RESEARCH ARTICLE

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Establishment of Epithelial Cell Culture from Ovarian Cancer Tissues: A Method Comparison Study

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Abstract

Objective: This study aimed to determine the most effective method in establishing primary cell culture from epithelial serous ovarian cancer tissues with the highest yield of cells and percentage of epithelial cells. **Methods:** Primary and metastasis tissues from three serous ovarian cancer patients were processed using 18 different combinations of methods based on different factors: the source of tissue (primary site or metastasis site), the cell suspension method (explant method, enzymatic methods, or the addition of Percoll), and the alternatives of three different media. We compared the total count of cells, the percentage of epithelial cells, and the estimated number of epithelial cells per observation field. The calculation of cells from primary tissues were compared to metastasis tissues, and the difference was statistically analyzed using Mann Whitney-U test on SPSS software. **Result:** The groups that were processed using dispase and trypsin resulted higher number of cells and higher percentage of epithelial cells when compared to the explant method. Among all media, we found that DMEM:F12 and McCoy's 5A media as equally useful in isolating and culturing epithelial cells. Statistically, the metastasis tissue derived more epithelial cells when compared to the primary tissue (102.32±82.65 vs 22.6±23.81, p=0.001). **Conclusion:** The use of metastasis tissue processed with trypsin or dispase and cultured in DMEM:F12 or McCoy's 5A media was found to be the most efficient way to produce the highest amount of cells with high percentage of epithelial cells.

Keywords: Ovarian Cancer- epithelial cell- epithelial-mesenchymal transition- cell culture- metastasis

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Introduction

Ovarian cancer is one of the most common gynecologic cancers with the highest mortality rate. Around 185,000 deaths among women occurred due to ovarian cancer globally, accounting up to 4,4% cancer-related mortality among women (Momenimovahed et al., 2019). In the United States alone, it was estimated that the new cases of ovarian cancer may reach up to 22,240 cases with mortality of up to 14,070 cases annually (Torre et al., 2018). In Asia, the incidence of ovarian cancer accounts for 111,887 cases with 66,215 related deaths, with China as the country with the highest incidence (World Ovarian Cancer Coalition, 2018). The trend of ovarian cancer incidence was estimated to increase reaching up to 371,000 in the year of 2035 with death cases of up to 254,.000 or 67% of all the cases (World Ovarian Cancer Coalition, 2018).

Epithelial ovarian cancer is often diagnosed at advanced stages due to its nature that does not show any specific symptoms except swelling of the abdomen caused by the accumulation of ascites at the later stages. The majority of epithelial ovarian cancers are of the serous subtype, which comes primarily as high-grade. The prognosis of ovarian cancer patients also worsen along the progression of the disease, shown by the fact that the survival rate of the patients decreases as the stadium gets higher (Torre et al., 2018). Moreover, effective screening to detect ovarian cancer is not yet available in the laboratory setting, so most of the patients are diagnosed in the more advanced stages with poorer prognosis.

Considering its elusiveness to be detected in early stage, disease models to study the nature of ovarian cancer is strongly needed. There are currently several types of cell lines that were raised from ovarian cancer tissues such as SKOV-3 and A2780 lines (Hasan et al., 2015). However, those cell lines could not resemble the complex heterogeneity and micro-environment of the real ovarian cancer tissue (O'Donnell et al., 2014).

In searching the most suitable models for ovarian

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cancer, we investigated the feasibility of developing primary cell cultures directly from patients' tissues, expecting that the cultures will highly mirror the real molecular events of the cancer. Previous studies have established (O'Donnell et al., 2014)methods to obtain primary cell culture from ovarian cancer tissues by utilizing combination of enzymes, such as Dispase and Trypsin, and culture media, such as DMEM:F12 and McCoy's 5A (Kar et al., 2017; Pribyl et al., 2014; Shepherd et al., 2007; Thériault et al., 2013). However, the epithelial cells of many tissues, including ovarian cancer, are often difficult to be cultured and oftentimes became outgrown by the fast-growing fibroblast (Chen et al., 1993; Hongpaisan, 2000). This study aims to develop the most efficient protocol to raise epithelial cells from serous ovarian cancer tissue by comparing several combinations of cancer tissue site (primary or metastasis), different cell suspension methods, the use of percoll, and the type of culture media, that were demonstrated separately in the previous studies (Kar et al., 2017; Pribyl et al., 2014; Shepherd et al., 2007; Thériault et al., 2013).

Materials and Methods

Ethical Clearance

This study was ethically approved by the Scientific and Ethical Committee of the Cipto Mangunkusumo Hospital in affiliation with the Faculty of Medicine, Universitas Indonesia, under the letter no. 102/UN2.F1/ETIK/2019. All patients were well informed about the research before consenting to participate.

Sample Collection

Cancer tissue samples were collected from both primary site and metastatic site, specifically on the omentum, at the time of primary surgery from three consenting advanced ovarian cancer patients. The tissues were confirmed by pathologist as serous ovarian cancer prior to collection. Once resected, approximately 5g of tissues were transported from the operating chamber in transport medium consisting of DMEM:F12 (Gibco) supplemented with 200U/ml Penicilin-Streptomycin (Gibco) and 5ug/ml Amphotericin B (Sigma-Aldrich). The samples were processed within 24 hours to anticipate loss of cell viability.

Cell Culture Experiments

All cancer tissues were processed to make cell suspension and later to be derived into primary cell cultures using several combinations of methods , performed by previous studies (Kar et al., 2007; Pribyl et al., 2014; Thériault et al., 2013). The primary and metastatic tissues were experimented according to Figure 1a (for primary site) and figure 1b (for metastatic site). Prior to the experiments, tissues were washed with phosphate buffer saline (PBS) supplemented with 2.5ug/ml Amphotericin B (Sigma-Aldrich) and 100U/ml Penicillin-Streptomycin (Gibco), and then diced into fine tiny cuts using sterile scissor, tweezers, scalpel, and blade.

Cell Suspension Methods

In this study, we used three different methods of cell suspension process, namely explanting method, dispase treatment, and tripsin treatment. The explanting method does not include enzymatic process, and the process is as follows: after the tissues were diced and prepared, the cells were filtered using 100 μm-mesh cell strainer. Following cell filtering using cell strainer, the cells were then precipitated by centrifugation at 1,000 g, 4°C for 5 minutes and diluted using preferred medium according to the experimental groups.

The enzymatic process involves two different enzymes, namely 2,4U/ml Dispase (Gibco) and 0,25% Tripsin (Gibco). The diced tissues were soaked in 5ml of chosen enzyme, according to the experimental grouping, and then the cells were incubated in 37°C for 40minutes and homogenized every 10 minutes. After incubation, 5ml of complete medium, containing 10% foetal bovine serum (FBS; Sigma-Aldrich), were added to the mixture to inactivate the enzymes. Afterwards the cells were filtered using 100 μm-mesh cell strainer, precipitated by centrifugation at 1,000 g, 4°C for 5 minutes, diluted using chosen medium, and seeded in culture flasks. When both enzymes were used, a ratio of 1:1 was used in combining Dispase (2,4U/ml) and Tripsin (0,25%).

Percoll Gradient Centrifugation

Gradual centrifugation utilized different concentrations of Percoll to separate cells based on their density. Inside the same conical tube, Percoll 90% was placed on the bottom followed by Percoll 45%, and then the cells were placed on top of both Percoll concentrations. Afterwards, the tube was centrifuged at 1,500 g, 4°C, for 30minutes, then the epithelial cells were obtained from the 45% Percoll phase. The epithelial cells were then washed using DMEM:F12 medium, precipitated, and seeded in culture flask using the chosen medium according to the experimental grouping.

Cell Culture Maintenance

Three basal media were used in this study, namely DMEM:F12 (Gibco), referred as medium A; MCDB105:M199 (1:1) (Gibco) referred as medium B; and McCoy's 5A (Gibco), referred as medium C. Prior to usage, media were supplemented with 10% foetal bovine serum (FBS, Sigma-Aldrich), 2.5ug/ml Amphotericin B (Sigma-Aldrich), 2mM Glutamax (Gibco), and 100 U/ml Penicillin-Streptomycin (Gibco).

The cells were plated in the 6-well plates and incubated for six days at 37°C with 5% CO_2. All experiments were done in duplicates, and five pictures were taken for analysis from each group after the experiments were terminated on the sixth day. Each of the steps that were performed in this study is detailed in the supplement attached to this manuscript.

Cell Calculation

Six days after cell cultures were initiated, five microscopic photographs were taken randomly from each of the cell culture groups with 100x magnification, , and then the cells were analyzed using the ImageJ software

(Bourne and Bourne, 2010; Schneider et al., 2012). The epithelial and fibroblastic cells were separately counted, from which the total count of cells, the percentage of epithelial cells, and the estimated total epithelial cells (percentage of epithelial cells × total count) were calculated. The formulations for each of the calculations are attached in the supplement.

Finally, we compared the estimated number of epithelial cells per observation field of the primary tissue groups with the metastasis tissue group, and the difference was analyzed using Mann Whitney-U test on SPSS Software. The statistical analysis is attached in the supplement.

Results

Patients' Demography

All three patients that were included in this study were diagnosed with malignant epithelial tumor, stadium IIIc based on FIGO guidelines, and had metastasis on the omentum. The patients underwent complete surgical staging followed by optimal surgical debulking. The complete data of the patients are detailed in the supplement. All patients were verbally informed about the research before consenting to participate and signing the informed consent form.

Morphological Observation

We observed each of the culture 6 days after seeding. Each of the cultures showed different combinations of both epithelial and fibroblastic cells. The epithelial cells showed round and cuboid form with firmer structure (Figure 2a), whereas the fibroblastic cells were seen as more elongated, smaller, and gathered in a denser population (Figure 2b).

Cell Calculation

Five random shots from each group were analyzed using the ImageJ software (Bourne and Bourne, 2010; Schneider et al., 2012). After counting the epithelial and fibroblastic cells separately, we calculated the average total count of cells, the percentage of epithelial cells, and the estimated total epithelial cells in every observation field of every group.

We counted the cells in each group, and based on our calculations, the highest total count was placed by the MDC group, followed by MDA (Figure 3a). However, that result is not consistent in the calculation of epithelial cells percentage (Figure 3b). In the percentage data, the group with highest percentage is the MDTPC group, followed by the MDPA group. That result might be caused by the effect of using percoll in the experiment. Percoll distinguishes cells gradually based on their mass and density, therefore it is possible to differentiate epithelial cells from fibroblastic cells using gradient concentration of Percoll.

In this study, we aimed to get the best method, not only to get the most abundant cells, but we wanted to also get a culture with the highest percentage of epithelial cells during the culturing period. Therefore, we multiplied the total count of cells with the value of epithelial cells percentage, as can be observed in Figure 3c. In this observation, metastatic tissues produced more epithelial cells compared to the tissue from primary cancer site, as the groups begun with the letter M dominated the highest ranks. The highest places were also dominated by the groups that were processed using Dispase and Trypsin. The use of Percoll was placed as high as the 5th rank, since Percoll may separate the cells more specifically based on size, shape, and density, although the total count might have to be compromised.

A Primary Site

Cell Suspension Method	Medium: DMEM:F12 (A)	Medium: MCDB:M199 (B)	Medium: McCoy's 5A (C)	Percoll Usage
Explant	P-E-A	P-E-B	P-E-C	Non-Percoll
Dispase	P-D-A	P-D-B	P-D-C	Non-Percoll
	P-D-P-A	P-D-P-B	P-D-P-C	Percoll
Trypsin	P-T-A	P-T-B	P-T-C	Non-Percoll
	P-T-P-A	P-T-P-B	P-T-P-C	Percoll
Dispase-Trypsin	P-DT-P-A	P-DT-P-B	P-DT-P-C	Percoll

Experiment Codings

B Metastasis Site

Cell Suspension Method	Medium: DMEM:F12 (A)	Medium: MCDB:M199 (B)	Medium: McCoy's 5A (C)	Percoll Usage
Explant	M-E-A	M-E-B	M-E-C	Non-Percoll
Dispase	M-D-A	M-D-B	M-D-C	Non-Percoll
	M-D-P-A	M-D-P-B	M-D-P-C	Percoll
Trypsin	M-T-A	M-T-B	M-T-C	Non-Percoll
	M-T-P-A	M-T-P-B	M-T-P-C	Percoll
Dispase-Trypsin	M-DT-P-A	M-DT-P-B	M-DT-P-C	Percoll

Experiment Codings

Figure 1. Experiments that were Performed based on Combinations of Site of Tissue Source, cell suspension method, usage of percoll, and type of medium. A. Experiments performed to the tissues from the primary site; and B. Experiments performed to the tissues from the metastasis site. The experiment groups were coded based on the steps taken. The coding starts with the source of the tissue (P for primary site; M for metastatic site), continued with code for the cell suspension methods (E for explant without cell suspension process; D for dispase; T for trypsin; DT for both dispase and trypsin), the usage of percoll (P for when percoll was used), and lastly followed with the code of the medium (A for DMEM:F12; B for MCDB:M199; and C for McCoy's 5A).

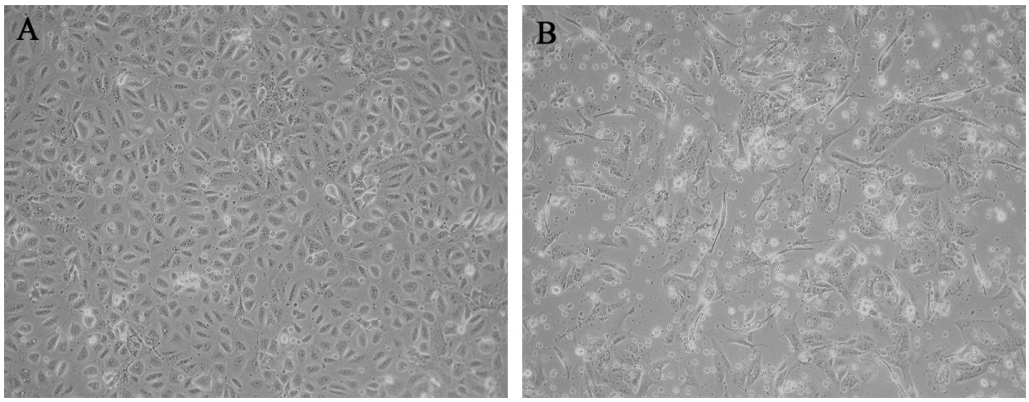

Figure 2. Pictures of Cell Culture 6 Days after Cell Suspension Treatment and Seeding (100X magnification). Picture A was taken from the culture of metastasis tissue that was treated with dispase and cultured in McCoy's 5A Medium (M-D-C group), which showed that the culture was dominated by Epithelial cells. On the other hand, Picture B was taken from the culture of primary tissue that was treated with dispase and cultured in DMEM:F12 medium (P-D-A group), showing that the culture was dominated by fibroblastic cells.

Primary Vs Metastasis

Based on the cell calculation, we found that the metastasis groups dominated the highest ranks in resulting more epithelial cells in culture. Therefore, we calculated the metastasis group and primary group and made a comparison on the estimated number of epithelial cells based on the morphological observation (Figure 4). We found that the metastasis tissue produced more epithelial cells in culture compared to the primary tissue regardless of the methods and media used in the experiment. The

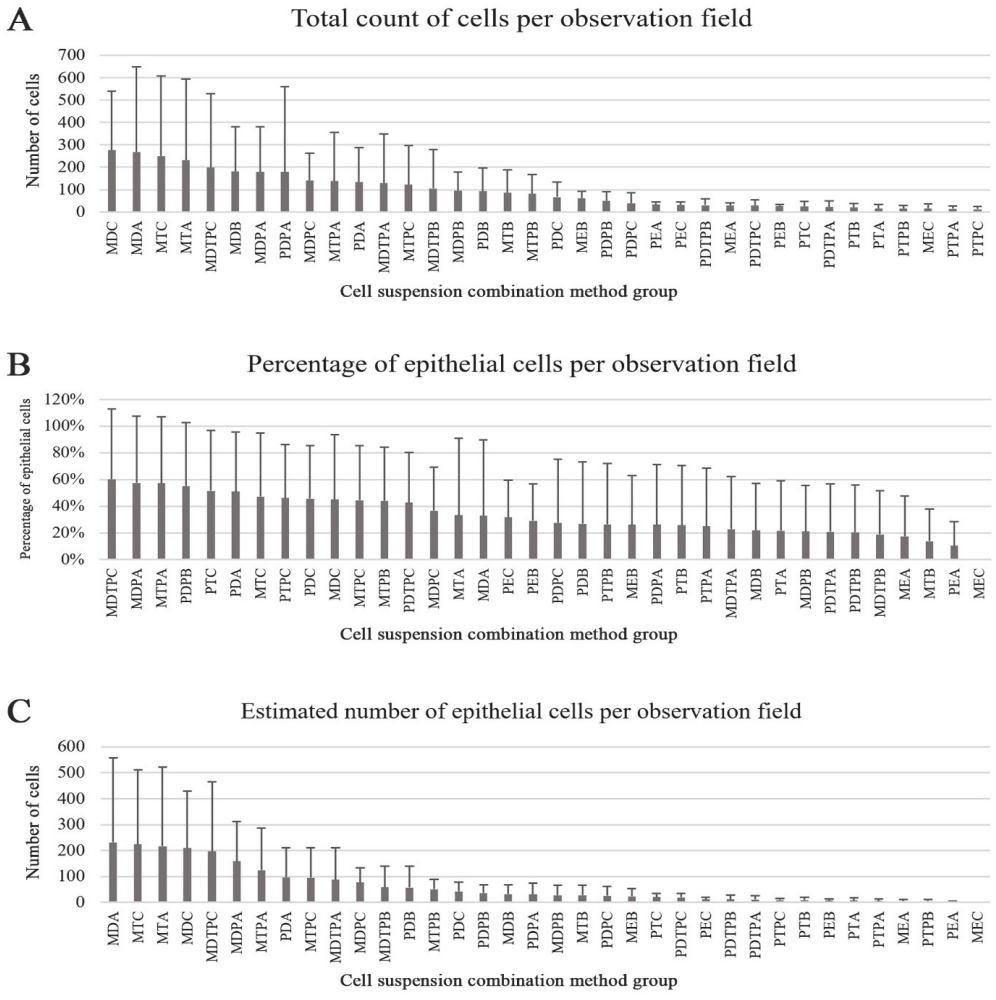

Figure 3. Cell Calculations of the Cell Culture Experiments. A. Total count of cells was obtained by adding the number of epithelial cells and fibroblastic cells. B. Percentage of Epithelial Cells was obtained by dividing the number of epithelial cells by the total count of cells. C. Estimated Number of Epithelial Cells per Observation Field.

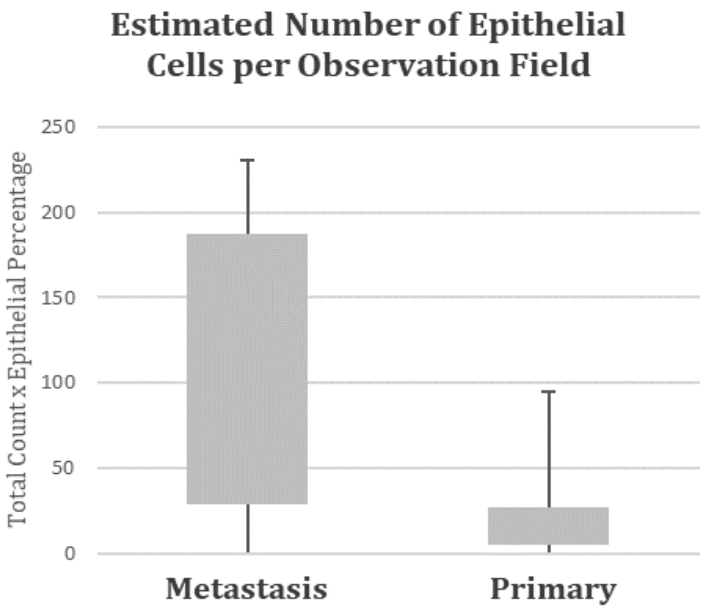

Figure 4. Comparison of Metastasis Tissue and the Primary Site Tissue based on the Estimated Number of Epithelial Cells per Observation Field. Metastasis tissue produces more epithelial cells in culture, compared to its primary counterpart.

statistical analysis using Mann Whitney-U test found that the metastasis tissue derived more epithelial cells significantly compared to the primary tissue (102,32±82,65 vs 22.6±23.81, p=0.001).

Discussion

The previous studies of epithelial ovarian cancer cell culture were performed using different methods of cell dissociation and different media. Studies conducted by Pribyl et al.,(2014) and Kar et al., (2017) utilized Dispase and DMEM:F12 medium to establish the ovarian cancer primary cell culture. In another study conducted by Shepherd et a., (2007) the ovarian tissues were processed into cell suspension using Dispase and precipitated using Percoll, and then the cells were cultured in MCDB105:M199 medium. In another study performed by Thériault et al., (2013), epithelial ovarian cell culture was established by performing trypsinization of the tissue and the cells were cultured in McCoy's 5A medium. In our study, we tried to compare each of the steps by combining the cell suspension methods, the use of Percoll, and the types of media. In addition, we also compare d the tissue sources of the culture cells, which are the primary and the metastatic site, to determine the best method to culture the epithelial cells from serous ovarian cancer tissue.

We compared several methods in transforming the ovarian cancer tissues into cell suspension, before being seeded into cell culture. Firstly, we used the explant method, where the tissue was diced into smaller pieces and seeded directly in the culture dish. The explanting method is the earliest method used in culturing cancer cells, dating back to 1914 when the first sarcomatous tissue cells were cultured (Losee and Ebeling, 1914). This method is simple and only requires good aseptic techniques and saline as buffer supplemented with antibiotic to avoid unwanted microbial contamination. This method has already been used previously to culture human ovarian cells (Siemens and Auersperg, 1988). However, this method could not warrant the type of cells that will be cultured since it depends strongly on the composition of cells in the diced tissue surface. The explant method typically needs longer time to culture as the cells need to detach itself from the tissue and to spread across the culture flask surface. Moreover, the physiology and morphology of the epithelial cells, as a more rigid type of cells, hardly allow the epithelial cells to grow quicker than the fibroblastic cells. It is well illustrated in our experiments as the explant method came in the last ranks in producing epithelial cell culture when compared to the other cell suspension methods.

We also compared two different enzymes in disintegrating the tissue: Trypsin and Dispase. Trypsin is a pancreatic serine protease specific for peptide bonds involving the carboxyl group of the basic amino acids, arginine and lysine. Based on our results, it was observed that enzymatic process was more effective than using explant to dissociate cancer tissue. Although Trypsin is tolerated by many cell types, because Trypsin cleaves any arginine and lysine, long term incubation with high trypsin concentration damages cells by stripping cell surface and cleaving cytoplasmic proteins, and further may kill the cells (Tsuji et al., 2017).

Dispase is a natural protease that is isolated from a bacteria, Bacillus polymyxa (Stenn et al., 1989). This enzyme is widely used for dissociating tissues for cell culture application including epidermis (Stenn et al., 1989), pancreas (Ono et al., 1977), spleen (Fujiyama et al., 2018), brain cells (Volovitz et al., 2016; Zhu et al., 2012), stem cells (Jager et al., 2019), and cancerous tissues

such as the mammary (Nishikata et al., 2013), lung (Kim et al., 2013), and ovary (Pribyl et al., 2014; Shepherd et al., 2007). Dispase specifically breaks down fibronectin, which typically assembles integrins that bind cells in the tissues, and type-IV collagen, which constructs the basal lamina of tissues, indicating that this enzyme has higher specificity compared to trypsin (Stenn et al., 1989; Zhu et al., 2012). More recent study demonstrated that dispase was also able to cleave serine-phenylalanine interaction (Weimer et al., 2006). For its high specificity, dispase was used as a powerful tool to dissect interactions between epithelial and mesenchymal cells. In our study the use of Dispase produced slightly higher yield of cells and more abundant epithelial cells in the culture when compared to Trypsin.

To get higher variety of methods, we also included Percoll in the combination. Percoll was firstly introduced in 1977 as silica-based colloidal medium for cell separation based on density gradient centrifugation (Cytiva, 2007; Pertoft et al., 1977). Percoll exploits differences of density in cells to separate them from one type to another, including epithelial and fibroblastic cells (Pertoft et al., 1977). Utilization of Percoll in harvesting epithelial cells from ovarian cancer tissues seems to produce higher percentage of epithelial cells as it is demonstrated in our study that based on the result of epithelial cells percentage (Figure 3b) four groups that were separated using Percoll reached the highest ranks compared to those processed without Percoll. However, in our study, the total count of cells was lower when Percoll was used compared to other groups that utilized only Trypsin or Dispase. This indicated that the use of Percoll might result more specific type of cell, although the yield might have been compromised in order to get more specific type of cells.

We compared three different kinds of media, namely DMEM:F12 (1:1), MCDB105:M199 (1:1), and McCoy's 5A. Based on our experiments, DMEM:F12 and McCoy's 5A Media were more preferable to grow human epithelial ovarian cancer cells shown by the fact that the top 11 ranks on the estimated epithelial cell number graph (Figure 3c) were occupied by either DMEM:F12 or McCoy's 5A media. Those media gave more abundant cells and higher percentage of epithelial cells when compared to the MCDB105:M199 Medium.

DMEM

F12 medium is one of the most widely used basal media for serum-free culture, constituting a 50:50 mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F12 medium (Yao and Asayama, 2017). Quirk et al., (1997) used the DMEM:F12 medium with addition of supplements such as transferrin, insulin, hydrocortisone, and the factor of epidermal growth for culturing epithelial cells. In contrast, in this study, by using medium DMEM:F12 supplemented with FBS at 10%, without the addition of other growth factors and hormones, we managed to establish the primary epithelial cell culture. This study showed that both DMEM:F12 and McCoy's 5A media were better than MCDB105:M199 in isolating and culturing epithelial cells from ovarian cancer tissues. Because DMEM:F12 medium did not need any additives or additional growth factors, it made the isolation and culture of epithelial cells easier and inexpensive. Therefore, DMEM:F12 can be a candidate basal medium for primary ovarian cancer epithelial cell culture, along with McCoy's 5A medium.

McCoy's 5A medium is generally purposed medium that supports the propagation of many types of primary cells, established cell lines, and explants from biopsy tissues. Previous researches had used McCoy's 5A medium to grow ovarian cancer cell line SKOV3 (Bu et al., 2020; Mielczarek-Palacz et al., 2016; Yao et al., 2015). Therefore, we picked this basal medium as an alternative to propagate ovarian cancer derived cells. Combination between enzymatic process using McCoy's 5A medium resulted high estimated number of epithelial cells (Figure 3c).

Medium M199 was initially developed by Morgan, Morton, and Parker in 1950, and was made specifically to culture primary chick embryo fibroblasts (Gruber and Jayme, 1994; Morgan et al., 1950). It was observed that explanted tissue might survive in this medium without the addition of serum in the short term of around 4-5 weeks (Morgan et al., 1950). Whereas, MCDB105 medium is a basal nutrient medium formulated by Ham and McKeehan (1979). In combination, the MCDB105:M199 medium was utilized widely as a basal medium for the growth of ovarian cancer cells (Berchuck et al., 1992; Kar et al., 2017; Ortolan et al., 2010; Shepherd et al., 2007; Walker, 2013). However, our study did not show MCDB105:M199 to be the best choice in separating and culturing the epithelial cells from ovarian cancer tissues.

In our study, we sampled both the primary and metastatic tissues from the same ovarian cancer patients and we found that the metastasis tissues gave rise to more epithelial cells compared to the primary tissues. Most studies comparing the nature of primary and metastasis tissues of cancer emphasizes the genomic and mutational differences (Baldus et al., 2010; Diep et al., 2006; T. M. Kim et al., 2015; Li et al., 2017; Mũoz-Bellvis et al., 2012; Yamamoto et al., 2010). One study showed that in colorectal cancer the mutational concordance between the primary and metastasis sites of the same patient accounts for up to 88% (Mekenkamp et al., 2014), and higher differences were found in comparison of cancer tissues between different patients (Kim et al., 2015).

One study based on immunohistochemistry found that there were higher expressions of epithelial markers such as E-cadherin and Catenins in the metastasis tissue (Imai et al., 2004). Another study performing qPCR, western blotting, and immunohistochemistry of epithelial cell adhesion molecule (EPCAM), an epithelial marker, also demonstrated that metastasis tissues expressed higher EPCAM compared to the primary counterpart. The loss of EPCAM is one of the hallmark on epithelial-mesenchymal transition (EMT), which in cancer might lead to metastasis (Hyun et al., 2016), however, the opposite phenomenon, called the mesenchymal-epithelial transition (MET), gains higher EPCAM expression, strengthening the cells in anchoring to its microenvironment and leading to more committed epithelial cells. Higher epithelial markers found in metastasis tissue indicated that there were more

cells committing to epithelial form when compared to the tissue of the primary site, indicating MET in the metastasis site and EMT in the primary site.

The process of EMT and MET is related to several cellular modification such as basal membrane remodeling, cell-cell adhesion, and apical constriction (Jolly et al., 2017). Those changes can be observed by the gene expression changes of epithelial markers, such as EPCAM (Hyun et al., 2016) and E-cadherin (Fenouille et al., 2012); and mesenchymal markers, such as ZEB1 (Das et al., 2009), Vimentin (Mendez et al., 2010), Snail1 (Sonego et al., 2019), Snail2/Slug (Haslehurst et al., 2012), and Twist (Haslehurst et al., 2012). However, as the limitation of our study, we observed only the morphological feature of the cells without measuring the changes in gene expression level. In the future, research on the gene expression level and also epigenetic characteristics on the comparison between primary and metastasis sites of ovarian cancer may lead to more accurate method on how to culture epithelial cells from ovarian cancer tissue as disease model.

In Conclusion, the use of metastasis tissue processed enzymatically using Dispase or Trypsin and cultured in DMEM:F12 or McCoy's 5A media was demonstrated to be the most efficient way to produce the highest amount of cells with high percentage of epithelial cells. Even though the use of Percoll might increase the percentage of epithelial cells in the culture, it reduces the overall amount of the cells.

Author Contribution Statement

Tricia Dewi Anggraeni as the main researcher of the study; Ariananda Hariadi contributed in planning and performing the experiments in the laboratory, collecting and analyzing data, and also in establishing and completing the scientific article; Tera Dria Kispa contributed in planning and performing the experiments in the laboratory, collecting data, and also in writing the scientific article; Jenivia Thiono contributed in performing the experiments in the laboratory; Hariyono Winarto as the consultant from the clinical point of view. Marselina Irasonia Tan as the consultant from the biological, conceptual, and technical point of view.

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Ethics Approval and Consent

This study was a part of a doctoral thesis and was ethically approved by the Scientific and Ethical Committee of the Cipto Mangunkusumo Hospital in affiliation with the Faculty of Medicine, Universitas Indonesia, under the letter no. 102/UN2.F1/ETIK/2019. All patients were well informed about the research before consenting to participate.

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Conflict of Interest

All authors hereby declare that there is no conflict of interest in this study and publication.

All authors agreed and consented to publish this manuscript to the Asia Pacific Journal of Cancer Prevention.

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