Advanced-stage gynecologic cancer remains a life-threatening disease. Here, we present a protocol for establishment of stable in vitro 3D spheroid cells derived from human uterine endometrial and ovarian cancer tissues. The tumor-derived spheroid cells have cancer stem cell-related characteristics, including tumorigenesis, and can be used for biological and biochemical analyses and drug efficacy assays. Because these cells possess the biological characteristics of original human tumors, spheroid cells and spheroid-derived xenografts will have applications in personalized medicine in the future.
Establishment of in vitro 3D spheroid cell cultivation from human gynecologic cancer tissues

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SUMMARY

Advanced-stage gynecologic cancer remains a life-threatening disease. Here, we present a protocol for establishment of stable in vitro 3D spheroid cells derived from human uterine endometrial and ovarian cancer tissues. The tumor-derived spheroid cells have cancer stem cell-related characteristics, including tumorigenesis, and can be used for biological and biochemical analyses and drug efficacy assays. Because these cells possess the biological characteristics of original human tumors, spheroid cells and spheroid-derived xenografts will have applications in personalized medicine in the future.

For complete details on the use and execution of this protocol, please refer to Ishiguro et al. (2016) and Mori et al. (2019).

BEFORE YOU BEGIN

Note: This protocol focuses on tumor-derived spheroids, also known as tumorospheres, under floating culture conditions. As it is well established that normal neural and mammary stem cells can be propagated in sphere cultures (Chakrabarti et al., 2018), spheroid cultivation is, thus, suitable for in vitro cancer stem cell cultivation (Pastrana et al., 2011). As such, tumor-derived spheroids can enrich cancer stem cells or cells with stem cell-related characteristics, a feature distinct from that of other 3D cultivated cancer cells, including patient-derived organoids (Ishiguro et al., 2017). Evidently, spheroid culture is suitable for cancer stem cell-related studies. Additionally, spheroid culture requires relatively simple culture conditions and incurs lower costs compared to organoid culture.

1. Perform all procedures under aseptic conditions in order to avoid exposure to bacteria.
2. Prepare the reagents in the Materials and equipment section before tumor sample collection.
3. Cells are cultured at 37°C in a 5% CO2 humidified incubator.
4. It is essential to confirm the pathological diagnosis of the tumors and avoid isolating nearby non-tumor regions, although a long-term culture of spheroid cells can only be established from uterine endometrial and ovarian malignant tissues and not from uterine endometrial hyperplasia, ovarian low potential malignant tumor, or normal tissue.
## Key Resources Table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Biological samples** | | |
| Ovarian cancer tissue samples | This study | N/A |
| Uterine endometrial cancer tissue samples | This study | N/A |
| **Chemicals, peptides, and recombinant proteins** | | |
| Collagenase/hyaluronidase | STEMCELL Technologies | Cat#7912 |
| Penicillin-streptomycin | Thermo Fisher Scientific (Gibco) | Cat#1540122 |
| Amphotericin B | Thermo Fisher Scientific (Gibco) | Cat#15290018 |
| StemPro hESC SFM* | Thermo Fisher Scientific (Gibco) | Cat#A1000701 |
| FGF-basic recombinant human protein | Thermo Fisher Scientific (Gibco) | Cat#AA10-155 |
| 2-Mercaptoethanol | Thermo Fisher Scientific (Gibco) | Cat#21985023 |
| Y-27632 | FUJIFILM Wako Pure Chemicals | Cat#036-24023 |
| Insulin | Roche | Cat#11376497001 |
| DMEM/F-12 | Thermo Fisher Scientific (Gibco) | Cat#11320033 |
| Histodenz | Sigma-Aldrich | Cat#D2158 |
| D-PBS (-) | FUJIFILM Wako Pure Chemicals | Cat#045-29795 |
| HBSS (1×) calcium, magnesium, no phenol red | Thermo Fisher Scientific (Gibco) | Cat#14025092 |
| HEPES (1 M) buffer solution | Thermo Fisher Scientific (Gibco) | Cat#15630106 |
| ACK lysis buffer | Thermo Fisher Scientific (Gibco) | Cat#A1049201 |
| Accumax | Innovative cell technologies | Cat#AM105 |
| StemPro Accutase cell dissociation reagent | Thermo Fisher Scientific (Gibco) | Cat#A1110501 |
| STEM-CELL BANKER DMSO free GMP grade | ZENOAQ | Cat#ZR645 |
| Trypan Blue Stain 0.4% | Thermo Fisher Scientific (Invitrogen) | Cat#T10282 |
| **Other** | | |
| BioShaker | TAITEC | BR-21UM |
| EASY strainer 100 μm | Griner | Cat#542 000 |
| EASY strainer 70 μm | Griner | Cat#542 070 |
| Disposable vacuum filter/storage systems | Corning | Cat#431096 |
| Costar 96-well clear flat bottom ultra-low attachment microplate | Corning | Cat#3474 |
| Costar 24-well clear flat bottom ultra-low attachment multiple well plates | Corning | Cat#3473 |
| Costar 6-well clear flat bottom ultra-low attachment multiple well plates | Corning | Cat#3471 |
| Corning 60 mm ultra-low attachment culture dish | Corning | Cat#3261 |
| Corning 100 mm ultra-low attachment culture dish | Corning | Cat#3262 |

*StemPro hESC SFM (Thermo Fisher Scientific (Gibco), Cat#A1000701) contains DMEM/F-12 with GLUTAMAX (Cat#10565018), StemPro hESC Supplement (Cat#A1000601), and Bovine Serum Albumin 25% (Cat#A1000801).

## Materials and Equipment

### Preparation of reagents and equipment

#### Spheroid culture medium

| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| DMEM/F-12 with GLUTAMAX | 1× | 500 mL |
| STEMPRO hESC supplement | 1× | 11 mL |
| Bovine serum albumin (25%) | 1.8% | 39.6 mL |
| FGF-basic recombinant human protein (100 μg/mL) | 8 ng/mL | 44 μL |
| 2-Mercaptoethanol (55 mM) | 0.1 mM | 1 mL |
| Penicillin-streptomycin (10,000 U/mL, 10,000 μg/mL) | 100 U/mL, 100 μg/mL | 5.6 mL |
| **Total** | n/a | 557 mL |

Divide the spheroid culture medium into 50 mL tubes, and it can be stored at −20°C for up to 1 month. Before use for cultivation, thaw the medium in a 37°C water bath and mix by gently
inverting the tubes. After thawing, store the remaining unused medium at 4°C for 1 week with 2-mercaptoethanol added daily at volumes listed in the above table.

### Other recipes and reagents

#### Tissue processing buffer

| Reagent                      | Final concentration | Amount     |
|------------------------------|---------------------|------------|
| HBSS (1x) calcium, magnesium, no phenol red | 1x                  | 495 mL     |
| HEPES (1 M) buffer solution  | 10 mM               | 5 mL       |
| Total                        | n/a                 | 500 mL     |

Tissue processing buffer can be stored at 4°C.

#### Density gradient centrifugation buffer

| Reagent | Final concentration | Amount     |
|---------|---------------------|------------|
| Histodenz | 30% (w/v)         | 30 g       |
| D-PBS (-) | 1x                  | Up to 100 mL |
| Total   | n/a                 | 100 mL     |

Dissolve Histodenz in D-PBS (-) to a final concentration of 30% (w/v) using a magnetic stirrer, filter through a 0.22-μm filter (Disposable Vacuum Filter/Storage Systems), and store at 4°C.

### Others

1. Thaw Y-27632 with distilled water to a final concentration of 10 mM.
2. Dispense diluted Y-27632, insulin, and FGF in volumes of 100 μL each to new Eppendorf tubes and store at −20°C.

3. Dispense collagenase/hyaluronidase, penicillin/streptomycin, and amphotericin B to a new Eppendorf tube, and store at −20°C. Just before use, thaw at 15°C–25°C.

### STEP-BY-STEP METHOD DETAILS

#### Collection of tumor tissue samples

**Timing:** 10 min

Acquisition of good-quality tissue samples is one of the most important steps to establishing spheroid cells. In this step, we describe the points to which attention should be paid in collecting samples.

1. Collect tumor tissue for cell cultivation, and extract DNA or RNA as soon as possible after surgical resection (Figure 1).

2. Tumor samples for cultivation are placed on ice in phosphate-buffered saline (PBS) and transported to the laboratory. Samples for DNA or RNA extraction are washed with PBS, cut into small pieces, placed in cryopreserved tubes, quickly frozen in liquid nitrogen, and stored at −80°C.

**Critical:** Cancer tissues always contain necrotic tissues. It is important to collect fresh and viable tissue samples for efficient cultivation and further analyses. To obtain high-quality samples, contact well-versed gynecologic oncologists or pathologists.

**Note:** Because failure of spheroid cultivation can result from insufficient sample size, more than 1 cm³ sample size, if available, is preferable for cell culture.
Isolation of cancer cells from tissue samples

© Timing: 3 h

In this step, the procedure performed aims to make the collected tissue samples suitable for initial cultivation.

△ CRITICAL: This procedure should be started immediately after the surgical resection.

Alternatives: When unable to immediately perform the procedure after the resection, store the sample at 4°C or on ice in DMEM/F12 and penicillin-streptomycin. Over-storage is not recommended, although viable cells can be obtained from tissue samples after 14–17 h storage.

3. Warm a bioshaker to 37°C.

Note: The bioshaker for cell isolation should be discriminated from that for bacterial cultivation.

4. Wash the samples with PBS 2 to 3 times for a few seconds.

△ CRITICAL: Uterine endometrial cancer tumor samples are sometimes contaminated with bacteria because of pyometra. In such cases, wash samples intensively with PBS to remove surface fragile tissue and liquid to prevent contamination.

5. Mechanically cut the tumor into small pieces (approximately ~0.5 cm in size) and mince with a disposable sterile scalpel or razor blade.
6. Transfer the all tumor to a 50-mL tube and add 10 mL tissue processing buffer, 500 µL collagenase/hyaluronidase (finally 0.5×), 100 µL penicillin/streptomycin (finally 100 U/mL penicillin and 100 µg/mL streptomycin), and 200 µL amphotericin B (finally 5.0 µg/mL).

Optional: When a large sample can be obtained, the digestion solution should be proportionally increased.

We checked the adequate concentration of collagenase/hyaluronidase to dissociate ovarian and endometrial tumor samples, and similar effects were obtained with collagenase/hyaluronidase concentrations ranging from 0.25× to 1×.

7. Wrap the tube to prevent bacterial contamination.
8. Shake the tube in the bioshaker at 37°C at 200 rpm for 60 to 120 min.

△ CRITICAL: To avoid over dissociation, check the sample conditions at 60, 90, and 120 min after the commencement of shaking. Over dissociation is one of the causes of failure of spheroid cultivation. Therefore, when the over-dissociated sample solution appears more turbid and the tumor volume is decreased to approximately one-third of the initial volume, dissociation should be stopped.

9. Sequentially filter the sample through 100-µm and 70-µm cell strainers, rinse the tube with tissue processing buffer, and filter the rinses through the same cell strainer.
10. Centrifuge at 2673 g for 10 min at 15°C–25°C and discard the supernatant.
11. Resuspend the pellet in DMEM/F12 medium to a total of 1 mL.
12. Add 4 mL density gradient centrifugation buffer and vortex for a few seconds.
13. Gently overlay 2 mL DMEM/F12 medium on the solution.
14. Centrifuge at 2673 g for 10 min.
15. During this period, add 5 mL DMEM/F12 medium in a new 15-mL tube.
16. Check the white layer between upper and lower solutions (Figure 1). This layer contains viable cancer cells.
17. Carefully transfer the upper solution and the white layer (approximately 2.5 mL) to the new 15 mL tube prepared in step 15 by pipetting with a 1,000 µL tip not to collect the lower density gradient centrifugation buffer layer.
18. Centrifuge at 2673 g for 10 min and discard the supernatant.

Optional: Check the cells, and repeat steps 10–18 when many dead cells remain.

Optional: When many red blood cells form red pellets, add 1 mL ACK lysing buffer into the cell pellet and pipette several times for 10 min. Following this, add 5 mL PBS, and repeat step 18.

19. Resuspend the pellet in 1 mL spheroid culture medium and count viable cells with trypan blue staining under a light microscope.
20. Adjust concentration at 1 × 10⁴–10⁶ cells/mL in the spheroid culture medium and seed cells to the adequately sized dish.
21. Incubate at 37°C in a 5% CO₂ humidified incubator.

△ CRITICAL: Y-27632 can drastically improve the expansion of colon cancer spheroids (Ohata et al., 2012). Like colon cancer spheroid cells, it is important to add Y-27632 (final concentration of 20 µM) and insulin (10 µg/mL) to the cultivation medium for ovarian cancer spheroid cells (Ishiguro et al., 2016). In most cases, Y-27632 and insulin are not required for proliferation and maintenance of endometrial cancer spheroid cells (Mori et al., 2019).
**Note:** Low cell density (i.e., \(<1 \times 10^4 \text{ cells/mL}\)) is one of the causes of failure of spheroid cultivation. The appropriate size of culture dishes should be selected to maintain cellular concentration.

**Cultivation of spheroid cells**

⊙ **Timing:** at least 1 month

After the above initial cultivation, cells should be carefully cultivated until a continuous spheroid cell growth is achieved. This step describes the important points to be noted for continuous cultivation.

22. Tip the dish for 30 s, remove one-third of the volume of the medium, and add an equal volume of fresh medium every 2–3 days. Fresh medium containing Y-27632 and insulin should be used for ovarian cancer cells.

**Alternatives:** Transfer all cells and medium to a 10-mL tube, centrifuge at 267 \(\times\) g for 5 min, remove one-third of the medium, and add an equal volume of fresh medium. We recommend step 22 rather than this alternative method to avoid stress to cells.

**Note:** In most cases, all dissociated cells formed sphere-like structures within the first 1–2 weeks.

In cases where sphere-like structures are not formed within 2 weeks, cells generally cannot form spheroid structures even after extended cultivation.

23. When large sphere-like structures are formed, mechanically dissociate them by gently pipetting up and down with a 1-mL pipette tip.

△ **CRITICAL:** In most cases, the formation and expansion of spheroids should be carefully observed for 1–3 months to confirm stable cell growth. Cell density should be maintained at approximately \(1 \times 10^5–1 \times 10^6 \text{ cell/mL}\). Enzymatic dissociation to single cells, which applies significant stress to cells, is not recommended at this initial stage of the spheroid expansion.

**Passage of spheroid cells with enzymatic dissociation**

⊙ **Timing:** 30 min to 2 h

To maintain the spheroid cells, regular passaging is critical. In this step, the process for passaging the spheroid cells is described.

**Note:** When cells achieve continuous growth, test the possibility of passage with enzyme using some of the growing cells. For initial some passages, cells should be diluted at a ratio of 1:2 to 1:3. Verify the established spheroid cell lines when cells stably grow after enzymatic dissociation multiple times. Once cultivation is stable, change the medium every 2–3 days described as above (step 22) and passage the spheroid cells every 10–14 days. The dilution rate (1:2 to 1:5) is selected according to the cell condition.

24. Collect cells in 15 mL tube, centrifuge at 267 \(\times\) g for 5 min, and discard the supernatant.

25. Add adequate dissociation reagent solution (approximately 1 mL per \(1 \times 10^5–1 \times 10^6 \text{ cells}\)).

**Note:** Because conditions required for cellular dissociation are different among spheroid cells, dissociation conditions (we use Accumax (Innovative cell technologies) and Accutase
(Thermo Fisher Scientific), and only pipetting) should be determined for each spheroid at the initial passage. Accumax and Accutase are the most efficient reagents we have tried for our endometrial and ovarian cancer spheroid cells.

26. Pipette cells up and down 10–20 times with a 200-μL tip and incubate at 37°C for 5–10 min. Repeat this procedure until the cells are mostly dissociated.

**Alternatives:** When the cell aliquot is over 200 μL, a 1000-μL or 5-mL pipette combined with a 200-μL tip is useful.

**Optional:** Density gradient centrifugation (steps 10–18) is useful to remove non-viable cells after dissociation.

27. Add 3–5 volumes of PBS, centrifuge at 267 × g for 10 min, discard the supernatant, resuspend with new spheroid culture medium, and seed in a new cultivation dish (Figure 2).

**Cryopreservation of spheroid cells**

**Timing:** 1 day

Spheroid cells can be stored in liquid nitrogen. This step describes the process for cryopreservation of spheroid cells.

28. Collect cells in a 15-mL tube, centrifuge at 267 × g for 5 min, and discard the supernatant.
29. Suspend the pellet in STEM-CELL BANKER.
30. Dispense the cell suspension to cryopreservation vials.
31. Store at −80°C freezer for 14–17 h, following to transfer in liquid nitrogen.

**Δ CRITICAL:** We generally used small-sized spheroid cells (i.e., spheroids formed 3 to 5 days after passage). Storage at low density (less than 1 × 10⁴ cells/mL) may cause recovery failure after thawing. Supplementation with Y-27632 (20 μM) in STEM-CELL BANKER seems beneficial for ovarian cancer cell viability. Cells can be stored for over 1 year in liquid nitrogen.

**Revival of spheroid cells**

**Timing:** 10 min
Cryopreserved spheroid cells can be easily revived according to the below process.

32. Remove the frozen vials from liquid nitrogen and thaw quickly in a 37°C water bath for 5 min.
33. Centrifuge at 267 × g for 10 min and gently remove the supernatant.
34. Gently suspend the cell pellets in 1 mL spheroid culture medium and plate in a new culture dish.
35. Continue further cultivation according to the Cultivation and Passage of spheroid cells (steps 22, 24–27).

**Alternatives:** To remove enough STEM-CELL BANKER, the below-mentioned procedure can be performed before step 33.

*Transfer the dissolved cell solution into a new 15-mL tube containing 5 mL of spheroid culture medium.*

**Note:** The first passage with enzymatic dissociation after the revival of spheroid cells should be performed with the longer interval (2–3 weeks) than that for the regular passage.

**Preparation of spheroid cell block**

**Timing:** 1 day

Histological examination, including hematoxylin & eosin staining and immunostaining, can show the characteristics of spheroid cells. For this purpose, a spheroid cell block can be prepared using the protocol below.

**Note:** The paraffin-embedded cell blocks with standard procedures are a useful strategy to stain spheroid cells. The cells can be evaluated by hematoxylin & eosin staining and immunostaining with the standard protocol for paraffin-embedded sections. See Figure 1 and Supplementary Figure 1 in our original report (Ishiguro et al., 2016).

36. Collect cells in a 15-mL tube, centrifuge at 267 × g for 5 min, and discard the supernatant.
37. Suspend the cell pellet in 4% paraformaldehyde (PFA) for 1 to 3 h.
38. Centrifuge at 267 × g for 5 min, and discard the supernatant.
39. Embed the cell pellet into paraffin blocks according to the standard protocol for paraffin embedding of tissue samples.

**EXPECTED OUTCOMES**

Established spheroid cells can be propagated in vitro for over 1 year with continuous passaging. To avoid changes of spheroid cell characteristics due to long-term cultivation, it is important to store cells at early-passage stages after establishment to monitor any morphological alterations and count passage numbers. It is imperative to not use the same cells for more than 6 months, that is, more than 20 passages. The growth rate of spheroid cells varies, and the CellTiter-Glo Luminescent Cell Viability Assay (Promega) is useful for determining cell growth quantitively in vitro.

Tumor-derived spheroid cells have cancer stem cell-related characteristics, including tumorigenesis (Ishiguro et al., 2017). After injecting the cells into immunodeficient mice, these cells can generate xenograft tumors, which are pathologically similar to the original clinical tumor. The growth rate of xenograft tumors in vivo is more varied than cell growth in vitro. Some spheroid cell lines can generate xenograft tumors over 6 months after injection. From about 3 to 7 days in vitro cultivation after passage, small-sized spheroid cells can generate xenograft tumors more rapidly than single cells after dissociation.
The established spheroid cells are used for various in vitro assays. Genomic DNA is extracted from spheroid cells and spheroid-derived xenograft tumors, as from the original clinical tumor for sequencing. Examination of cancer-related genes in spheroid cells, spheroid-derived xenograft tumors, and original clinical tumor cells indicate that these cells share similar mutation profiles. See Figure 1 and Supplementary Figure 1 in our original report (Mori et al., 2019). In addition, RNA is extracted from the spheroids for microarray analysis, quantitative real-time PCR analyses, RNA-seq, and protein is extracted for Western blotting analyses. Moreover, the cells can be subjected to cell sorting using FACS Aria II and III Cell Sorter (BD Bioscience) after staining of cell surface markers or ALDEFLUOR reagent (STEMCELL Technologies). See Figure 2 in our original report about ovarian cancer (Ishiguro et al., 2016) and Figures 1 and 6 in our original report about uterine endometrial cancer (Mori et al., 2019). Metabolic states of the cells are measured by using Seahorse XFe-24 extracellular flux analyzer (Agilent). See Figure 6 in our original report (Mori et al., 2019). The spheroid cells are also suitable for other applications, including examination of chemotherapeutic drug sensitivity assays using the CellTiter-Glo luminescent cell viability assay (Promega). For the in vitro cell growth assays, cell samples should be extensively dissociated to single cells to measure accurate cell numbers.

LIMITATIONS

The limitation of our protocol is a moderate success rate of establishment of spheroid cells (approximately 30%–40%). Tumor samples from high-grade tumors and advanced-stage disease cases tend to be capable of generating spheroid cells more easily than low-grade and early-stage disease cases. Hence, the spheroid experiments may be more suitable for examining advanced high-grade tumors, which is in contrast to an organoid culture that, in general, allows the cultivation of various stages of tumors (Boretto et al., 2019, Maenhoudt et al., 2020).

TROUBLESHOOTING

Problem 1
Decreased cell viability and cell loss

Potential solution
For the purpose of passaging, complete dissociation to single cells is not recommended because the over-dissociation procedure causes decreased cell viability and cell loss. Additional Y-27632 (20 μM) in the dissociation reagent solution has a positive effect on the viability of some ovarian cancer spheroids. During dissociation by pipetting, the pipette tip should not be held directly against the tube or dish to avoid unnecessary mechanical stress to cells.

Problem 2
Contamination of non-cancer cells

Potential solution
During initial cultivation, non-cancer cells, especially macrophages, attach to the dish soon after cultivation. Collection of the unattached cells and multiple changes of the dish can facilitate the removal of the macrophages. Other non-cancer cells, especially fibroblasts, cannot propagate in the spheroid medium because they require a serum-containing medium. As a result, established spheroids over-propagate these non-cancer cells. Staining with an epithelial cell marker is useful to determine the purity of cancer cells.

Problem 3
Cell loss during preparation of spheroid cell block
Potential solution

Small amounts of spheroid cells cause failure in the preparation of spheroid cell block. When it is difficult to embed the small cell pellet into a paraffin block, embed spheroids into iPGell (GenoStaff, Tokyo, Japan) before PFA-fixation, according to the manufacturer’s instruction.

RESOURCE AVAILABILITY

Lead contact

Enquiries should be directed to and will be answered by the lead contact, Tatsuya Ishiguro (tishigur@med.niigata-u.ac.jp).

Materials availability

This study did not generate novel unique reagents.

Data and code availability

This study did not generate datasets.

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AUTHOR CONTRIBUTIONS

Y.M., T.I., and K.O. designed the research. H.U., Y.M., K. Yamawaki., T.I., K. Yoshihara., H.O., and A.S. performed the research and analyzed the data. H.U., Y.M., K. Yamawaki, T.I., K. Sugino, N.Y., M.Y., K. Suda, and R.T. collected samples. H.U and T.I. wrote the paper. K.O. and T.E. supervised the study and final approval of the manuscript. All authors reviewed, approved, and commented on the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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