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Supplemental information

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

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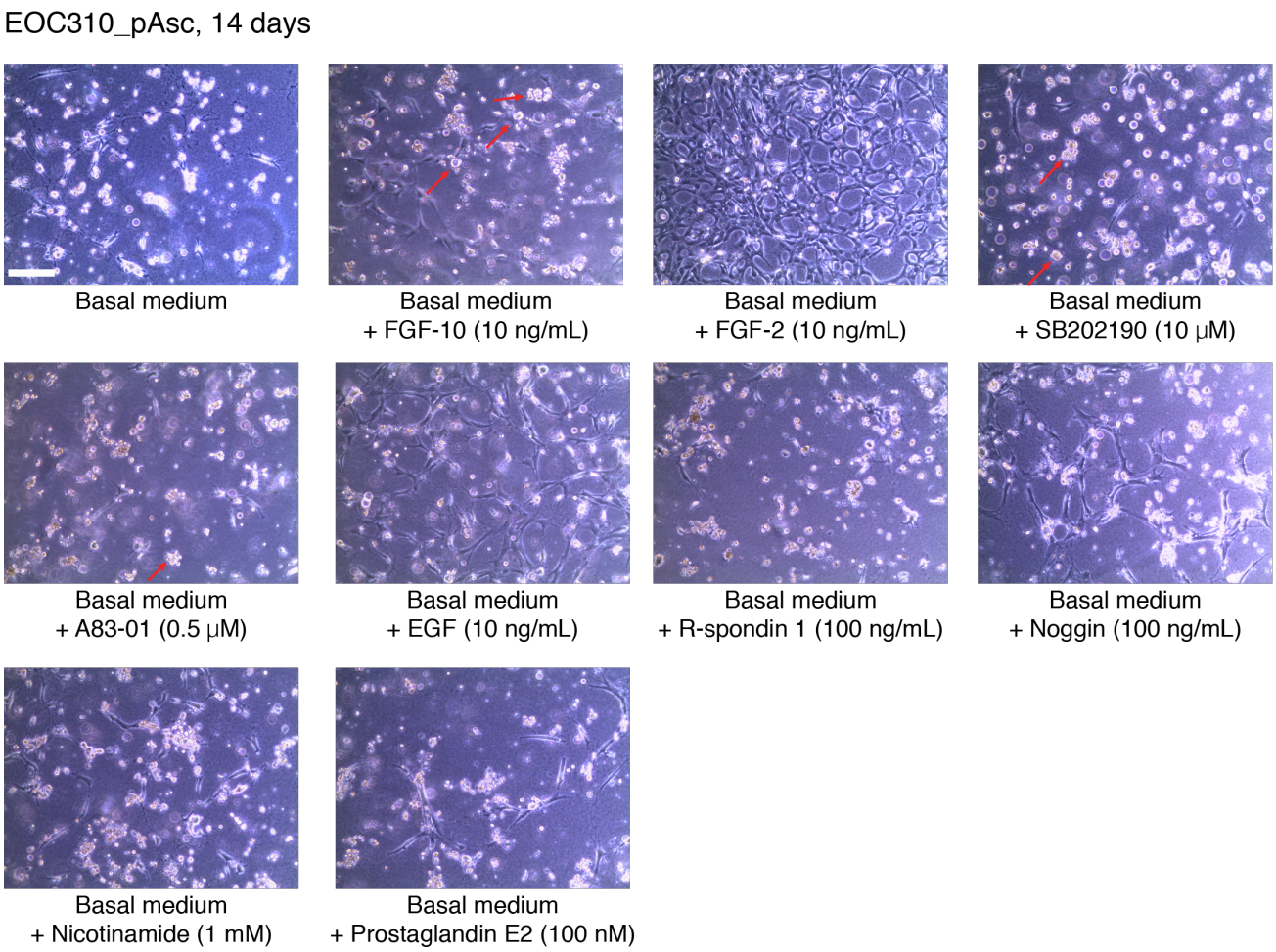


Figure S1. Influence of individual additives on short-term HGSC organoid formation. Related to Figure 1.
Phase-contrast images of EOC310_pAsc cells, embedded in BME and cultured for 14 days in the Basal Medium, supplemented with individual additives, as indicated. Red arrows indicate formation of coherent, three-dimensional multicellular clusters. 10X magnification; scale bar, 100 μm.

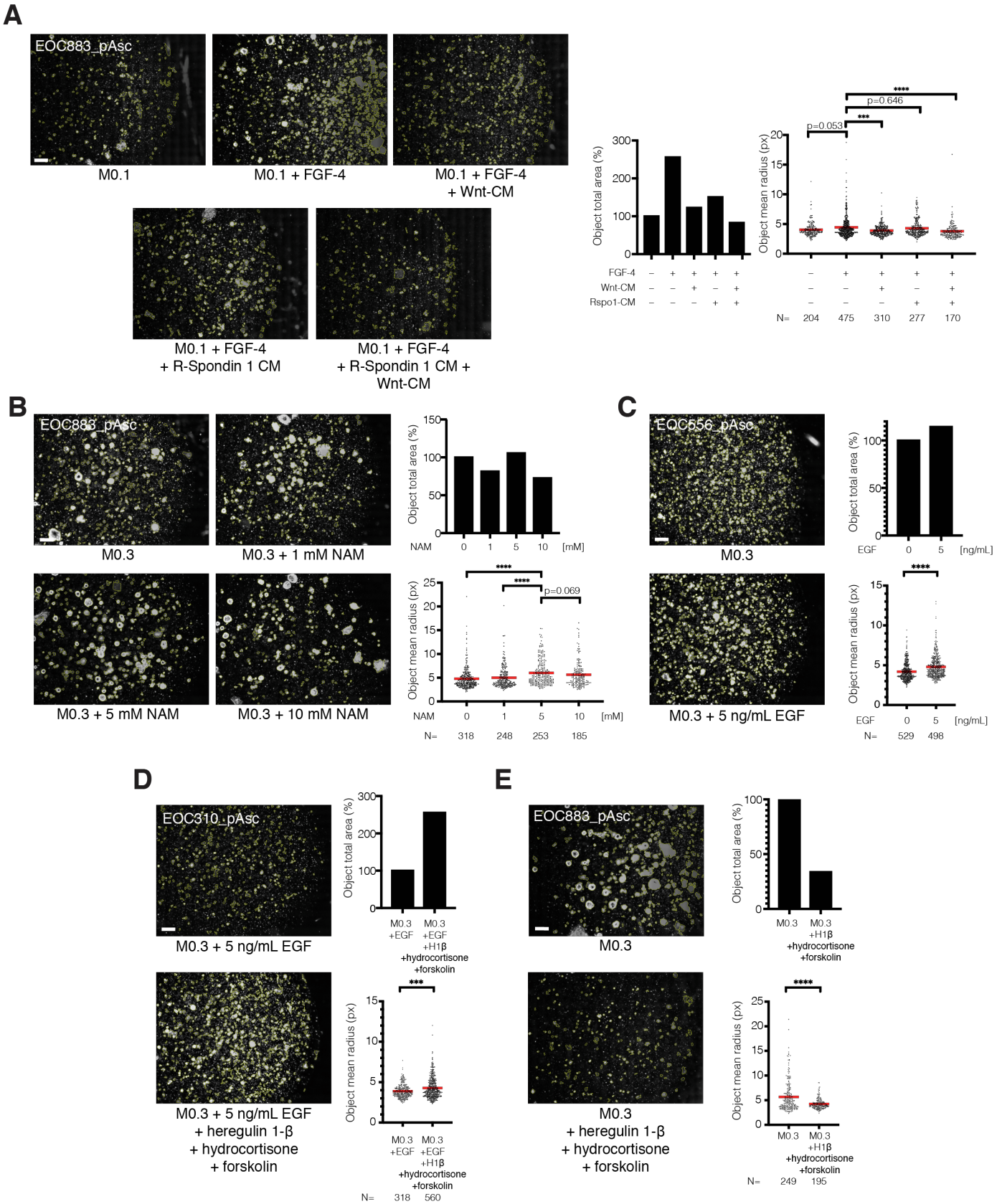
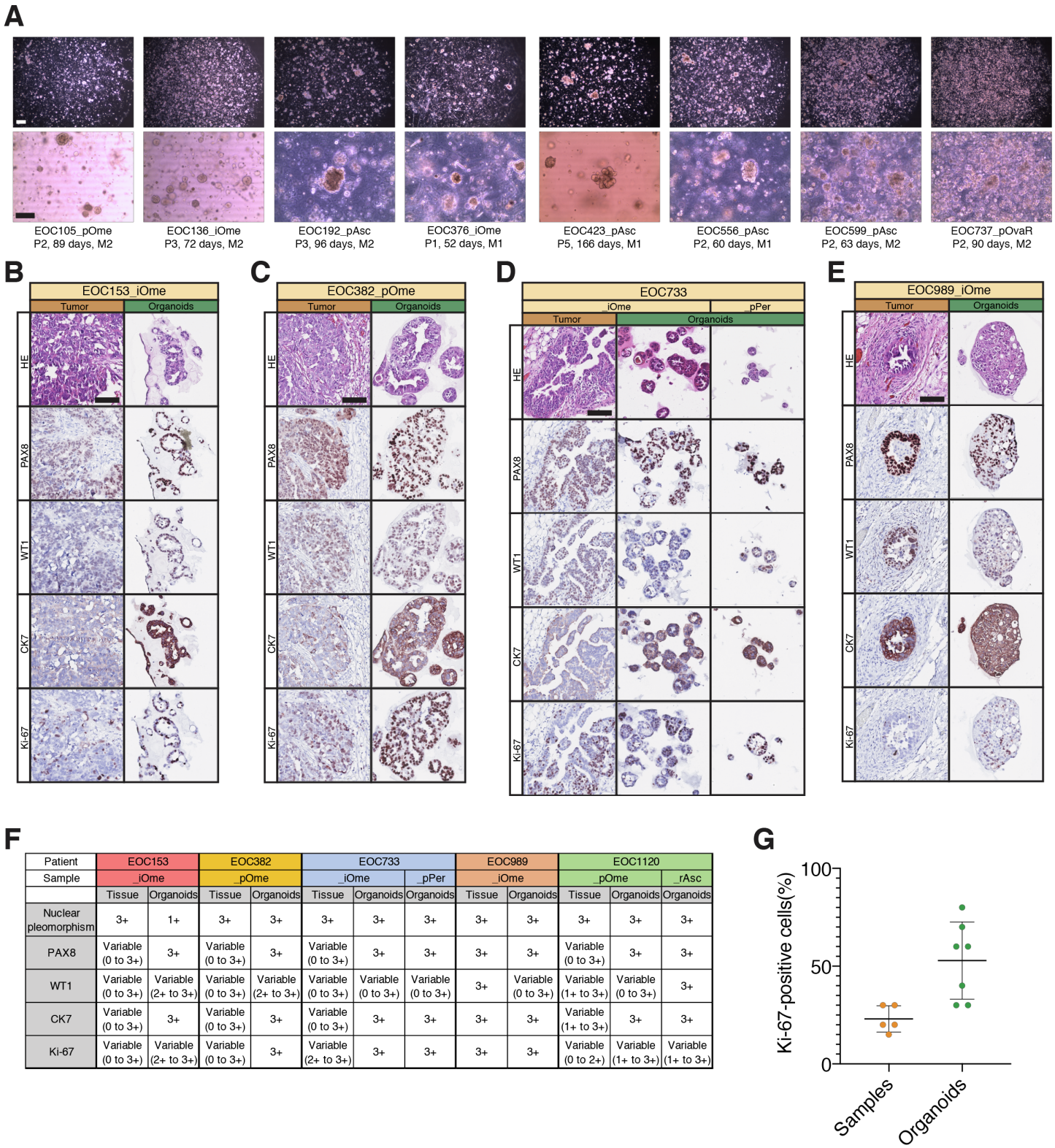


Figure S2. Establishment of new HGSC organoid media formulations. Related to Figure 1.

(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 µm. *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 µm. *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 µm. *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-1β, 0.5 µg/mL hydrocortisone and 5 µM forskolin for 35 days (passaged once on day 19, (D)) or 39 days (passaged once on day 19, (E)). Scale bar, 200 µm. *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.



F

Patient	EOC153		EOC382		EOC733			EOC989		EOC1120		
Sample	_iOme		_pOme		_iOme		_pPer	_iOme		_pOme		_rAsc
	Tissue	Organoids	Tissue	Organoids	Tissue	Organoids	Organoids	Tissue	Organoids	Tissue	Organoids	Organoids
Nuclear pleomorphism	3+	1+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
PAX8	Variable (0 to 3+)	3+	Variable (0 to 3+)	3+	Variable (0 to 3+)	3+	3+	3+	3+	Variable (0 to 3+)	3+	3+
WT1	Variable (0 to 3+)	Variable (2+ to 3+)	Variable (0 to 3+)	Variable (2+ to 3+)	Variable (0 to 3+)	Variable (0 to 3+)	Variable (0 to 3+)	3+	Variable (0 to 3+)	Variable (1+ to 3+)	Variable (0 to 3+)	3+
CK7	Variable (0 to 3+)	3+	Variable (0 to 3+)	3+	Variable (0 to 3+)	3+	3+	3+	3+	Variable (1+ to 3+)	3+	3+
Ki-67	Variable (0 to 3+)	Variable (2+ to 3+)	Variable (0 to 3+)	3+	Variable (2+ to 3+)	3+	3+	3+	3+	Variable (0 to 2+)	Variable (1+ to 3+)	Variable (1+ to 3+)

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 µm (*top*) and 100 µm (*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 µm. (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 ± s.d.

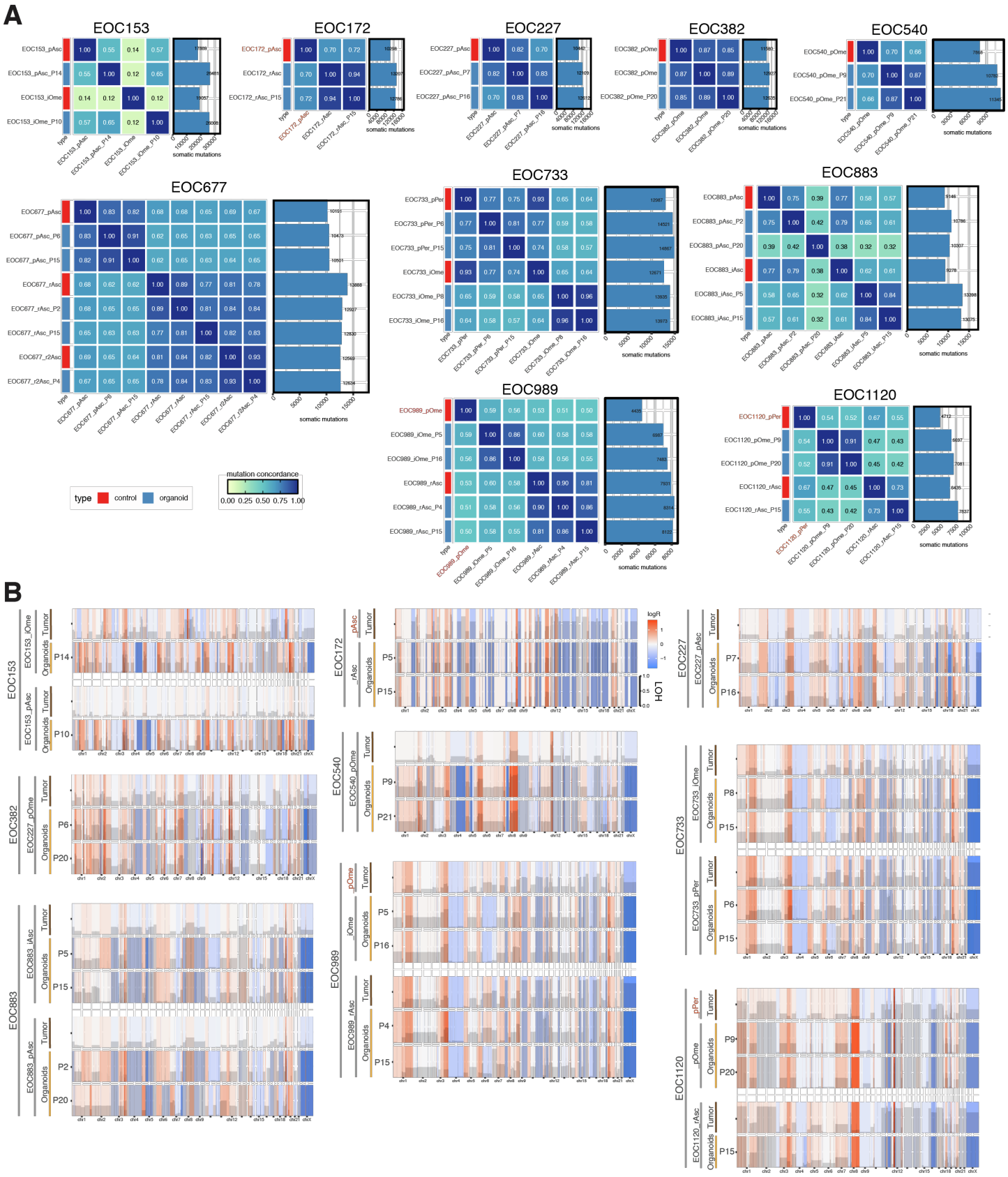
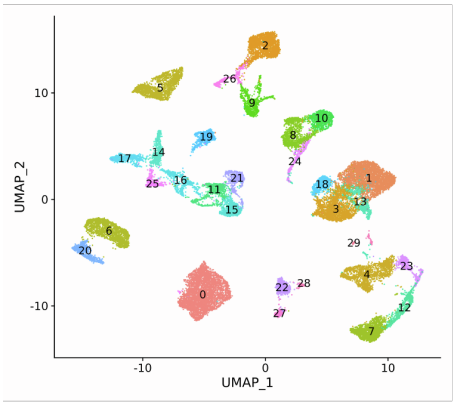


Figure S4. Comparison of genomic landscapes of organoids and original tumor tissues. Related to Figure 4.
(A) Total number of detected somatic mutations indicated for each sample. Sample names are typed in orange, where tumor tissue from a different metastatic location/clinical progression stage than the one used for organoid derivation is presented (due to limited matching tissue availability for sequencing. (B) Genome-wide CNV analysis of tumor tissue and corresponding organoids. Copy number changes are expressed as logR and color-coded. The extent of LOH is displayed with grey bars. Passage numbers (P) at sequencing are indicated for organoid cultures. Sample names are 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).

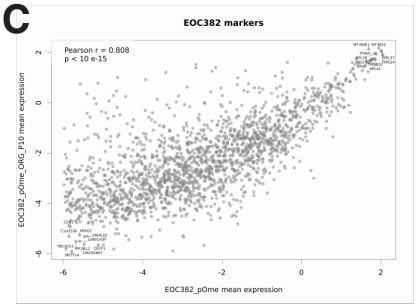
A



B

		Cancer	Immune	Stromal
EOC382	EOC382_pOme	662	3932	490
	EOC382_pOme_ORG_P10	1571	0	0
EOC540	EOC540_pOme	311	1356	327
	EOC540_pOme_ORG_P10	327	1	1
EOC677	EOC677_pAsc	421	1362	39
	EOC677_pAsc_ORG_P13	669	5	0
	EOC677_rAsc	258	6	0
	EOC677_r2Asc_ORG_P13	714	4	1
EOC733	EOC733_pPer	3552	450	75
	EOC733_pPer_ORG_P9	1464	0	0
	EOC733_iOme	493	3250	23
	EOC733_iOme_ORG_P11	664	0	0
EOC883	EOC883_pAdn	749	89	2320
	EOC883_pAsc	698	3865	9
	EOC833_pAsc_ORG_P4	334	0	0

C



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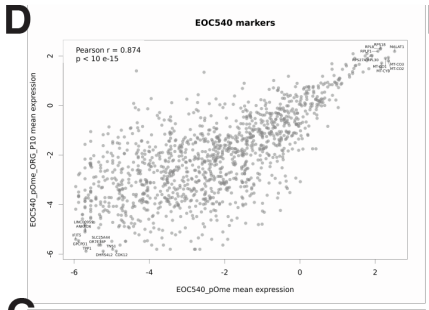

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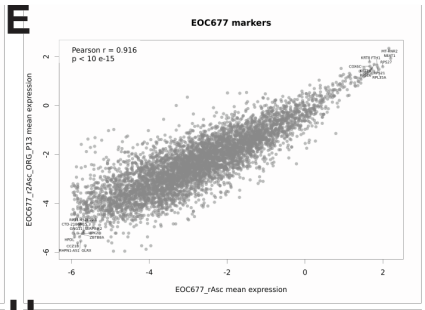

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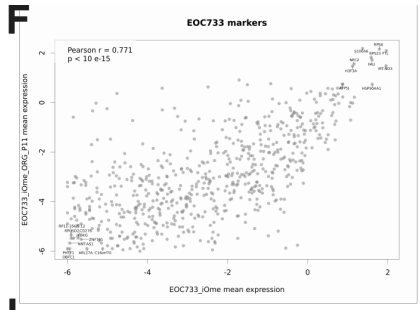

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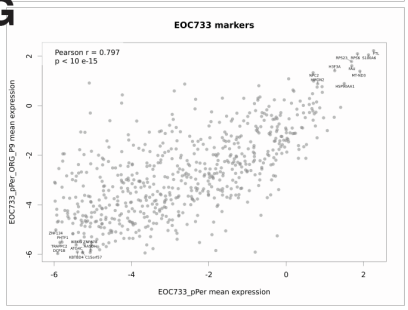

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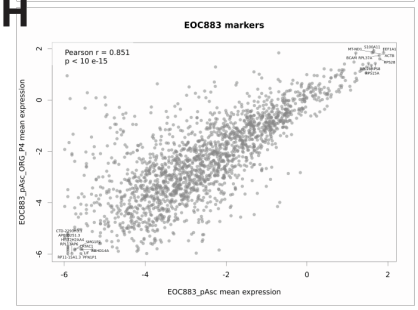
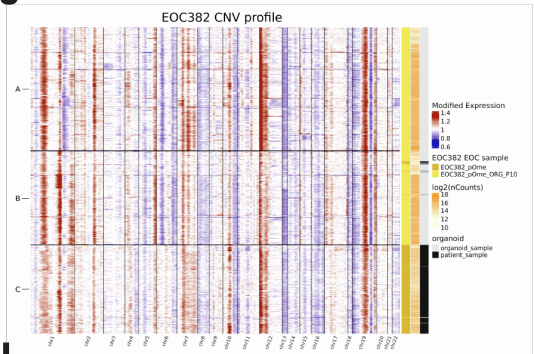


I

		Sample-specific subclone occupancy (%)				
		A	B	C	D	E
EOC382	EOC382_pOme	0.11	3.61	98.76	N/A	N/A
	EOC382_pOme_ORG_P10	99.89	96.39	1.24	N/A	N/A
EOC540	EOC540_pOme	0.00	99.51	96.40	N/A	N/A
	EOC540_pOme_ORG_P10	100.00	0.49	3.60	N/A	N/A
EOC677	EOC677_pAsc	13.19	3.81	63.72	N/A	N/A
	EOC677_pAsc_ORG_P13	72.42	6.73	2.72	N/A	N/A
	EOC677_rAsc	8.15	6.48	31.52	N/A	N/A
	EOC677_r2Asc_ORG_P13	6.24	82.97	2.04	N/A	N/A
EOC733	EOC733_pPer	86.45	0.08	85.98	0.15	0.00
	EOC733_pPer_ORG_P9	1.27	98.57	2.51	2.58	100.00
	EOC733_iOme	12.15	0.00	11.51	0.00	0.00
	EOC733_iOme_ORG_P11	0.13	1.35	0.00	97.27	0.00
EOC883	EOC883_pAdn	0.30	83.33	73.84	2.62	N/A
	EOC883_pAsc	89.53	12.23	6.27	8.90	N/A
	EOC833_pAsc_ORG_P4	10.17	4.43	19.89	88.48	N/A

J



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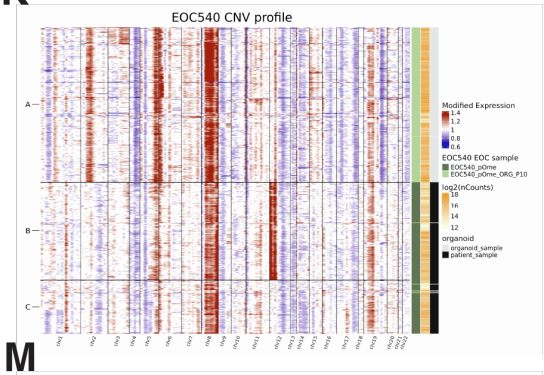

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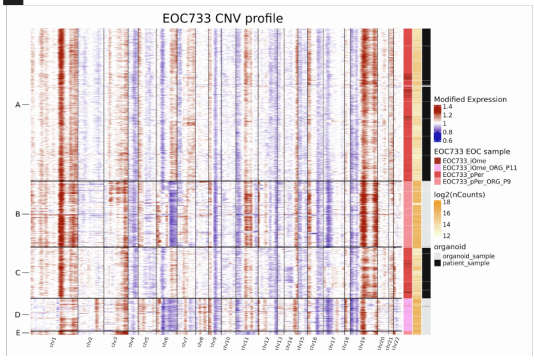


M

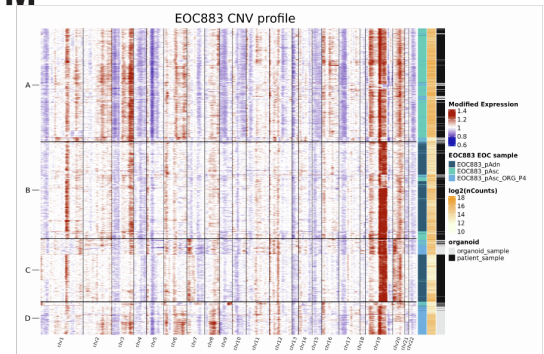
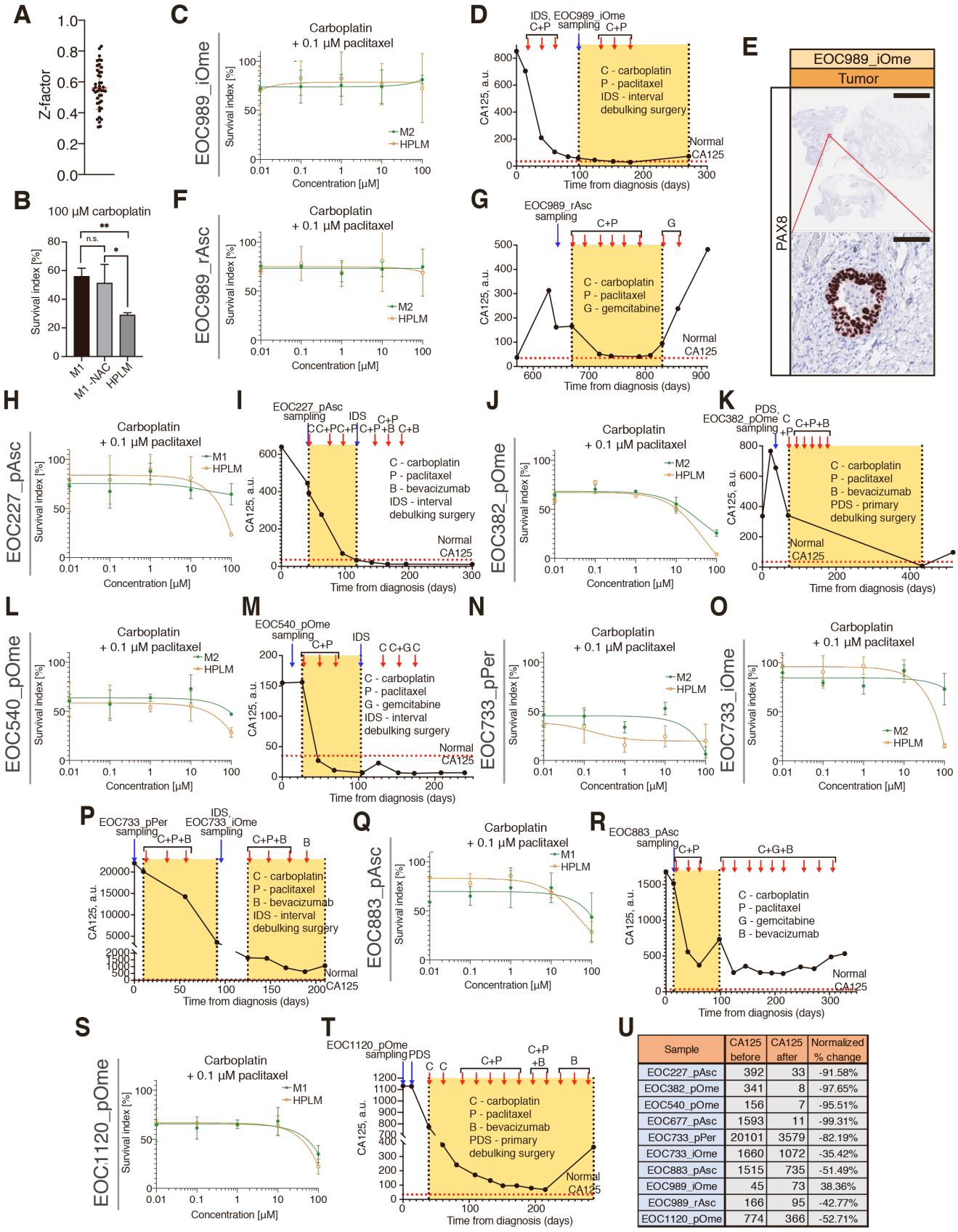


Figure S5. scRNA-seq characterization of HGSC organoids. Related to Figure 5.

(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), EOC667_rAsc (E), EOC733_iOme (F), EOC733_pPer (G) or EOC833_pAsc tumor samples and corresponding organoids. (J-M) Single-cell CNV plots from EOC382 (J), EOC540 (K), EOC733 (L) or EOC833 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.



Sample	CA125 before	CA125 after	Normalized % change
EOC227_pAsc	392	33	-91.58%
EOC382_pOme	341	8	-97.65%
EOC540_pOme	156	7	-95.51%
EOC677_pAsc	1593	11	-99.31%
EOC733_pPer	20101	3579	-82.19%
EOC733_iOme	1660	1072	-35.42%
EOC883_pAsc	1515	735	-51.49%
EOC989_iOme	45	73	38.36%
EOC989_rAsc	166	95	-42.77%
EOC1120_pOme	774	366	-52.71%

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 ± s.d. (n=42). (B) Survival index of EOC677_pAsc organoids exposed to 100 µM carboplatin for 96 hours in M1, M1 deprived of N-acetylcysteine (M1 -NAC) or HPLM. Data presented as mean ± 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 µM paclitaxel, in M1/M2 or HPLM. Results are shown as mean of 2 biological replicates (each with 2-3 technical replicates) ± 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 µm (*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	Ascites	Primary	12%	No			
			EOC136_iOme	Omentum	Interval	72.9%	No			
3	EOC153	IVA	EOC153_pAsc	Ascites	Primary	15.5%	Yes	100%	120	Yes
			EOC153_iOme	Omentum	Primary	70.5%	Yes	99%	185	Yes
4	EOC172	IVA	EOC172_pOme	Omentum	Primary	40.5%	No			
			EOC172_rAsc	Ascites	Recurrence	0%*	Yes	100%	100	Yes
5	EOC192	IIIC	EOC192_pAsc	Ascites	Primary	35.5%	No			
			EOC192_pOvaL	Left ovary	Primary	Unknown	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	Ascites	Primary	Unknown	No			
			EOC423_pOme	Omentum	Primary	70.2%	No			
			EOC423_iOvaR	Right ovary	Interval	100%	No			
10	EOC473	IVB	EOC473_pAdn	Adnex	Primary	72%	No			
			EOC473_iPer	Peritoneum	Interval	Unknown	No			
11	EOC540	IIIC	EOC540_pOme	Omentum	Primary	22%	Yes	99.50%	120	Yes
12	EOC556	IIIC	EOC556_pAsc	Ascites	Primary	17.8%	No			
			EOC556_iBow	Bowel	Interval	8.5%	No			
13	EOC599	IVA	EOC599_pAsc	Ascites	Primary	18%	No			
			EOC599_iOme	Omentum	Interval	10%	No			
14	EOC677	IIIC	EOC677_pAsc	Ascites	Primary	45.4%	Yes	100%	86	Yes
			EOC677_rAsc	Ascites	Recurrence	40.8%	Yes	97.5%	26	Yes
			EOC677_r2Asc	Ascites	2nd recurrence	67.5%	Yes	100%	49	Yes
15	EOC737	IIIC	EOC737_pOvaR	Right ovary	Primary	50.7%	No			
16	EOC733	IVA	EOC733_pPer	Peritoneum	Primary	97%	Yes	96.5%	91	Yes
			EOC733_iOme	Omentum	Interval	51.5%	Yes	100%	140	Yes
17	EOC883	IIIC	EOC883_pAsc	Ascites	Primary	18.5%	Yes	100%	35	Yes
			EOC883_iAsc	Ascites	Interval	27.9%	Yes	99.00%	85	Yes
18	EOC989	IVA	EOC989_iOme	Omentum	Interval	5%	Yes	100%	55	Yes
			EOC989_rAsc	Ascites	Recurrence	90.8%	Yes	99.50%	49	Yes
19	EOC1120	IVB	EOC1120_pOme	Omentum	Primary	Unknown	Yes	100%	177	Yes
			EOC1120_rAsc	Ascites	Recurrence	80%	Yes	99%	70	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
VEGF	Peprotech, #AF-100-20	10 ng/mL	Neutral
Other proteins			
Heregulin-1β	Peprotech, #100-03	5 nM	Harmful or beneficial
BMP-2	Thermo Fisher, #PHC7145	10 ng/mL	Neutral
Jag-1	AnaSpec, #AS-61298	1 μM	Neutral
R-Spondin 1	Peprotech, #120-38	100 ng/mL	Neutral
		400 ng/mL	Neutral
		1 μg/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	10 nM	Neutral
	#HY-101952	1 mM	Harmful
Small-molecule inhibitors			
A83-01	Sigma, #SML0788	0.5 μM	Beneficial
SB202190	MedChemExpress, #HY-10295	0.5 μM	Beneficial
		3 μM	Beneficial
		10 μM	Beneficial
CHIR-99021	MedChemExpress, #HY-10182	2.5 μM	Harmful
Idasanutlin	MedChemExpress, #HY-15676	0.1 μM	Harmful
Forskolin	MedChemExpress, #HY-15371	5 μM	Harmful or beneficial
		10 μM	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

Contents

***Patient sample:** count cells.
Frozen organoids: don't

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 37ºC.
- 6-well culture plates Nunc Cell-Culture Treated (ThermoFisher: #140685).
- Multistep electronic pipette and tips.
- Cold, sterile 1X PBS [-] $CaCl_2$ [-] $MgCl_2$.
- 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 (37ºC).

Beginning of the protocol

4. Aliquot 20 mL per sample of M1 in 50-mL Falcon tubes.
5. Warm up the media at 37ºC 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 ≈ 100-200 µL 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 µL per 10 droplets per single well of 6-well plate + 50-60 µL 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 µL/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 5µM) and mix.
12. Warm up the media at approx. 37ºC 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 [-] $CaCl_2$ [-] $MgCl_2$.
- 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 (37ºC).
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. 37ºC 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 µM).
- 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 organoid 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

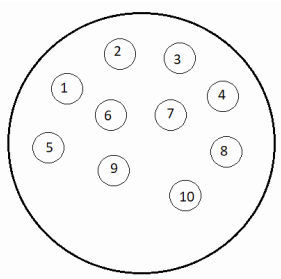
6. Aspirate the media from all the wells.
7. Wash the gels with room-temerature sterile PBS with a serologic pipette.
8. Add 2 mL of TrypLE Express per well.
9. Scrape the gels off and detach them from the plate with a cell scraper.
    a. Remember to use a different cell scraper for each sample.
10. 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 µL per 10 droplets per single well of 6-well plate + 50-60 µL 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 µL/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 5µM) and mix.
3. Warm up the media at approx. 37ºC 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 decribed 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 µL 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 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.

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 ºC
*** only for 2-3 days after seeding	