in two different media—M1 and Medium 2 (M2; M1 supplemented with EGF, heregulin β -1, hydrocortisone, and forskolin).

We tested this strategy by culturing three HGSC samples in parallel in M1, M2, and previously published HGSC organoid media. As expected, EOC883_pAsc could only be successfully cultured in M1 (Figure 2A). In other formulations, the growth was limited and followed by the eventual collapse of the culture (Figure 2B). In contrast, EOC382_pOme could be cultured in M2 and the formulations from Maenhoudt et al.¹⁹ and Hoffmann et al.²⁰ (Figure 2A). Notably, in the two latter formulations, which both contain higher concentrations of EGF than M2, the growth was accelerated when compared to M2 (Figure 2C), suggesting that EOC382_pOme benefits from growth-factor-rich conditions. Finally, EOC136_pAsc failed to stably grow over passaging in any of the tested medium formulations (Figure 2A), but short-term growth was observed to the largest extent in M2 (Figure 2D). Taken together, these results demonstrated that culturing HGSC primary cells in parallel in M1 and M2 enables successful derivation of organoids from samples, which would otherwise fail if cultured using previously published methods.

This conclusion is corroborated by comparing previously reported HGSC organoid long-term culture success rates to the success rate of M1/M2 culture. Hill et al. only attempted short-term cultures¹⁷ and we therefore excluded this report from the comparison. Maenhoudt et al.¹⁹ succeeded in culturing organoids from five different HGSC patients (defined by the authors as >4 passages), out of attempted 22 patients, resulting in an overall success rate of 23% (for data source in Maenhoudt et al.,¹⁹

see Table S3; Figure 2E). Hoffmann et al.²⁰ successfully grew organoids for 13 out of 45 patients (min. 6 passages in all reported cultures), with a success rate of 29% (Table S3; Figure 2E). Kopper et al.¹¹ reported successful development of organoids that had not shown growth arrest and reached at least passage 8 for 11 patients out of 29 attempted (success rate of 38%; Figure 2E; Table S3). We used even more stringent criteria and defined a successful organoid culture if four conditions were fulfilled: (1) we managed to grow the cells for at least 10 passages; (2) we did not observe growth arrest in the sample; (3) we expanded the cancer cells in the sample; (4) the cultured cells carried the same *TP53* mutation as the original sample. Overall, we attempted to culture organoids from cancer material of 19 different patients. Using the M1/M2 method, we derived organoid cultures for 10 patients, and achieved a success rate of 53% (Figure 2E).

Establishment and characterization of expandable long-term HGSC organoid collection

In total, we developed 17 stable, long-term HGSC organoid cultures—7 cultures in M1 and 10 in M2 (Figure 3A). The time required to reach a phase of sustained expansion varied across the organoid cultures, ranging from 26 days for EOC677_rAsc to 185 days for EOC153_iOme, and on average was 89 days (Table S1). Importantly, even though we observed cell growth and 3D structure formation in samples from 19 out of 20 patients (Figure S3A), initial material expansion did not always result in a stable organoid culture. For instance, EOC136_iOme ceased to expand beyond passage 8 and the culture collapsed (Figure 3A). However, in most cases, samples that reached a stable passaging rate (every 9–16 days, at a ratio ranging from 1:2 to 1:6) expanded throughout the tested period (up to passage 20). All organoid cultures were cryopreserved and resumed growth after resuscitation (100% success rate, $n = 17$; Table S1).

We derived organoids from patients sampled at different clinical course phases: before chemotherapeutic treatment (“primary, p”; $n = 8$), during chemotherapy (“interval,” $n = 4$) and at relapse (“recurrence, r,” $n = 5$) (Figure 3B; Table S1). For 6 patients, we were able to derive multiple organoid cultures. For instance, for patient EOC677 we developed a treatment-naive organoid model (EOC677_pAsc) and organoid models from the first and second relapse (EOC677_rAsc and EOC677_r2Asc, respectively). Sampled patients were diagnosed at stages (according to The International Federation of Gynecology and Obstetrics, FIGO) IIIC ($n = 4$), IVA ($n = 5$) or IVB ($n = 1$) (Figure 3C; Table S1). Using WGS data, we estimated the cancer cell content of the organoids and the original samples. Organoids were characterized by high tumor purity ($99.2\% \pm 1.1\%$), contrary to the original samples they were derived from ($44.1\% \pm 29\%$) (Figure 3D). In contrast to earlier reports,^{11,19} we did not observe any significant contamination with normal cells in any of the organoid models.

HGSC organoids exhibited broad morphologic heterogeneity in culture, at both inter- and intra-patient levels (Figure 3E). For instance, EOC677_pAsc organoids grew as small, densely packed aggregates, while EOC677_rAsc and EOC677_r2Asc formed loosely aggregated, cystic structures. Other observed structures include spheroid-like aggregates (for instance, EOC172_rAsc or EOC733_iOme) or large, irregular, densely packed aggregates (for instance, EOC733_pPer, EOC1120_pOme or EOC1120_rAsc).

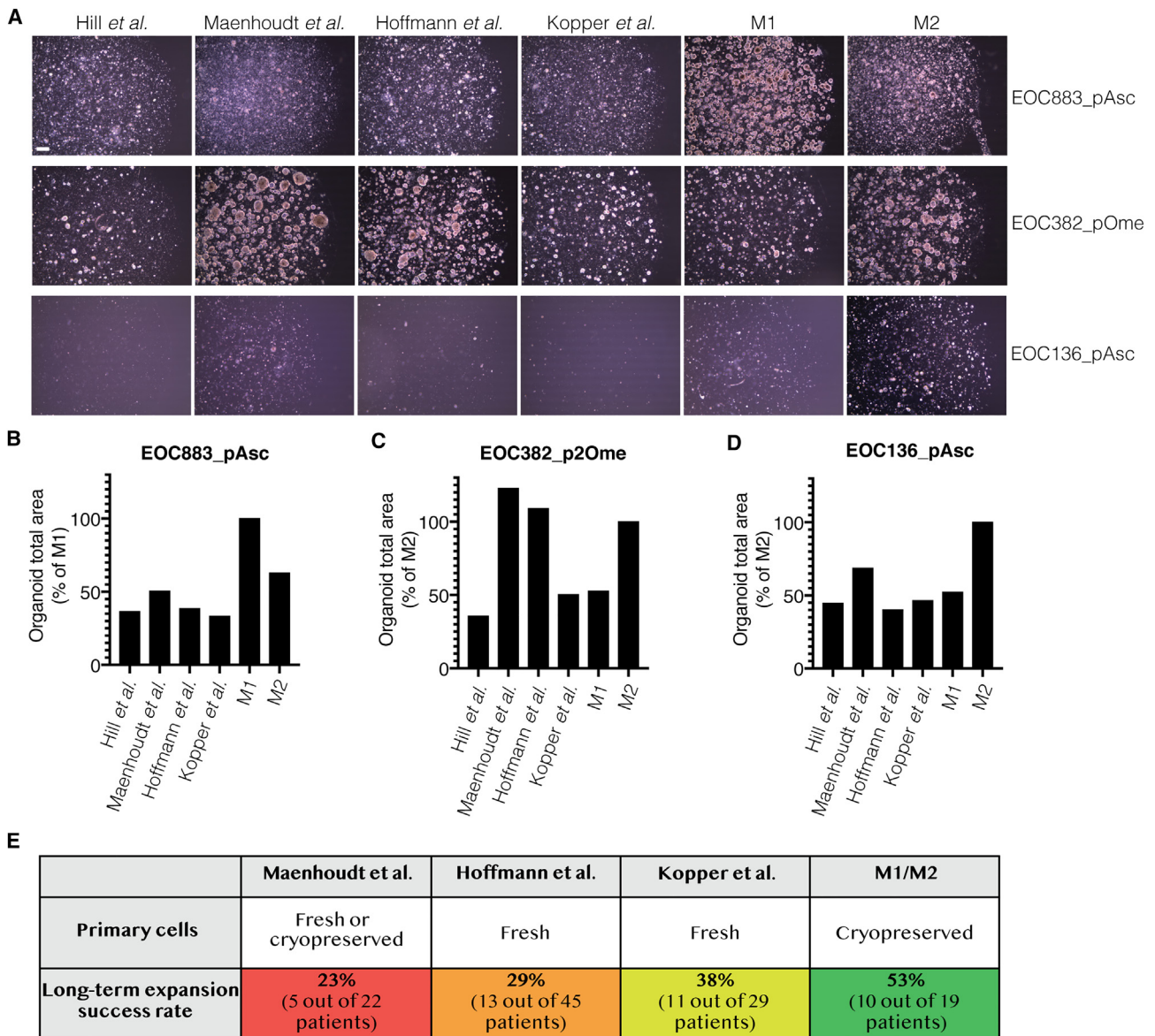


Figure 2. The M1/M2 method provides greater success rate of HGSC organoid culture than previously published protocols

(A) Phase-contrast images of EOC883_pAsc, EOC382_pOme and EOC136_pAsc cultures in previously published HGSC organoid media or M1/M2. EOC883_pAsc, EOC382_pOme or EOC136_pAsc cells were cultured for 36 days (passaged once on day 20), 31 days (passaged once on day 17) or 51 days (passaged once on day 29), respectively. Scale bar, 200 μ m.

(B–D) Total area of objects identified with CellProfiler in images presented in (A), for EOC883_pAsc (B), EOC382_pOme (C) and EOC136_pAsc (D).

(E) Comparison of HGSC organoid culture establishment success rates reported previously (for source of the data in relevant publications, see Table S3) to the success rate of M1/M2 organoid culture.

See also Table S3.

To compare the organoids' internal phenotypes to those of corresponding patient samples, we performed hematoxylin and eosin (H&E) and IHC stainings. Overall, organoids exhibited morphological features of the matching tumors' epithelia, including adenopapillary growth pattern and severe (3+) nuclear pleomorphism (Figures 3F and S3A–S3E). All tested organoid-tissue pairs ($n = 5$) were also concordant in the expression of HGSC IHC markers—paired box gene 8 (PAX8), Wilms' tumor protein (WT1) and cytokeratin 7 (CK7) (Figures 3F and S3A–S3E)—with organo-

ids showing more homogeneous staining intensity and higher percentage of cells positive for each marker, compared with the more variable expression patterns in tumor tissue samples. Interestingly, IHC features of EOC1120_pOme tissue and organoids were preserved in organoids derived from the patient's recurrent disease ascites (EOC1120_rAsc, Figures 3F and S3E). As expected, organoids exhibited stronger staining (both in intensity and percentage of positive cells) for the cellular proliferation marker Ki-67 than cancer cells in the original tumor tissue (Figures 3F and S3A–S3F).

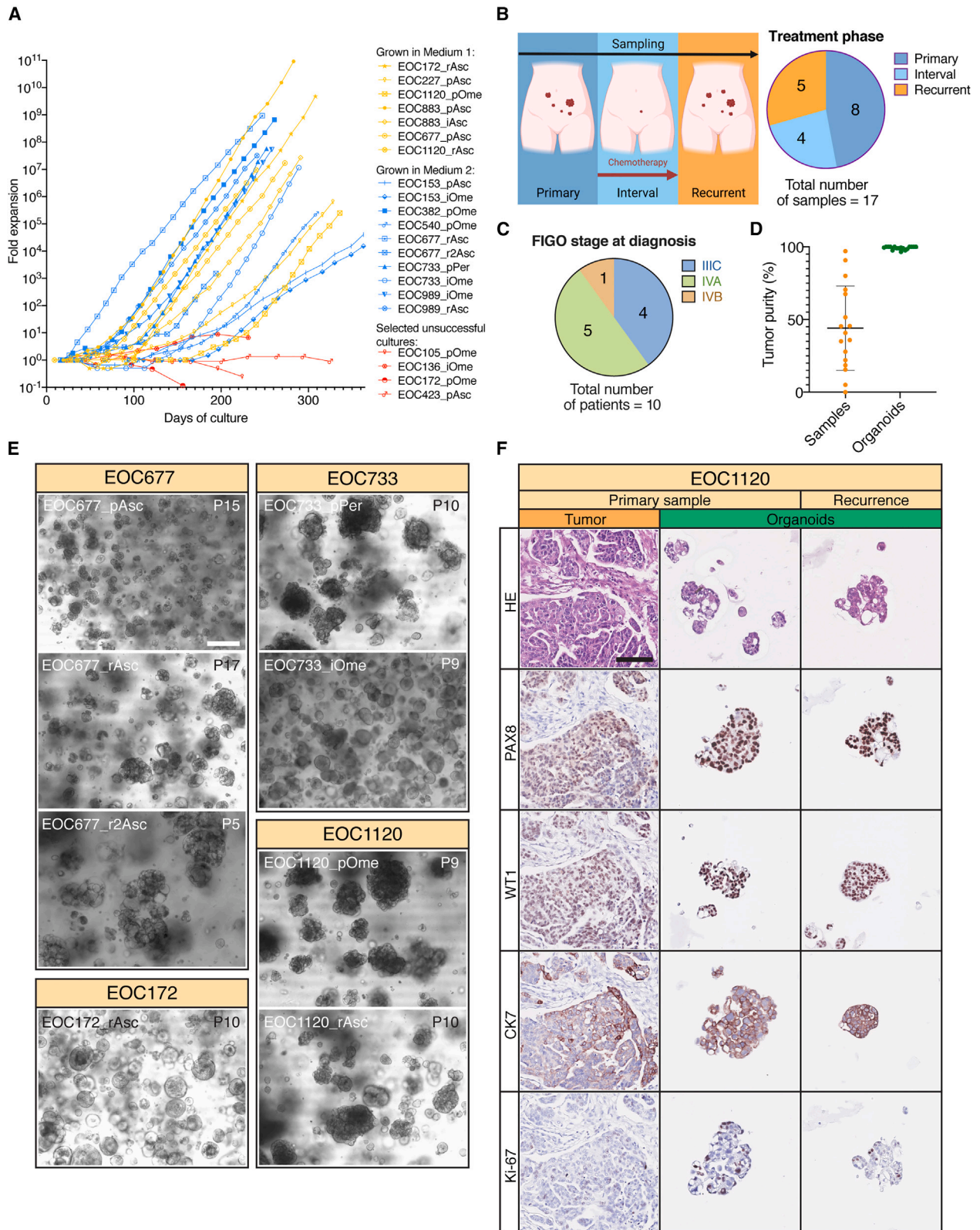


Figure 3. Overview of the HGSC organoid collection

(A) Growth curves of successful organoid cultures in M1 (n = 7), M2 (n = 10) and selected unsuccessful cultures (n = 4).

(B and C) Categorization of established HGSC organoid cultures according to the clinical course phase at sampling (B) and FIGO stage at diagnosis (C).

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HGSC organoids retain the genomic landscape of patient samples over long-term culture

We performed WGS analysis to investigate whether the organoids recapitulated the genomic profiles of original patient tumors. All organoid models harbored *TP53* mutations matching those observed in corresponding tumor tissues (Figure 4A). Notably, the variant allele frequency (VAF) of *TP53* mutations was 1 for all organoid cultures, confirming that organoids comprised only cancer cells. Other genetic aberrations characteristic for HGSC included amplification of *CCNE1*, *KRAS*, *MYC*, and *MECOM* and point mutations in *RB1*, *CSMD3*, *CDK12*, *KMT2B*, *KMT2C*, and *CCNA2* (Figure 4A). In general, these were conserved between original tumor samples and organoids and over long-term passaging. The use of samples from different clinical course phases of the same patient enabled us to derive organoids representing tumors' genetic evolution (for example, models with *de novo* *CDK12* mutation in patient EOC677, acquired at the first tumor recurrence) (Figure 4A). However, in some organoid cultures, we observed new mutations that appeared at late passages, suggesting genetic evolution or clonal selection during culture, as described in previous long-term organoid culture reports.^{9,11} Overall, organoids exhibited very high mutation concordance with the original patient samples, even when compared to control material from other tumor sites (Figures 4A and S4A).

Defects in the homologous recombination DNA-repair pathway are present in around 50% of HGSC cases. Alexandrov et al. have identified a number of mutational signatures associated with specific mutational processes (COSMIC v3.1), including single-base substitution signature 3 (SBS3) and small insertion and deletion signature 6 (ID6), that are associated with homologous recombination deficiency (HRD).^{25,26} We fitted the COSMIC v3.1 signatures to mutational profiles of tumor samples and matching organoids and assessed their contribution to the overall mutational burden. SBS3 and/or ID6 signatures were identified in 12 out of 17 patient samples and their contributions were well reflected in matching organoid cultures (Figure 4A). Notably, organoids also recapitulated the emergence of SBS3 and ID6 signatures during clinical progression (for instance, SBS3 signature that emerged at recurrence in patient EOC989).

To compare genomic landscapes of organoids and matching tumor material, we performed copy-number variation (CNV) analysis. Organoids maintained the CNV profiles of original tumors over long-term passaging (Figures 4B and S4B). In addition, organoids from different clinical course phases of the same patient maintained the genomic changes acquired during tumor evolution (for instance, gain in chromosome 7 fragment at the first recurrence in patient EOC677, Figure 4B). The only exception was the EOC153_iOme organoid culture, which was more similar to the primary tumor sample EOC153_pAsc than the interval deposit it was derived from (Figure S4A). Overall, the present organoid collection recapitulated interpatient

genomic heterogeneity and accurately mirrored disease evolution. To allow closer examination of the genomic landscapes, we provide the mutation, copy-number and signature profiles through an interactive visualization in the online tool GenomeSpy (<https://genomespy.app/>), accessible at: https://csbi.itdk.helsinki.fi/p/senkowski_et_al_2022/.

HGSC organoids recapitulate tumor's transcriptomic features

Transcriptomic profiling of HGSC organoids has only been performed on bulk cell mass before.¹¹ Importantly, single-cell analysis is critical to analyze not only how well organoids represent transcriptional heterogeneity but also the patient-specific transcriptional profile of each original tumor. This is because tumor- or ascites-derived bulk transcriptomes intermix immune and stromal expression signals, making them poorly comparable to those from pure-cancer organoids. The only attempt to study HGSC organoids at a single-cell level performed recently has been focused on cell aggregates merely 96 h after tissue dissociation.²⁷ However, naming these short-term cultures "organoids" is problematic according to the established definition that organoids are three-dimensional, self-organizing, self-renewing cultures derived from stem cells that recapitulate aspects of original tissue structure and function.^{15,28,29} Thus, it has remained unclear whether stable, long-term HGSC organoid cultures represent patient tumors in terms of transcriptional features at the single-cell level. To address this, we performed scRNA-seq of 7 organoid samples from 5 patients and the tumor/ascites material they were derived from, as well as an additional, adnexal tumor sample for patient EOC883. We performed unsupervised clustering on the total of 30,492 cells, resulting in 26 subclusters (Figure S5A). Cells from organoid samples formed organoid-line-specific clusters, while cells from patient samples formed multiple mixed clusters (Figure 5A). Marker expression analysis revealed that mixed clusters consisted of stromal or immune cells, while cancer cells formed patient-specific clusters (Figures 5B and 5C). As expected, nearly all cells from organoids were categorized as cancer cells (Figure S5B). We then asked whether expression of genes identified as patient-specific markers in tumor samples was reflected in the corresponding organoids. We observed very strong correlations for the tumor samples and organoids of patients EOC677, EOC540 and EOC883, (Pearson $r > 0.85$, Figures 5C, 5D, and S5C–S5H). For patients EOC733 and EOC382 organoid- and tumor-specific markers correlated to a slightly lesser extent, but strongly and in patient-specific manner (Person $r > 0.77$).

Next, we investigated whether HGSC organoids represent the original patient samples at the cell subpopulation level. For this, we used the InferCNV,³⁰ which infers genomic copy-number variation changes at a single-cell resolution and identifies subclusters of similarly altered cells within a population. Using

(D) Tumor purity of tumor samples and corresponding organoid cultures presented as mean \pm SD.

(E) Bright-field images of selected organoid cultures depicting various organoid morphologies. Passage numbers (P) indicated in top-right corners. Scale bar, 100 μ m.

(F) HE and IHC staining of EOC1120_pOme tumor tissue, organoids derived from it and organoids derived from relapsed tumor (EOC1120_rAsc). Organoids demonstrate morphological features similar to the original tissue, including nuclear pleomorphism, adenopapillary growth pattern, and positive staining for PAX8, WT1, and CK7. They are also more proliferative than the original tissue, depicted by higher Ki-67 expression. Scale bar, 100 μ m. See also Figure S3.

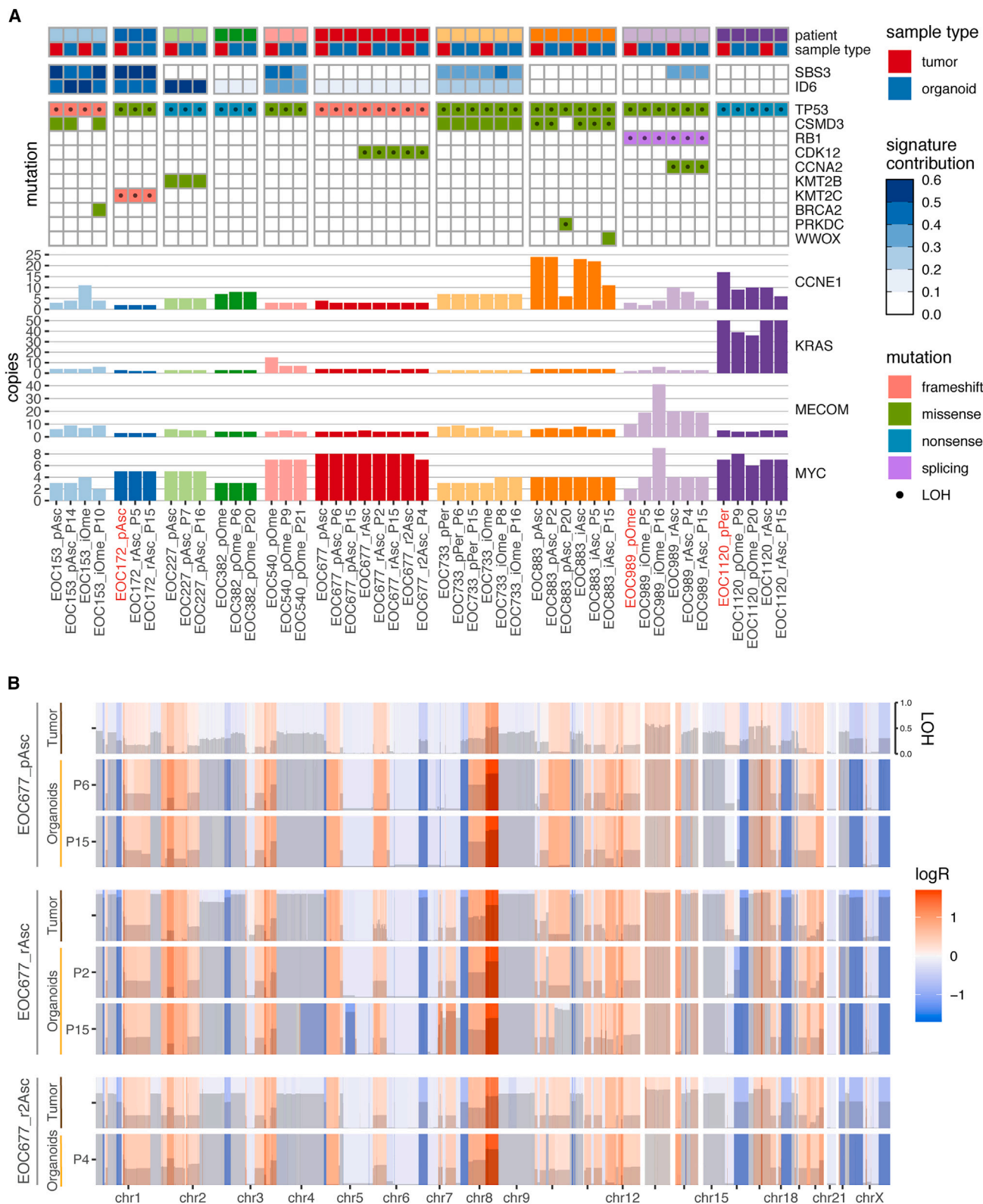


Figure 4. HGSC organoids cultured with M1/M2 recapitulate genomic landscapes of original tumor tissues over long-term culture

(A) Chart displaying somatic mutations and amplifications of selected, HGSC-relevant genes and contribution of HRD-associated mutational signatures (ID6, SBS3) in patient tumor tissue and corresponding organoid cultures. Passage numbers (P) at sequencing are indicated for organoid cultures. Sample names are

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InferCNV, for each patient we analyzed tumor sample and organoid single-cell profiles and identified 3–5 main genetic subpopulations. For patient EOC677, all 3 subclusters contained cells from all 4 analyzed samples (primary and relapse ascites and ascites-derived organoids) (Figures 5F and S5I). Subclusters identified for other patients demonstrated various degrees of sample representation, with EOC883 samples being most equally represented throughout identified subclusters and EOC733_iOme organoids the least (Figures S5I–S5M). Nevertheless, subpopulations from all patients, when analyzed jointly, showed patient-specific clustering (Figure 5G). Taken together, these data demonstrate that organoids are transcriptionally highly similar to original tumor samples at the single-cell level. Consistent with genomic characterization of the organoids, subcluster analyses of scRNA-seq data indicated subclonal drift over several months of *in vitro* culture in a subset of organoids. Notably though, the new subpopulations that emerged from this drift retained patient-specific transcriptional features of the original sample, reflecting robust fidelity of the established organoid models.

HGSC organoid drug responses correlation to patient clinical outcomes is culture medium dependent

Finally, we performed drug-response profiling of eleven organoid cultures to investigate whether organoid drug responses correlate with those previously observed in patients. For this, we seeded organoid fragments, suspended in a basement membrane extract, into ultra-low attachment 384-well microplates and covered the cultures with sample-appropriate growth medium (Figure 6A). We and others have previously demonstrated that cell culture conditions, including non-physiologic concentrations of glucose, glutamine, and other nutrients, can drastically influence drug responses in functional assays.^{31–34} Thus, to explore whether culturing conditions impact the correlation between organoid drug responses and clinical responses, we exchanged the growth medium to HPLM in half of the seeded plates after an initial period of organoid growth. HPLM mimics the metabolic composition of human plasma and has been shown in cancer cell lines to provide a more physiologically relevant environment for assessing drug responses.³³

We exposed organoids in both growth and HPLM media to a panel of drugs used in HGSC clinical treatment (chemotherapeutics: carboplatin, paclitaxel, carboplatin/paclitaxel combination and gemcitabine; PARP inhibitors: olaparib, niraparib and rucaparib) and Wee1 inhibitor adavosertib. We assessed the cytotoxicity by dead cell fluorescent staining and high-throughput confocal imaging (Figure 6B). Overall Z-factor across the experimental plates was 0.56 (± 0.14), confirming the suitability of the assay (Figure S6A).

Organoids demonstrated differential responses to the drug panel (Figure 6C). Sensitivity of particular organoid cultures to gemcitabine, PARP inhibitors and adavosertib was similar in

the M1/M2 and HPLM in most cultures. However, responses to first-line chemotherapeutics carboplatin, paclitaxel, and their combination often differed between the growth medium and HPLM. N-acetylcysteine, present in M1/M2, is a precursor of intracellular glutathione, which has been found to influence *in vitro* sensitivity of cancer cells to carboplatin.³⁵ However, we did not observe an increased carboplatin sensitivity in M1/M2 deprived of N-acetylcysteine (Figure S6B), suggesting that the basis of the differences between carboplatin sensitivity in M1/M2 and HPLM is multi-factorial. We then explored whether the *in vitro* drug responses matched those recorded in corresponding patients in the clinic. For example, patient EOC677 demonstrated clinical response to carboplatin/paclitaxel combination, indicated by reduction of CA125 blood level (from 1,593 to 11 a.u.) and its subsequent stabilization in the normal range (<35 a.u.). This more closely corresponded to the organoid drug response in HPLM than in the growth medium (Figures 6D and 6E). At relapse, the patient was treated with dose-dense paclitaxel, inducing a transient response followed by resistance to the therapy, which matched the EOC677_rAsc partial resistance to paclitaxel both in growth medium and in HPLM (Figures 6F and 6G). Subsequent treatment with gemcitabine induced longer-lasting normalization of CA125 and complete radiological response, which corresponded to sensitivity of organoids (notably, both EOC677_pAsc and EOC677_rAsc) in both media (Figures 6C, 6F, and 6G). Accordingly, EOC989_iOme organoids, derived from a sample containing only small deposits of tumor cells after 3 cycles of neo-adjuvant chemotherapy, were most resistant to the carboplatin and paclitaxel combination (Figures S6C–S6E). Similarly, EOC989_rAsc organoids, derived from a relapsed sample from the same patient, exhibited resistance to carboplatin/paclitaxel combination (Figures S6F and S6G).

We then asked if there was a correlation between sensitivity to carboplatin/paclitaxel combination and clinical outcome of chemotherapy (expressed as a percentage of CA125 blood level change, attributable to chemotherapeutic treatment in the period relevant for a particular sample/therapy (Figure S6U)). For the analysis, we chose only organoid cultures derived from samples acquired directly before the carboplatin/paclitaxel combination treatment ($n = 10$, Figures 6D, 6E, and S6C–S6T). For organoids exposed to the combination in M1/M2, the correlation was moderate and not statistically significant (Spearman $r = 0.552$, $p = 0.105$) (Figure 6K). In contrast, organoid drug responses in HPLM strongly correlated to CA125 reduction in corresponding patients (Spearman $r = 0.770$, $p = 0.013$). Taken together, these results underscore the importance of careful consideration of experimental medium conditions for attempts to correlate organoid-based functional assay results with clinical outcomes. They also suggest that further research on using organoid-based assays and physiologic-like culture media for prediction of clinical outcomes is warranted.

typed in orange, where tumor tissue from a different metastatic location/clinical progression stage than the one used for organoid derivation is presented (due to limited matching tissue availability for sequencing). LOH, loss of heterozygosity.

(B) Genome-wide CNV analysis of tumor tissue and corresponding organoids from patient EOC677, derived from material sampled at diagnosis, first and second recurrence. Copy-number changes are expressed as logR and color-coded. The extent of LOH is displayed with gray bars. Passage numbers (P) at sequencing are indicated for organoid cultures.

See also Figure S4.

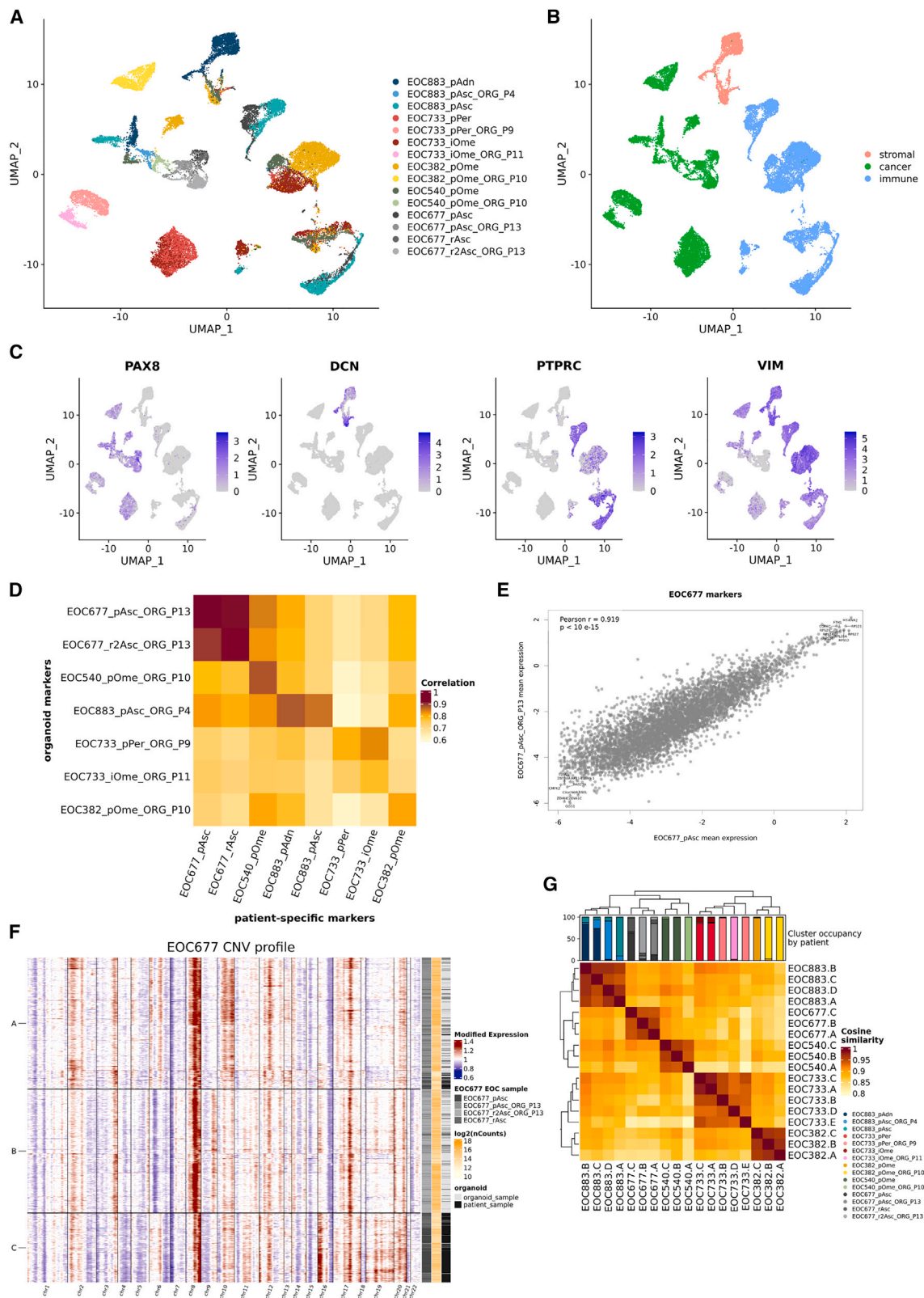


Figure 5. HGSC organoids preserve transcriptomic features of original tumors

(A) UMAP visualization of 30,492 cells from 7 organoid cultures and their tissue controls (with addition of EOC883_pAdn tumor sample) with color-coded assignment to particular sample.

(legend continued on next page)

DISCUSSION

Efficient establishment of long-term HGSC organoid cultures is essential for their broad application in OC research. Some studies have reported high success rates of short-term primary HGSC cell culture,^{17,18} but reported success rates of stably expanding HGSC organoid culture establishment were markedly lower.^{11,19,20} In this context, our study confirms that a wide variety of culturing conditions allow for short-term HSGC cell survival and limited expansion from the majority of HGSC samples (in our case, samples from 95% patients attempted), but also that the long-term, robust HGSC organoid expansion is only possible under a restricted set of growth conditions. We present a method for HGSC culture, which enabled the generation of expandable HGSC organoid collection with a 53% success rate, markedly higher than in previous reports. We established all cultures from cryopreserved material, demonstrating the feasibility of using viably biobanked tissue for HGSC organoid derivation with a high success rate—a major advantage over published protocols, as the common problem of the limited availability of fresh cancer tissue can be circumvented. Notably, Maenhoudt et al. also reported long-term HGSC organoid cultures developed from frozen tissue, but with a low success rate (2 cultures developed out of 11 attempted). Extensive testing of organoid culture media components resulted in two different medium formulations—M1 and M2. Seven samples in our collection grew in M1, which is relatively scarce in growth factors, compared with other previously published OC organoid media. Notably, unlike any previously published OC organoid medium, M1 does not contain EGF, which we found to be harmful for some HGSC samples. In contrast, 10 samples were sustained by the growth-factor-rich M2. Furthermore, we found FGF-4, previously not used in cancer organoid culture, to be beneficial for HGSC organoid growth. Interestingly, if we had used only the EGF-containing M2, we would only have been able to derive cultures from 6 patients with a success rate of 32%, similar to other reports. These findings suggest that HGSC samples exhibit differential needs for sustained growth and that organoid media design should address this heterogeneity.

Importantly, HGSC organoids in our collection were pure cancer cell cultures, with >99% tumor purity based on CNV analysis and harbored the correct *TP53* mutation with VAF = 1. We have not encountered any significant contamination with normal cells, a problem described in previous HGSC organoid reports.^{11,19} Presumably, this can be attributed to the lack of Wnt-pathway stimulants in M1 and M2. Wnt signaling inducers are essential components of media used for culturing organoids from normal epithelia,^{36,37} but multiple reports indicated that they often are redundant in cancer organoid cultures.^{23,38,39} Detrimental ef-

fects of Wnt on HGSC organoid culture have been previously described.²⁰ However, other HGSC organoid media included Wnt-activating molecules in their composition.^{11,19} Tumor purity of HGSC organoids in these reports ranged from 46% to 100% and reported was a development of one organoid culture that eventually turned out to be derived from healthy tissue.¹⁹ Together, these findings indicate that HGSC organoids require different niche factors than the matching healthy tissue and that supplementing the HGSC media with Wnt activators may provide a selective growth advantage to normal cells, resulting in decreased organoid tumor purity.

We established an organoid collection from samples acquired at different times of clinical progression. This enabled us to derive sequential organoid models from six patients reflecting their clinical history—“primary” organoids representing treatment-naive tumor; “interval,” from tumor cells that survived neo-adjuvant chemotherapy and organoids from the relapsed disease (“recurrence”). This approach provides a collection of models that could be used to study differences in drug responses, changes in genetic dependencies and drug resistance mechanisms over disease progression, which are the most significant challenges in the HGSC patient treatment. Based on available consents from patients, we have deposited two organoid cultures—EOC733_p-Per and EOC733_iOme in a public biobank (Auria Biobank, Turku, Finland, <https://www.auria.fi/biopankki/en/>), from which they are available to the research community. Genomic data, including mutational, CNV and HRD-related signature profiles from all organoid cultures are available to explore through the online visualization tool GenomeSpy (https://csbi.itdk.helsinki.fi/p/senkowski_et_al_2022/).

We characterized the organoid collection using WGS and HGSC-relevant IHC. Overall, organoids were representative of their tissue of origin and genetically stable over long-term passaging. The only exception was the EOC153_iOme line, which was more similar to the primary tumor than the interval sample. Genetic analysis demonstrated that it was likely derived from a small cell subpopulation that survived the selection pressure of the *in vitro* culture. This observation highlights the need for in-depth validation of all experimental models in organoid research. We also characterized seven organoid cultures and their tissues of origin using scRNA-seq. The analysis demonstrated that HGSC organoids represent the phenotypic and patient-specific expression features of the original tumors, supporting the suitability of the model for organoid-based functional assays.

We also observed major differences in organoid drug responses in different culturing media. When organoids were exposed to the drugs in the growth medium, drug responses only weakly correlated with clinical responses to chemotherapy.

(B) The same UMAP visualization with color-coded assignment to particular cell type (tumor, stromal, or immune).

(C) Single-cell expression of *PAX8*, *DCN*, *PTPRC*, or *VIM* visualized in the same UMAP plot.

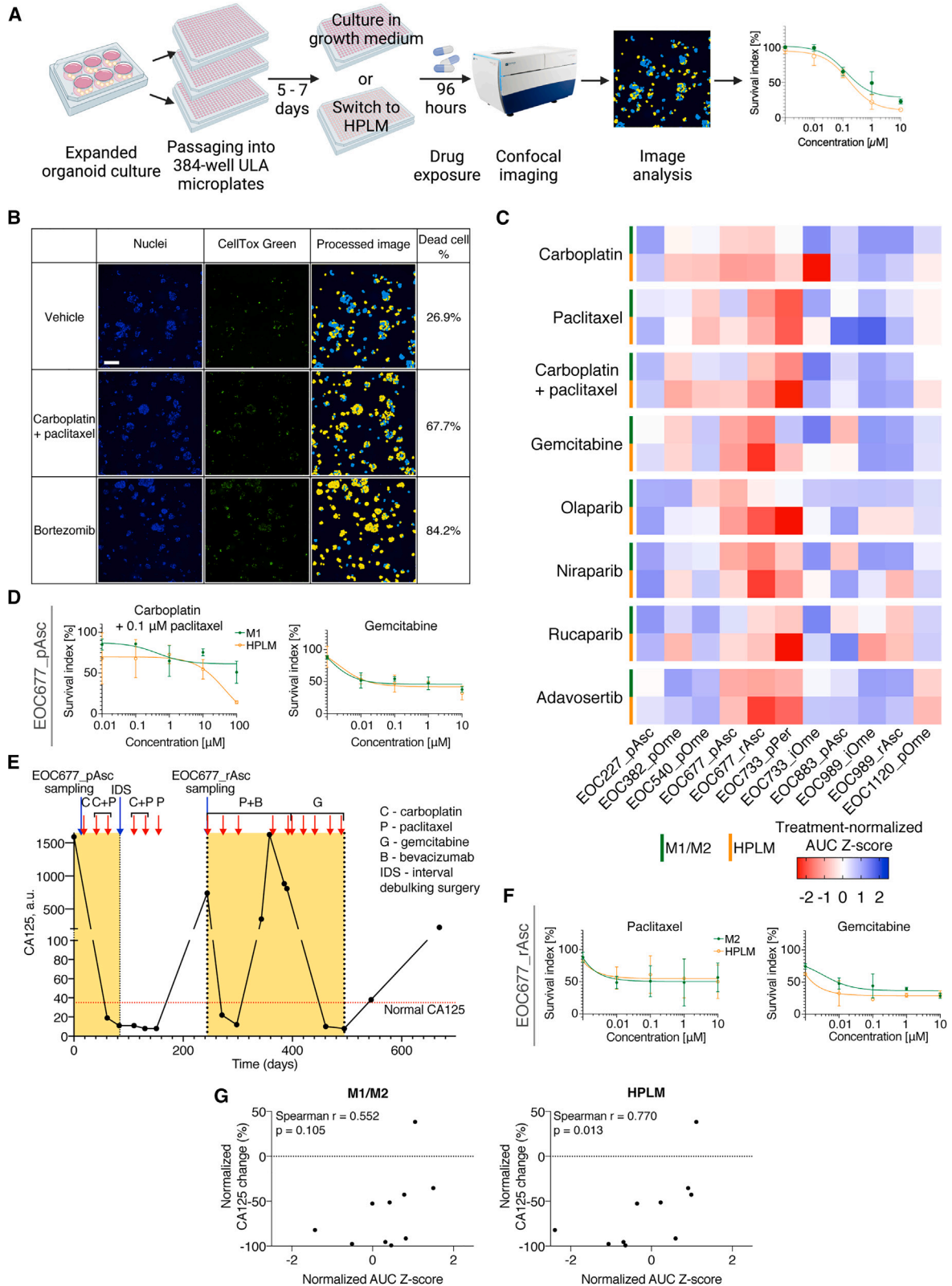
(D) Heatmap displaying overall correlation scores between the patient-specific marker expression derived from organoids vs. the original patient material.

(E) Pearson correlation plot of patient-specific markers expression in EOC677_pAsc tumor sample and corresponding organoids.

(F) Single-cell CNV plots from EOC667_pAsc and EOC667_r2Asc organoids and EOC667_pAsc and EOC677_rAsc tumor samples, inferred using InferCNV and classified into 3 subclusters. All analyzed samples are represented in each of the subclusters.

(G) Heatmap displaying cosine distances between all subclusters in all samples, demonstrating patient-specific clustering of organoids and matching tissue samples.

See also Figure S5.



(legend on next page)

This contrasts with previous reports, where strong correlations were found in HGSC⁴⁰ and in other tumors.^{13,41–43} On the contrary, *in vitro* drug responses in HPLM correlated very well with *in vivo* CA125 level change, highlighting the impact of culture conditions on organoid drug responses. However, similarly to previous work from de Witte et al.,⁴⁰ we have not found any correlation between organoid drug responses and progression-free survival (PFS). This suggests that PFS might not be a good comparative measure, since this outcome is dependent both on the extent and success of the surgery and the response to the chemotherapy, where the former has a very big impact. Taken together, as cancer organoids have been proposed as predictive models for personalized medicine, our results suggest a need for large-scale evaluation of the physiological relevance of organoid cell culture conditions.

In summary, we present a method for efficient HGSC organoid derivation and long-term culture. We provide a comprehensive validation of the models, demonstrating that they preserve the patient-specific genetic and phenotypic characteristics. We also highlight the need for further evaluation of culture conditions to facilitate broad application of HGSC organoids in cancer research and their relevance in personalized cancer medicine.

Limitations of the study

Despite the success rate improved over previous methods, we were not able to establish long-term organoid cultures for 47% of patients. One reason for this may be poor viability of some cryopreserved samples upon resuscitation. However, it is likely that the pro-growth signaling needs of these samples are yet to be discovered and additional medium formulations could further increase the success rate of HGSC organoid culture establishment. Interestingly, we noted that successfully grown samples carry *CCNE1* amplification, a negative prognostic factor in HGSC,⁴⁴ more often than the failed-to-grow samples, indicating that this genetic aberration may facilitate cell growth *in vitro*. We were unable to find any other significant difference between the two groups, presumably due to a moderate number of established cultures. Nevertheless, when generating *in vitro* cellular models that might yield clinical implications, it is worthwhile to consider niche factor dependencies of particular tumors, as they have been found to impact not only *in vitro* culture take rates, but also drug sensitivities.⁴⁵

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.devcel.2023.04.012>.

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Figure 6. Correlation of HGSC organoid drug responses to clinical outcomes is culture medium dependent

- (A) Overview of the drug-response profiling assay.
- (B) Image-based cytotoxicity assay. After drug exposure, cells' nuclei in organoids are stained with Hoechst 33342 (blue, Nuclei) and dead cells are stained with CellTox Green (green). Organoids are imaged using automated confocal fluorescence microscopy, dead cell percentage is estimated using image analysis and normalized to negative (vehicle) and positive (bortezomib) control values. Scale bar, 100 μ m.
- (C) Heatmap displaying treatment-normalized AUC Z score values, showing responses of particular organoid cultures in growth medium (M1/M2) or HPLM to a panel of HGSC-relevant drugs.
- (D) Dose-response curves of EOC677_pAsc organoids treated with carboplatin + 0.1 μ M paclitaxel or gemcitabine, in M1 or HPLM. Results are shown as mean of 2 biological replicates (each with 2–3 technical replicates) \pm SD.
- (E) CA125 blood levels of patient EOC677 over time. Periods relevant for comparisons with *in vitro* drug response indicated with yellow rectangles. Normal CA125 range (<35 a.u.) indicated with a red dotted line.
- (F) Dose-response curves of EOC677_rAsc organoids treated with paclitaxel or gemcitabine, in M2 or HPLM. Results are shown as mean of 2 biological replicates (each with 2–3 technical replicates) \pm SD.
- (G) Spearman correlation plots between normalized AUC Z score for carboplatin + 0.1 μ M paclitaxel combination in organoids and normalized change (expressed as % of maximal patient-specific CA125 level in the relevant period) of blood CA125 in corresponding patients after carboplatin + paclitaxel chemotherapeutic treatment. Number of samples = 10.
- See also Figure S6.

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AUTHOR CONTRIBUTIONS

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DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-PAX8	Abcam	Cat# ab191870; RRID: AB_2728805
Rabbit anti-WT1	Abcam	Cat# ab89901; RRID: AB_2043201
Anti-Ki67	Cell Marque	Cat# 275R-16; RRID: AB_1158037
Anti-CK7	Abcam	Cat# ab183344; RRID: AB_2936915
Goat anti-rabbit IgG	Dako	Cat# K4003; RRID: AB_2630375)
Biological samples		
Ovarian cancer tissue and ascites	Turku University Hospital	NA
Chemicals, peptides, and recombinant proteins		
Collagenase/Hyaluronidase	Stemcell Technologies	Cat# 07912
DMEM/F12	Lonza	Cat# BE12-719F
B-27 Supplement	Gibco	Cat# 17504044
hEGF	Sigma-Aldrich	Cat# E9644
FGF-b	Gibco	Cat# PHG0023
Histopaque-1077	Sigma-Aldrich	Cat# 10771
Toluidine blue	Sigma-Aldrich	Cat# 89640
STEM-CELLBANKER	Amsbio	Cat# 11890
Advanced DMEM/F12	Gibco	Cat# 12634010
Primocin	Invivogen	Cat# ant-pm1
HEPES	Gibco	Cat# 15630080
N-acetyl-cysteine	Sigma-Aldrich	Cat# A7250
GlutaMAX	Gibco	Cat# 35050061
SB202190	MedChemExpress	Cat# HY-10295
A83-01	Sigma-Aldrich	Cat# SML0788
Recombinant hFGF-10	Peptotech	Cat# 100-26
Recombinant hFGF-4	Peptotech	Cat# 100-31
β -estradiol	Sigma-Aldrich	Cat# E2758
Nicotinamide	Sigma-Aldrich	Cat# N0636
Cultrex Reduced Growth Factor Basement Membrane Extract, Type 2, Pathclear	R&D Systems	Cat# 3533-010-02
Y-27632	MedChemExpress	Cat# HY-10583
TrypLE Express	Gibco	Cat# 12604013
Animal-free recombinant hEGF	Peptotech	Cat# AF-100-15
Recombinant human heregulin 1- β	Peptotech	Cat# 100-03
Hydrocortisone	Sigma-Aldrich	Cat# H0888
Forskolin	MedChemExpress	Cat# HY-15371
FGF-basic	Peptotech	Cat# 100-18B
Recombinant human R-Spondin 1	Peptotech	Cat# 120-38
Recombinant human Noggin	Peptotech	Cat# 120-10C
Prostaglandin E2	MedChemExpress	Cat# HY-101952
Recombinant hFGF-7	Peptotech	Cat# 100-19
Recombinant hIGF-1	Peptotech	Cat# 100-11
Animal-free recombinant hVEGF	Peptotech	Cat# AF-100-20
BMP-2	ThermoFisher	Cat# PHC7145
JAG-1	AnaSpec	Cat# AS-61298

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
R-Spondin-3	Peprotech	Cat# 3500-RS-025
Follicle-stimulating hormone	R&D Systems	Cat# 5925-FS-010
Gonadotropin-stimulating hormone	Sigma-Aldrich	Cat# L8008
Triiodothyronine	Sigma-Aldrich	Cat# T6397
CHIR-99021	MedChemExpress	Cat# HY-10182
Idasanutlin	MedChemExpress	Cat# HY-15676
Formaldehyde	Sigma-Aldrich	Cat# 252549
HistoGel	ThermoFisher	Cat# HG-4000-012
Tissue clear	Sakura	Cat# 1466
Hematoxylin	Histolab Products AB	Cat# 01820
Eosin	Histolab Products AB	Cat# 01650
Antibody Diluent	Agilent	Cat# S302283-2
NovaRED	Vector Laboratories	Cat# SK-4805
Human Plasma-Like Medium	ThermoFisher	Cat# A4899101
Carboplatin	MedChemExpress	Cat# HY-17393
Paclitaxel	MedChemExpress	Cat# HY-B0015
Gemcitabine	MedChemExpress	Cat# HY-17026
Olaparib	ChemieTek	Cat# CT-A2281
Niraparib	MedChemExpress	Cat# HY-10619
Rucaparib	MedChemExpress	Cat# HY-10617
Adavosertib	ChemieTek	Cat# CT-MK1775
Bortezomib	ChemieTek	Cat# CT-BZ001
Hoechst 33342	Sigma-Aldrich	Cat# B2261
<i>Critical commercial assays</i>		
DNAeasy Blood & Tissue Kit	QIAGEN	Cat# 69504
AllPrep DNA/RNA mini kit	QIAGEN	Cat# 80204
Chromium Single Cell 3' Reagent Kit	10x Genomics	Cat# 1000424
CellTox Green	Promega	Cat# G8743
<i>Deposited data</i>		
Genomic data (visualization)	This paper; https://csbi.ltdk.helsinki.fi/p/senkowski_et_al_2022/	NA
Genomic data	European Genome-phenome Archive (EGA)	EGA: EGAS00001004714
Transcriptomic data	European Genome-phenome Archive (EGA)	EGA: EGAS00001006246, EGA: EGAS00001005010 (for samples from patient EOC733, samples IDs EGA: EGAN00002853421 and EGA: EGAN00002853407)
scRNA-seq count data	Gene Expression Omnibus	GEO: GSE202263
<i>Software and algorithms</i>		
CellProfiler v3.1.9	McQuin et al. ⁴⁶	RRID: SCR_007358
Anduril 2	Cervera et al. ⁴⁷	NA
BWA-MEM	https://github.com/lh3/bwa	RRID: SCR_022192
Picard Tools	Broad Institute; http://broadinstitute.github.io/picard/	RRID: SCR_006525
GATK	McKenna et al. ⁴⁸ ; De Pisto et al. ⁴⁹	RRID: SCR_001876
GATK Mutect2	Benjamin et al. ⁵⁰	RRID: SCR_000559
CADD	Rentzsch et al. ⁵¹	RRID: SCR_018393
ANNOVAR	Wang et al. ⁵²	RRID: SCR_012821
ClinVar	Landrum et al. ⁵³	RRID: SCR_006169

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
COSMIC v3.1 signatures	Alexandrov et al. ²⁵ ; Tate et al. ²⁶	RRID: SCR_002260
ENCODE blacklist	Amemiya et al. ⁵⁴	RRID: SCR_015482
ASCAT	Van Loo et al. ⁵⁵	RRID: SCR_016868
Cell Ranger v5.0.0	10x Genomics	RRID: SCR_017344
Souporcell	Heaton et al. ⁵⁶	NA
Seurat v3.2.2	Hao et al. ⁵⁷	RRID: SCR_007322
InferCNV	Trinity CTAT Project; https://github.com/broadinstitute/inferCNV	RRID: SCR_021140
GraphPad Prism	GraphPad	RRID: SCR_002798
Other		
EOC733_pPer and EOC733_iOme organoid cultures	This paper	Contact the Auria Biobank for access at: https://www.auria.fi/biopankki/en/
All other organoid cultures	This paper	NA

RESOURCE AVAILABILITY

Lead contact

For inquiries regarding the availability of materials and resources, contact Krister Wennerberg (krister.wennerberg@bric.ku.dk).

Materials availability

Organoid cultures EOC733_pPer and EOC733_iOme and associated clinical data are available to the research community through the Auria Biobank (<https://www.auria.fi/biopankki/en/>), based on patients' informed consents. Organoid cultures from nine patients are not shareable due to the lack of patient consents for this purpose.

Data and code availability

Genomic data, including mutation, copy-number and signature profiles are available through an interactive visualization in GenomeSpy, accessible at: https://csbi.ltdk.helsinki.fi/p/senkowski_et_al_2022/. Genomic sequence data has been deposited at the European Genome-phenome Archive (EGA), which is hosted by the EBI and the CRG, under accession number EGA: EGAS00001004714. Transcriptomic sequence data are available from the EGI under accession numbers EGA: EGAS00001006246 and EGA: EGAS00001005010 (the EOC733 tumor samples, sample IDs EGA: EGAN00002853421 and EGA: EGAN00002853407). Count data from scRNA-seq for all samples are available in Gene Expression Omnibus (GEO) with accession number GEO: GSE202263.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

HGSC tumor samples and clinical data

The study used tumor material from female patients who participated in the European Union's Horizon 2020-funded HERCULES study (ID: 667403). All patients consented to collection, storage and research use of their tumor material and related data. The collection and storage of the material has been approved by The Ethics Committee of the Hospital District of Southwest Finland (ETMK): ETMK 53/180/2009 § 238. All samples used in the study are listed in [Table S1](#). The patients were treated at The Department of Gynecology and Obstetrics at Turku University Hospital, Finland between 2016 and 2019. The patient outcomes were followed prospectively and their clinical outcome parameters such as platinum free interval (PFI) and response to chemotherapy were defined with RECIST 1.1 and GCIg criteria. Fresh tumor samples were obtained during tumor debulking surgeries, laparoscopic biopsies or ascites paracentesis. After acquisition, whenever possible, samples were divided in a few parts. A fraction of the sample was frozen at -150 °C for subsequent WGS and the rest was immediately processed.

HGSC tissue processing

Solid fresh tissue samples were minced with scalpel and enzymatically digested overnight using 0.1X Collagenase/Hyaluronidase (#07912, Stemcell Technologies) in DMEM/F12 (#BE12-719F, Lonza) supplemented with 1X B-27 Supplement (#17504044, Gibco), 20 ng/mL hEGF (#E9644, Sigma) and 10 ng/mL FGF-b (#PHG0023, Gibco). Detached cells were purified with 70 μm and 40 μm filtering followed by gradient centrifugation with Histopaque-1077 (#10771, Sigma-Aldrich), according to the manufacturer's instructions. Gradient centrifugation was repeated when necessary. Cell integrity and purity were assessed on cell smears on X-tra Adhesive Slides (#3800200, Leica) stained with toluidine blue (#89640, Sigma). Fresh ascites samples were centrifuged at 475 rcf for 15 min,

followed by gradient centrifugation with Histopaque-1077 and freezing similarly to the solid tissue derived cells. Finally, all samples were washed with PBS and frozen in STEM-CELLBANKER (#11890, Amsbio) and stored at -150°C .

Organoid media establishment experiments

Cryopreserved vials containing processed HGSC tumor material were shipped to the laboratory in dry ice and stored in liquid nitrogen until resuscitation for culturing. On the day of seeding, vials were taken out of the liquid nitrogen and thawed immediately in a 37°C water bath. Then, cell suspensions were mixed with minimal growth medium (depending on the medium establishment progress – Basal Medium, M0.1, M0.2, M0.3 or M1), that is: Basal Medium: Advanced DMEM/F12 (#12634010, Gibco), supplemented with 100 $\mu\text{g}/\text{mL}$ Primocin (#ant-pm-1, Invivogen), 10 mM HEPES (#15630080, Gibco), 1 mM N-acetyl-cysteine (#A7250, Sigma), 1X GlutaMAX (#35050061, Gibco) and 1X B-27 Supplement; M0.1: Basal Medium, supplemented with 0.5 μM SB202190 (#HY-10295, MedChemExpress), 0.5 μM A83-01 (#SML0788, Sigma) and 10 ng/mL recombinant human FGF-10 (#100-26, Peprotech); M0.2: M0.1, supplemented with 10 ng/mL recombinant human FGF-4 (#100-31, Peprotech); M0.3: M0.2, supplemented with 100 nM β -estradiol (#E2758, Sigma); M1: M0.3, supplemented with 5 mM nicotinamide (#N0636, Sigma). After mixing, cells were centrifuged at 200 rcf and washed with the minimal medium again. Next, cells were counted and viability was assessed using Countess Automated Cell Counter (Thermo Fisher). Then, cells were resuspended on ice in cold 7.5 ng/mL (concentration adjusted with cold PBS) Cultrex Reduced Growth Factor Basement Membrane Extract, Type 2, Pathclear (BME-2 #3533-010-02, R&D Systems) at a minimum of 10^6 live cells/mL and seeded into pre-warmed 6-well cell culture plates (#140675, Nunc) in 20 μL droplets (10 droplets/well). Gel droplets were solidified in a humidified cell culture incubator at 37°C for 30–45 min and gently covered with 3 mL of the minimal or experimental culture medium (full list of tested supplements and manufacturers is available in [Table S2](#)). For the first 2–3 days after cell seeding, 5 μM Y-27632 (ROCK-I and -II inhibitor, #HY-10583, MedChemExpress) was added to each of the media. Cultures were maintained in a humidified cell culture incubator at 37°C and culturing media were exchanged every 2–3 days. Cultures were regularly assessed using phase-contrast microscopy to monitor growth and organoid formation. Once pronounced growth was observed, cultures were photographed using a CCD camera (EC3, Leica) coupled to a phase-contrast microscope and passaged. Briefly, cultures were washed with PBS, covered with 2 mL of TrypLE Express (#12604013, Gibco), scraped off the cell culture plate surface and dissociated by vigorous pipetting. Then, the suspensions were incubated at 37°C for 15 min, transferred to 15 mL conical tubes and centrifuged at 300 rcf for 5 min. The supernatant was removed, cell pellets were resuspended in BME-2 and suspensions were seeded into 6-well culture plates and cultured as above. The passaging ratio for medium optimization experiments was 1:2. After 10–30 days of growth, cultures were photographed for image analysis.

Organoid derivation and long-term expansion

Cells from patient samples were resuscitated from frozen aliquots and seeded into 6-well culture plates and cultured as described above. Number of wells and gel droplets seeded were dependent on the total number of live cells after resuscitation (10–60 droplets per sample). Each sample was initially cultured in M1 and M2 in parallel. Cultures were regularly assessed using phase-contrast microscopy to monitor growth and organoid formation. Once pronounced growth was observed, cultures were photographed using a CCD camera (EC3, Leica) coupled to a phase-contrast microscope and passaged. Initial passaging ratio was decided on a case-by-case basis, depending on the number of growing organoids observed. When very few organoids were observed, cultures were often initially passaged at 1:0.5–1 ratio, in order for organoids to reach higher density, which frequently resulted in increased expansion in subsequent passages. For densely growing cultures, passaging ratios were steadily increased until the cultures reached a stable expansion rate. In most cases, after 3–7 passages culture was continued only in M1 or M2, based on the observed growth and successful organoid formation. Cultures where no further growth was observed, were terminated. Overall fold-expansion was calculated as the initial number of seeded wells (counting 1 BME droplet as 0.1 well) multiplied by all previous passaging ratios. Organoids that reached a stable expansion rate were cryopreserved. Briefly, cultures were washed with PBS, covered with 2 mL of TrypLE Express (#12604013, Gibco), scraped off the cell culture plate surface and dissociated by vigorous pipetting. Then, the suspensions were incubated at 37°C for 15 min, transferred to 15 mL conical tubes and centrifuged at 300 rcf for 5 min. The supernatant was removed, cell pellets were resuspended in 1 mL of STEM-CELLBANKER and immediately frozen at -80°C . After 24 h, the frozen vials were transferred to liquid nitrogen tanks for long-term storage. Organoid cultures were expanded until passage 20. Based on available biobanking consents, two organoid cultures –EOC733_pPer and EOC733_iOme – were deposited in Auria Biobank (Turku, Finland) and are available to the research community.

For step-by-step protocols for organoid culture, please see [Methods S1](#).

METHOD DETAILS

Image-based organoid growth assessment

All phase-contrast images used for the analysis were taken at 2.5X magnification. Photographs were analyzed using CellProfiler v3.1.9.⁴⁶ First, to make separate images comparable, illumination was equalized using “CorrectIllumination” modules with Gaussian filter. Subsequently, objects (organoids/cell clusters) were identified using “IdentifyPrimaryObjects” module in each image, within borders of the gel droplet (using a pre-prepared mask image for each analysis to define the droplet area). Objects were identified and single cells/large artifacts excluded from the analysis using Otsu three-class adaptive thresholding (typical diameter 20–400,

adaptive window 150, smoothing 2, correction 1, smoothing filter 30). Then, total object area was estimated as a fraction of total area of all the objects divided by the area of the gel droplet. Object mean radius was also estimated for each object.

Comparison of M1/M2 with previously published media

Previously published media were reproduced according to authors' instructions. From Kopper et al. report, only the formulation without the Wnt-conditioned medium was used, due to previously observed negative impact of Wnt conditioned medium on HGSC samples. Cells from EOC883_pAsc, EOC382_pOme and EOC136_pAsc patient samples were resuscitated from frozen aliquots and seeded into 6-well culture plates and cultured as described above. 1 well/sample/medium was seeded, each containing 10 separate BME droplets. For the first 2-3 days after cell seeding, 5 μ M Y-27632 was added to each of the media. Culturing media were exchanged every 2-3 days. EOC883_pAsc, EOC382_pOme or EOC136_pAsc cells were cultured for 36 days (passaged once on day 20, 1:1 ratio), 31 days (passaged once on day 17, 1:1 ratio) or 51 days (passaged once on day 29, 1:1 ratio), respectively. Then, cultures were photographed using a CCD camera (EC3, Leica) coupled to a phase-contrast microscope at 2.5X magnification and photographs analyzed as above. Organoid long-term culture success rate for HGSC in previous reports was calculated using published data (Table S3). Overall, all previously reported cultures were deemed long-term and expandable, unless stated otherwise. For the exact definition of a long-term, stable culture in each report, see the results section.

Histological analysis and immunohistochemistry

BME domes with organoids were covered with 4% solution of formaldehyde (#252549, Sigma) for 10 min at room temperature. Then, BME domes were scraped off the plates, suspensions were transferred to conical tubes and centrifuged at 300 rcf for 5 min. Supernatants were removed and pellets were gently resuspended in hot HistoGel (#HG-4000-012, ThermoFisher). HistoGel was solidified at room temperature, embedded in paraffin and sectioned. Tissue or organoid sections (3.5 μ m) were de-paraffinized in tissue-clear (#1466, Sakura) and hydrated in ethanol solutions at decreasing concentrations before further treatment. H&E staining was performed using Mayer's hematoxylin (5 min) and eosin (5 min) (01820 and 01650, Histolab Products AB). For immunohistochemical stainings, heat-induced antigen retrieval was performed at 95°C for 15 min in 10 mM sodium citrate, pH 6.0 (for Ki67 and CK7) or at 95°C for 20 min in Tris-EDTA buffer (10 mM Tris base, 1 mM EDTA, 0.05% Tween-20, pH 9.0) (for PAX8 and WT1). Endogenous peroxidase activity was blocked by incubation in 1% (v/v) hydrogen peroxide for 15 min. The sections were then incubated overnight in Shandon racks (Thermo Shandon) at 4°C with the primary antibodies (100 μ L/section) diluted in Antibody Diluent (#S302283-2, Agilent) to the following concentrations; 1:1000 mAb PAX8 (#ab191870, Abcam); 1:100 mAb WT1 (#ab89901, Abcam); 1:500 mAb Ki67 (#275R-16, Cell Marque) and 1:100 mAb CK7 (#ab183344, Abcam). Envision horseradish peroxidase-labeled anti-rabbit IgG (K4003, Dako) was used for detection (100 μ L/section) by incubating 45 min at room temperature, followed by development with NovaRED (Vector Laboratories) for 9 min as specified by the manufacturer. The sections were then counterstained with Mayer's hematoxylin for 30 seconds. Both H&E and immunoperoxidase stained sections were finally dehydrated in ethanol solutions before mounting in tissue mount (#1467, Sakura).

Morphological features such as growth pattern and nuclear pleomorphism, in both tissue and organoid sections, were compared by two pathologists using a light microscope (Olympus BX46 Clinical Upright Microscope). Nuclear pleomorphism was scored as weak (1+), moderate (2+) or severe (3+). Similarly, the expression of each IHC marker was assessed in a blinded manner and independently by the two observers using light microscopy. After verifying the specificity of each immunostaining in the samples (nuclear stain for PAX8, WT1 and Ki-67; membranous and cytoplasmic for CK7), the staining intensity (negative = 0, weak = 1+, moderate = 2+, strong = 3+), the degree of homogeneity/variability of the immunostainings and the percentage of stained cells were recorded for each marker. Consensus on discrepant scores was obtained by simultaneous observation using a double-arm microscope.

Whole-genome sequencing and analysis

Genomic DNA was extracted from snap-frozen organoids using DNAeasy Blood & Tissue kit (#69504, QIAGEN) and from snap-frozen tumor tissue or ascites cells, as well as from whole blood buffy coat (germline) samples using AllPrep DNA/RNA mini kit (#80204, QIAGEN). DNA quality was assessed using a Qubit fluorometer (Invitrogen). The samples were sequenced with Illumina HiSeq X Ten, BGISEQ-500 or MGISEQ-2000 at BGI Genomics Europe (Copenhagen, Denmark) and processed at the Anduril 2 platform.⁴⁷ Short-reads were aligned to human genome GRCh38.d1.vd1 using BWA-MEM⁵⁸ followed by duplicate removal with Picard Tools (<http://broadinstitute.github.io/picard/>) and base quality score recalibration with GATK.^{48,49}

Somatic mutations were called using GATK Mutect2⁵⁰ with joint calling. A panel of normals generated from 105 HERCULES/DECIDER and 99 TCGA blood-derived normal samples was utilized. Mutations were annotated using CADD,⁵¹ ANNOVAR⁵² and ClinVar.⁵³ Non-silent or splicing variants pathogenic in ClinVar or non-benign with CADD phred score > 10 were considered functionally relevant. Mutation concordance was defined between two samples as Jaccard index using the number of shared and private mutations, where a mutation is present in a sample as long as at least one ALT read is observed.

Germline mutations used in CNV analysis come from a callset of 106 HERCULES/DECIDER normals using GATK.⁵⁹ Variant quality score recalibration was allele-specific.

Mutational signature analysis was performed using COSMIC v3.1 signatures^{25,26} adjusted for GRCh38 nucleotide frequencies. Mutations without VAF > 0.05 in any sample as well as those that only had ALT reads in one strand were excluded. Sample mutational profiles comprised mutations with at least one ALT read in that sample.

CNV analysis

Copy-number segmentation was done with GATK.⁴⁸ To collect the minor allele counts, all filtered biallelic (VAF 0.4-0.6) germline SNPs from each patient were used. Read-count collection was used at one-kilobase intervals. Both read and allelic count collection excluded regions listed in the ENCODE blacklist⁵⁴ and internal HERCULES/DECIDER blacklist, which includes regions that have $abs(\log R) > 0.2$ in at least three of the 114 available normal samples.

After the segmentation, a reimplemented ASCAT algorithm⁵⁵ was used to estimate purity, ploidy, and allele-specific copy numbers. The original ASCAT R package was not directly applicable because it does not accept data segmented using external tools. Our implementation also uses VAF of homozygous TP53 mutations as additional evidence for the ploidy/purity estimates.

Single-cell RNA sequencing and analysis

Cryopreserved tissue samples were thawed and resuspended in culture medium immediately before sample processing for scRNA-seq and subsequently processed for scRNA-seq in the same way as dissociated organoid samples. Organoid cultures were dissociated in 2 mL of TrypLE Express for 25 min with occasional trituration by pipetting. The dissociated organoid cells were washed twice with 10 mL ice-cold PBS, and resuspended in 1 mL of PBS. The live cells were counted by Trypan blue exclusion method using Countess II counter (ThermoFisher). scRNA-seq libraries were prepared with Chromium Single Cell 3' Reagent Kits (v.2.0 or v.3.1, 10x Genomics) and samples were sequenced on an Illumina NovaSeq 6000 instrument (Sequencing Unit of the Institute for Molecular Medicine Finland). The fastq files obtained from sequencing were processed using 10x Genomics Cell Ranger v5.0.0 for de-multiplexing, alignment, barcode processing and UMI quantification. GRCh38.d1.vd1 genome was used as reference and GENCODE v25 for gene annotation. Organoid samples from EOC540 and EOC733 (interval) and all samples from EOC677 were multiplexed prior to sequencing. In order to demultiplex these samples, SoupCell⁵⁶ was used. Count matrices were loaded in Seurat (v3.2.2)⁵⁷ and initial filtering was applied to remove cells with <20% of mitochondrial reads. Next, uniform manifold approximation and projection (UMAP) was used for dimensionality reduction and initial clustering with k-means. Here, three major cell types were assigned based on the marker expression: epithelial tumor cells (WFDC2, PAX8, MUC16), stromal cells (COL1A2, FGFR1, DCN) and immune cells (CD79A, FCER1G, PTPRC). To ensure the quality of the cells used in subsequent analysis, further filtering was performed by removing tumor cells with <12 and immune and stromal cells with a number of UMI (in log scale) <11 and, resulting in total 12887, 14320 and 3285 cells of particular type, respectively.

Patient-specific molecular markers for tumor samples and their derived organoids were extracted using a logistic regression. The normalized average expression of all the patient-specific markers for each of the tumor samples were compared against all organoid samples using a Pearson correlation.

CNV profiles for the individual cancer cells were obtained with InferCNV of the Trinity CTAT Project³⁰ using the stromal and immune cells as reference. Next, Leiden algorithm was used for the determination of the underlying subclonal populations based on the CNV profiles. The similarity matrix for the subclones was calculated by measuring the cosine distance between the consensus CNV status of each subclone.

Drug response profiling

Fully grown organoids were extracted from BME-2 domes as described above. Cell pellets were resuspended in BME-2 and 10 μ L of the suspension was plated into each well (omitting outside border wells) of pre-cooled 384-well Ultra-Low Attachment microplates. Seeding density was adjusted to the expansion rate of particular organoid culture using the following formula: Number of organoid culture droplets seeded into a single microplate = culture passaging ratio \times 30. Gel droplets were solidified in a humidified cell culture incubator at 37°C for 30-45 min and covered with 40 μ L of culture-appropriate growth medium. Culturing medium was exchanged every 2-3 days using EL406 plate washer (BioTek). 2-3 days before drug addition, in half of the plates, M1/M2 medium was exchanged to HPLM (#A4899101, ThermoFisher), supplemented with relevant niche factors from M1/M2, omitting GlutaMax, nicotinamide and N-acetylcysteine, which majorly impact cell metabolism. All organoid cultures maintained growth in HPLM over the investigated period (10 days). After 7-10 days of growth post-seeding (depending on the growth rate of each organoid culture), experimental drugs, DMSO (vehicle control) or 10 μ M bortezomib (positive control) were added to the microplates using Echo 550 Liquid Handler (Labcyte). Experimental compounds were added in five 10-fold dilutions (carboplatin at 10 nM-100 μ M, other compounds at 1 nM-10 μ M). Microplates were incubated at 37°C for 96 h. Subsequently, 25 μ L of the culture medium was exchanged to staining solution, containing CellTox Green reagent (#G8743, Promega, final concentration 1X) and Hoechst 33342 (#B2261, Sigma, final concentration 5 μ g/mL). Plates were incubated for 2 h at room temperature and imaged in an ImageXpress Micro Confocal automated fluorescence microscope (Molecular Devices). A single image per well for each fluorophore was taken using 10X objective. Survival indices were estimated by image analysis using MetaXpress (Molecular Devices) software. Nuclei were identified as individual objects based on Hoechst 33342 staining and computationally enlarged. Objects which overlapped with CellTox Green signal were counted as dead cells and negative as viable cells. Data points coming from microplate wells that lost the BME gels with embedded organoids during plate processing (for example, during media exchanges) were removed from the analysis. Fraction of viable cells was estimated in each well. Survival index in each well was estimated by normalization to negative (100% viability) and positive (0%) controls. Dose-response curves were generated using non-linear $\log(\text{inhibitor})$ vs response (three parameters) fit and AUC calculated in GraphPad Prism using the trapezoid rule. For AUC calculation for combination of carboplatin and 0.1 μ M paclitaxel, only three highest concentrations of carboplatin were used (1-100 μ M) in order to avoid exaggerated effect of 0.1 μ M paclitaxel on overall drug combination effect at low carboplatin concentrations (10-100 nM). To simplify comparisons between different samples and medium conditions, AUCs were transformed for particular treatments using Z-score normalization. For correlation of drug response to patient outcomes, normalized CA125

change was calculated as a difference between last CA125 level before chemotherapeutic treatment with carboplatin + paclitaxel combination and first measured post-treatment CA125 level in that patient, expressed as a percentage of the maximal, patient-specific CA125 blood level in the relevant period (relevant period marked with yellow rectangles in the figures).

QUANTIFICATION AND STATISTICAL ANALYSIS

Mean organoid radius values were compared with unpaired two-tailed Mann-Whitney test using GraphPad Prism. Correlations between single-cell expression levels of organoid markers and tumor markers were calculated using Pearson's R , assuming linear relationship (testing for normality was not performed as it is not necessary with large number of data points, such as in this case). Correlations between normalized blood CA125 level change in patients and organoid-based normalized AUC Z-score were not assumed to be linear (data were not normally distributed according to D'Agostino-Pearson and Shapiro-Wilk normality tests) and were calculated using rank-based Spearman's r . P values <0.05 were considered significant. Definitions of n and details of statistical analyses are provided in relevant figure legends.

ADDITIONAL RESOURCES

Genomic data, including mutation, copy-number and signature profiles are available through an interactive visualization in GenomeSpy, accessible at: https://csbi.ltdk.helsinki.fi/p/senkowski_et_al_2022/.

Supplemental information

**A platform for efficient establishment
and drug-response profiling of high-grade
serous ovarian cancer organoids**

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EOC310_pAsc, 14 days

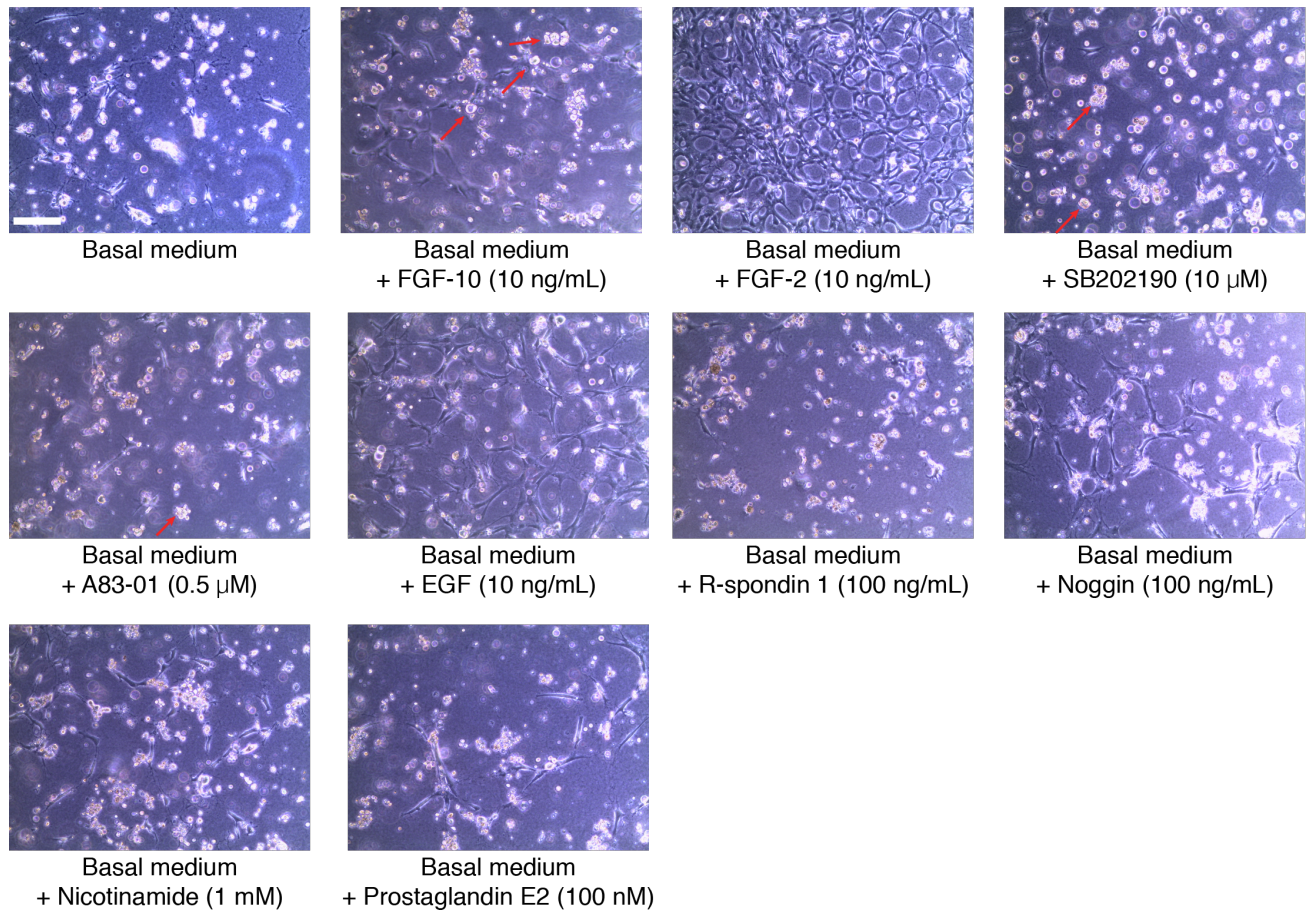


Figure S1. Influence of individual additives on short-term HGSC organoid formation. Related to Figure 1.

Phase-contrast images of EOC310_pAsc cells, embedded in BME and cultured for 14 days in the Basal Medium, supplemented with individual additives, as indicated. Red arrows indicate formation of coherent, three-dimensional multicellular clusters. 10X magnification; scale bar, 100 μ m.

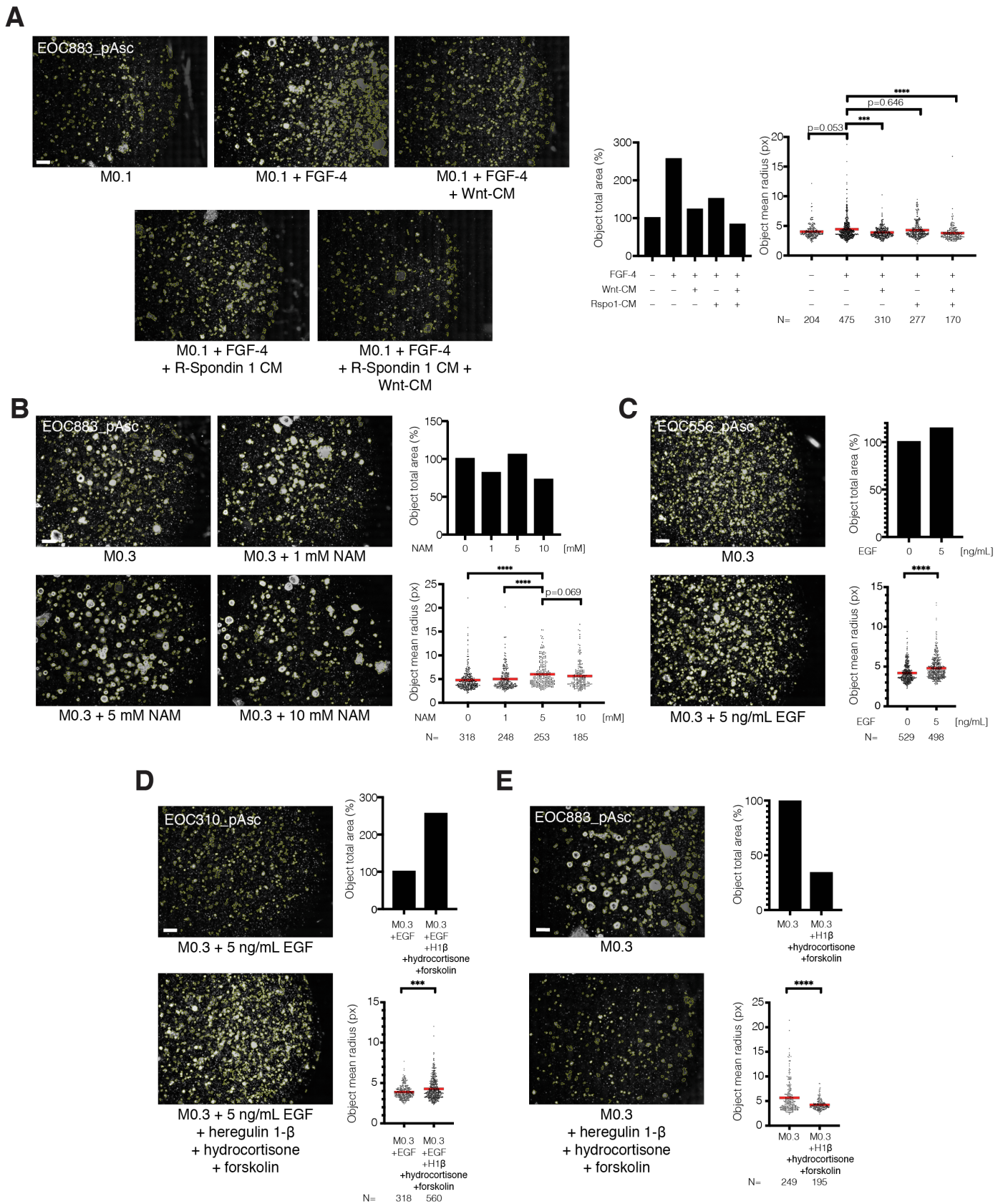


Figure S2. Establishment of new HGSC organoid media formulations. Related to Figure 1.

(A) *Left*: Phase-contrast images of basement membrane extract (BME) droplets with objects (outlined in yellow) identified with CellProfiler. EOC883_pAsc cells were cultured in M0.1 or M0.1 supplemented with FGF-4 (10 ng/mL), Wnt conditioned medium (Wnt-CM, 50% v/v) and/or R-Spondin 1 conditioned medium (R-Spondin 1 CM, 25% v/v) for 38 days (passaged once on day 17). Scale bar, 200 μ m. *Right*: Total area of objects and mean (marked with a line) object radius in the particular picture, estimated using CellProfiler. (B) *Left*: Phase-contrast images of BME droplets with objects identified as above. EOC883_pAsc cells were cultured in M0.3 or M0.3 supplemented with nicotinamide (NAM, 1, 5 or 10 mM) for 38 days (passaged once on day 19). Scale bar, 200 μ m. *Right*: Mean object radius in the particular picture, as above. (C) *Left*: Phase-contrast images of BME droplets with objects identified as above. EOC556_pAsc cells were cultured in M0.3 or M0.3 supplemented with EGF (5 ng/mL) for 33 days (passaged once on day 17). Scale bar, 200 μ m. *Right*: Mean object radius in the particular picture, as above. (D, E) *Left*: Phase-contrast images of BME droplets with objects identified as above. EOC310_pAsc (D) or EOC883_pAsc (E) cells were cultured in M0.3 supplemented with 5 ng/mL EGF (D) or M0.3 (E) or these formulations supplemented with 37.5 ng/mL heregulin-1 β , 0.5 μ g/mL hydrocortisone and 5 μ M forskolin for 35 days (passaged once on day 19, (D)) or 39 days (passaged once on day 19, (E)). Scale bar, 200 μ m. *Right*: Mean object radius in the particular picture, as above. * = $p < 0.05$; *** = $p < 0.001$; **** = $p < 0.0001$, unpaired two-tailed Mann-Whitney test.

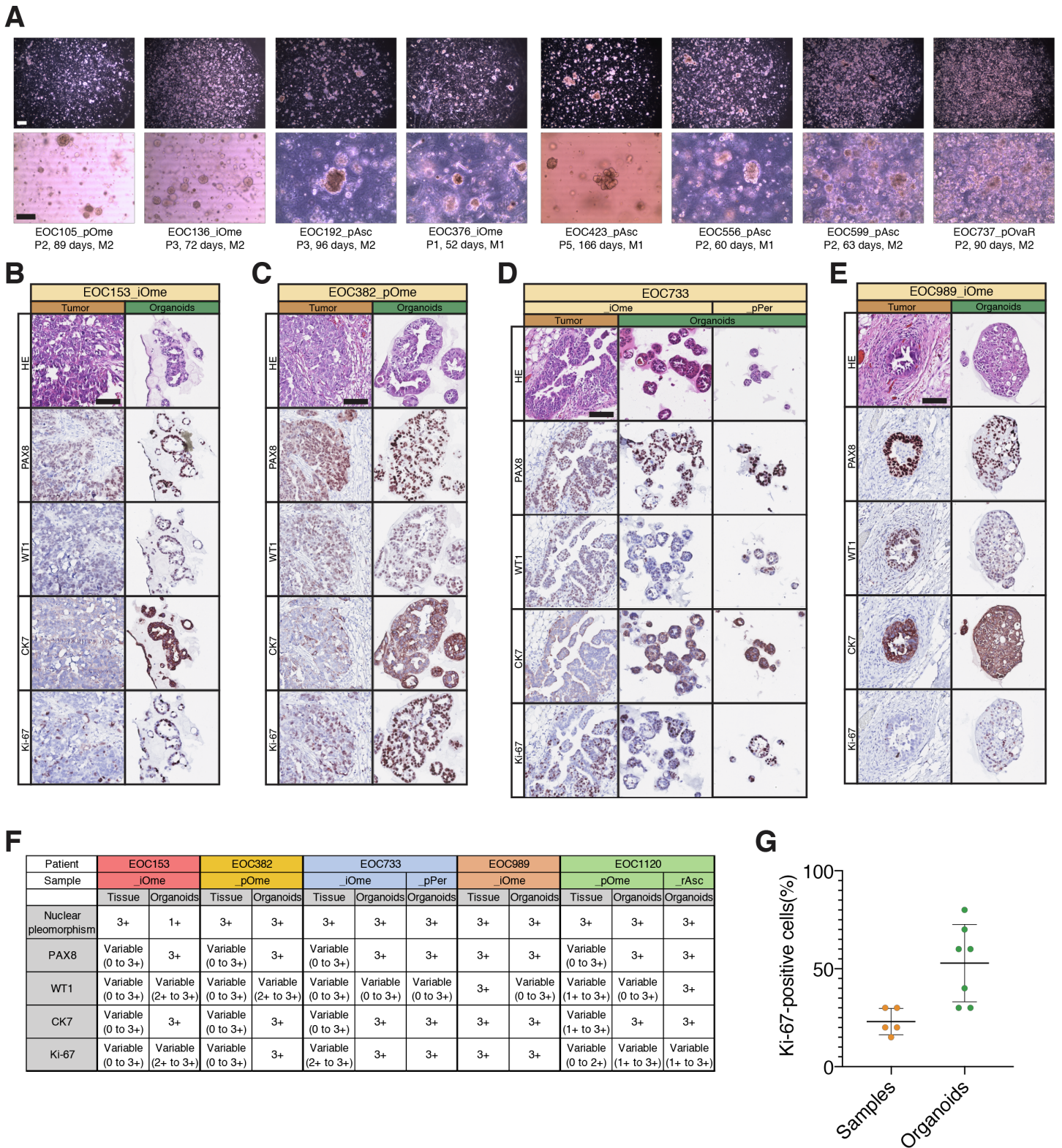
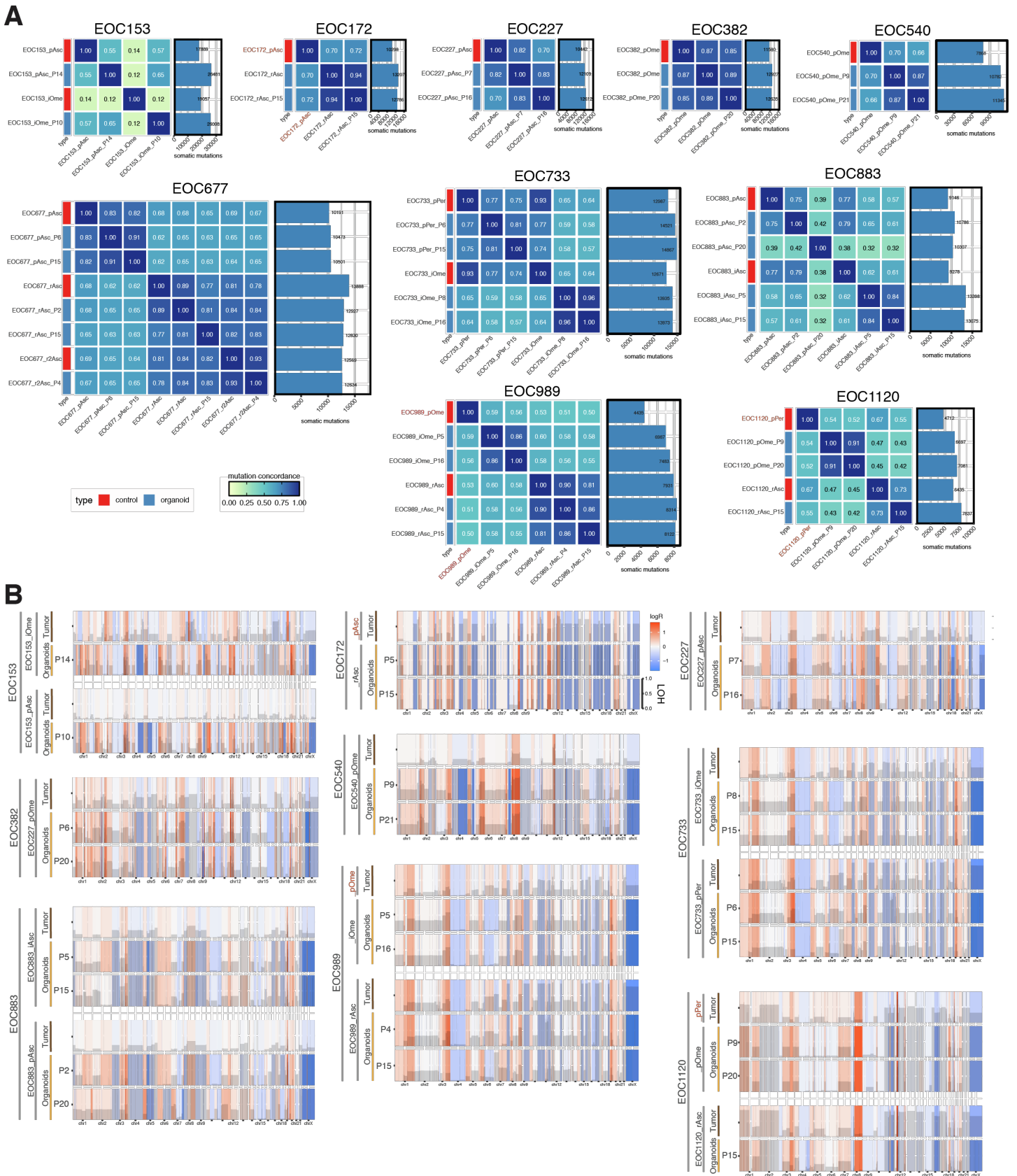


Figure S3. Immunohistochemical comparison of organoid models and tissues of origin. Related to Figure 3.

(A) Brightfield/phase-contrast images of failed cultures depicting initial 3D structure formation and cellular growth. Scale bars, 200 μ m (top) and 100 μ m (bottom). (B-E) HE and IHC stainings (for indicated markers) of EOC153_iOme, EOC382_pOme, EOC733_iOme and EOC989_iOme tumor tissues and matching organoids. Additionally, EOC733_pPer organoids were stained (C). Scale bar, 100 μ m. (F) Pathological assessment and scoring of the stained tissues. Organoids demonstrate morphological features similar to the original tissue, including nuclear pleomorphism, adenopapillary growth pattern and positive staining for PAX8, WT1 and CK7. They are also more proliferative than the original tissue, depicted by higher Ki-67 expression. (G) Comparison of estimated cancer cell Ki-67 positivity between organoids and original tissues, based on Ki-67 IHC staining, presented as mean \pm s.d.



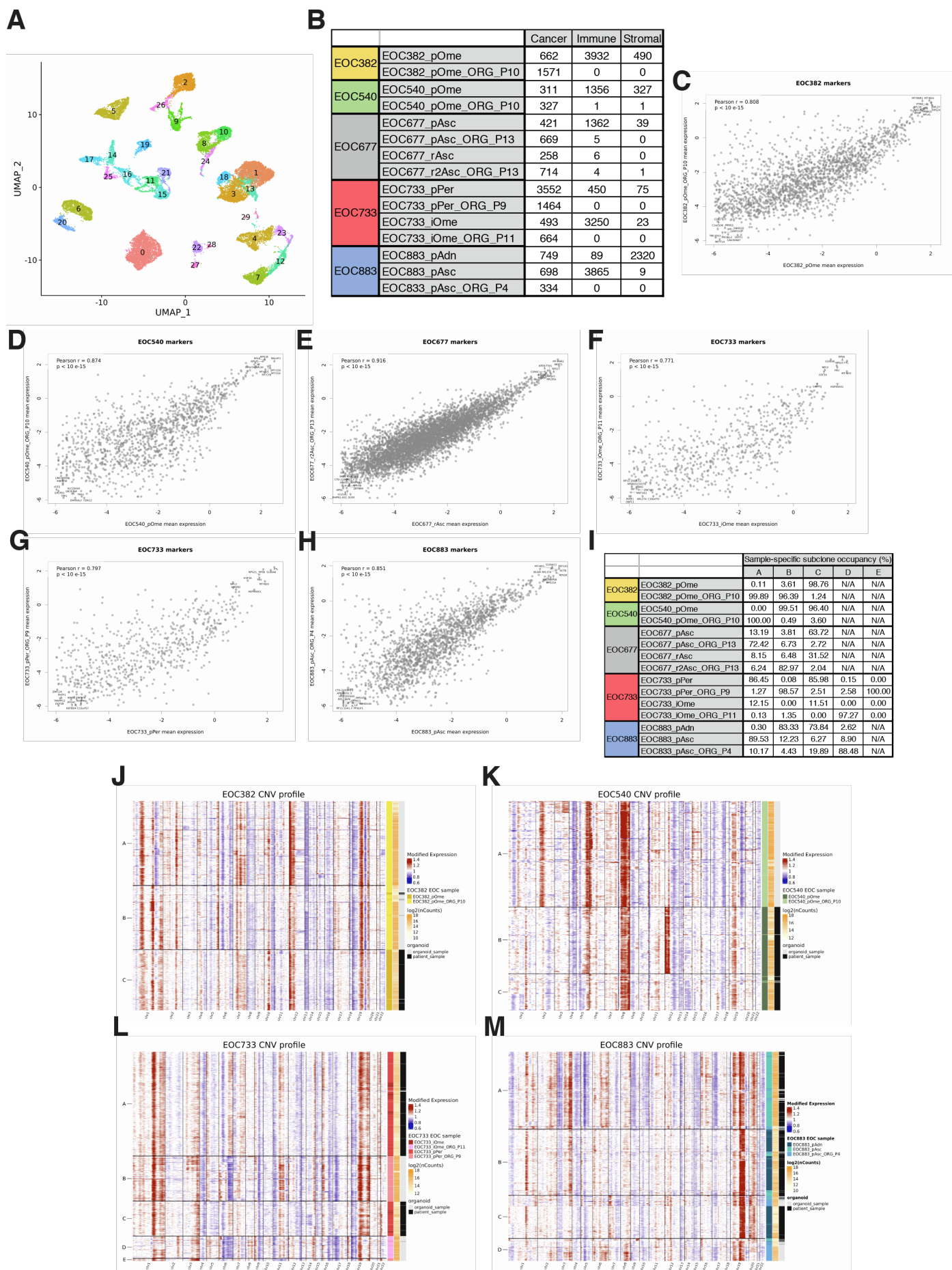


Figure S5. scRNA-seq characterization of HGSC organoids. Related to Figure 5.

(A) UMAP visualization of 30,492 cells from 7 organoid cultures and their tissue controls (with addition of EOC883_pAdn tumor samples), assigned to 26 subclusters (indicated by different colors and numbers) through unsupervised clustering. (B) Number of cells in analyzed samples assigned to a particular cell type (cancer, stromal or immune). (C-H) Pearson correlation plots of patient-specific markers expression in EOC382_pOme (C), EOC540_pOme (D), EOC677_rAsc (E), EOC733_iOme (F), EOC733_pPer (G) or EOC883_pAsc tumor samples and corresponding organoids. (J-M) Single-cell CNV plots from EOC382 (J), EOC540 (K), EOC733 (L) or EOC883 tumor samples and organoids, inferred using InferCNV and classified into 3-5 subclusters. (I) Occupancy of patient-specific subclones (as %) by individual cells from different samples.

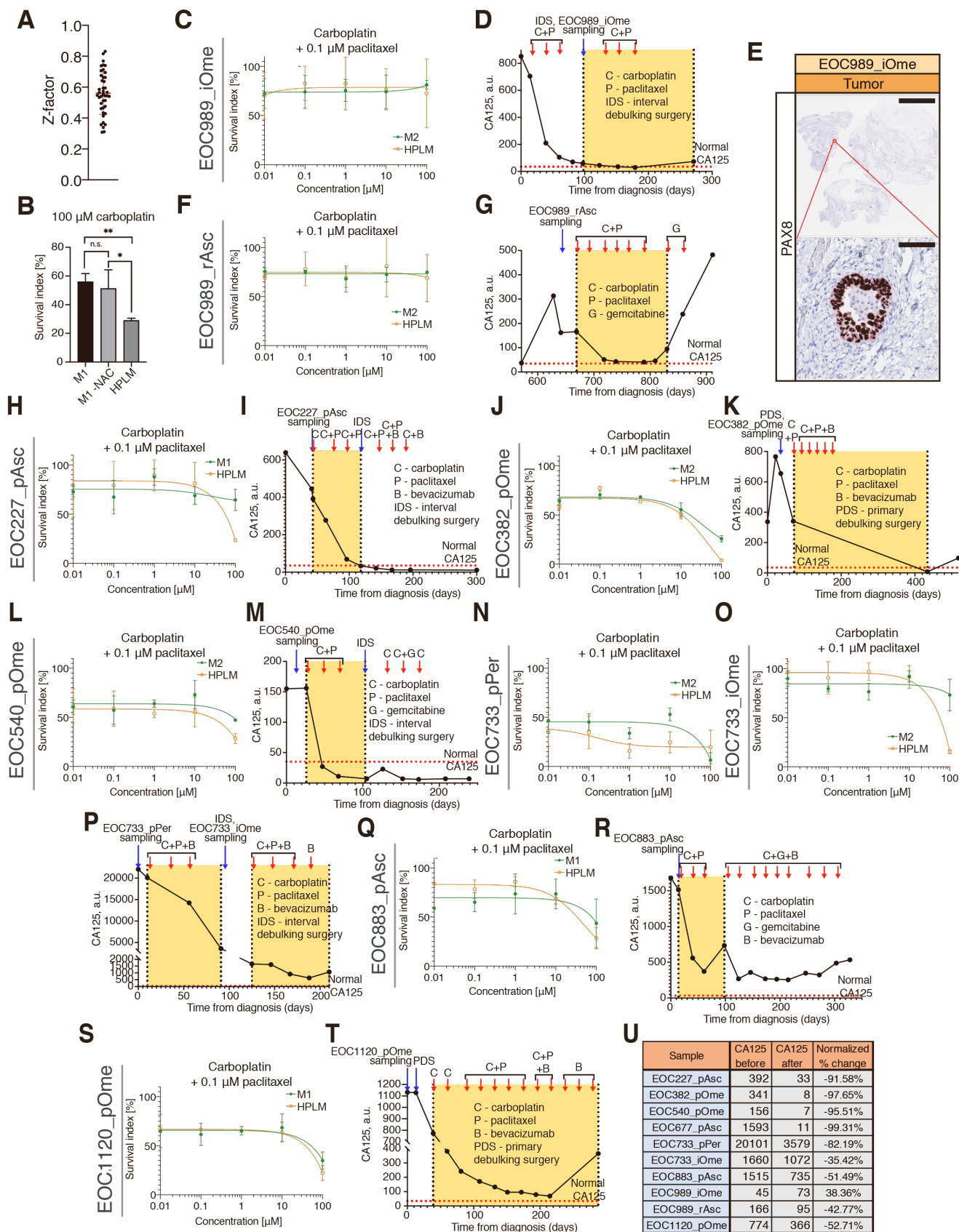


Figure S6. HGSC organoid drug responses and patient clinical outcomes. Related to Figure 6

(A) Z-factors in the drug response profiling experimental 384-well microplates. Presented as mean \pm s.d. (n=42). (B) Survival index of EOC677_pAsc organoids exposed to 100 μ M carboplatin for 96 hours in M1, M1 deprived of N-acetylcysteine (M1 -NAC) or HPLM. Data presented as mean \pm s.d. (n=3) * = p<0.05; ** = p<0.01; unpaired two-tailed t test. (C, F, H, J, L, N, O, Q, S) Dose-response curves of EOC989_iOme (C) EOC989_rAsc (F), EOC227_pAsc (H), EOC382_pOme (J), EOC540_pOme (L), EOC733_pPer (N), EOC733_iOme (O), EOC883_pAsc (Q) or EOC1120_pOme (S) organoids treated with carboplatin at indicated concentrations + 0.1 μ M paclitaxel, in M1/M2 or HPLM. Results are shown as mean of 2 biological replicates (each with 2-3 technical replicates) \pm s.d.

(D, G, I, K, M, P, R, T) CA125 blood levels of patients EOC898 (D and G), EOC227 (I), EOC382 (K), EOC540 (M), EOC733 (P) EOC883 (R) or EOC1120 (T) over time. Period relevant for comparison with *in vitro* drug response indicated with yellow rectangles. Normal CA125 range (<35 a.u.) indicated with red dotted lines.

(U) Last blood CA125 measurements before carboplatin + paclitaxel combination chemotherapy and first blood CA125 measurement after in indicated patients. The difference between the two measurements was normalized by maximal patient-specific CA125 level value in the relevant period.

(E) IHC staining of EOC989_iOme tumor tissue for PAX8. Scale bars, 5 mm (top), 100 μ m (bottom)

Samples used for organoid derivation and medium optimization										
Patient no.	Patient code	FIGO stage at diagnosis	Sample name	Tumor deposit source	Clinical progression stage at sampling	Sample tumor purity	Successful organoid derivation	Organoid tumor purity (latest available passage)	Time in culture to reach stable expansion (days)	Successful resuscitation from frozen organoids
1	EOC105	IIIC	EOC105_pOme	Omentum	Primary	Unknown	No			
2	EOC136	IVA	EOC136_pAsc EOC136_iOme	Ascites Omentum	Primary Interval	12% 72.9%	No No			
3	EOC153	IVA	EOC153_pAsc EOC153_iOme	Ascites Omentum	Primary Primary	15.5% 70.5%	Yes Yes	100% 99%	120 185	Yes Yes
4	EOC172	IVA	EOC172_pOme EOC172_rAsc	Omentum Ascites	Primary Recurrence	40.5% 0%*	No Yes	100%	100	Yes
5	EOC192	IIIC	EOC192_pAsc EOC192_pOvaL	Ascites Left ovary	Primary Primary	35.5% Unknown	No No			
6	EOC227	IVA	EOC227_pAsc	Ascites	Primary	35.9%	Yes	98.50%	102	Yes
7	EOC376	IIIC	EOC376_iOme	Omentum	Interval	Unknown	No			
8	EOC382	IIIC	EOC382_pOme	Omentum	Primary	35%	Yes	97.5%	30	Yes
9	EOC423	IIIC	EOC423_pAsc EOC423_pOme EOC423_iOvaR	Ascites Omentum Right ovary	Primary Primary Interval	Unknown 70.2% 100%	No No No			
10	EOC473	IVB	EOC473_pAdn EOC473_iPer	Adnex Peritoneum	Primary Interval	72% Unknown	No No			
11	EOC540	IIIC	EOC540_pOme	Omentum	Primary	22%	Yes	99.50%	120	Yes
12	EOC556	IIIC	EOC556_pAsc EOC556_iBow	Ascites Bowel	Primary Interval	17.8% 8.5%	No No			
13	EOC599	IVA	EOC599_pAsc EOC599_iOme	Ascites Omentum	Primary Interval	18% 10%	No No			
14	EOC677	IIIC	EOC677_pAsc EOC677_rAsc EOC677_r2Asc	Ascites Ascites Ascites	Primary Recurrence 2nd recurrence	45.4% 40.8% 67.5%	Yes Yes Yes	100% 97.5% 100%	86 26 49	Yes Yes Yes
15	EOC737	IIIC	EOC737_pOvaR	Right ovary	Primary	50.7%	No			
16	EOC733	IVA	EOC733_pPer EOC733_iOme	Peritoneum Omentum	Primary Interval	97% 51.5%	Yes Yes	96.5% 100%	91 140	Yes Yes
17	EOC883	IIIC	EOC883_pAsc EOC883_iAsc	Ascites Ascites	Primary Interval	18.5% 27.9%	Yes Yes	100% 99.00%	35 85	Yes Yes
18	EOC989	IVA	EOC989_iOme EOC989_rAsc	Omentum Ascites	Interval Recurrence	5% 90.8%	Yes Yes	100% 99.50%	55 49	Yes Yes
19	EOC1120	IVB	EOC1120_pOme EOC1120_rAsc	Omentum Ascites	Primary Recurrence	Unknown 80%	Yes Yes	100% 99%	177 70	Yes Yes
Samples used only for medium optimization										
20	EOC310		EOC310_pAsc	Ascites	Primary	Unknown	N/A			

Table S1. Overview of samples used in the study. Related to Figures 1-4

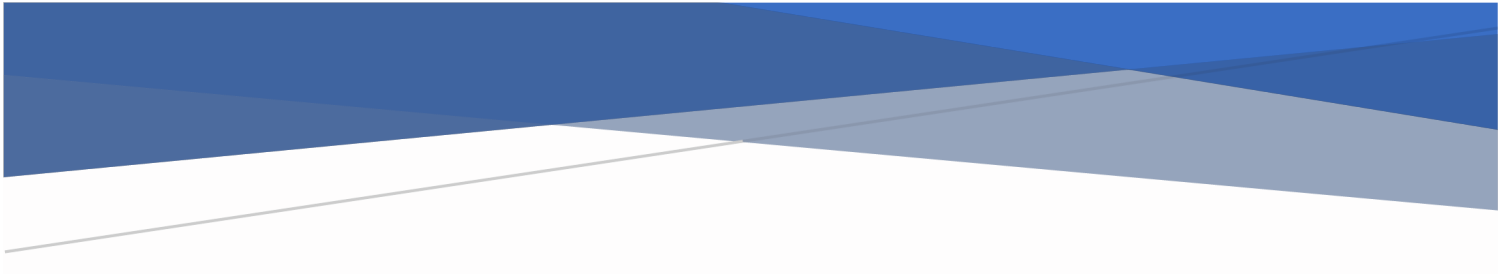
*Cancer cells not detectable in the sample using WGS

Media supplements tested for organoid establishment and long-term culture			
Growth factors	Manufacturer, product no.	Concentrations tested	Effect on organoid derivation
FGF-2	Peprotech, #100-18B	10 ng/mL	Harmful
FGF-4	Peprotech, #100-31	10 ng/mL	Beneficial
FGF-7	Peprotech, #100-19	10 ng/mL	Neutral
FGF-10	Peprotech, #100-26	10 ng/mL	Beneficial
EGF	Peprotech, #AF-100-15	5 ng/mL	Harmful or beneficial
		10 ng/mL	Harmful
		50 ng/mL	Harmful
IGF-I	Peprotech, #100-11	20 ng/mL	Neutral
		100 ng/mL	Neutral
		10 ng/mL	Neutral
VEGF	Peprotech, #AF-100-20	10 ng/mL	Neutral
Other proteins			
Heregulin-1 β	Peprotech, #100-03	5 nM	Harmful or beneficial
BMP-2	Thermo Fisher, #PHC7145	10 ng/mL	Neutral
Jag-1	AnaSpec, #AS-61298	1 μ M	Neutral
R-Spondin 1	Peprotech, #120-38	100 ng/mL	Neutral
		400 ng/mL	Neutral
		1 μ g/mL	Harmful
R-Spondin 3	Peprotech, #3500-RS-025	250 ng/mL	Neutral
Noggin	Peprotech, #120-10C	100 ng/mL	Harmful
Hormones			
β -estradiol	Sigma, #E8875	100 nM	Beneficial
Hydrocortisone	Sigma, #H0888	100 ng/mL	Harmful or beneficial
		500 ng/mL	Harmful or beneficial
Follicle-stimulating hormone	R&D Systems, #5925-FS-010	10 ng/mL	Neutral
		50 ng/mL	Neutral
Gonadotropin-stimulating hormone	Sigma, #L8008	10 ng/mL	Neutral
		50 ng/mL	Neutral
Triiodothyronine	Sigma, #T6397	0.1 ng/mL	Harmful
		1 ng/mL	Harmful
		10 ng/mL	Harmful
Prostaglandin E2	MedChemExpress #HY-101952	10 nM	Neutral
		1 mM	Harmful
Small-molecule inhibitors			
A83-01	Sigma, #SML0788	0.5 μ M	Beneficial
SB202190	MedChemExpress, #HY-10295	0.5 μ M	Beneficial
		3 μ M	Beneficial
		10 μ M	Beneficial
		2.5 μ M	Harmful
CHIR-99021	MedChemExpress, #HY-10182	0.1 μ M	Harmful
Idasanutlin	MedChemExpress, #HY-15676	5 μ M	Harmful or beneficial
Forskolin	MedChemExpress, #HY-15371	5 μ M	Harmful or beneficial
		10 μ M	Harmful or beneficial
Conditioned media			
Rspo1-conditioned medium	Gift from prof. Kim Jensen	25% v/v	Harmful
Wnt-conditioned medium	Gift from prof. Kim Jensen	20% v/v	Harmful
		50% v/v	Harmful
Other			
Nicotinamide	Sigma, #N0636	1 mM	Neutral
		5 mM	Beneficial
		10 mM	Beneficial

Table S2. Overview of tested media additives and their effects on HGSC organoid culture. Related to Figures 1-2.

Data for success rate calculation in previous studies come from:	
Maenhoudt et al. (2020)	Table 1
Hoffmann et al. (2020)	Table EV3
Kopper et al. (2019)	Extended Data Fig. 2a and Supplementary Table 4

Table S3. Data sources for the calculation of long-term organoid culture success rate in previous studies. Related to Figure 2.



METHOD S1, RELATED TO
STAR METHODS:
PROTOCOLS FOR HGSC
ORGANOID CULTURE

High-Grade Serous Ovarian Cancer Organoids

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Medium 2	9

Starting a culture from cryopreserved material (Patient Samples or Organoids)

Materials

- Dry ice and a Styrofoam box.
- Culture media (only M1).
- Y-27632 stock solution (10 mM)
- BME Type 2 (#3533-010-02, R&D Systems)
- 15-mL polypropylene snap cap Falcon tubes (1 per sample).
- 50-mL Falcon tubes.
- Pipette set and tips.
- Water bath set to 37°C.
- 6-well culture plates Nunc Cell-Culture Treated (ThermoFisher: #140685).
- Multistep electronic pipette and tips.
- Cold, sterile 1X PBS [-] CaCl₂ [-] MgCl₂.
- M1 and M2.
- 10-mL serological pipettes.

Instructions (this section covers how to thaw frozen material)

Before starting the protocol

1. Fill a Styrofoam box with dry ice for short-term patient samples storage.
2. Place 6-well cell culture plates in the incubator (they need to be pre-heated before pipetting the BME-2 to allow instant gel polymerization).
3. Turn on the water bath (37°C).

Beginning of the protocol

4. Aliquot 20 mL per sample of M1 in 50-mL Falcon tubes.
5. Warm up the media at 37°C by placing the Falcon tubes in the water bath for 10-15'.
6. Transfer 10 mL of **M1 (without Y-27632)** to the snap cap Falcon tubes.
7. Defrost the samples by placing the cryovials in the water bath for 1-2'.
 - a. Remember to swirl the cryovials so that the heat distributes homogeneously.
8. Add around 1 mL of M1 to each cryovial, mix 2-3 times, and transfer the content to the snap cap Falcon tubes. Rinse the cryovial with extra media.
9. Spin down the samples (200 or 300g, 5').
 - a. 200g for patient samples.
 - b. 300g for organoids.
10. Gently aspirate the supernatant.
 - a. Be **very careful** because the pellet is usually quite loose.
11. Prepare 10 mL **M1 + Y-27632 (Fc: 5 µM)** per sample.
12. Transfer **10 mL of M1 + Y-27632** to the tube with the pellet and re-suspend it by pipetting around 10 times with the P1000.
 - a. Remember not to over-pipette the cells too much when re-suspending. Having some cell clusters is beneficial for culturing.
13. If thawing a **patient sample**, count the cells. If thawing organoids, don't count them (just seed according to the split ratio).
14. **IMPORTANT: When thawing a cryopreserved organoids, reduce the initial passaging ratio by ½ (e.g. for organoid culture passaged at 1:4 ratio, seed the cryopreserved material from a single well to 2, instead of 4 wells). You will notice that there is an increased amount of dead cells after thawing and the organoid growth might be initially slower. This is normal and the culture should stabilize after 1-2 passages, returning to the old growth/passaging ratio.**
15. Spin down the sample (200 or 300G, 5').
16. Gently aspirate the supernatant.
17. Go to Sample Seeding.

Gel preparation (performed simultaneously with the procedure above)

1. Slowly thaw the BME-2 (recommended to be performed on ice, as the gel polymerizes at room temperature).
 - a. Remember to occasionally swirl the vial and place it in ice when defrosted. Never mix it by inversion.
2. Place 2 15-mL snap cap Falcon tubes in the ice bucket – one empty and the other with 1-2 mL of sterile PBS.
3. When the gel is defrosted, dilute it with **cold, sterile PBS**. Avoid introducing air bubbles. This step must be performed early so that any air bubbles have time reach the surface of the gel and disappear.
 - a. Take the BME vial and gently re-suspend the content with the P1000.
 - b. Transfer a desired amount of BME to a snap cap Falcon tube **together with the pipette tip** (since it contains a lot of product).
 - c. Add cold, sterile PBS to obtain final protein BME concentration of 7.5 mg/mL and mix it until obtaining a homogenous solution.
 - d. Remember to prep \approx 100-200 μ L of extra gel (as BME is a viscous solution, the volume indicated by the pipette is not exact and you will need some dead volume)
 - e. each gel batch (lot) has a different protein concentration. We try to work with a protein concentration of around 7.5 mg/mL. The ideal gel concentration is 7.5-8 mg/mL, and the minimum required is 7 mg/mL. Thus, every batch is diluted differently (usually 10-15% of PBS v/v).

Sample seeding

4. Gently mix the BME solution with the P1000. Avoid introducing air bubbles.
5. Take out 1 plate from the incubator and describe it with patient ID, sample, passage number, medium, and date.
6. Transfer the desired amount of the gel solution (200 μ L per 10 droplets per single well of 6-well plate + 50-60 μ L extra volume) to the cell pellet and gently re-suspend until obtaining a homogenous solution (pipette between 10-14 times).
 - a. **Patient samples:** re-suspend to obtain a density of min 10^6 live cells/mL of BME-2.
 - b. **Organoids:** follow the ratio on the tube.
7. Seed the cells with the electronic or manual pipette. **Seed 10 droplets of gel per plate (20 μ L/droplet).**
 - a. Remember to place the droplets far enough from each other and from the walls to avoid merging (see Organoid Passaging protocol).
 - b. **TIP:** decrease the aspiration speed to avoid air bubbles.
8. If seeding multiple samples, mix the gel between them.
9. Place the plates in the incubator for 45' to solidify the BME.

During the 45-minute break

10. Aliquot the desired amount (3 mL/well) of M1 and M2 media in Falcon tubes.
11. Add Y-27632 (F_c 5 μ M) and mix.
12. Warm up the media at approx. 37°C by placing the Falcon tubes in the water bath for approx. 10-15'.

After the 45 minute break

13. Take out the plates from the incubator and check the gels.
14. Finally, gently add 3 mL of media to each well (M1/M2 + Y-27632) with a **10-mL pipette**.
 - a. Fresh patient samples should be cultured in M1 and M2 in parallel in order to determine (over a few passages) which medium formulation is preferred by the particular sample.
15. Place the plates in the incubator.

Medium Change (6-well plates)

The media are exchanged 3 times per week, every 2-3 days (usually Mondays, Wednesdays and Fridays).

Materials

- Culture media (M1 and M2).
- 1X Sterile PBS [-] CaCl₂ [-] MgCl₂.
- 50-mL Falcon tubes.
- Serologic pipettes.
- Sterile, Pasteur glass pipettes for the vacuum pump.

Instructions

1. **Keep the media in the fridge while not using them.**
2. Turn on the water bath (37°C).
3. In the meantime, take out the plates from the incubator and examine the cells. Calculate the amount of media and PBS that will be required.
 - a. Each well must contain 3 mL of fresh media.
 - b. For the washes, approximately 1 mL of PBS per well is required.
4. Aliquot the desired amount of M1, M2, and sterile PBS in Falcon tubes, and **place the media bottles back in the fridge.**
5. Warm up the media and the sterile PBS at approx. 37°C in the water bath for approx. 10-15'.
 - a. The media and PBS must be warm to prevent the gels from depolymerizing.
6. Take out the plates from the incubator.
7. Gently aspirate the old media.
 - a. To aspirate, tilt the plate and aspirate from the well wall to avoid disrupting the gels.
8. Wash the gels by gently adding some **warm sterile PBS** to each well with a serological pipette.
 - a. Remember to dispense the PBS toward the wall of the well.
9. Gently aspirate the PBS.
10. Gently add fresh medium to each well with a serological pipette.

Organoid Passaging (6-well plates)

Materials

- Cultrex RGF Basement Membrane Extract, Type 2, Pathclear (3533-005-02).
- Bucket with ice.
- Y-27632 10 mM (F_c 5 μ M).
- 1X Sterile PBS [-] $CaCl_2$ [-] $MgCl_2$.
- M1 and M2.
- 15-mL PP snap cap Falcon tubes (1 for the gel, 1 for the cold PBS, one for each sample).
- 50-mL Falcon tubes.
- 6-well culture plates Nunc Cell-Culture Treated (ThermoFisher: 140685).
- TrypLE Express.
- Cell scrapers.
- 10-mL serological pipettes.
- Electronic pipette and tips.
- Pipette set and tips.
- Sterile, Pasteur glass pipettes for the vacuum pump.

Important Notes

Organoids must not be over-pipetted. This is particularly important when organoids cultures are in early phases. Cells usually give rise to organoids in presence of other cells. In early phases of culture development, you might notice that the organoid growth is very slow and organoids are scarce. In such case, do not try to expand the number of wells cultured – instead, try to concentrate the organoids in a smaller amount of gel and make the culture denser. HGSC organoids show preference for growth in high-density culture. Once the culture is dense, you will notice that passaging/expansion ratio and time between passages become stable.

Instructions

Early preparation

1. Take the plates out from the incubator, observe them under the microscope to make sure that the organoids reached the desired size/density for passaging and no contamination is present.
2. Place the new 6-well plates in the incubator for at least 30 minutes.
 - a. The BME should polymerize quickly in a warm plate, so that most cells don't attach to the plastic.
3. Aliquot some PBS in a 50 mL Falcon tube.
4. Place 2 snap cap tubes in the ice bucket (1 for the cold PBS and 1 for the gel).
5. Aliquot some PBS in 1 of the snap cap tubes and place it back on ice.

Cell harvest

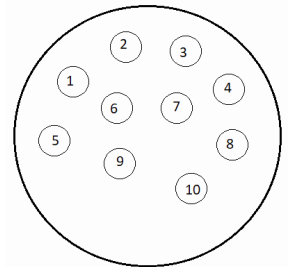
6. Aspirate the media from all the wells.
7. Wash the gels with room-temperature sterile PBS with a serologic pipette.
8. Add 2 mL of TrypLE Express per well.
9. Scrape the gels off and detach them from the plate with a cell scraper.
 - a. Remember to use a different cell scraper for each sample.
10. With the P1000, vigorously pipette the gels (**4-7 times depending on the cell density**) until disrupted while rinsing the whole well. The gels must be disrupted so that they digest well in TrypLE Express.
 - a. In case of a low cell density, pre-wet the pipette tip with TrypLE Express (by aspirating and dispensing back to the bottle)
11. **Incubate the plate for 15' in the 37 degrees incubator.** The organoids must not be incubated with TrypLE Express longer than 25-30 minutes because organoids could be over-digested as well.

During the 15-minute break prepare the gel

12. Prepare the gel as described in previous sections.

After the 15-minute break

13. Take the plates out of the incubator (one at a time).
14. Take the P1000 and pre-wet the pipette tip with TrypLE Express.
15. Transfer the cells to a 15-mL snap cap Falcon tube. Try to get most of the gel the first time. Rinse the well with the leftover liquid the second time.
16. Add 1 mL of PBS to each well to rinse it and transfer everything to the same tube in order to harvest the maximal number of organoids.
17. Spin down the organoids (300G, 5').
18. Gently aspirate the supernatant.
 - a. Start by aspirating the bubbles on the surface of the supernatant, as they might disrupt the pellet.
19. Gently mix the BME solution with the P1000.
20. Add desired amount of the gel solution (200 μ L per 10 droplets per single well of 6-well plate + 50-60 μ L extra volume. The more wells you seed, the less extra volume per well you will need) to the cell pellet and re-suspend it until obtaining a homogenous solution. **For delicate samples, pipette 10-12 times and around 14-18 for the sturdy ones.**
 - a. The thicker the gel, the less accurate the volume is.
21. Take a new 6-well plate out of the incubator.
22. Seed the gels with the electronic pipette (**20 μ L/droplet x 10 droplets/well**).
 - a. Be careful not to place the gels neither too close to each other nor to the walls.
 - b. Try to use as much of the organoid suspension as possible (e.g. seed the dead volume in the stepper pipette as well) – this is especially important when organoids are scarce in early culture development phase.
23. Describe the plates: patient ID, sample, passage, medium, and date.
24. **Incubate the plates for 45' in the incubator.**



During the 45'-minute break

1. Aliquot the desired amount (3 mL/well) of M1 and M2 media in Falcon tubes.
2. Add Y-27632 (F_c 5 μ M) and mix.
3. Warm up the media at approx. 37°C by placing the Falcon tubes in the water bath for approx. 10-15'.

After the 45'-minute break

25. **Carefully** add medium to the plates with a serologic pipette (do not pipette the medium directly on the gel domes – instead, dispense against the well wall, as the gels are delicate and disrupted easily).
26. Assess the seeding density and cell morphology under the microscope (it is important to observe every culture in order to adjust the passaging ratio for each one separately)
27. Place the plates in the incubator.

Organoid Cryopreservation and Biobanking

Materials

- Pipette set and tips.
- Cryovials for cell storage.
- Printed labels.
- Stem-Cellbanker (#11890, Amsbio (DMSO-free freezing solution. When using it,

samples can be transferred to -80 right away. Samples can be transferred to the nitrogen storage tank 24 h after being frozen).

Instructions

1. Label to the cryovials.

2. After harvesting the cells and aspirating the supernatant (as described in Organoid Passaging), add Stem-Cellbanker to the pellets as following:
 - a. If the pellet is from 1 well:
 - i. Add 1 mL of Stem-Cellbanker, take up the pellet, and transfer it to a cryovial.
 - ii. Transfer the rest of the Stem-Cellbanker to the cryovial.
 - iii. Should there be more cells in the tube, rinse it with 250 μ L of Stem-Cellbanker.
 - iv. Re-suspend the cells in the cryovial using a P1000 pipette (5-7 times) to reach a homogenous solution without large pellet fragments (these do not freeze well). Do not over-pipette the organoids.
 - b. If the pellet is from more than 1 well:
 - i. Add 1 mL (per each prepared cryovial) of Stem-Cellbanker to the cell pellet.
 - ii. Re-suspend to achieve a homogenous cell suspension, and aliquot it in the cryovials.
3. Place the samples at the -80 freezer.

IMPORTANT: When thawing a cryopreserved organoids, reduce the initial passaging ratio by $\frac{1}{2}$ (e.g. for organoid culture passaged at 1:4 ratio, seed the cryopreserved material from a single well to 2, instead of 4 wells). You will notice that there is an increased amount of dead cells after thawing and the organoid growth might be initially slower. This is normal and the culture should stabilize after 1-2 passages, returning to the old growth/passaging ratio.

Media Preparation

Materials

- Advanced DMEM/F12 (1X) (+NEAA; +sodium pyruvate; -L-Glutamine).
- Supplements, growth factors, and small molecule inhibitors.
- 1 15-mL Falcon tube.
- 1 50-mL Falcon tube.
- Plastic spoon and spatula.
- 250-mL sterile Corning bottle.
- Pipette set and tips.

Important Notes

Small molecules in DMSO can be refrozen up to 3 times.
FGF-4, FGF-10, EGF, neuregulin-1 and hormones cannot be re-frozen.

Instructions

Medium 1

1. Thaw the reagents in advance.
2. Weight the N-Acetyl-L-Cysteine and the Nicotinamide powders.
3. Spin down all the aliquots beforehand.
4. Add the different reagents into a bottle of Advanced DMEM/F12 Medium to prepare M1.
 - a. HEPES, GlutaMAX, and B-27 can be poured directly into the medium flask. Tubes must be rinsed with the medium
 - b. Add B-27 before the growth factors, as it contains BSA, which prevents growth factor molecules from attaching to plastic.
 - c. N-Acetyl-L-Cysteine and Nicotinamide can be dissolved by adding some media into the Falcon tubes and mixing.
 - i. N-Acetyl-L-Cysteine takes longer to dissolve, so repeat the operation as many times until there aren't any crystals left in the Falcon tube.
5. Once M1 is prepared, mix it thoroughly.

Medium 2

6. Pour the desired amount of M1 into a sterile plastic bottle.
7. Add EGF, Neuregulin-1, Forskolin, and hydrocortisone.
8. Mix thoroughly and store it in the fridge.

Medium 1	Stock concentration		Final concentration		Initial Volume	
	Value	Units	Value	Units	Amount	Units
Advanced DMEM/F12 (1X) (+NEAA; +sodium pyruvate; -L-Glutamine) ^F	-	-	-	-	500	mL
Primocin ^{Fz}	50	mg/mL	100	µg/mL	1	mL
HEPES ^{Fz}	1	M	10	mM	5	mL
GlutaMAX ^{Fz}	100	X	1	X	5	mL
N-Acetyl-L-Cysteine ^F	163,1951	g/mol	1	mM	0,08159755	g
Nicotinamide ^{RT}	122,12	g/mol	5	mM	0,3053	g
B-27 ^{Fz}	50	X	1	X	10	mL
β-estradiol ^{Fz}	10	mM	100	nM	5	µL
SB202190 ^{Fz}	10	mM	0,5	µM	25	µL
A83-01 ^{Fz}	10	mM	0,5	µM	25	µL
FGF-4 ^{Fz}	100	µg/mL	10	ng/mL	50	µL
FGF-10 ^{Fz}	100	µg/mL	10	ng/mL	50	µL
***Y-27632	10	mM	5	µM	250	µL
Medium 2	Stock concentration		Final concentration		Initial Volume	
	Value	Units	Value	Units	Amount	Units
Medium 1	-	-	-	-	300	mL
Neuregulin-1 ^{Fz}	50	µM	5	nM	30	µL
EGF ^{Fz}	100	µg/mL	5	ng/mL	15	µL
Forskolin ^{Fz}	10	mM	5	µM	150	µL
Hydrocortisone ^{Fz}	2	mg/mL	500	ng/mL	75	µL
Legend						
Blue: prepare 5-mL aliquots in advance			^{RT} room temperature			
Green: powder			^F fridge			
Red: the stock can be re-frozen after use (up to 3 times)			^{Fz} -20 °C			
*** only for 2-3 days after seeding						