Journal of Visualized Experiments

Evaluating the Angiogenetic Properties of Ovarian Cancer Stem-like Cells using the Three-dimensional Co-culture System, NICO-1

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE61751R2
Full Title:	Evaluating the Angiogenetic Properties of Ovarian Cancer Stem-like Cells using the Three-dimensional Co-culture System, NICO-1
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TITLE:

Evaluating the Angiogenetic Properties of Ovarian Cancer Stem-like Cells using the Three-dimensional Co-culture System, NICO-1

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KEYWORDS:

ovarian cancer, ascites, cancer stem cell, vascular niche, co-culture, angiogenesis

SUMMARY:

Ovarian cancer stem cells (OCSC) are responsible for cancer initiation, recurrence, therapeutic resistance, and metastasis. The OCSC vascular niche is considered to promote self-renewal of OCSCs, leading to chemoresistance. This protocol provides the basis for establishing a reproducible OCSC vascular niche model in vitro.

ABSTRACT:

Cancer stem cells (CSCs) reside in a supportive niche, constituting a microenvironment comprised of adjacent stromal cells, vessels, and extracellular matrix. The ability of CSCs to participate in the development of endothelium constitutes an important characteristic that directly contributes to the general understanding of the mechanisms of tumorigenesis and tumor metastasis. The purpose of this work is to establish a reproducible methodology to investigate the tumor-initiation capability of ovarian cancer stem cells (OCSCs). Herein, we examined the neovascularization mechanism between endothelial cells and OCSCs along with the morphological changes of endothelial cells using the in vitro co-culture model NICO-1. This protocol allows visualization of the neovascularization step surrounding the OCSCs in a time course manner. The technique can provide insight regarding the angiogenetic properties of OCSCs in tumor metastasis.

INTRODUCTION:

Ovarian cancer is the eighth most common malignancy in women worldwide, with approximately 300,000 new diagnoses and an estimated 180,000 deaths annually[1]. At initial diagnosis, ovarian cancer often presents with severe symptoms, with about 75% of patients already at stage III–IV. Accordingly, the 5-year survival rate is <30% and the mortality rate is the highest among gynecological cancers[2], with the efficiency of treatment for ovarian cancer being highly dependent on clinical factors such as the successful accomplishment of debulking surgery, resistance to chemotherapy, and recurrence after the initial therapy.

Ovarian cancer tissues are hierarchically organized, with not all tumor components being equally capable of generating descendants. The only cells able to self-renew and produce a heterogeneous tumor cell population are considered to represent cancer stem cells (CSCs)[3]. CSC self-renewal and tumor initiation are accompanied by the promotion of angiogenesis to remodel their tumor microenvironment for the purpose of maintaining a supportive niche. However, previous models could not be utilized for in vitro analyses because of the limited reproducibility of cultivating CSCs derived from clinical samples owing to the disruption of spheroids after multiple passaging. More recently, experimental methods to cultivate CSCs from patients have been developed for several applications[4-7]. In particular, by exploiting the characteristic of CSCs to grow by forming spheroids in ultra-low attachment plates with serum-free medium, the cultivated CSCs are induced to express a stem-cell surface marker that is not expressed in normal tumor cells with multilineage differentiation potential[8,9].

Recent data have shown that the persistence of dormant ovarian (O)CSCs visualized as dissemination at the peritoneum is associated with their regeneration as recurrent tumors[10]. Understanding the molecular and biological features of OCSCs may thus allow for effective targeting and eradication of these cells, resulting in potential tumor remission. In particular, little is known regarding the cellular and molecular mechanistic features of CSCs roles in angiogenesis[11]. Therefore, in the present protocol we used patient-derived OCSCs in an in vitro setting to investigate the angiogenic property of endothelial cells using the co-culture model, which may mimic the tumor microenvironment of CSCs and endothelial cells at the metastatic site in the clinical setting. Ultimately, as neovascularization constitutes a critical process

necessary to support tumor growth and metastasis, a better understanding of its mechanism will allow the development of a novel targeting therapy for OCSCs at the metastatic site.

Here, we present a protocol to visualize the neovascularization step surrounding the CSCs in a time course manner. The advantage of the protocol includes allowing fully reproducible investigations using the 3D co-culture system, NICO-1, thereby permitting observation of the effects on patients of the OCSC-derived tumor-initiation capability during endothelial cell angiogenesis.

PROTOCOL:

All procedures were performed under the protocol approved by the Ethics Committee for human welfare. All patients provided written informed consent for the research use of their samples, and the collection and use of tissues for this study were approved by the Human Genome, Gene Analysis Research Ethics Committee at Teikyo University.

1. Isolation and culture of ovarian cancer stem cells (OCSCs) from Patients with ovarian cancer and ascites in a level 2 biosafety cabinet

1.1. Isolate cancer stem cells from human ovarian cancer ascites obtained via paracentesis. Collect at least 100-250 mL of ascites from patients to take enough number of cancer stem cells. Additionally, evaluate the expression profiles of cancer stem cell markers (i.e., EpCAM, Calretinin, CD133, CD44, CD45, ALDH1, and Oct4) and ovarian cancer markers (pAX-8, WT-1) by flow cytometry.

1.1.1. Centrifuge the human ovarian cancer ascites at 300 x *g* for 10 min at room temperature within 24 h after ascites aspiration.

1.1.2. Remove the supernatant and add 2 mL of OCSC medium and 8 mL of 30% Histodenz/phosphate buffered saline (PBS, pH 7.4) solution.

1.1.3. Prepare OCSC medium: StemPro hESC supplement, DMEM/F-12 with L-glutamine (GlutaMAX medium), 25% BSA, 100 µM 2-mercaptoethanol, 8 ng/mL FGF BASIC, 10 µM insulin, and 20 µM Y-27632.

1.1.4. Carefully overlay 2 mL of OCSC medium to the cell solution in step 1.1.2 in a 15 mL tube and centrifuge at 450 x *g* for 20 min at room temperature in a swinging-bucket rotor without braking.

1.1.5. Carefully transfer the OCSC layer (undisturbed at the interphase) to a new 15 mL tube by transfer pipet.

1.1.6. Fill with PBS up to 15 mL. Centrifuge at 300 x *g* for 5 min at room temperature and remove the supernatant.

1.1.7. Resuspend the cell pellet in OCSC medium and seed on an ultra-low-attachment culture dish; cultures should be maintained at 37 °C in 5% CO_2.

1.1.8. Change the medium every three days. Carefully stand the culture dish for about 1 minutes, and discard part of the supernatant and add the new medium.

1.2. Passage of CSCs

1.2.1. Collect OCSCs in a 15 mL tube and centrifuge at 200 x *g* for 5 min at room temperature.

1.2.2. Remove the supernatant, fill with PBS, and centrifuge at 200 x *g* for 5 min at room temperature.

1.2.3. Remove the supernatant, add 1 mL of the cell detachment solution consisting of proteolytic and collagenolytic enzymes (e.g., AccuMax), and incubate at 37 °C for 10 min.

1.2.4. Mix well by pipetting and incubate at 37 °C for 5 min. Ensure cells are in a single suspension.

1.2.5. Mix well by pipetting, fill with PBS, and centrifuge at 300 x *g* for 5 min at room temperature.

1.2.6. Remove the supernatant and resuspend the cell pellet in OCSC medium for subsequent seeding on ultra-low-attachment culture dishes and maintenance at 37 °C in 5% CO_2.

2. HUEhT-1 endothelial cell culture

2.1. Passage of HUEhT-1 cells

2.1.1. Remove medium from the HUEhT-1 culture dish and wash the cells with PBS.

2.1.2. Add 1 mL of 0.025% trypsin and incubate for 3 min at room temperature.

2.1.3. Add 5 mL of Endothelial Cell Growth Medium 2, collect cells in a 15 mL tube, and centrifuge at 200 x *g* for 5 min at room temperature.

2.1.4. Remove the supernatant, resuspend the cell pellet in HUEhT-1 medium, and seed the cells on collagen-coated culture dishes followed by maintenance at 37 °C in 5% CO_2.

2.1.5. Change the medium every three days.

3. Preparation of the NICO-1 Coculture Plate for tube formation assay using HUEhT-1 cells

3.1 Assemble NICO-1 and coating with the extracellular matrix-based hydrogel (Matrigel Matrix).

3.1.1 Assemble one side of NICO-1 following the manufacturer's instructions and keep on ice.

3.1.2 Cover the surface of NICO-1 with 300 µL of cold PBS and then remove the buffer.

3.1.3 Add 300 µL of chilled extracellular matrix-based hydrogel and incubate at 37 °C for 60 min.

3.1.4 To equilibrate, immerse the filter with 100% ethanol, then wash a 13 mm ICCP Filter (0.6 µm) with PBS for 1 min.

3.1.5 Assemble the NICO-1 including both main body parts A (right chamber) and B (left chamber) along with the O ring and equilibrated filter.

4. Seeding HUEhT-1 Cells and CSCs onto the NICO-1 system

4.1.1 Prepare HUEhT-1 cell suspensions by trypsinizing the cell monolayers and resuspending the cells in endothelial cell growth medium with 2% fetal calf serum.

4.1.2 Add 1.2 mL of the cell suspension (1.5 x 10^5 cells) to each extracellular matrix-based hydrogel-coated well.

4.1.3 Add 1.5 mL of OCSCs cultured for five days to the other well.

4.1.4 Incubate NICO-1 at 37 °C in 5% CO_2; tube formation can be observed under the microscope and network formation on extracellular matrix-based hydrogel measured by means of the number of branches.

REPRESENTATIVE RESULTS:

We collected ascites fluids obtained from patients with advanced ovarian cancer during surgery or paracentesis for the purpose of performing a long-term stable culture for spheroids. Here, we present cases of a long-term spheroid culture of ovarian CSCs termed CSC1 and CSC2. Both cell lines carry the same diagnosis and histological profiles. The mechanistic roles of OCSCs underlying the interaction with endothelial cells necessary to induce the neovascularization of endothelial cells surrounding the OCSCs remain unknown. Therefore, we aimed to clarify the processes of the CSC vascular niche development at the metastatic sites. We examined the interaction between endothelial cells (HUEhT-1) and OCSCs using the in vitro coculture model NICO-1. **Figure 1** shows a comparison of tube formation activity induced by CSC1 and CSC2. The number of formed vascular tubes dramatically increased over time in the coculture with CSC2 (**Figure 2A**). For the positive control, we present **Figure 2B** which shows the angiogenic property of HuEhT-1 after the treatment of VEGF (10 ng/mL) without co-culture of CSC2. We are now clarifying the detailed mechanism underlying the result**. Figure 3** shows representative images of coculture model of OCSC with endothelial cells using the NICO-1. HUEhT-1 cells were cocultured with CSC2 for 20 hours, and the time-lapse video image was captured. **Figure 3A** shows the phenotype of CSC2 before and after coculture for 20 hours. **Figure 3B** shows HUEhT-1 cells cocultured at the

same time. It is noteworthy that HUEhT-1 cells formed vascular tubes during the coculture with CSC2 (**Figure 3C: Video clip**)

Figure 1: OCSCs vascular niche model. The mechanistic roles by which OCSCs induce the tube formation (vascularization) of endothelial cells remain unknown. We examined the interaction between endothelial cells (HUEhT-1) and OCSCs using an in vitro co-culture mode, NICO-1. The right compartment of this system is composed of an insert, which holds OCSCs with the cell medium. The left compartment consists of a well containing endothelial cells and HUVECs with the same medium as in the right well.

Figure 2: Comparison of the neovascularization activities induced by OCSCs. Over time, the number of formed vascular tubes dramatically increased upon coculture with CSC2.

Figure 3: The vascular formation of HUEhT-1 cells coculture with CSC2 using NICO-1. HUEhT-1 cells formed vascular tubes during the coculture with CSC2.

DISCUSSION:

The presented protocol describes how to mimic the tumor microenvironment of OCSCs in an in vitro setting. The primary component of the method constitutes the highly reproducible coculture model obtained using the NICO-1 system, an indirect Transwell co-culture system. Many of the currently available coculture models examine the effects of direct cell-cell contact on cocultured cell populations[12-18]. The simplest model that can be used for examining the effects of co-culture may reproduce by the direct mixing of two cell types, and the extent of heterotypic and homotypic interactions can be examined by altering the seeding densities of each cell type and relative seeding ratio of the subpopulations[19]. However, directly determining the relative contributions of OCSCs to any observed effects of coculture independently by microscopy is difficult owing to the precise invisibility of each cell; thus, these studies are often accompanied in parallel by conditioned media experiments. For example, a segregated coculture system can be utilized in studies in which the effects of paracrine signaling on tumor microenvironment are of interest[19]. In comparison, in the present method we describe a model that allows simultaneous evaluation of the effects of cell-cell contact and paracrine signaling, which mimic the architecture of the native tumor microenvironment.

Angiogenesis serves as a hallmark of ovarian cancer and plays a critical role in its progression, which involves interactions between cancer cells, endothelial cells, and the surrounding tumor microenvironment[20]. Bevacizumab is a key molecular targeting drug that has been widely accepted for use in combination chemotherapy for advanced ovarian cancer[21]. Specifically, bevacizumab constitutes a monoclonal antibody against vascular endothelial growth factor (VEGF). VEGF contributes to the development of peritoneal carcinomatosis, and formation of malignant ascites in advanced ovarian or peritoneal cancer by promoting neovascularization and enhancing vascular permeability[22]. Therefore, VEGF inhibition has been shown to inhibit ascites production and massive tumor growth at the metastatic site. Further investigation that effectively targets VEGF-stimulated vascular endothelial cells in the tumor microenvironment

level is thus warranted. However, it may be difficult to effectively utilize preclinical models, such as mouse models, for these studies. For example, it has been reported that the affinity of bevacizumab for human VEGF is high whereas that of the mouse protein is lower[23]. This enforces a limitation with regard to the application of bevacizumab within experiments designed to further investigate its anti-VEGF mechanism toward neovascularization in the tumor microenvironment as ascertained using mouse models. An appropriate model with well-controlled architecture is therefore needed to recapitulate the components of the in vivo tumor microenvironment. In such case, we note that our in vitro coculture model system permits the live tracking of cell behavior throughout the culture of the patient-derived OCSCs and endothelial cells and allows for the study of these individual cell subpopulations in response to coculture.

A better understanding the role of OCSCs and their vascular niche could provide new insights for developing therapeutic strategies for OCSCs. As shown in the representative experiments, the strengths of the model are that it provides a study platform that appears to be partly congruent with clinical settings, enabling the development and testing of better targeted and effective novel drugs. Further studies are needed with direct clinical replication of our results involving a more significant number of patient-derived OCSCs.

ACKNOWLEDGMENTS:

This work was supported by a Grant-in-Aid for Scientific Research C (grant no. 19K09834 to K.N.) from the Ministry of Education, Science, and Culture, Japan.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

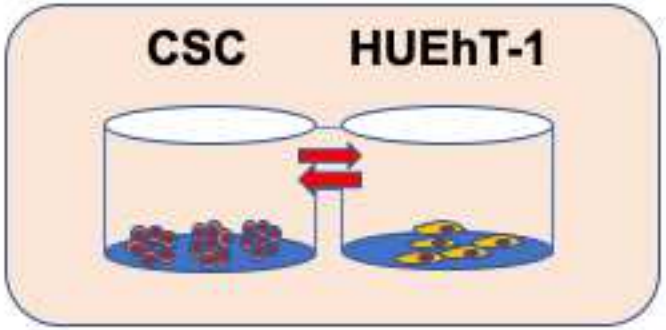


Figure 2

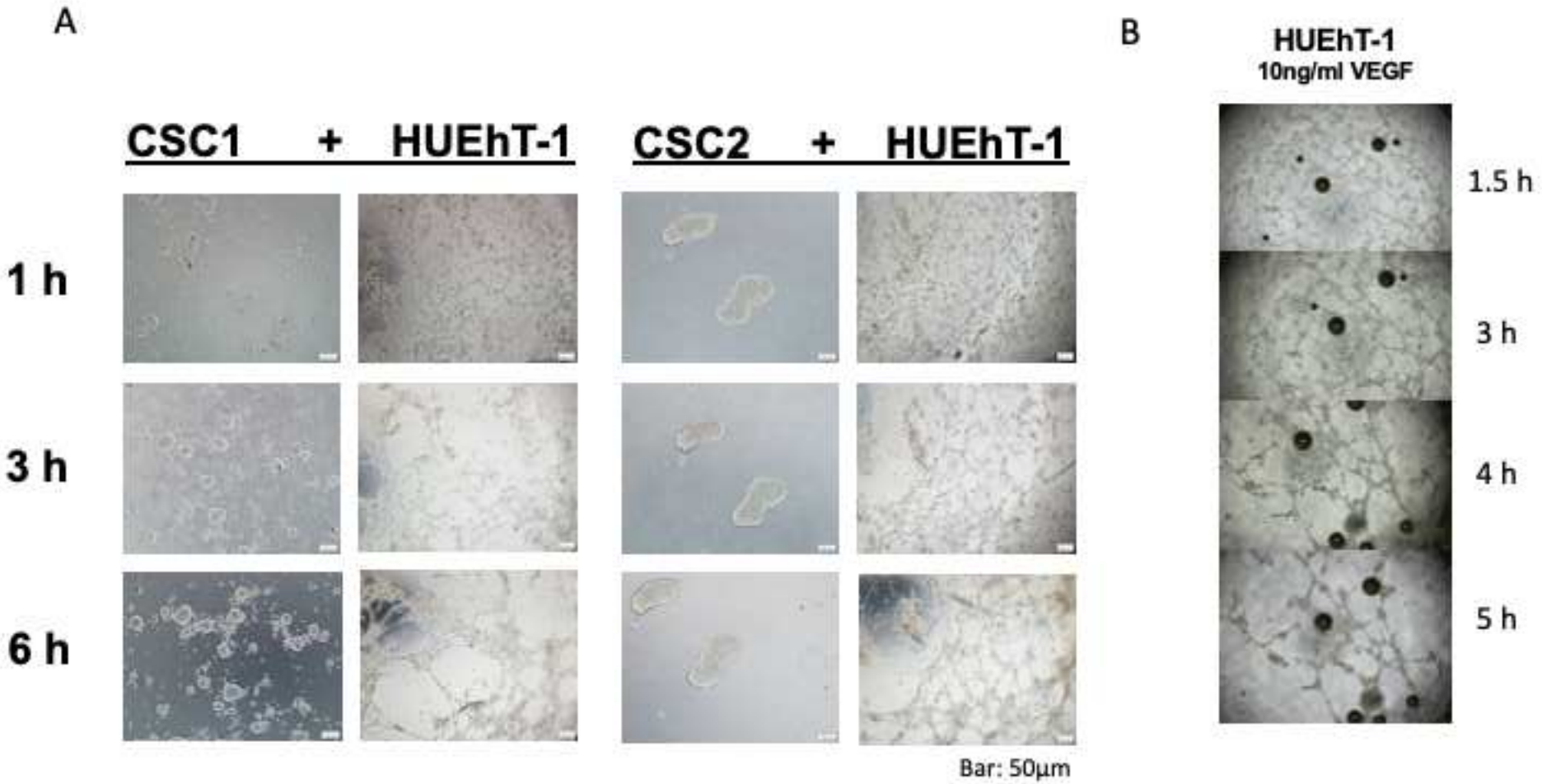


Figure 3

A

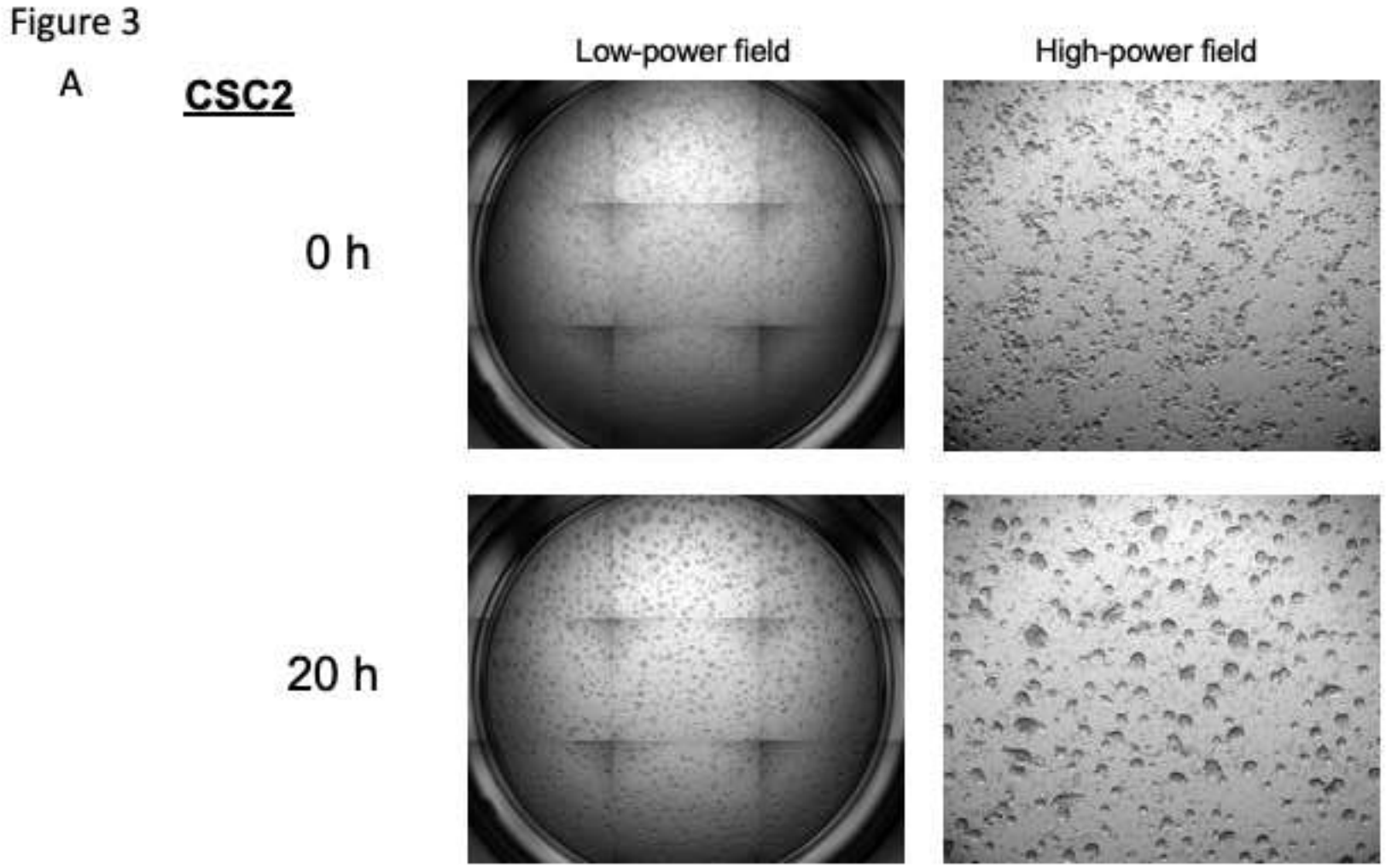


Figure 3

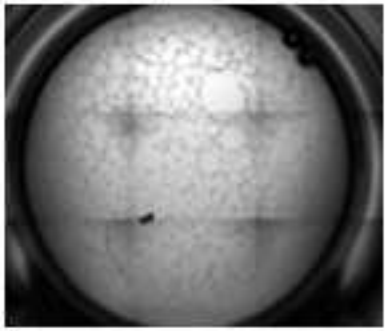
B

HUEhT-1

Low-power field

High-power field

20 h



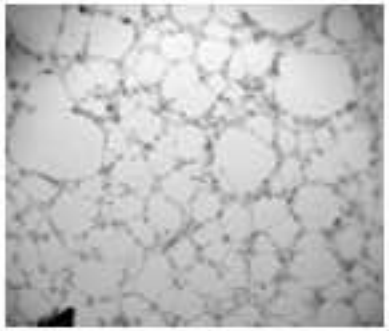
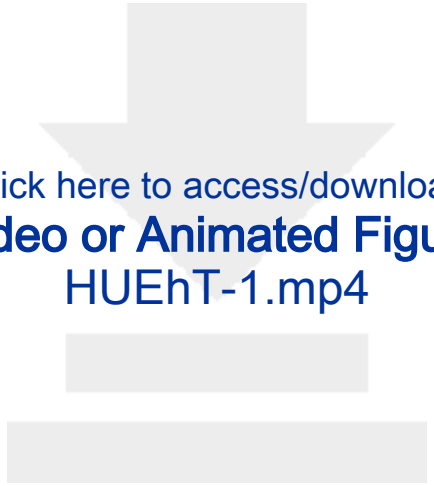


Figure 3C


Click here to access/download
Video or Animated Figure
HUEhT-1.mp4

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.025% Trypsin	Thermo	R001100	
10 mL Pipet	Thermo	170356N	
1250 µL Pipet tip	QSP	T112XLRS-Q	
15 mL tube	Nunc	339650	
200 µL Pipet tip	QSP	T110RS-NEW	
2-Mercaptoethanol	Thermo (Gibco)	21985023	
5 mL Pipet	Thermo	170366N	
50 mL tube	Corning	430290	
AccuMAX	Innovative Cell Technologies	AM105	
BioCoatTM Collagen I 60mm Dish	Corning	356401	
Centrifuge	KUBOTA	2800	
Costar 6 Well Clear Flat Bottom Ultra Low Attachment Multiple Well Plates	Corning	3471	
Endothelial Cell Growth Medium 2	PromoCell	C-22011	
Ethanol	WAKO	057-00456	
FGF-Basic	Thermo (Gibco)	PHG0021	
Histodenz	SIGMA	D2158	
HUEhT-1 cell	JCRB Cell Bank	JCRB1458	
ICCP Filter 0.6 µm	Ginrei Lab.	2525-06	
Insulin, human	SIGMA (Roche)	11376497001	
Luminometer	PerkinElmer	ARVO MX-flad	
Matrigel Matrix	Corning	356234	
Microscope	Yokogawa	CQ-1	
NICO-1	Ginrei Lab.	2501-02	
OptiPlate-96	PerkinElmer	6005290	
P1000 Pipet	Gilson	F123602	
P200 Pipet	Gilson	F123601	
PBS	Thermo (Gibco)	14190-144	
StemPro hESC SFM	Thermo (Gibco)	A1000701	

Transfer Pipet	FALCON	357575	
Y-27632	WAKO	253-00513	

October 30, 2020

Ronald Myers, Ph.D.
Director of Editorial
Journal of Visualized Experiments

Dear Dr. Myers:

Thank you very much for the comments regarding our manuscript

entitled 'Evaluating the Angiogenetic Properties of Ovarian Cancer Stem-like Cells using the Three-dimensional Co-culture System, NICO-1.'
We have carefully revised the manuscript again according to all the provided comments in red text, as detailed in the attached document (wherein the reviewers' comments are shown in the blue italicized text).

We thank you and the reviewers for your thoughtful suggestions and insights, which have enriched the manuscript and produced a more balanced and better account of the research.

We hope that the revisions have clarified and improved the manuscript such that it is now acceptable for publication in your esteemed journal.

I look forward to your reply.

Sincerely,
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Editorial comments:

1. Figure 2: Please use Si abbreviations for time: h instead of hr. Please include a space between all numbers and their corresponding unit: 1 h instead of 1h
2. Please provide the representative images of the vascular formation as a Figure in the manuscript. Please note that the figures in the manuscript and the video should be the same. We can accept a short video clip as a file as well.

Response:
Thank you very much for these suggestions. We have corrected Si abbreviations for time. Regarding the second comment, we have newly added a representative video clip as a file (Figure 3) that shows the time-rasped vascular formation movie using the 3D co-culture system, NICO-1, and added the explanation of Figure 3 in line 227-231, and the tile in line 243-244.

Reviewers' comments:

Reviewer #3:

Manuscript Summary:

The authors have addressed the comments on the details of the methods. However the phenotypic characterisation of cells in the ascites is not presented or referenced.

Major Concerns:

1. The cells which are separated by density gradient centrifugation of ascites were termed as OCSCs, which is not correct. More characterisation of these cells is required. They should be positive for epithelial markers and ovary specific markers like CA125, pAX-8, WT-1 etc. How are we sure that they represent CSCs?

Response:
Thank you very much for the comments. We apologize for you that we have not mentioned how we decide the characterisation of these cells. After we separated the ascites cells by density gradient centrifugation, we evaluated the expression profiles of EpCAM, Calretinin, CD133, CD44, CD45, ALDH1, and Oct4 in spheroid cells by flow cytometry. We have not shown in the manuscript, but we found CD44, EpCAM (widely used cancer stem cell markers) were highly positive in both CSC1 and CSC2 cells (data not shown). We have also checked pAX-8 or WT-1 were positive in the ascites cells and confirmed that the cells were derived from ovarian cancer. We have added further statements in line 114-115 in the text.

2. Angiogenic property was evaluated by tube formation assay. It would be better if other functional assays like uptake of acetylated low density lipoprotein could also be used. This is important otherwise one is not sure that cells are endothelial even if they start with that.

Response:
Thank you very much for the useful comments. We have confirmed the cells are endothelial by staining for CD31. We also will investigate uptake analysis using Ac-LDL and will explain it in the video.

Reviewer #6:

Manuscript Summary:

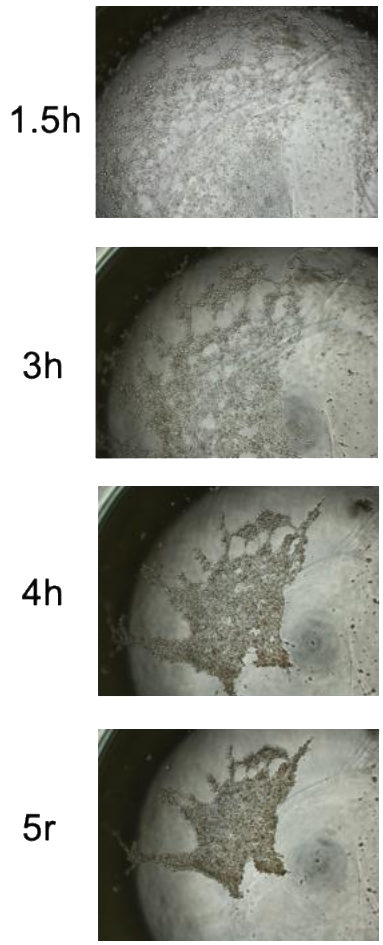
This manuscript presents a method to examine angiogenesis through the formation of vascular tubes after co-culturing ovarian cancer cells and endothelial cells. Specifically, the protocol utilizes ovarian cancer stem cells and the immortalized endothelial cell line HUehT-1. A 2-chamber cell culture system is used to facilitate the cell culture crosstalk. While the protocol and cell culture system are interesting there are some concerns that fall into both the major and minor categories that should be addressed.

Major Concerns:

While positive control data in the form of VEGF addition to the culture media is presented to show vascular tube formation without cancer cells, a negative control is lacking in the data set. Will the endothelial cells form vascular tubes without the presence of cancer cells?

Response:

Thank you very much for your suggestion. We agree with the reviewer that we have not mentioned the negative control. For this protocol, we have evaluated the vascular formation of HUEhT-1 cells with and without the presence of cancer cells. HUEhT-1 cells cannot form a vascular tube, for example, as attached below.



Minor Concerns:

1. There needs to be some clarity in the following steps:

1.1.3 This should maybe stated differently "Carefully overlay 2 mL OCSC medium on to the cell solution in 1.1.2....."

Response:
We have modified the sentence according to the suggestion in line 123.

1.1.7 Is it necessary to state how to change the media since these will in essence be suspension cultures and not typical adherent cultures?

Response:
We have added further information for the medium change in line 144-145 in the text.

3.1.2 Cover the surface of both chambers?
3.1.3, is the hydrogel going in both chambers or only one?

Response:
We apologize for the lack of explanation. Regarding the both chambers, as we mentioned in 3.1.1, we first assemble one side of NICO-1 to seed HUEhT-1 cells for tube formation assay. We have modified the title according to the suggestion in line 181 in the text.

3.1.4, a timeframe for 100% ethanol evaporation should be included to ensure that the system is not assembled/closed with potential ethanol contamination.

Response:
We apologize for this mistake and correct the sentence correctly in line 193-194 in the text.

4.1.3, how many OCSC cells are being added? Are they being added to chamber without hydrogel? What media is being used here? Are the 2 different medias mixing or is only one being used?

Response:
Thank you very much for your comments. As OCSC cells began to form spheroid after cultured five days, we have used a 3D cell titer for compensation. We will explain it in detail in the video. As explained above, we have added OCSC medium without hydrogel in the OCSC side of NICO-1. Also, the NICO-1 prevents cross-contamination of cells between both chambers, and the media will not be shared totally during the experimental procedure.

2. We would include a statement that all work should be performed under sterile conditions in a level 2 BSC especially when working with large volumes of ascites.

Response:
Thank you very much for your comments. We have added the statement in line 110 in the text.

3. The second paragraph of the discussion needs a number of references to support its statements.

Response:
Thank you very much for your comments. We have added three references (ref.20-22) in the text.

Reviewer #7:
Manuscript Summary:

Miyagawa et al reported a useful work showing three-dimensional Co-culture System of Ovarian Cancer Stem-like Cells to investigate the angiogenetic properties of OCSCs in tumor metastasis. The protocol is interesting and potentially important to understand cell co-culture modeling in 3D cell system, which might visualize the neovascularization step surrounding the CSCs. The paper is well written and understandable to a wide audience. Nonetheless, I'm not fully convinced based on the current submission due to the following reasons.

Major Concerns:

(1)My first major issue is about Isolation of Ovarian Cancer Stem Cells (OCSCs) from Patients with Ovarian Cancer and Ascites.The authors should report the accurate characterization of OCSCs due to other cell types easily participating in the stem cell popuplation. It will be possible to influence the following experimental steps and uncertainty by this protocol. It is not clear how the OCSCs is obtained in the work. The authors should state certain step to make sure the ovarian cancer stem cell type.

Response:
Thank you very much for the comments. I apologize for you not mentioning the characterisation of OCSC cells. We have evaluated the expression profiles of OCSC cells using EpCAM, Calretinin, CD133, CD44, CD45, ALDH1, and Oct4 by flow cytometry. We found CD44, EpCAM were highly positive in both CSC1 and CSC2 cells (data not shown). We have also checked pAX-8 or WT-1 as positive in the ascites cells and confirmed the cells were derived from ovarian cancer. We believe both CSC1 and CSC2 cells have a phenotypic characteristic of cancer stem cells. We have added further statements in line 114-115 in the text.

(2)Another issue is that Comparison of the neovascularization activities induced by OCSCs. In Figure 2, the number of formed ascular tubes dramatically increased upon co-culture with CSC2, where the authors only showed 6hours results that cannot represent the real situation for 3D co-culture model.

Response:
Thank you very much for the comments. To explain the real situation for the 3D co-culture model, We agree with the 6h time-lapse culture is too short, so we have added a video clip showing the 20h culture as an additional file. We also will explain it in the video.

(3)Reviewer is especially concerned about the confounding effects within this NICO-1 System and the accuracy in the reported methodology. According to the results presented by authors, this OCSCs vascular niche model needs furthermore data to illustrate the effect of OCSCs co-cultured with endothelial cells to induce the tube formation (vascularization) by a accurate experimental methodology.

Response:
Thank you very much for your beneficial suggestions. We currently work on to investigate further data to see the effect of OCSCs co-culture model. To further explain our methodology, we have added a video clip (Figure 3) showing the effect of OCSCs in vascularization.

Minor Concerns:

(1) line 195, what is results for day 5 co-culture model?

Response:

Thank you very much for the comments. After day 5, OCSCs form larger spheroids and proliferate, but some cells weaken gradually. We consider that day 5 must be appropriate timing for the analysis.