

The effect of hypoxia and 3D culture conditions on heterogeneous ovarian cancer spheroids

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Abstract

Epithelial ovarian cancer (EOC) is the leading cause of death from gynecological malignancy due to the insufficient accurate screening programs for the early detection of EOC. To improve the accuracy of the early detection, there is a need to deeply understand the mechanism of EOC progression and the interaction between cancer cells with their unique microenvironment. Therefore, this work investigated the metabolic shift in the mouse model for progressive ovarian cancer, and evaluated the effects of hypoxic environment, spheroid formation as well as stromal vascular fractions (SVF) on the metabolic shift, proliferation rate, drug resistance and protein markers in functional categories. The results demonstrated an increasingly glycolytic nature of MOSE cells as they progress from a tumorigenic (MOSE-L) to a highly aggressive phenotype (MOSE-FFL), and also showed changes in metabolism during ovarian cancer spheroid formation with SVF under different oxygen levels. More specifically, the hypoxic environment enhanced glycolytic shift by upregulating the glucose uptake and lactate secretion, and the spheroid formation affected the cellular metabolism by increasing the lactate secretion to acidify local environments, modulating the expression of cell adhesion molecules to enhance cell motility and spheroids disaggregation, and up-regulating invasiveness markers and stemness makers to promote ovarian cancer aggressive potential. Hypoxia and spheroid formation decreased ovarian cancer cells growth but increased the chemoresistance, which leads to the promotion of aggressiveness and metastasis potential of ovarian cancer. SVF co-cultured spheroids further increased the glycolytic shift of the heterogeneous of ovarian cancer spheroids, induced the aggressive phenotype by elevating the

corresponding protein markers. Decreasing the glycolytic shift and suppression of the proteins/pathways may be used to inhibit aggressiveness or metastatic potential of ovarian cancer heterogeneous of ovarian cancer spheroids, induced the aggressive phenotype by elevating the corresponding protein markers. Decreasing the glycolytic shift and suppression of the proteins/pathways may be used to inhibit aggressiveness or metastatic potential of ovarian cancer.

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General audience abstract

Epithelial ovarian cancer (EOC) is the leading cause of death from gynecological malignancy due to the usually late detection when the cancer has already spread throughout the peritoneal cavity. Physical, cellular and chemical factors can contribute to EOC progression and metastasis. Critical physical factors are the low oxygen content in the peritoneal cavity (hypoxia) that promotes tumor cells survival, and the formation of tumor spheres, which have been demonstrated to have a more aggressive phenotype. Moreover, obesity has been proposed to support ovarian cancer development and progression. Therefore, this work investigated the impact of oxygen deprivation, sphere formation, and white adipose tissue-derived stromal cells on ovarian cancer cells progression. The results showed that all these factors contribute to the aggressive potential of ovarian cancer cells by increasing the drug resistance, and modulation of cellular metabolism. The understanding of the interactions between ovarian cancer and other cells within their unique microenvironment may provide critical targets for chemotherapeutic interventions that are aimed to control the aggressiveness of ovarian metastases in their hypoxic tumor microenvironment, and enhance the life of women afflicted with ovarian cancer.

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Chapter 1 Introduction

1.1. Background

Epithelial ovarian cancer (EOC) has a high malignancy. It is the fifth leading cause of cancer death among women the United States based on the statistics from Center for Disease Control and Prevention (CDC). Each year, more than 20,000 women in the US get EOC and an estimated 14,240 deaths are expected in 2016^{1,2}. The overall 5-year relative survival rates of EOC is about 46%, but women aged beyond 65 have a 50% lower chance to survive¹. Substantial research efforts have focused on improvement of detection and treatment of EOC, however, the death rate has only declined by 2% per year. One of the reasons is insufficiently accurate screening programs for the early detection of EOC, which results in the EOC often being diagnosed at an advanced stage, with metastasis and malignant progression already initiated in the peritoneal cavity. Thus, it is critical to improve the accuracy of screening programs for the early detection of EOC. To address this research need, identifying biomarkers for screening programs has been demonstrated as a potential way². For that purpose, there is a need to deeply understand the mechanism of EOC progression and the interaction between cancer cells with their unique microenvironment.

There are four aspects needing to be explored for understanding the mechanism of EOC and the interaction between cancer cells with their unique microenvironment. First, invasion and metastasis play crucial roles in promoting EOC progression, but the mechanisms involved in the spread of ovarian carcinoma remain unknown. Second, the unique metastatic mechanism of EOC shows that cancer cell spheroids formed and circulated through peritoneal fluid to the

secondary lesions³. Thus, using 3-dimensional (3D) culture model to study EOC metastatic mechanisms can better reflect the true tumor microenvironment. Third, metabolic shifting of EOC under hypoxic condition are known to affect tumor cell invasion and metastasis, the extent of their effects need to be studied both qualitatively and quantitatively. Lactate, the end product of glycolysis, has been recognized as an alternative energy source for cancer⁴ and an inducer of tumor progression⁵ can be used to reflect the influence of hypoxia on EOC progression. Also, the maintaining of cancer stem-like characteristic is enhanced under hypoxia⁶, which contributes to tumor progression. Thus, the effects of hypoxia on lactate secretion and the formation of cancer stem cells need to be studied in more detail. Last, obesity is reported to promote tumor invasion through providing a chronic systemic inflammatory microenvironment⁷ and supportive cells^{7,8}. The white adipose tissue-derived stromal vascular fraction (SVF) contain different types of cells that can be recruited by cancer cells to potentially increase tumor burden. Therefore, effects of SVF interacting with EOCs on metabolic shifting need to be elucidated to further address the mechanism of EOC progression.

1.2. Invasion and metastasis

Invasion and metastasis are responsible for the high morbidity and mortality in EOC patients⁹⁻¹¹. The most common invasion-metastasis cascade includes of the following steps: 1) shedding cells from primary tumor, 2) breaching of the basement membrane, 3) gaining access to blood vessels (extravasation), 4) interacting with platelets, lymphocytes and other blood components, and 5) passaging of tumor cells through the circulation, lodging and extravasation, colonization and expansion¹².

It is well recognized that EOC cells can go through epithelial-mesenchymal transition (EMT) to fulfill the ability of metastasis and invasion as shown in Figure 1-1¹³. EMT is a process by which epithelial cells lose cell-cell and cell-matrix adhesion and acquire a motile mesenchymal phenotype. Epithelial cancer cells at the leading edge of a primary carcinoma undergo an EMT and invade the tissue below the basement membrane by triggering the signals from the nearby tumor-associated stromal cells¹³. These tumor cells are recruited through circulation to the secondary normal tissue site. However, the normal stroma does not release EMT-inducing signals, which allows these tumor cells and their daughter cells to go back to an epithelial phenotype via a mesenchymal–epithelial transition (MET). Cancer cells activate the expression of mesenchymal genes such as Vimentin, and lose the epithelial gene expression such as E-cadherin for the EMT to change morphology and acquire motility and invasiveness¹⁴. EOC cells over-express mesenchymal markers (cadherin and vimentin), when cultured in low-adherent plates as spheroids¹⁵. Therefore, understanding the mechanism underlying the change of organization of cytoskeleton of the ovarian cancer cells is helpful to study the metastasis of EOC.

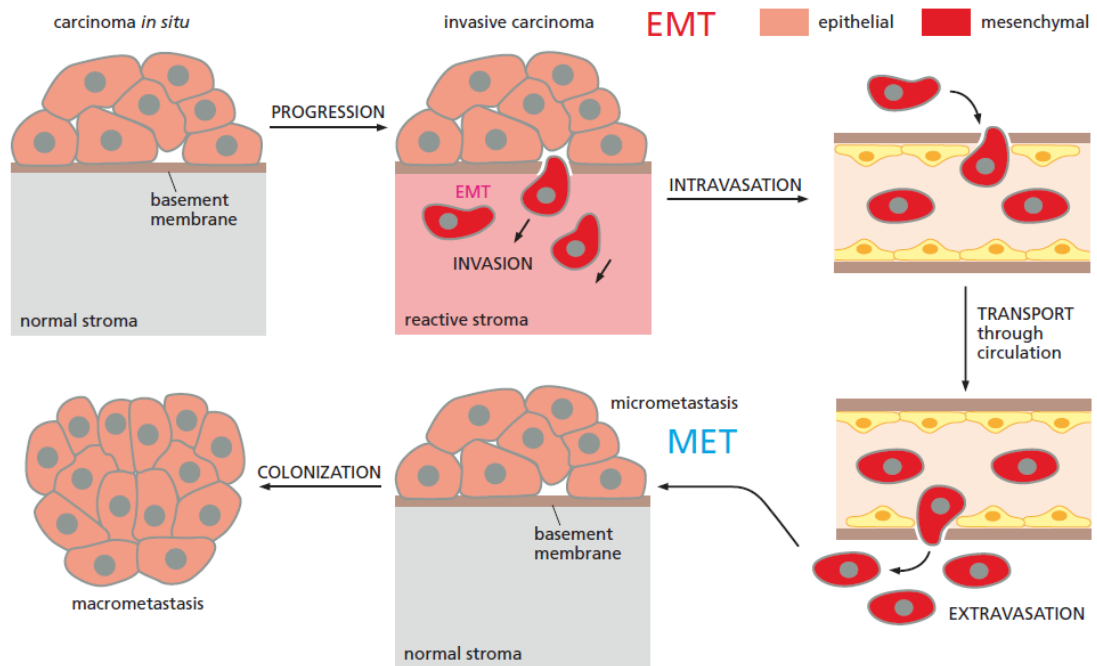


Figure 1-1 **Representation of the epithelial mesenchymal transition.** Tumor cells with epithelial characteristics acquire mesenchymal characteristics through the activation of EMT effectors, such as vimentin, Oct4 and Nanog, causing them to become motile and invasive. These cells leave the microenvironment of the primary tumor and invade the peritoneal cavity. (Figure reproduced from Ref.¹⁴)

EOC tumors have a unique progression compared to other cancer types as shown in Figure 1-2. The primary ovarian tumors originate from the surface of ovary, or the fallopian tube (serous ovarian cancer). Then exfoliated individual tumor cells from primary tumor sites can form homogenous or heterogeneous multicellular spheroids that are carried by the peritoneal fluid, being disseminated throughout the peritoneum and omentum. The development of peritoneal metastases in EOC requires the release tumor cells into the local environment and potential spaces of peritoneal cavity and the ability of shed tumor cells to survive, attach to and engraft onto the surface of other organs or the lining of the peritoneal cavity¹⁶. EOC uses multiple forms of adhesion into its survival dissemination activities. Cell-cell adhesion, mediated by cadherins, is disrupted to acquire mesenchymal phenotype during tumor cells

migration to make multicellular spheroids prevalent in peritoneal cavity³. Integrin-mediated cell-matrix adhesion is remodeled during peritoneal anchoring¹⁷, and enhances spheroids disaggregation on extracellular matrices (ECM)¹⁸ and promotes a sustained invasion of EOC spheroids into mesothelial cells¹⁷. Cancer cells and the surrounding peritoneal resident cells (i.e., T cells, B cells macrophages, and lymphocytes) and mesothelial cells lining the peritoneum secrete cytokines and growth factors^{19,20}, which in turn enhance vascular permeability²¹, lymphatic obstruction²².

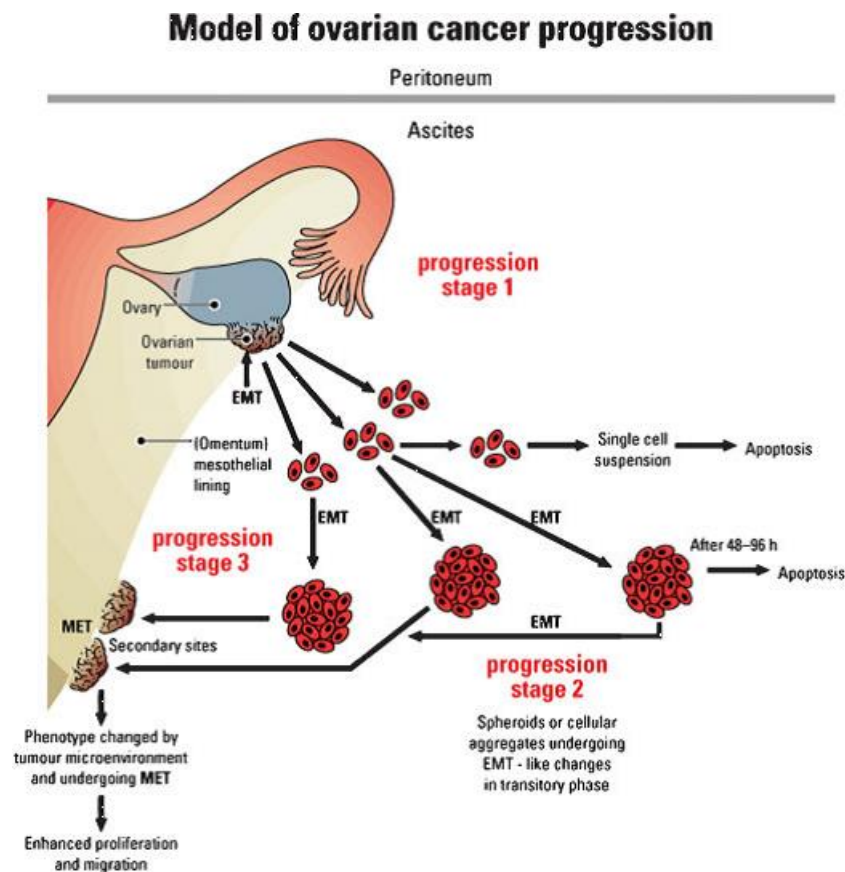


Figure 1-2 **Progression of epithelial ovarian cancer.** During ovarian cancer progression, epithelial ovarian cancer cells growing on the surface of the ovary undergo EMT to gain motile phenotype, result in shedding of tumor cells into the peritoneal cavity as single cells or cellular aggregates/spheroids. Tumor cells or spheroids remodel integrin-matrix contacts, attach to and infiltrate the mesothelial lining of the abdominal cavity to proliferate and establish the secondary sites. (Figure reproduced from Ref.²³)

1.3. The spheroids formation and EOC

EOC cells exfoliated from the primary tumor site survive in a non-adherent state within the peritoneal fluid as single cells or three-dimensional multicellular structures, termed multicellular aggregates or spheroids¹⁹. There are several studies demonstrate that EOC spheroids are commonly found in peritoneal cavity can have a sustained and longer growth than a normal ovarian cell line¹⁸, and are capable of tumorigenesis *in vivo*²⁴. Because EOC metastasis involves multiple steps, each of which occurring in a distinct microenvironment with distinct impacts on tumor behavior, these 3D spheroids structure would be an important target to treat EOC. However, most current models and research are based on 2D cell culture results to study the anti-cancer agents. Given that most ovarian cancer patients are diagnosed after the cancer has already metastasized, and forming 3D structure is a key mechanism for EOC metastasis, a better understanding of spheroid biology may contribute to the identification of new treatment opportunities for the sustained treatment of metastatic EOC. Together, 3D culture of EOC cells *in vitro* would be a better model for micro-metastases study because: 1) the 3D cellular context allows cancer cells have interactions in all directions with other cells such as SVFs, 2) the 3D structure can better mimic tumor microenvironment in order to more accurately reflect clinical expression profiles.

A 3D microenvironment can alter protein expression and chemosensitivity of epithelial ovarian cancer cells *in vitro*. Lee, J.M. et al.²⁵ cultured 31 EOC cell lines on non-adherent culture dishes to generate spheroids and evaluate changes in protein expression of cell adhesion molecules and ovarian cancer biomarkers. They found that the cell adhesion marker E-cadherin was upregulated in 3D, whereas, vimentin, an intermediate filament protein expressed in

mesenchymal cells, tended to be down-regulated in those cell lines compared with 2D²⁵. E-cadherin is expressed in many normal epithelial tissues. Loss of E-cadherin-mediated adhesion characterizes the transition from benign to invasive, metastatic ovarian cancer. Furthermore, EOC cells cultured in 3D were less chemo-sensitive than the same cells grow as 2D monolayers²⁵. EOC cells had increased resistance to both a DNA-damaging agent (cisplatin) and microtubule-stabilizing agent (paclitaxel). The possible mechanisms how ovarian cancer spheroid formation contributes to chemotherapy resistance are the presence of hypoxia zones, and enriched cancer stem-like cells in spheroids²⁶. Liao et al. ²⁶demonstrated that ovarian cancer spheroids expressed stem cell genes such as CD34 and Nanog. Those spheroid cells also showed a significant increase in anaerobic glycolysis and defective aerobic glucose metabolism, increased in glucose uptake and the shuttling of carbons to the pentose phosphate pathway, suggesting that ovarian cancer spheroids are likely to survive in hypoxic microenvironments²⁶. Collectively, these results indicate the importance of studying ovarian cancer progression and interaction with its microenvironment by using 3D culture models.

1.4. Metabolic shifting of EOC and hypoxia

The ability that cancer cells switch their metabolic ways from mitochondrial oxidation to glycolysis even in presence of oxygen is a key characteristic, which in turn affects tumor cell invasion and metastasis. This alteration of cancer cell metabolism was first observed and published by Otto Warburg²⁷. His initial findings²⁸ included two major metabolic processes: dramatically increased rates of glucose uptake and lactic acid production even under adequate oxygen. It seems that cancer cells prefer aerobic glycolysis to use high levels of glucose and convert it to lactate rather than to ATP via oxidative phosphorylation. This phenomenon is

termed as Warburg effect. Glucose is taken up into cells and converted to pyruvate, producing 2 ATP. In benign cells, pyruvate enters the mitochondria and is oxidized to HCO_3 with generating 36 additional ATP per glucose molecule. For anaerobic metabolism, pyruvate is converted to lactic acid without producing more energy. This shows that cancer cells prefer to use an inefficient way to produce energy, and generate an acidic microenvironment. However, previous research has shown that the upregulated glycolysis in cancers has an enormous advantage to promote the malignant phenotype and metastasis²⁹, which is related to hypoxia.

Hypoxia, or the state of low oxygen (O_2) levels, is known to exist in solid tumors with the size more than 1 mm^3 because of the slower blood vessel growth³⁰, which results in the formation of O_2 and nutrients gradients in the microenvironment³¹. Tumor cells need to respond to these distinct conditions and adjust their metabolism. Rapid cancer cells proliferation needs extra blood supply to provide adequate O_2 and nutrients. Angiogenesis, a pivotally natural process that new blood vessels grow from pre-existing vessels used for healing and reproduction, is activated under hypoxia. There are two different ways of angiogenesis: incorporating preexisting normal vessels into tumor tissue and arising new microvessels through the influence of tumor angiogenesis factors³². However, the newly formed vascular network shows significant differences from that formed in normal tissue, displaying structural and functional alterations³³. These abnormalities include immature, leaky, tortuous, and dilated vascular structures, which lead to a reduction of O_2 and nutrient supply³². Hypoxia and nutrients deprivation then activate hypoxia-inducible factor 1 (HIF1).

HIF1 is an essential regulator of cellular O_2 homeostasis and hypoxic stress adaptor³⁴. It has been shown that transcription factor HIF-1 plays a key role in reprogramming pyruvate

catabolism and oxidative phosphorylation to aerobic glycolysis³⁵. In addition, HIF-1 regulates the expression of genes coding for proteins involved in glucose metabolism and glycolytic enzymes³⁶.

Hypoxia may be involved in tumor progression and metastasis because hypoxic cells are more resistant to traditional cancer therapeutics (such as chemotherapy and radiation) and have more invasive and metastatic potential^{37,38}. Some crucial pathophysiological conditions associated with hypoxia include the induction of angiogenesis³⁴, activation of glucose transporters³⁹, increasing lactic acid secretion, and the maintenance characteristics of cancer stem cells⁴⁰. Thus, understanding those functions and characteristics of EOC cells under hypoxic conditions is highly attractive for exploring the new therapeutic methods for EOC.

Therefore, the major part of my experimental study will focus on the exploration of the lactate glycolysis and the stemness characteristics on EOC.

1.4.1. Lactate with cancer

Lactate, the end product of glycolysis, is recently recognized as an alternative energy source⁴ and an inducer of tumor progression⁵. Previous study has documented that lactate can be up-taken by glycolytic cancer cells in the absence of glucose in the culture medium⁴¹. Lactate accumulation results in acute and chronic acidification which can be toxic to normal cells but relatively harmless to cancer cells. Lactate appears to serve several different functions as listed below in the tumor environment including but not limited to energy production, promotion of necrosis or apoptosis, and immune invasion effects:

- 1) Cancer cells can recycle part of their own lactate production to generate energy through

oxidative metabolism without requiring initial energy input by glucose⁴. Sonveaux et al.⁴ found that lactic acid is an important energy source for tumor cells. Tumor cells in hypoxic condition inefficiently use glucose and produce lactate. However, when there is a good oxygen supply, cancer cells actually prefer to utilize lactate, which spares extra glucose for the less-oxygenated cells, as shown in Figure 1-3. It is known that a tumor's oxygen level and blood flow can fluctuate rapidly⁴². Under these conditions, cancer cells can accumulate lactate during hypoxia and switch using glucose to lactate during normoxia, which gives those tumor cells a way to save and produce enough energy. 18 ATP can be yielded per lactate molecule through respiration of lactic acid, which is used to support glycolytic enzymes⁴, allowing cancer cells to still getting enough energy for their growth through lactate oxidation. The monocarboxylate transporter 1 (MCT1), a protein at the plasma membrane of oxidative fibers, mediates lactate uptake for oxidative metabolism. MCT1 inhibition can disrupt lactate-fueled respiration and push cells toward inefficient glycolysis. Because glucose can be used more abundantly by better oxygenated cells, those cancer cells used most of glucose before it could reach the hypoxic cells, which starved hypoxic cells to death⁴³. Their research showed a promising target, MCT1, may be used to sensitize the conventional anticancer therapies.

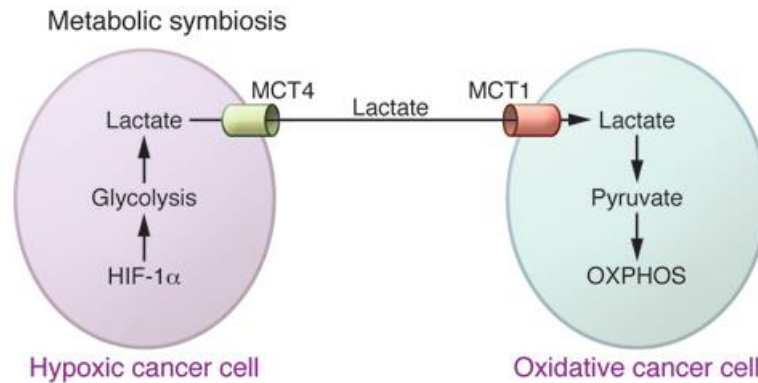


Figure 1-3 **The lactate shuttle as a form of metabolic symbiosis.** Tumor cells in hypoxic regions of the tumor export lactate via MCT4, which is then imported by tumor cells that are more oxidative through MCT1. This shuttling facilitates the delivery of glucose to the hypoxic tumor cells. (Figure reproduced from ref. ⁵)

- 2) Tumor acidity from lactate accumulation can also result in a significant decrease in local extracellular pH, which will lead to prolonged exposure of adjacent normal populations to an acidic microenvironment and cause necrosis or apoptosis through p53-dependent and caspase-dependent mechanisms^{44,45}. The extracellular pH of tumors can be as low as 6-6.5⁴⁶. With such acidic environment, Park et al.⁴⁴ demonstrated that acidic stress stimulate apoptosis through caspases by increasing the level of Bax, a protein that has been shown to induce apoptosis and is regulated by p53⁴⁷. p53 is a tumor suppressor gene, which can response to DNA damage through cell cycle arrest or apoptosis. A loss of function is commonly associated with colorectal epithelial carcinoma. Based on this knowledge, Williams et al.⁴⁵ found that acidic environment selectively inhibited cell growth with functioned p53 gene through apoptosis, but cells with no p53 function were resistant to the acidification of the microenvironment. Thus, normal cells, by the virtue of lacking a mechanism to adapt to extracellular acidosis (like p53 mutation) cannot survive under such conditions of microenvironmental acidosis, whereas tumor populations continue to

proliferate.

- 3) High lactate levels predict metastases, tumor recurrence and indicate a correlation with the clinic outcome of some cancer types⁴⁸⁻⁵⁰. The diffusion of acid from the tumor to host induces alteration of microenvironmental pH, provides a specific mechanism promoting tumor invasion: either through inducing the secretion of inflammatory factor or vascular endothelial growth factor (VEGF)⁵¹, then leads to increased migration and angiogenesis in solid tumors, as shown in Figure 1-4 or through degradation of ECM.

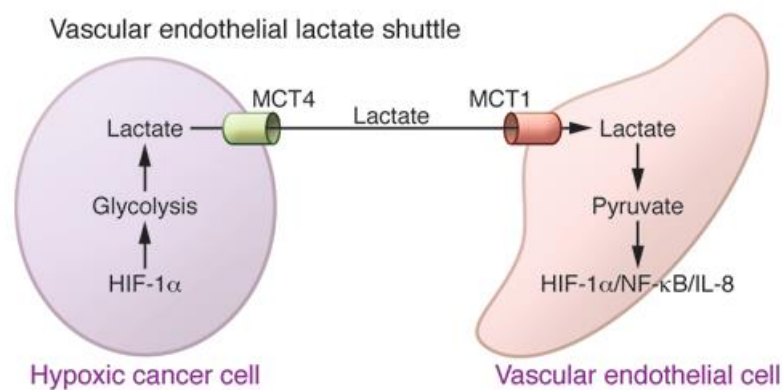


Figure 1-4 **The vascular endothelial lactate shuttle.** The interplay of tumor cells with vascular endothelial cells induces lactate signaling pathway, leads to endothelial cell migration and tumor angiogenesis (Figure reproduced from ref. ⁵).

Lactate has also been demonstrated as a signaling molecule in endothelial cells through HIF-dependent mechanisms. Hunt et al.⁵¹ reported that lactate accumulation in the presence of oxygen can drive new vessel formation through increasing VEGF levels. The enhanced VEGF production can further regulate the cell migration⁵². A lactate-enriched environment in solid tumors can also promote tumor progression by adding the ability of immune surveillance escape and increasing mobility of cancer cells through modulation of potential immune cells (such as dendritic cells and T cells) and cytokine release (such as tumor

necrosis factor and interleukin-8⁵³, interleukin -6)⁵⁴. The data presented in these studies suggested lactate as an active metabolite in cell signaling, playing an important role in regulating tumor environment.

Furthermore, research has documented that tumors acidified the extracellular space of normal tissue around the tumor leading edge and showed a degradation of ECM, which supports the hypothesis that glycolysis lactate plays a role in promoting tumor invasion⁵⁵. This phenomenon can be driven through different ways: inducing the adjacent normal cells to secrete cathepsin B⁵⁶, or increasing the circulation of lysosome⁵⁷. Cathepsin is reported to degrade ECM⁵⁸. Highly malignant cells had greater secretion of active cathepsin B, and the cathepsin B was redistributed toward the cell surface with the lower the pH of the medium; fibroblasts and macrophages were found to be involved in this process⁵⁶. On the other hand, the role of lysosomes in degradation of ECM proteins is supported by the research of Glunde et al.⁵⁷. Larger lysosomes were found to be increased with extracellular acidification in highly malignant breast cancer cells.

In summary, these studies demonstrate a critical impact of lactate on EOC induced by hypoxia and identify lactate metabolism as target for interventions.

1.4.2. Hypoxia and cancer stem cells

EOC are believed to arise from multiple mutations or other genetic alterations of normal epithelia. The key to tumor progression is that these mutations need to be maintained and passed to their daughter tumor cells. In other words, tumor cells need to be self-renewal and remain a stem cell-like type to contribute to tumor progression. Figure 1-5 illustrates this

processes. This type of tumor cells are generally called cancer stem cells (CSCs). CSCs are similar to normal stem cells with the ability to self-renewal, and share the similar phenotype through activation of stemness markers like Oct4⁵⁹ and Nanog⁶⁰. CSCs can undergo EMT to initiate and maintain tumors⁶¹. Breast cancer stem cells express genes related to EMT to become invasive, metastasis and resistant to chemotherapy and drug treatments⁶².

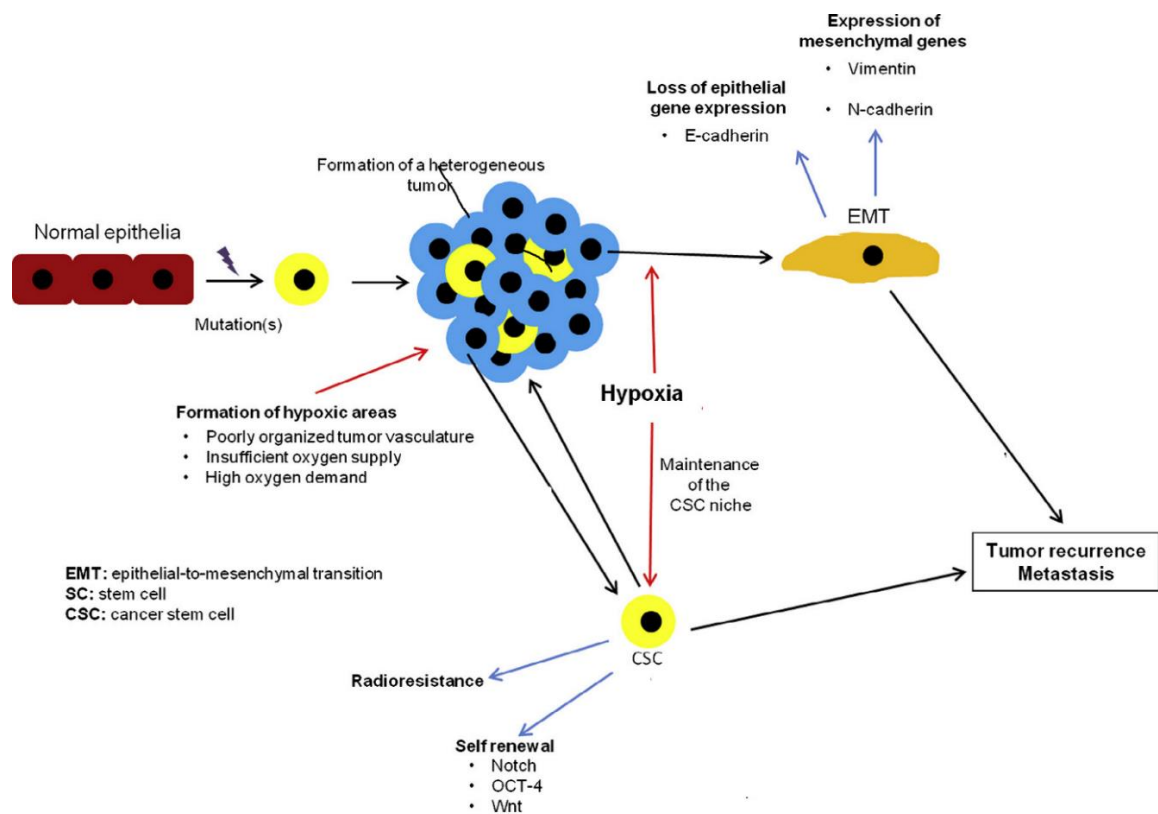


Figure 1-5 The formation of cancer stem cells (Figure reproduced from ref. ⁶³)

Recent evidence has highlighted a link between hypoxia and cancer stem cells⁴⁰. Hypoxia has been shown to inhibit cell proliferation in a wide variety of cancer cell lines^{64,65}. Thus, hypoxic conditions may regulate cell differentiation in order to facilitate the maintenance of CSC characteristics and thus contribute to the evolution of malignant tumor cells. Evidence is now accumulating in support of the hypothesis that hypoxia and HIFs are involved in maintaining stem-like state in cancer. HIFs can regulate stem cell phenotype through signaling

pathway like Notch and Oct4⁶. Culturing glioma cells under hypoxia leading to activation of HIF1 α , the sub-population of the cells positive for cancer stem cell marker is expanded ⁶⁶. Furthermore, Nanog and Oct4, the critical cellular reprogramming transcription factors, transcriptionally regulated by HIF2 α ⁶⁷, are often used as stemness markers, are also found to be part of unique of genetic signature of cancer cells^{68,69}. Together these studies suggest that EOC stem-like characteristics can be a critical part of a hypoxic response by regulating pathways associated with enhanced HIFs and activated EMT.

1.5. Obesity and EOC

An increasing number of studies point to an association between obesity and EOC development and progression. Obesity is characterized as a state of chronic low-level inflammation, which can promote cancer progression by providing a beneficial microenvironment. Adipose tissue is a metabolically active endocrine organ that secretes hormones and inflammatory cytokines. In obese individuals, there is an elevated concentrations of nutrients circulating, such as glucose⁷⁰ and free fatty acids (FFAs)⁷¹. FFAs are pro-inflammatory in many cell types^{7,72} through binding to toll-like receptor to induce a pro-inflammatory response⁷³. The activated inflammatory pathways by FFAs also involves the production of reactive oxygen species (ROS) by further activating interleukin (IL)-1 system⁷⁴. In addition to FFAs, elevated glucose levels may also trigger a systemic inflammatory response⁷⁰ via activating the pro-inflammatory transcription factor Nuclear factor- κ B (NF- κ B) and stress kinases ERK1 and ERK2 and further inducing ROS production⁷⁵. The inflammatory state of obesity is also characterized by the increased infiltration of immune cells into the metabolic tissues. For example, macrophage numbers in adipose tissue is increased with

obesity⁷⁶. Of particular note, Nishimura et al. reported that large numbers of CD8(+) effector T cells infiltrated obese epididymal adipose tissue in mice fed a high-fat diet, which, in turn, promoted the accumulation and activation of macrophages. Chronic inflammation has been associated with an increased risk of numerous cancers including colon⁷⁷, gastric⁷⁸, lung⁷⁹, and potentially ovarian cancer⁸⁰. Therefore, specific subpopulations in the adipose tissue may engage in an extensive and dynamic crosstalk with cancer cells by providing an inflammatory microenvironment.

There is another factor that may contribute to a correlation between obesity and ovarian cancer risk: the heterogeneous of tumors. Tumors are not homogenous populations of mutant cells but contain various supporting cells such as progenitor or stem cells, immune cells, fibroblasts and endothelial cells⁸¹. Analysis of carcinomas shows that neoplastic epithelial cells co-exist with those cells¹⁴. Those stromal cells can be modulated into a tumor-associated phenotype that benefit cancer progression. In addition, adipose tissue is composed of not only adipocytes but also many different cell types⁸². These include stem and progenitor cells, fibroblasts, endothelial cells, macrophages, and lymphocytes^{83,84} and collectively are referred to as Stromal Vascular Fraction (SVF). SVF can be recruited directly from adipose tissues by tumors and impact tumor formation⁸. The importance of SVF involvement in cancer progression has been reported by Zhang et al.⁸ who found that white adipose tissue (WAT)-derived SVF from mice that constitutively express green fluorescent protein (GFP) in all cells, can home to tumors to become tumor-associated stroma and form tumor vasculature, suggesting the endothelial stem cells within the SVF can be induced to differentiate. When transplanting of GFP whole WAT into nude mice, and cancer cells were grafted 2cm away from

the fat pad, tumors grew significantly increased in mice carrying fat pad implants (REF). When SVF cells were consistently administered in low doses over six weeks in mice with tumors, tumor growth rate were accelerated. Thus, SVF can provide a growth advantage to tumors and the different SVF populations may independently contribute to tumor progression. The crosstalk between ovarian cancer cells and SVF play a crucial role in ovarian cancer formation growth and metastasis. By studying the interaction of SVF and EOC cells and involved signaling events, we will have a better understanding of influence of microenvironment on EOC and potential targets for treatment options.

1.6. Summary

EOC is the leading cause of death from gynecological malignancy in Western societies. Efficient methods of early detection by using accurate molecular markers are critical to increase survival rates.

Essentially, cancer is a metabolic disease involving disturbances in energy production through respiration and fermentation. The alteration of metabolism as described by the Warburg effect is still a key characteristic of cancer cells. This alteration in metabolism is necessary for cancer's progression. In most mammalian cells, glycolysis is inhibited by the presence of oxygen, which allows mitochondria to oxidize pyruvate to CO₂ and H₂O. However, cancer cells converse of glucose to lactic acid even in the presence of oxygen. This aerobic glycolysis adaptation is likely caused by the limited blood supply as cancer develops. When pre-malignant lesions grow progressively, the blood supply remains separated from the growing cells by the intact basement membrane, which induces the hypoxia regions. Cancer cells have to response

to this hypoxic condition by upregulation of glycolysis, which results in the increase in glucose uptake and shuttling more glucose to pentose phosphate pathway to fulfill the lipid and nucleic acid synthesis for proliferation.

To support this shift in metabolism, HIFs are often activated under hypoxic environment to confer a significant, identifiable growth advantage. HIFs affect the process of angiogenesis and vascularization by promoting VEGF expression in tumor. HIFs also promotes the glucose uptake levels by activation of glucose transporter GLUT1. All of those in return enhance glycolysis in tumors. Constant upregulation of glycolysis increases the acid production through conversion glucose to lactate, which not only provides cancer cells an extra energy source but also leads to the microenvironmental acidosis to favorite the growth of cancer cells.

Although metabolic changes in many cancers are well documented, ovarian cancer metabolism is still not well explored. The interaction of EOC cells with SVF or other cells within the microenvironment can support the growth of tumors. In addition, the unique metastasis mechanism results in the formation of spheroids, which further promotes anaerobic glycolysis, invasion. Consequently, a deeper understanding of the mechanism underlying the EOC progression, invasion and metastasis will allow us to develop novel strategies to prevent aggressive ovarian cancer focusing on the effect of WAT-derived stem and progenitor populations on ovarian cancer under hypoxic condition with 3D culturing.

1.7. Specific Aims

Cancer cells switch from mitochondrial oxidation to glycolysis even in presence of oxygen and this is well known as The Warburg Effect. This ability helps cancer cells meet unrestricted

growth demands through the uptake of high levels of glucose to synthesis lipids, nucleotides and proteins, conversion of pyruvate to lactate to produce alternative energy and immune invasion effects, and induction of a hypoxic state to promote a malignant phenotype. Furthermore, the hypoxic microenvironment plays a major role in controlling the tumor stem cell phenotype and in return, enhances this metabolic shift. Since the EOC cells detach from the primary sites and become independent in the peritoneal cavity either as single cells or multicellular aggregate/spheroids during dissemination, using 3D culture to mimic tumor microenvironment is considered important in the study of ovarian cancer because it allows the cancer cells to interact with the microenvironment in all directions. As adipose tissue serves as a reservoir for various stem and progenitor populations, it is an important source of cells to be recruited for tumor support. SVF of WAT can be exploited by tumor cells incorporated into the stroma or vasculature of tumors where they proliferate or differentiate into various cell types and produce growth factors, chemokines, and anti-apoptotic signals to enhance tumorigenesis and metastatic potential⁸⁵⁻⁸⁸.

This study aims to determine how culture conditions and cellular heterogeneity affect cancer cells aggressive potential.

The specific aims of this thesis project are:

SPECIFIC AIM 1: To determine the influence of hypoxia on factors promoting aggressiveness and metastasis potential of ovarian cancer.

Hypothesis: Hypoxia and multicellular aggregation will affect cellular metabolism to promote ovarian cancer aggressive potential.

Objective 1: Determine metabolic changes that take place during MOSE progression under hypoxia and spheroid formation by measuring lactate secretion and glucose uptake.

Objective 2: Determine the impact of spheroid formation and hypoxia on MOSE cell response to the chemo-therapeutic agents cisplatin and paclitaxel.

Objective 3: Determine protein markers and signaling pathways that may increase the aggressive potential of ovarian cancer spheroids.

Rationale: Cancer cells prefer aerobic glycolysis to use glucose at a high level and convert it to lactate than to oxidative phosphorylation. Lactate secretion can be used as an aggressiveness indicator since cancer cell growth by providing alternative energy resource and promote apoptosis of normal tissue as well tumor migration. Also, the spheroid EOC model can truly reflect the peritoneal microenvironment as it mimics how EOC cells circulating in peritoneal cavity interact with its microenvironments.

SPECIFIC AIM 2: Determine changes in cellular metabolism in response to heterogeneous spheroid formation. To identify protein markers and signaling pathways that may increase the aggressive potential of ovarian cancer spheroids.

Hypothesis: Heterogeneous of ovarian cancer spheroids will contribute to the aggressive potential of MOSE cells.

Objective 1: Determine the impact of SVF from obese mice on glucose uptake and lactate secretion of heterogeneous spheroids. Compare the impact on tumorigenic (MOSE-L) and

highly aggressive (MOSE-L FFL) spheroids under normoxic and hypoxic conditions.

Objective 2: Determine the impact of heterogeneous composition of ovarian cancer spheroids on the expression of proteins that are associated with a more aggressive phenotype.

Rationale: Adipose-derived SVF contains many cell types that may contribute to cancer progression by serving as a reservoir of recruitable cells that can be incorporated into tumors.

Aim 1B will investigate the *in vitro* interactions of SVF and MOSE-L cells to determine if their association initiates changes in lactate secretion levels, which further enhances cancer invasion.

The heterogeneous composition of ovarian cancer spheroids may promote glycolytic shift glucose uptake by activation of glucose transporter GLUT1 and induces lactate export and uptake to fuel cancer cells through lactate transporter MCT1&MCT4. Hypoxia and SVF cells can regulate stemness signaling pathways like Notch and Oct4 in cancer. The EMT process in EOC interferes with cytoskeleton organization and intercellular cell-cell communication. Suppression of these proteins/pathways may be used to inhibit aggressiveness or metastatic potential of ovarian cancer.

Chapter 2 Methodology

This chapter introduces the methods and materials used in this work.

Cell Culture: Mouse ovarian surface epithelial (MOSE) cells at different stages of tumorigenic potential (early, late and MOSE cells transfected with firefly luciferase (FFL) harvested from mice post injection with MOSE-L) will be used as a model for progressive ovarian cancer. The MOSE cell model was established by harvesting surface epithelial cells from the ovaries of C57BL/6 mice⁸⁹. This model is an ovarian cancer progression model which captures both the cellular and molecular changes in the progression of ovarian cancer⁹⁰. As the cells are passaged in cell culture, MOSE cells show a reduction in cell size and an increase in growth rate, gain the ability to grow in multiple layers and form spheroids, and acquire capacity to form tumors *in vivo*. The tumors formed with MOSE-L cells *in vivo* with 1×10^6 cells can cause lethal disease in about 80 days. However, 1×10^4 MOSE-FFL cells causes lethal disease in about 21 days. MOSE-E, MOSE-L, and MOSE-L FFL cells were routinely maintained in high glucose Dulbecco's Modified Eagle Medium (Sigma), supplemented with 5% fetal bovine serum (Atlanta Biologicals), 100mg/ml penicillin and streptomycin. MILE SVEN 1 (MS1), mouse pancreatic endothelial cells, were obtained from ATCC. MS1 cells were routinely maintained in high glucose DMEM, supplemented with 5% FBS and 100mg/ml penicillin and streptomycin. MS1 cells were transfected with mCherry for monitoring cells in mixed cultures.

Oxygen deprivation: To induce hypoxia (1% O₂), cells were cultured in a sealed chamber which connected to a carbon dioxide & oxygen controller (BioSpherix, Ltd). The set point of hypoxia is 1% O₂ because research showed that most of the oxygen percentage in human tumors is between 0.3–4.2% and with the median oxygen levels <2%⁹¹. Although the level of

oxygen in normal tissues averages about 5% oxygen⁹¹, our set point of normoxia is 20% oxygen. We maintained our cells in the incubator with the specific O₂ level in order to not change their exposure to O₂ levels. There was no cell manipulation (medium changing or imaging) when the cells were in the hypoxic chamber during the experiment period.

Isolation of SVF progenitor cells: Female C57BL/6 mice (Harlan Laboratories) were housed five per cage in a controlled environment (12 hour light/dark cycle at 21°C) with free access to water and a high-fat diet (60% Kcal from fat, Teklad Diets) to induce obesity. Mice were sacrificed at 31 weeks of age (45g average body weight) by CO₂ asphyxiation. All animal procedures were approved by the Virginia Tech Institutional Animal Care and Usage Committee.

SVF cells were isolated from the peritoneal white adipose tissue according to a standard protocol⁹². Briefly, peritoneal WAT was rinsed in Krebs-Ringer bicarbonate buffer (118 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 2 mM KH₂PO₄, 2 mM MgSO₄, 25 mM NaHCO₃, 5 mM glucose, pH 7.4.), minced, and placed into a 50ml centrifuge tube with digest buffer (1:1 ratio of Krebs-Ringer bicarbonate buffer and collagenase solution (1 mg type IV collagenase, 10 mg BSA, in 1 ml PBS)). Tubes were incubated in a water bath at 37°C for 45 min with agitation every 5–10 min, then centrifuged to separate the SVF from adipocytes. The oil layer on top and the primary adipocytes were aspirated, then the remaining cells were washed in warm PBS containing 1% BSA and centrifuged again. The remaining cells were then plated in tissue-culture treated flasks in high glucose DMEM (Sigma) supplemented with 5% FBS (Atlanta Biologicals), 100mg/ml penicillin/streptomycin and 1x ciprofloxacin. After 24 hours, the medium was aspirated to remove dead cells. Attached cells were washed and fresh medium

was added. Once cells proliferated to ~80% confluence, they were trypsinized and frozen back to be used for experiments.

Co-culture: MS1 cells or SVF cells were co-cultured with MOSE cells in tissue culture treated dishes to have monolayer structures or in ultra-low attachment plates to form different spheroids structures.

Lactate Assay: For adherent cell assays, MOSE cells and MS1 cells were seeded at a density of 2.5×10^5 cells/well separately or together in 1:1 and 2:1 ratios in 6-well cell culture plates. After 3 h, the cells were washed with PBS and fresh phenol red-free, serum-free DMEM (Gibco) was added. For cell spheroids, cells were seeded at a density of 2.5×10^5 cells/well with or without other cells (MS1 or SVF cells) in 6-well ultralow attachment plates and incubated for 24 h to form spheroids. Sterile transfer pipets were used to move cell solution to eppendorf tubes and centrifuged 2min at 900 RPM. The supernatant was removed and the cell pellets were washed in prewarmed PBS and centrifuged again. Phenol red-free, serum-free DMEM (Gibco) was added and the spheroids were transferred back to the ultralow attachment plates. The medium was collected after 8 h of incubation then assayed for lactate concentration using a colorimetric kit according to the manufacturer's instructions (BRSC, University of Buffalo). Data presented are the mean from three independent experiments performed in replicates of six biological replicates, normalized to protein content. Data are presented as mean \pm SEM.

Glucose Uptake Assay: For adherent cells, MOSE cells were seeded at 1.25×10^5 cells/well, SVF cells were seeded at 2.5×10^5 cells/well in 6-well cell culture plates. After 24 h, cells were washed with PBS and incubated in serum-free glucose-free DMEM (Gibco) for 1

h. For cell spheroids, MOSE cells were seeded at a density of 2.5×10^5 cells/well with or without SVF cells in 24-well ultralow attachment plates and incubated for 24 h to form spheroids. 6 wells of spheroids were combined by using sterile transfer pipets and moved to eppendorf tubes. The tubes were centrifuged 2min at 900 RPM, the supernatant was removed and the cell pellets were washed in prewarmed PBS and centrifuged again. Glucose-free, serum-free DMEM (Gibco) was added and cells were incubated for 1 h. Glucose uptake was assessed in adherent MOSE cells or SVF cells in Krebs-Ringer HEPES buffer with the addition of 10 M 2-deoxy-glucose (1 μ Ci/ml 2-deoxy-[3 H] glucose) and 10 M cytochalasin B (negative control). After 15 min of incubation, plates or eppendorf tubes were placed on ice, washed three times with ice-cold PBS, and harvested in 400 μ l of RIPA lysis buffer for cell lysis. Glucose uptake was calculated based on specific activity and total cellular protein concentration was measured by the BCA protein assay method (PIERCE) to normalize the glucose uptake. Data presented are the mean from at least two independent experiments performed on 4 replicates. Data are presented as mean \pm SEM.

Western Blotting: For adherent cells, cells were treated as indicated, harvested by scraping into RIPA lysis buffer⁹⁰, supplemented with Protease and Phosphatase Inhibitor Mini Tablets (Pierce), homogenized by placing sample and beads in eppendorf tubes in a Bullet Blender for 4 min, and centrifuged at 12,000g for 10 min. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL). Proteins (10–20 μ g/lane) were separated on 7.5-10% SDS polyacrylamide gels and transferred to a PVDF membrane (BioRad, Hercules, CA). PVDF membranes were blocked with 5% non-fat milk in washing buffer [50mM Tris-HCl, 150mM NaCl, 0.1% Tween-20 pH 7.4]. Blots were

probed with primary antibodies against BRCA1, matrix metalloproteinase-2 (MMP-2), HIF-1 α , Glut1 MCT4 (Santa Cruz); E-cadherin and Vimentin (BD Bioscience); γ -tubulin and Vinculin (Sigma); Integrin α 5 (Cell Signaling); Focal adhesion kinase (FAK) (Upstate); connexin43 (ZYMED); or Oct 4 (abcam), followed by incubation with the appropriate secondary antibodies. Proteins were visualized and quantitated using the Odyssey Infrared Imaging System (Licor).

Alamar Blue assay: For viability tests with adherent cells, MOSE cells were seeded into 96-well plates 24 h prior to treatment at the cell density of 3000/well/100 μ l. Plates were washed with PBS and media with the indicated substrates were added for 24 h. For assays with cell spheroids, MOSE cells with or without SVF cells were seeded into 96-well ultra-low attachment round bottom plates 24 h prior to treatment at the cell density of 3000/well/100 μ l. Centrifuged plates for 2min at 900 RPM after seeding. For both monolayer and spheroid treatment, stock solutions of the anti-cancer compounds were prepared and diluted to obtain the indicated concentration. One hundred microliter of dilution were added to each well, and plates were incubated for 72 h. At 72 h, 20 μ l of alamar blue was added and plates were incubated at 37 $^{\circ}$ C for 4 h (monolayer experiments) or overnight (spheroid experiments). The levels of reduced alamar blue were measured by a fluorescence plate reader using an excitation wavelength at 560nm and an emission wavelength of 590nm.

Chapter 3 Results

3.1. Effects of hypoxia and spheroid formation on the glycolytic shift of ovarian cancer cells.

To determine metabolic changes that are taking place during MOSE progression under hypoxia and spheroid formation, we analyzed the effect of hypoxia (1% O₂) on glucose uptake and the production of lactate by the malignant MOSE cells. Randomly sized spheroids were made in 6-well and 24-well plates

3.1.1. Hypoxic environment promotes the glycolytic shift of ovarian cancer cells

As mentioned in Chapter 1, the glycolytic shift can be determined by the measurements of glucose uptake and lactate secretion levels. Thus, the glucose uptake and lactate secretion levels were measured under the hypoxia/normoxia environment, respectively, to study its effect on the glycolytic shift.

Glucose uptake:

As shown in **Error! Reference source not found.**(A), hypoxia significantly enhanced glucose uptake in adherent MOSE-L ($P=9.3\times 10^{-7}$) and MOSE-FFL ($P\leq 0.001$). Hypoxia stimulated about 200% increase in glucose uptake over normoxia for both MOSE cells. Our previous study⁹³ has shown that glucose uptake was increased as MOSE cells progressed from pre-malignant (MOSE-E) to aggressive (MOSE-L). The results shown in **Error! Reference source not found.**(A) further confirmed the dependency of progression on aggressive phenotype since the highly aggressive MOSE-FFL cells had an even higher glycolytic nature. It was also observed in **Error! Reference source not found.**(A) that the glucose uptake level o

f highly aggressive MOSE-FFL cells was higher than that of the MOSE tumorigenic (MOSE-L) cells, illustrating that the MOSE progression can enhance the glucose uptake levels. Hypoxia significantly increased the glucose uptake in all cells irrespective of their tumorigenic potential. Glucose uptake was also measured in the benign SVF cells; in contrast to benign MOSE-E with a lower glucose uptake than MOSE-L⁹³, SVF cells had a comparable glucose uptake level than MOSE-L cells. Hypoxia also significantly increase the glucose uptake level for SVF cells ($P \leq 7.2 \times 10^{-6}$).

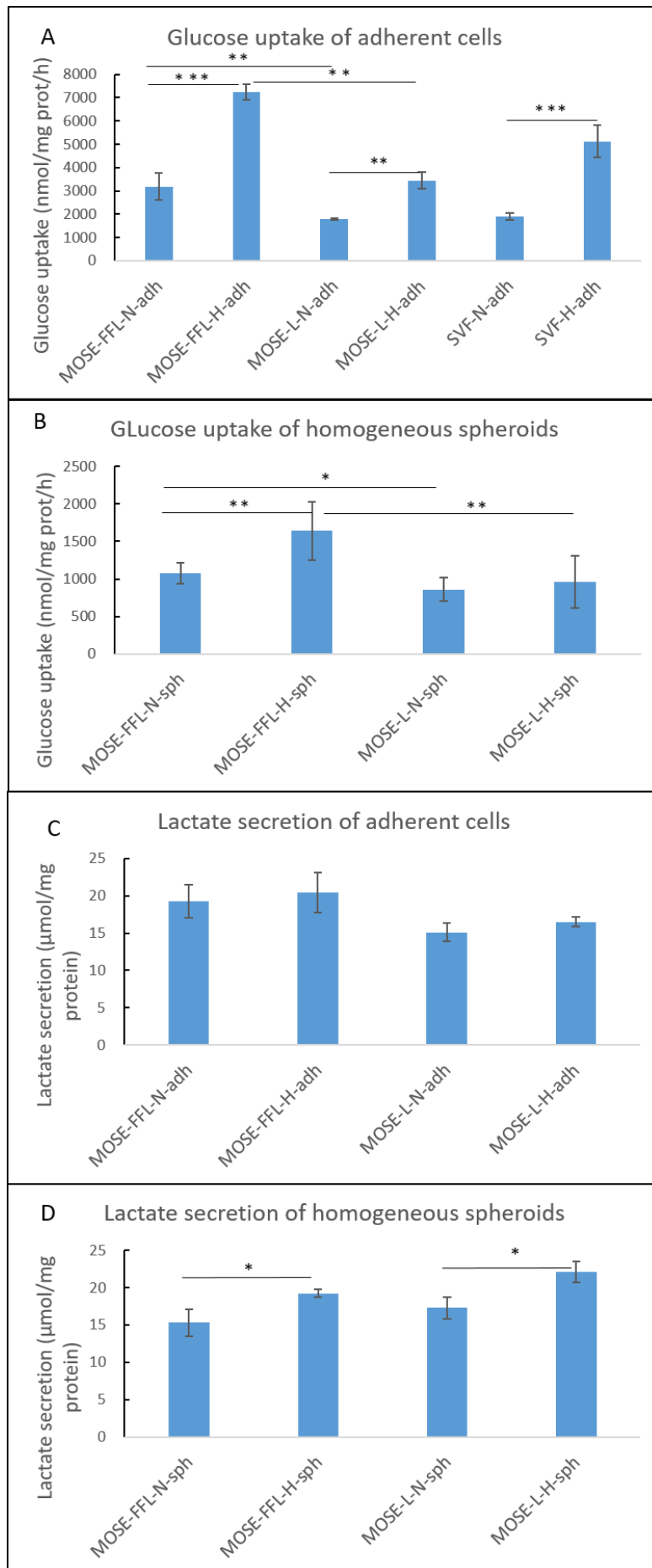


Figure 3-1 **The glycolytic shift results of MOSE cancer cells in 3D culture under hypoxia.** (A) and (B) glucose uptake at 15 min, (C) and (D) lactate secretion at 8 h in late (MOSE-L) stages and highly aggressive (MOSE-FFL) cell as adherent cell (adh) or as homogeneous spheroids (sph) under hypoxia (H) or normoxia (N). Data are presented as mean \pm SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

To test the effect of hypoxia on the spheroid (3D) cells, we generated MOSE cell 3D spheroids by culturing cells on ultra-low attachment plates and measured their glucose uptake levels under hypoxia and normoxia. The results are shown in **Error! Reference source not found.**(B). Glucose uptake of MOSE-FFL cells in spheroids was higher than that of MOSE-L cells in spheroids, illustrating that the MOSE progression can also enhance the glucose uptake levels in spheroids. This enhanced glucose uptake level of MOSE-FFL cells was significant under both hypoxic or normoxic conditions ($p=0.029$ and $p=0.0072$, respectively) compared to MOSE-L cells. The glucose uptake in MOSE-L was not significantly increased under hypoxic conditions but a significant increase was observed in MOSE-FFL cells ($p=0.0067$).

Lactate secretion:

Previous studies have demonstrated that cells under hypoxic conditions produce more lactate than under normoxic conditions^{4,94}. To test if the MOSE cells respond in a similar manner, we measured the lactate secretion for the adherent and spheroid cells under hypoxia and normoxia, respectively. **Error! Reference source not found.**C shows results that although the increased lactate secretion was observed under hypoxia for both adherent MOSE-L and MOSE-FFL cell, the increase did not reach significance compared to normoxia ($P=0.067$ and $p=0.071$ for MOSE-L and MOSE-FFL, respectively). In contrast, a significant increase of lactate secretion under hypoxia was observed for spheroid cell as shown in **Error! Reference source not found.**D, in which the P-value was calculated to be 0.023 and 0.014 for MOSE-FFL

and MOSE-L cells respectively. Spheroid formation of MOSE cells changes cellular glucose metabolism.

3.1.2. Spheroid formation of MOSE cells changes cellular glucose metabolism

As mentioned in Chapter 1, the cancer cells do not grow in a monolayer *in vivo*, thus, experiments with the adherent (2D) cell culturing cannot reflect the real situation during the treatment process. In 3D, the degrees of oxygenation and nutrient availability for cells can vary. Therefore, this section analyzes the effect of spheroid formation on glucose uptake and lactate secretion of cancer cells. The measurements were performed under normoxia and hypoxia.

Glucose uptake:

Figure 3-2A and B show the comparison of the glucose uptake between 2D and 3D culturing MOSE cells (Figure 3-2A shows the comparison on MOSE-FFL cells while Figure 3-2B shows the comparison on MOSE-L cells). Spheroid formation down-regulated the glucose uptake under both hypoxic and normoxic conditions. MOSE-L and MOSE-FFL cells cultured as multicellular aggregates had a significant decrease in glucose uptake compared to monolayers ($p < 0.0001$ for all conditions). Spheroids formation resulted in a ~50% decrease of glucose uptake of MOSE cells under normoxia and a ~75% decrease under hypoxia compared to cells cultured as monolayers.

Lactate secretion:

As shown in Figure 3-2C, for MOSE-L cells, the lactate accumulation increased significantly in 3D compared to 2D under hypoxia ($p=0.0028$), indicating that spheroid formation can enhance the lactate formation for MOSE-L cells. The increase in lactate secretion

of MOSE-L cells under normoxia was no significant between 2D and 3D (Figure 3-2C).

Figure 3-2D shows the comparison of lactate secretion levels of MOSE-FFL cells between 2D and 3D. The result shows a decrease of lactate secretion in spheroids, which is not consistent with the results of MOSE-L cells. One possible reason for this observation is that the passage number of MOSE-FFL cells used for 2D experiments was much higher (about 50 more passages) than the ones used for 3D experiments. In contrast, the passage numbers of MOSE-L cells for 2D and 3D tests were closer (within 5 passages). Thus, we continued to passage 30 times of MOSE-FFL cells and performed the same experiments under normoxia to test the effect of passage number on lactate secretion. As can be seen from Figure 3-2D, the spheroid formation did promote the lactate secretion by using the closer passage number cells. This demonstrates that the cells still are transitional and change their phenotype over time.

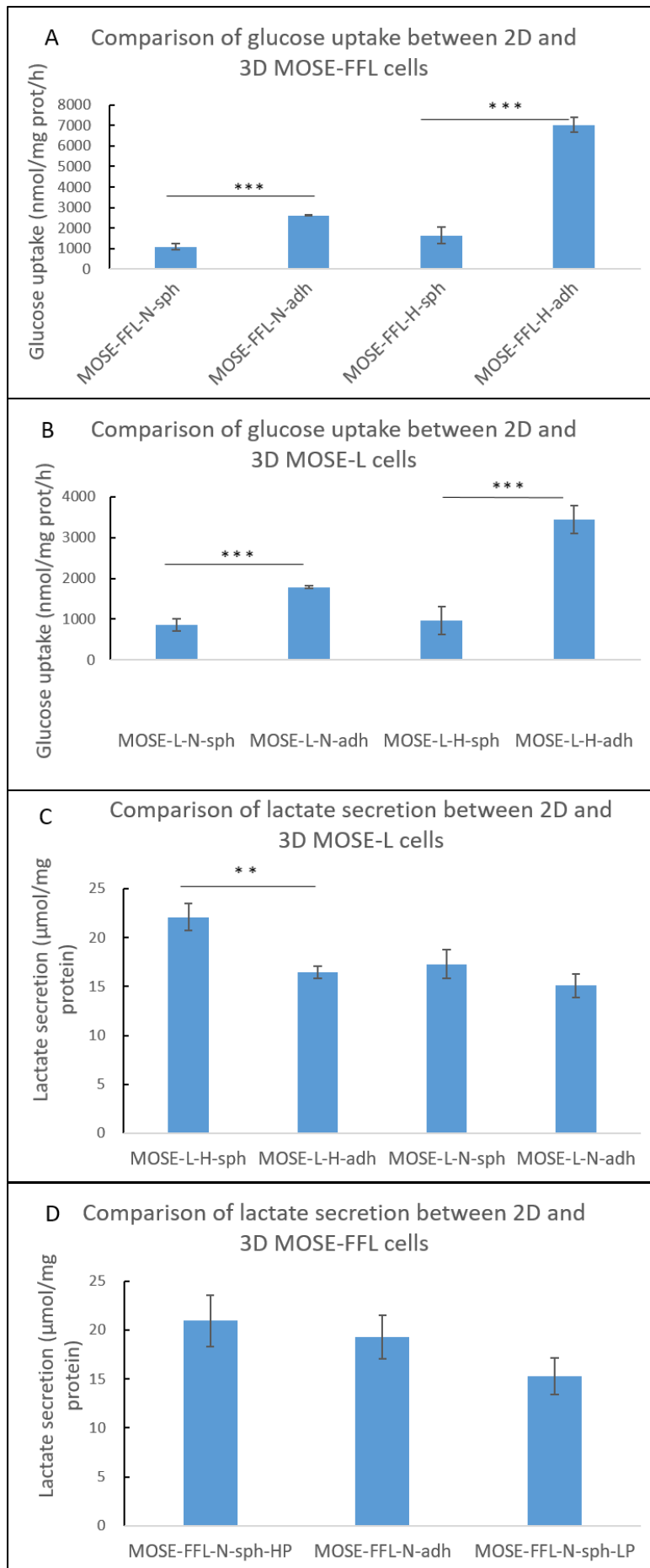


Figure 3-2 **The comparison of glycolytic shift results of MOSE cancer cells in 3D and 2D.** (A) and (B) glucose uptake at 15 min, (C) and (D) lactate secretion at 8 h in late (MOSE-L)stages and highly aggressive (MOSE-FFL) cell as adherent cell (adh) or as homogeneous spheroids (sph) under hypoxia (H) or normoxia (N). Data are presented as mean \pm SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

3.2. The effect of heterogeneous composition on metabolic changes

3.2.1. Heterogeneous spheroid formation affects the metabolism of ovarian cancer

Different cell types can be recruited into tumors and play various roles to facilitate the growth of tumors. Past efforts have demonstrated that endothelial cells play a predominant role in the pathological angiogenesis and cancer metastasis^{95,96}. The interaction of tumor cells with vascular endothelial cells induces the lactate signaling pathway and the promotion of secretion of inflammatory factors and/or VEGF, which leads to the increased migration and angiogenesis in tumors⁵. Furthermore, the stem cells from adipose tissues can be recruited to the tumors and differentiated into a variety of cells⁹⁷ to promote angiogenesis, which supports the structure of tumors or suppress cytotoxic immune responses. Our previous study showed that conditioned medium from MS1 and SVF cells were able to increase the proliferation of MOSE-FFL cells and MS1 cells were also capable of forming vasculature-like networks within the center of the tumor spheroids⁸². This demonstrate that the heterogeneity of tumors may promote the invasiveness of cancer growth; it also could be the reason why obese women have the increased stromal cell number⁹⁸ and the increased risk of ovarian cancer. Thus, the impact of endothelial cells (MS1) and SVF cells on MOSE spheroids was studied.

Lactate secretion:

As shown in Table 3-1, SVF cells survived when they were cultured in 2D monolayers and secrete lactate in amounts lower to MOSE cells. In contrast, most SVF cells were dead after

overnight incubation with a lower level of the lactate secretion when cultured as 3D spheroids. The SVF cells were then co-cultured with MOSE cells to investigate the impact of heterogeneous spheroid formation on lactate secretion level. As shown in Figure 3-3A and B, when co-cultured with MOSE-L or MOSE-FFL cells in 3D spheroids, the SVF cells survived with a higher level of lactate secretion, illustrating that combining SVF and MOSE cells highly enhanced the lactate secretion levels. The level was even higher than the corresponding homogenous spheroids under both hypoxia and normoxia as shown in Figure 3-3A and B, and more pronounced in MOSE-FFL.

Upon recruitment, endothelial stem- or progenitor cells can differentiate into endothelial cells and promote angiogenesis. Therefore, we investigated the impact of different number of MS1 cells in MOSE spheroids. We also included SVF to investigate a potential dependency on cell number. As shown in Figure 3-3C and D, there was no effect of an increased number of MS1 cells incorporated into the spheroids; however, lactate accumulation increased significantly under hypoxia compared to normoxia in 3D cell culture medium of all heterogeneous spheroids, indicating the same glycolytic shift of heterogeneous spheroids in a hypoxic environment. A higher up-regulation of lactate secretion was observed in SVF heterogeneous ovarian cancer spheroids than any combination ratio of MS1 heterogeneous spheroids.

Glucose uptake:

The effect of SVF on the metabolism of ovarian cancer heterogeneous spheroids was further studied by determining glucose uptake. Figure 3-3E and F show that glucose uptake

was highly upregulated in the co-culturing of both MOSE-L and -FFL cells and SVF spheroids together under hypoxia while there was only a small but not significant difference in uptake between homogeneous and heterogeneous MOSE-FFL spheroids under normoxic conditions. The presence of SVF cells did not affect the glucose uptake of MOSE-L spheroids.

Table 3-1 **Lactate secretion of SVF cells.** Lactate secretion at 8 h in SVF cells as 2D adherent cells (adh) and 3D spheroids (sph) under hypoxia (H) and normoxia (N) was measured.

culture condition	lactate (uMole/mg protein)
SVF-Adh-N	12.368
SVF-Sph-N	3.794
SVF-Adh-H	15.011
SVF-Sph-H	7.815

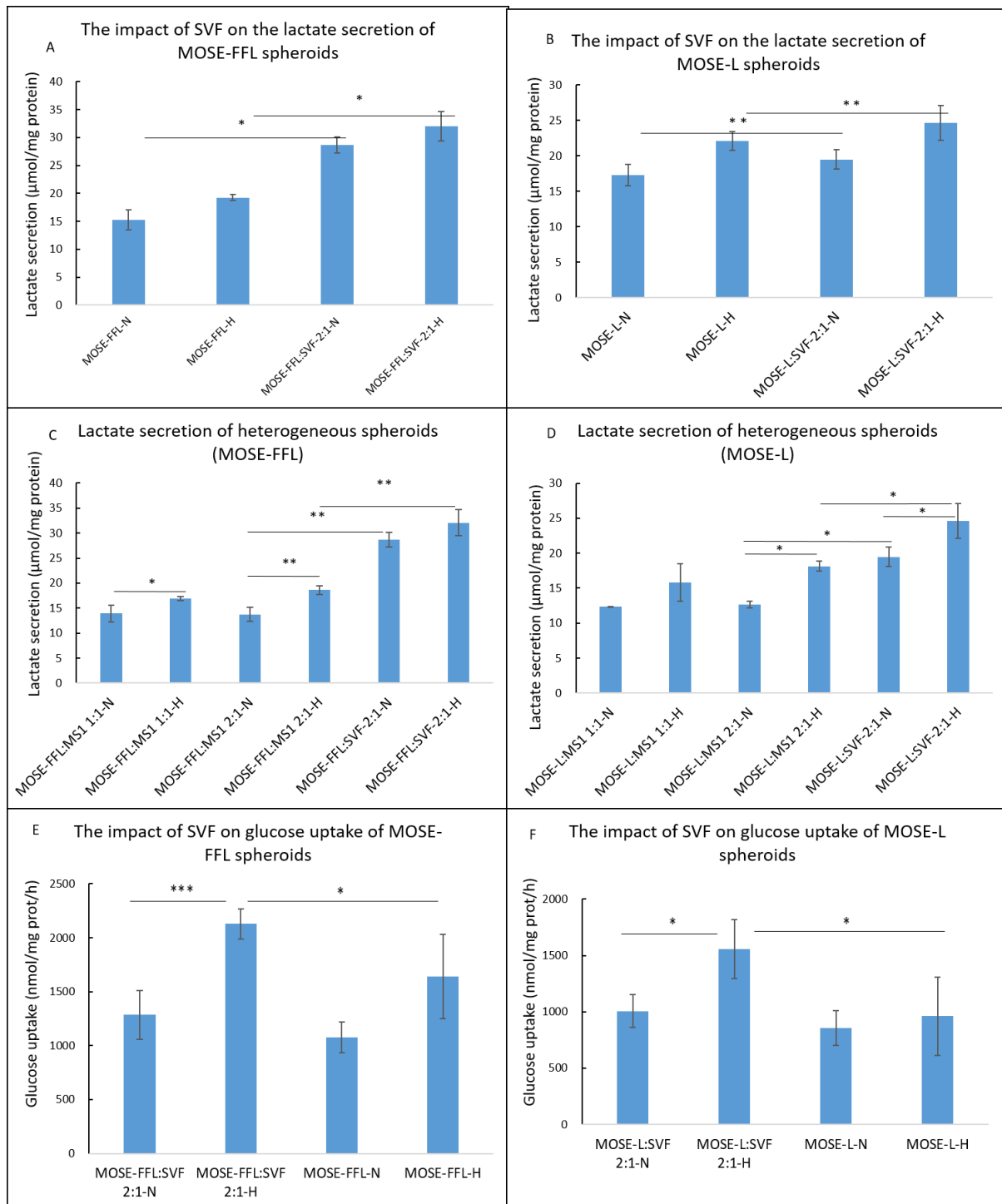


Figure 3-3 The impact of SVF cells and MS1 cells on glycolytic shift of MOSE cancer cells in 3D culture under hypoxia and normoxia. (A) and (B) The comparison of lactate secretion at 8 h in late (MOSE-L)stages and highly aggressive (MOSE-FFL) homogeneous MOSE cancer spheroids and heterogeneous spheroids that co-cultured with SVF as 2:1 ratio, (C) and (D) lactate secretion at 8 h in different cell combination: MOSE cancer cells with MS1 1:1 ratio, with MS1 2:1 ratio, and with SVF 2:1 ratio under hypoxia (H) and normoxia (N), (E) and (F) The comparison of glucose uptake at 15 min in late (MOSE-L)stages and highly aggressive (MOSE-FFL) homogeneous MOSE cancer spheroids and heterogeneous spheroids that co-cultured with SVF as 2:1 ratio. Data are presented as mean \pm SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

3.2.2. Comparison of the experimental and calculated results

There are two potential ways for the mixed cell types affecting the lactate secretion level. First, the effect of the mixed cells may just be an algebraic accumulation of the effect of each cell type when cultured separately. Second, there might be interactions between different cells thus the effect is an interactive effect. To determine if there are interactions between our MOSE cells and MS1/SVF cells, we compared the experimental results illustrated in section 3.2.1 with the calculated results (which are mathematical accumulation of the effect of each cell type). The results of MOSE-FFL heterogeneous spheroids were shown in Figure 3-4 A. As seen, the experimental results of heterogeneous MOSE-FFL spheroids show a significantly higher lactate secretion level than the calculated results for all mixtures under hypoxia and SVF mixture under normoxia, suggesting there are interactions between MOSE-FFL and MS1/SVF that increase the lactate secretion above the calculated value. Figure 3-4B shows the results of MOSE-L heterogeneous spheroids. As seen, the experimental results were significantly higher than the calculated results for SVF mixture under both hypoxia and normoxia. Over all, the averaged difference of experimental and calculated results for MOSE SVF mixed cells was ~50% while the averaged difference for MOSE MS1 mixed cells was ~21%, suggesting that the interaction between MOSE and SVF cells is larger than that between MOSE and MS1 cells.

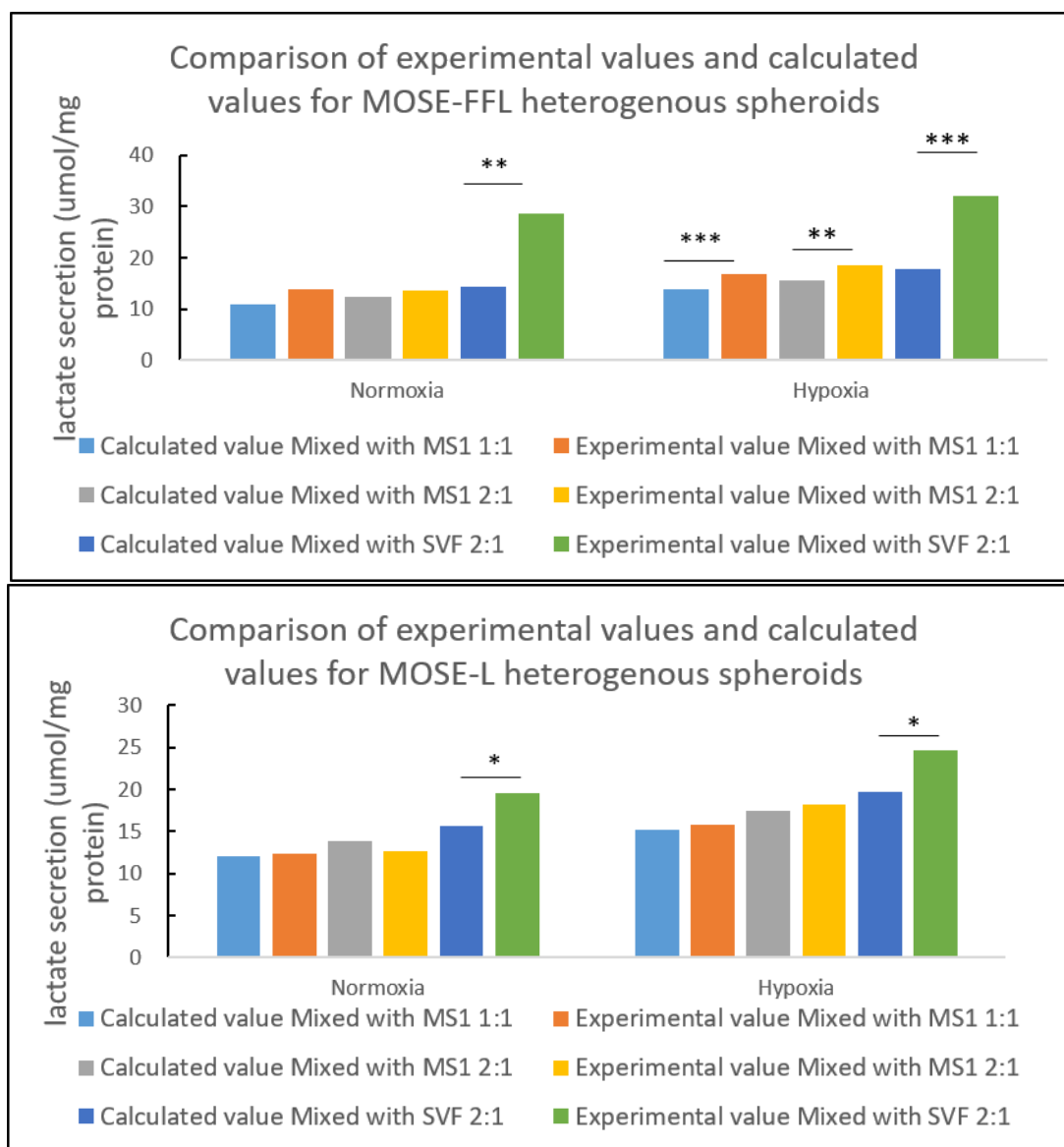


Figure 3-4 **The comparison of experimental and calculated results of heterogeneous spheroids.** Comparison of experimental values and calculated values for MOSE-FFL heterogeneous spheroids(A) and for MOSE-L heterogeneous spheroids(B) under hypoxia and normoxia.

3.3. Effects of SVF and spheroid formation on cell proliferation rate and drug resistance

Both spheroid formation and hypoxia have been reported to reduce cell proliferation^{99,100,101,64,65}. To validate and confirm the above findings on our MOSE cells, alamar blue assay was performed and the fluorescence intensity was measured to investigate the changes of the cell proliferation rate and drug resistance on our MOSE cells under different conditions.

3.3.1 Spheroid formation decreased cell proliferation rate

Figure 3-5 shows the impact of spheroid formation and hypoxia on cell proliferation. Spheroid formation significantly reduced proliferation compared to adherent MOSE cells under both hypoxic and normoxic condition. On the other hand, cell proliferation rates were also reduced in cells incubated under hypoxic compared to normoxic conditions for both 2D adherent cells and 3D spheroids cells. There was no difference in the response of MOSE-FFL compared to MOSE-L, suggesting that the disease stage has no impact on cell growth. All of the comparisons in Figure 3-5 have highly significant difference (with the P-value below to 0.001). Hypoxia decreased the proliferation rate about 3.5-fold compared to normoxia for the 3D spheroids but only decreased about 1.2-fold for the 2D adherent cells, indicating the remarkably effects of hypoxia on 3D cells proliferation.

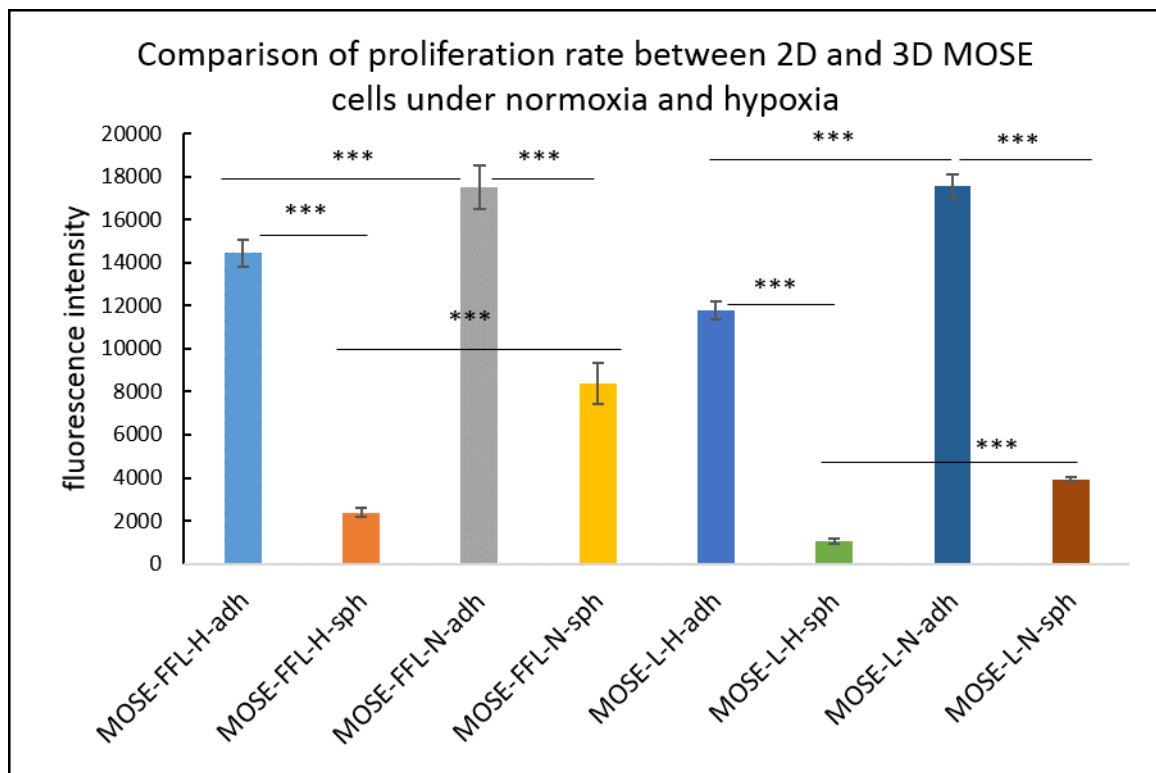


Figure 3-5 The comparison of proliferation rate of MOSE cancer cells in 2D and 3D culture under hypoxia and normoxia. Fluorescence intensity of alamar blue assay was read in late (MOSE-L) stages

and highly aggressive (MOSE-FFL) cell as adherent cell (adh) or as homogeneous spheroids (sph) under hypoxia (H) or normoxia (N). Data are presented as mean \pm SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

3.3.2 Spheroid formation increased drug resistance

Drug resistance is a common problem in EMT, a process in the progression of EOC and formation of multicellular spheres that has been associated with the chemo-resistance in EOC cells¹⁰². Thus, the effective chemotherapies are strongly needed. Multicellular spheroids are thought as a physiologically relevant model to test drug delivery and efficiency¹⁰³. We are interested in the sensitivity to paclitaxel and cisplatin, the two common anti-cancer drugs for ovarian cancer treatment, in 2D and 3D culture systems and under hypoxia and normoxia. Cisplatin is a DNA-damaging agent that causes the DNA strands to crosslink and triggers cells to die in a programmed way. Paclitaxel is an anti-microtubule agent, which inhibits the dynamic microtubule structures within the cell.

In 2D cultures, paclitaxel reduced cell viability of MOSE-L cells >90% in concentrations from 2.5 μ M to 0.25 μ M, whereas MOSE-FFL cells were rather resistant to the treatment, a reduction of viability 60-70% as shown in Figure 3-6A. MOSE-FFL cells representing highly aggressive disease were more resistant to the treatment under both hypoxia and normoxia in 2D. The same drug concentrations were also tested in 3D cultures. As shown in Figure 3-6 B, spheroid formation reduced the toxic response by at least 6-fold compared to 2D MOSE-L cells. Only MOSE-FFL spheroids under normoxia had reduced viability compared to 2D culture and the rest all had viability above 50% as shown in Figure 3-6 B.

Most adherent cells were resistant to cisplatin treatment in low concentrations; treatment with 1 μ M cisplatin only reduced the viability by 20% with no significant difference

between normoxia and hypoxia (Figure 3-6 C). Similar to the paclitaxel treatment, MOSE-FFL grown in monolayers were more resistant to the treatment under both hypoxia and normoxia than MOSE-L cells when treated at the higher concentration of the anti-cancer drug (i.e., 2.5 μ M and 5 μ M). In 3D cultures, MOSE cells showed an enhanced viability and had the viability above 65% in all cases except for MOSE-FFL spheroids under normoxia. Among these, 3D culture benefited MOSE-L most and significantly increased cell viability (as shown in Figure 3-6 D). We observed the significant differences of viability of cisplatin treatments between adherent cells and spheroids for MOSE-L cells under both normoxia and hypoxia and for MOSE-FFL cells under hypoxia.

It is noticed that hypoxia improved the viability of both MOSE-FFL and MOSE-L cells in 2D and 3D for both drugs, and the improvement was more obvious under the high concentrations of cisplatin treatments as shown in Figure 3-6C and D.

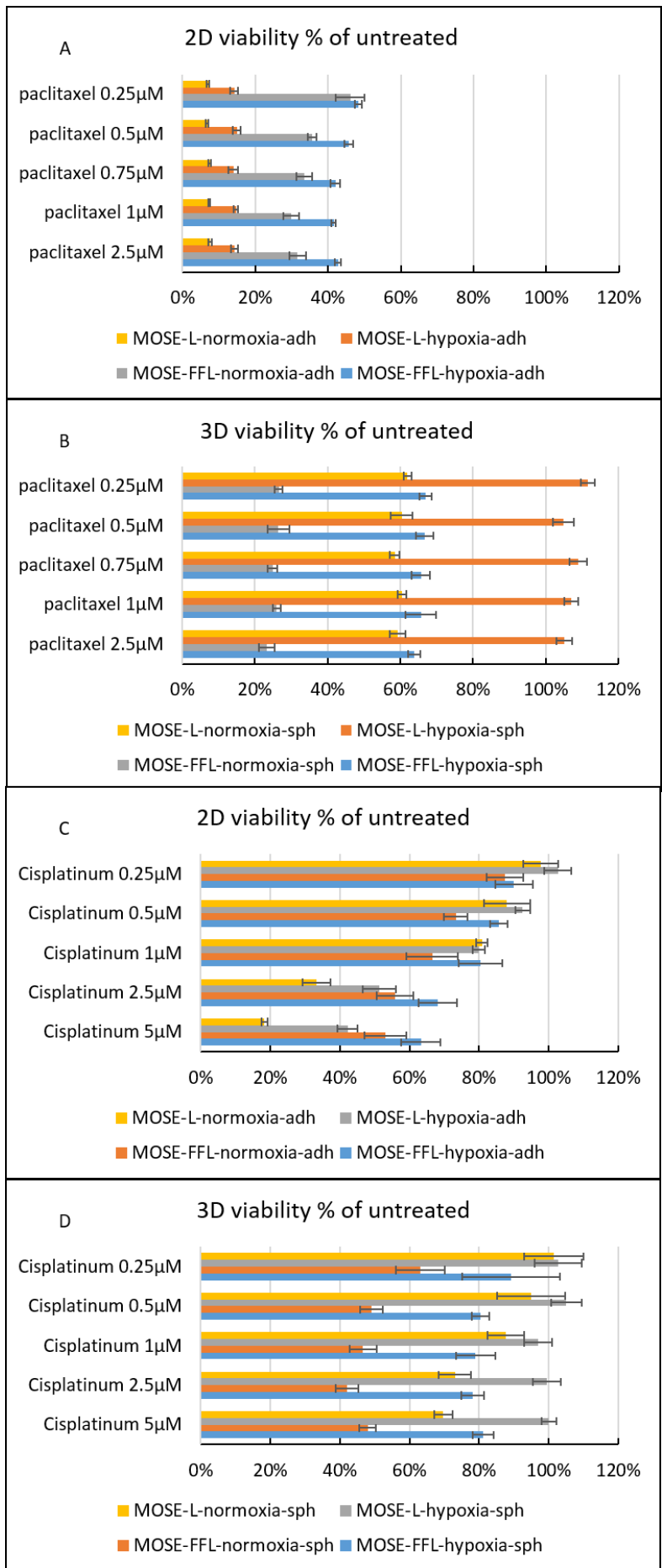


Figure 3-6 **The viability comparison of 2D and 3D of MOSE cancer cells under hypoxia and normoxia in response to anti-cancer drug treatment.** (A) and (B) a series concentration of paclitaxel treatment, (C) and (D) a series concentration of cisplatin treatment. Fluorescence intensity of alamar blue assay was read in late (MOSE-L) stages and highly aggressive (MOSE-FFL) cell as adherent cell (adh) or as homogeneous spheroids (sph) under hypoxia (H) or normoxia (N). Data are presented as mean \pm SEM.

3.3.3 SVF differentially modulate the response of MOSE cells to chemotherapeutic treatments.

After studying the influences of SVF on the MOSE cells metabolism in section 3.2, we next determined the impact of SVF on the chemoresistance of MOSE cells. Here, we co-cultured MOSE cells with SVF cells in 3D culture and treated the spheroids with the same concentrations of cisplatin and paclitaxel as above. Including SVF cells at a ratio of 2:1 into MOSE-FFL spheroids did not alter the sensitivity to either cisplatin or paclitaxel at any concentration tested (Figure 3-7A, C). In contrast, SVF cells increased the resistance to cisplatin significantly at concentrations of 0.25 μ M, 0.5 μ M and 1 μ M in MOSE-L heterogeneous spheroids. . SVF-containing MOSE-L spheroids showed an opposite pattern of the drug resistance in the paclitaxel treatment compared to the cisplatin treatment as these were more sensitive to the paclitaxel anti-cancer treatment than homogeneous spheroids as shown Figure 3-7B, D. It was also observed that MOSE-L spheroid cells were more resistant to paclitaxel than MOSE-FFL spheroid cells at all concentrations used.

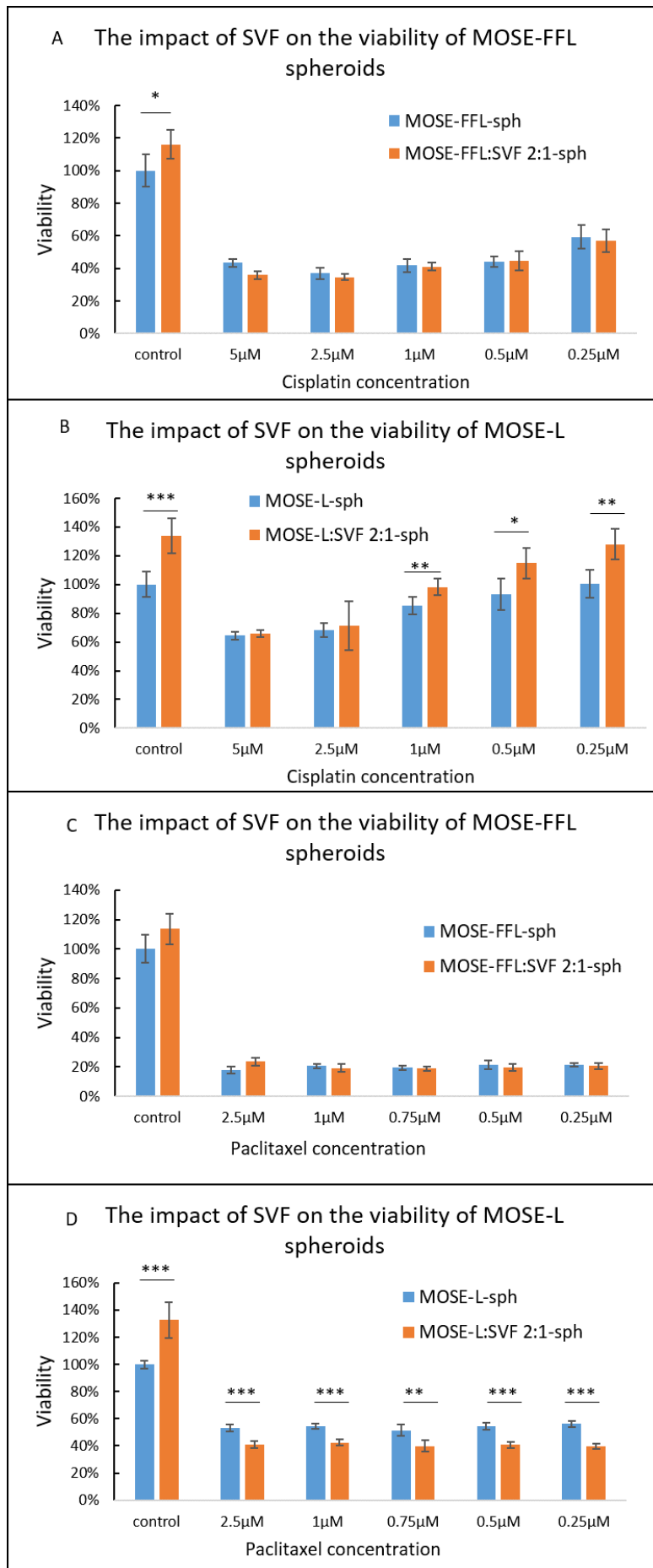


Figure 3-7 **The viability comparison of homogeneous and heterogeneous MOSE cancer cells under normoxia in response to anti-cancer drug treatment.** (A) and (B) a series concentration of cisplatin treatment, (C) and (D) a series concentration of paclitaxel treatment. Fluorescence intensity of alamar blue assay was read in late (MOSE-L)stages and highly aggressive (MOSE-FFL) cell as homogeneous spheroids or heterogeneous that mixed with SVF as 2:1 ratio under normoxia. Data are presented as mean \pm SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

3.4. Protein markers and signaling pathways that may increase the aggressive potential of ovarian cancer spheroids

In order to corroborate the changes in metabolism and aggressiveness of ovarian cancer spheroids under hypoxia, the expression of protein markers in functional categories that may regulate those changes was investigated to reveal the signaling pathways that may increase the aggressive potential of ovarian cancer spheroids.

3.4.1 Cell adhesion molecules that influence the EMT process

To determine the molecular mechanism responsible for the difference in 3D spheroid and 2D adherent cells among our MOSE cells, the expression of the cellular adhesion molecule E-cadherin and Integrin $\alpha 5$ were analyzed by the western blot. E-cadherin plays an important role in cell-cell adhesion, and integrins are a set of cell surface proteins that mediate adhesion of cells onto ECM. Both of them are interrupted and remodeled during the EMT process and the peritoneal anchoring¹⁰⁴ of ovarian cancer cells. As shown in Figure 3-8, as MOSE cells progressed from the late stage cells to highly aggressive tumorigenic cells (MOSE-FFL), the expression of E-cadherin increased in both 2D or 3D cultured cells with adherent cells expressing more E-cadherin than 3D spheroid cells. In contrast, the expression of Integrin $\alpha 5$ was higher in 3D heterogeneous spheroids than in 2D adherent cells.

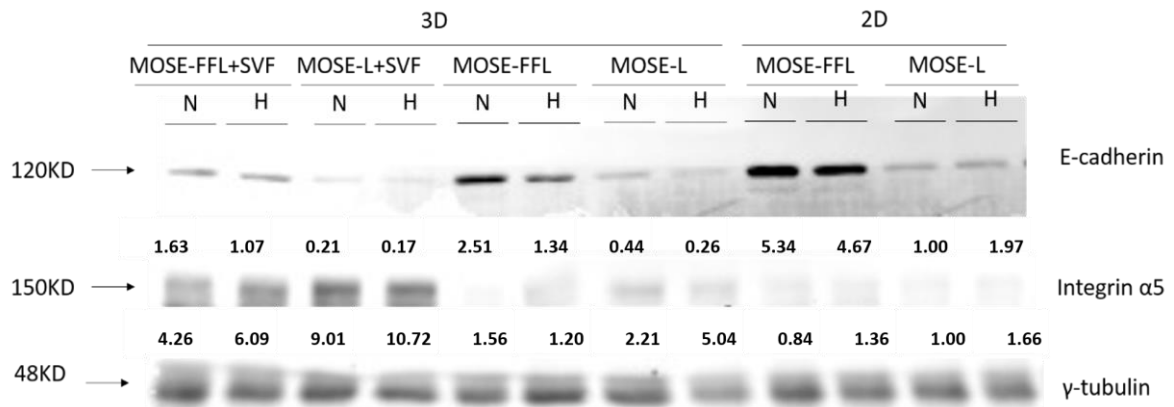


Figure 3-8 **E-cadherin and integrin $\alpha 5$ protein contents under different culture conditions.** MOSE-FFL and MOSE-L cells cultured with SVF as 2:1 ratio in 3D (first 4 bands), MOSE-FFL and MOSE-L cells cultured in 3D (middle 4 bands), and MOSE-FFL and MOSE-L cells cultured in 2D (last 4 bands) under hypoxia (H) or normoxia (N). Data are presented as fold-differences from 2D MOSE-L cells under normoxia.

3.4.2 Lactate & glucose transporters

The increasingly glycolytic nature of MOSE cells during progression has been reported previously⁹³ demonstrating significant increases in glucose uptake and lactate secretion in the late stage of our ovarian cancer cells. Thus, there is a need to explore the changes of the transporter proteins (GLUT and MCT) that facilitate glucose and lactate release and uptake. MCT4 is mainly expressed in glycolytic cells¹⁰⁵, and one hypothesis is that the expression of MCT4 may increase in hypoxia to enable the export of the increased amount of extracellular lactic acid. Also, GLUT1 is responsible for basal glucose uptake which is required to maintain the respiration in cells. The high level expression of GLUT1 in tumors have been demonstrated to relate to the poor survival of cells¹⁰⁶. Thus, the effect of these two transporters on our MOSE cells were analyzed here and the results are shown in Figure 3-9. There was a higher expression of GLUT1 in MOSE-FFL cells compared to MOSE-L cells in both 2D and 3D that was elevated under hypoxia. In contrast, spheroid formation did not affect the GLUT1 expression. Co-culturing MOSE cells with SVF decreased GLUT1 protein expression. In contrast, the MCT4

protein expression was observed to change under different culture conditions. A hypoxic environment strongly up-regulated the MCT4 expression in 2D adherent cells, but had no effect on spheroid cells. In addition, there was a decrease of MCT4 protein expression in 3D cells compared to 2D cells. Co-culturing SVF with heterogeneous spheroids resulted in a slightly increase of MCT4 expression compared to co-culturing SVF with homogeneous spheroids.

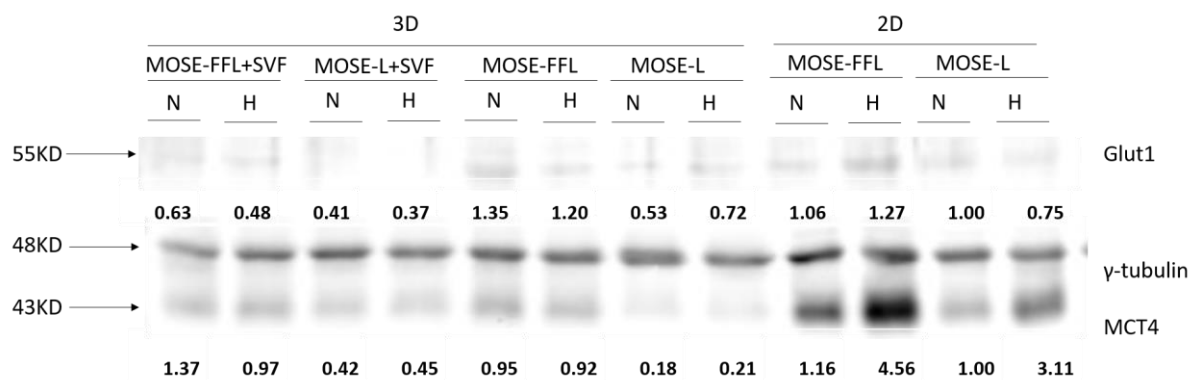


Figure 3-9 **GLUT1 and MCT4 protein contents under different culture conditions.** MOSE-FFL and MOSE-L cells cultured with SVF as 2:1 ratio in 3D (first 4 bands), MOSE-FFL and MOSE-L cells cultured in 3D (middle 4 bands), and MOSE-FFL and MOSE-L cells cultured in 2D (last 4 bands) under hypoxia (H) or normoxia (N). Data are presented as fold-differences from 2D MOSE-L cells under normoxia.

3.4.3 Invasiveness markers that changed under different culture conditions

Osteopontin is a non-collagenous matricellular protein¹⁰⁷ which can facilitate cell–matrix interactions and promote tumor progression¹⁰⁸. Osteopontin is overexpressed in ovarian cancer, which is recently thought to represent a key prognostic marker of ovarian cancer progression¹⁰⁹. Matrix-metalloproteinase (MMPs) can cleave osteopontin and disrupt integrin binding, which ultimately alters cellular responses¹¹⁰. MMP-2 has the ability to degrade extracellular matrix macromolecules, but also plays a critical role in cell migration and invasion¹¹¹. Clinical studies have shown that the activation of MMP-2 was mostly found in breast cancer cells at advanced stages¹¹². Thus, evaluation of the MMP-2 expression and activity may provide valuable

information in the detection and treatment of cancers.

We used western blotting to study the effect of the hypoxia and spheroid formation on osteopontin and MMP-2 protein expressions. Osteopontin was detected at ~66 (full-length) and 33 (MMP-3-cleaved) kDa; spheroid formation increased the levels of both the full-length and the cleaved osteopontin when compared to 2D cells as shown in Figure 3-10. In SVF co-cultured spheroids, osteopontin expression levels were lower compared with homogeneous spheroids. Hypoxia increased the expression of the cleaved form in all conditions. To investigate whether hypoxia and spheroid formation affect MMP-2 expression, the pro-MMP-2 and active MMP-2 were measured. As shown in Figure 3-11, there was an increase in active MMP-2 protein level in 3D cells compared to 2D cells. SVF co-cultured spheroids had a further increased expression in active form of MMP-2. Among homogeneous MOSE spheroids, the active MMP-2 expression was higher under hypoxic environment than under normoxia.

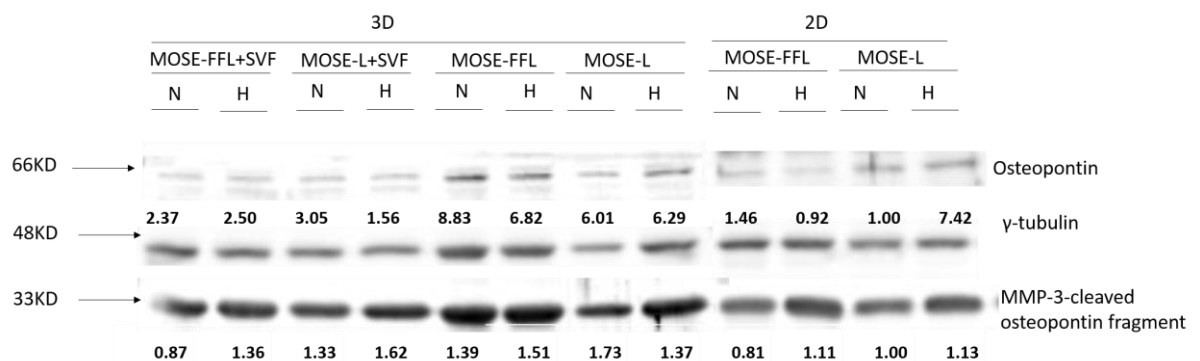


Figure 3-10 **Full length Osteopontin and cleaved osteopontin fragment protein contents under different culture conditions.** MOSE-FFL and MOSE-L cells cultured with SVF as 2:1 ratio in 3D (first 4 bands), MOSE-FFL and MOSE-L cells cultured in 3D (middle 4 bands), and MOSE-FFL and MOSE-L cells cultured in 2D (last 4 bands) under hypoxia (H) or normoxia (N). Data are presented as fold-differences from 2D MOSE-L cells under normoxia.

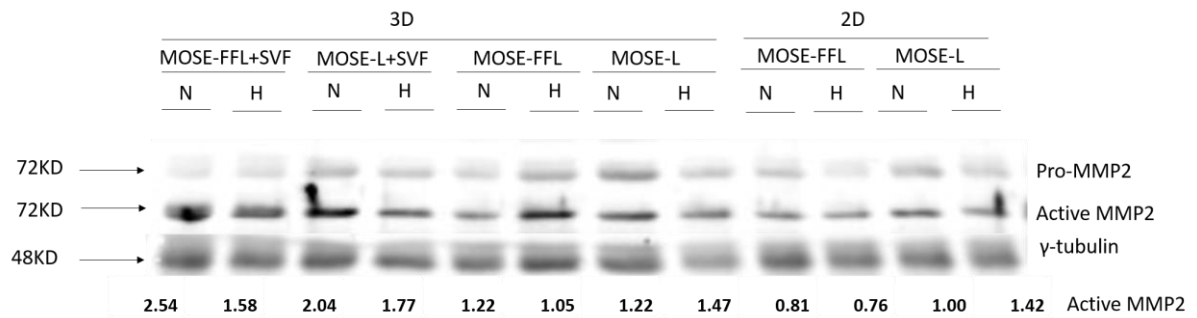


Figure 3-11 **Pro-MMP2 and active MMP2 protein contents under different culture conditions.** MOSE-FFL and MOSE-L cells cultured with SVF as 2:1 ratio in 3D (first 4 bands), MOSE-FFL and MOSE-L cells cultured in 3D (middle 4 bands), and MOSE-FFL and MOSE-L cells cultured in 2D (last 4 bands) under hypoxia (H) or normoxia (N). Data are presented as fold-differences from 2D MOSE-L cells under normoxia.

3.4.4 Changes in cytoskeleton organization under different culture conditions

To determine whether there are changes in cytoskeletal organization under different MOSE culture conditions, we investigated the expression levels of two regulatory proteins: vinculin and focal adhesion kinase (FAK). These proteins were chosen because of their involvement in cytoskeleton regulation, cell motility, and cancer progression/ metastasis. FAK stabilizes the cytoskeleton¹¹³. A previous study showed that constitutively activating FAK on the plasma membrane protected MDCK cells from apoptosis caused by the loss of anchorage (anoikis)¹¹⁴. In addition, it has been shown that vinculin worked as an inhibitor in cell motility and the amount of vinculin accounts for the migration speed of cells¹¹⁵. Our past work has also reported that an increase of cell proliferation during MOSE progression correlates with the increase in FAK expression, the decrease in vinculin expression, and the changes in the cytoskeleton architecture⁹⁰.

Here we observed an increased expression in FAK as MOSE cells progressed from tumorigenic (MOSE-L) to highly malignant (MOSE-FFL). Also, there were differences for the expression of FAK between hypoxia and normoxia in MOSE-FFL cells under both 2D and 3D

cell culture as shown in Figure 3-12. A hypoxic environment increased the expression of FAK in MOSE-FFL cells, while its effect on MOSE-L spheroids was less apparent. Furthermore, SVF co-culture increased FAK protein expression in both MOSE spheroids compared to homogeneous spheroids. Although FAK can increase protein levels during malignant progression, vinculin protein expression is often reduced. Spheroid formation stimulated its expression by at least 1.4-fold, as compared with adherent cells. SVF co-culture can also increase the expression of vinculin.

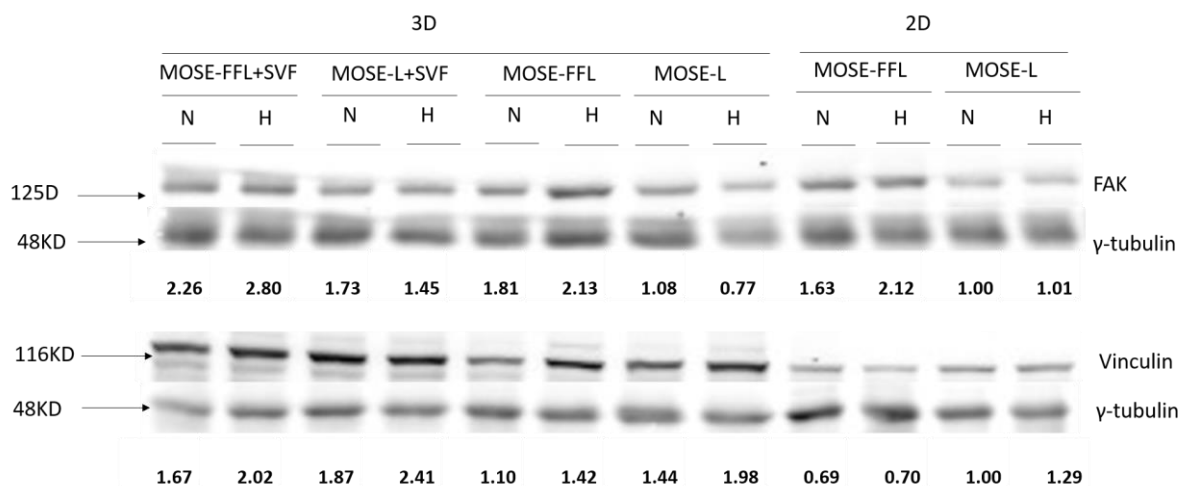


Figure 3-12 **FAK and vinculin protein contents under different culture conditions.** MOSE-FFL and MOSE-L cells cultured with SVF as 2:1 ratio in 3D (first 4 bands), MOSE-FFL and MOSE-L cells cultured in 3D (middle 4 bands), and MOSE-FFL and MOSE-L cells cultured in 2D (last 4 bands) under hypoxia (H) or normoxia (N). Data are presented as fold-differences from 2D MOSE-L cells under normoxia.

3.4.5 Changes in stemness marker under different culture conditions

OCT4, a key transcriptional regulator, enables the self-renewal of cells and involves in the maintaining of “stemness”⁵⁹. The particular characteristics of the stem cells can cause the highly tumorigenic phenotype and the resistance to chemotherapy. The gradual increase of its expression was found to be associated with the malignancy of cervical carcinoma¹¹⁶. Besides, overexpression of OCT4 has shown to inhibit the cells apoptosis¹¹⁶. Here, we evaluated the

OCT4 expression in our MOSE cells in 2D and 3D under hypoxia and normoxia. As shown in Figure 3-13, 2D adherent MOSE-FFL cells expressed extremely low levels of OCT4 and OCT4 could not be detected in MOSE-L cells. Spheroid formation resulted in nearly a 2-fold increase of OCT4 protein expression as compared with adherent cells. SVF co-culture did not show any further increase in the OCT4 expression as compared with homogeneous spheroids.

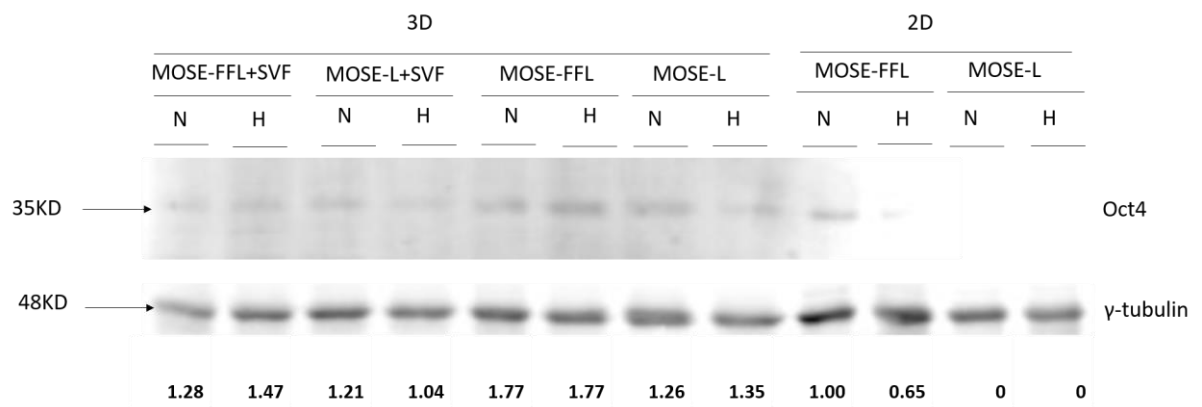


Figure 3-13 **Oct4 protein content under different culture conditions.** MOSE-FFL and MOSE-L cells cultured with SVF as 2:1 ratio in 3D (first 4 bands), MOSE-FFL and MOSE-L cells cultured in 3D (middle 4 bands), and MOSE-FFL and MOSE-L cells cultured in 2D (last 4 bands) under hypoxia (H) or normoxia (N). Data are presented as fold-differences from 2D MOSE-L cells under normoxia.

Chapter 4 Discussion

An increasingly glycolytic metabolism in cancers is thought to provide an advantage to cancer cells to promote a malignant phenotype and metastasis. The glycolytic shift is enhanced in response to hypoxia. Hypoxia is believed to be involved in tumor progression and metastasis³⁷ as EOC spheroids are commonly found in abdominal cavity¹⁸ where low oxygen levels in the abdominal cavity are reported¹¹⁷. Hypoxic and necrotic areas found in tumor spheroids have been associated with chemoresistance¹¹⁸. Thus, using 3D culture as our research model is necessary. Our present studies investigated the metabolic shift in our mouse model for progressive ovarian cancer, and evaluate the effects of hypoxic environment, spheroid formation as well as SVF on the metabolic shift, proliferation rate, drug resistance and protein markers.

Here we demonstrated the increasingly glycolytic nature of MOSE cells progress from tumorigenic (MOSE-L) to highly aggressive (MOSE-FFL). We observed a significant changes in glucose uptake and lactate secretion under different culture conditions. Hypoxic environment enhanced glycolytic shift by upregulating the glucose uptake and lactate secretion. Spheroid formation reduced the glucose uptake of both MOSE late stage cells and highly aggressive FFL cells, but further elevated the lactate production of MOSE cells. However, the facilitative transporter (GLUT1 and MCT4) proteins that mediate glucose and lactate metabolism did not show the same pattern as we saw in glucose uptake and lactate secretion assay. There was no apparent change of GLUT1 protein expression between hypoxia and normoxia, as well as between 3D and 2D culture, suggesting the involvement of other glucose transporters. Although there was an increase in MCT4 protein expression under hypoxia in 2D

cells, spheroid formation did not affect its protein level. This is in contrast to the study on breast cancer cells showing that MCT4 was necessary for the growth of spheroid cells.

Lactate can support tumor progression⁵. Its accumulation results in acute and chronic acidification which can be toxic to normal cells but relatively harmless to themselves. Tumor acidity from lactate accumulation leads to significant decreases in local extracellular pH, which causes adjacent normal populations to go necrosis or apoptosis^{44,45}. Many different types of cancer show a high incidence of P53 mutations¹¹⁹. The aberrant functioning p53 loses its role in the inhibition of glycolysis¹²⁰, which can induce the expression of GLUTs and further increase glucose uptake¹²¹. The hypoxic environment and spheroid formation increased lactate production in MOSE cells. Lactate induces the mutation of p53, which promotes cells to be resistant to anoikis, and help cells to survive when they lose contact with the matrix as spheroids. Future studies will investigate p53 function to further the understanding the mechanism of lactate induced invasiveness.

Furthermore, we show a decreased cell proliferation rate and increased chemoresistance under hypoxia and in 3D culture. The results validated and extended the findings that tumor spheroids are hypoxic inside, which ceases the proliferation of the spheroids cells, and coinciding with the previous studies that showed an inverse correlation between regions of proliferation and regions of hypoxia^{122,123}, and aligns with the decreased drug-induced senescence of breast cancer cells exposure to hypoxia¹²⁴. Tumor cells have high proliferation rates and, thus, a high demand for lipid, nucleotides and protein synthesis. However, in a hypoxic environment, cell proliferation needs to be slowed in an effort to conserve energy and adapt to reduced supplies of oxygen, micronutrients, and fuel sources¹²⁵. Besides, the

quiescence state within the spheroids and the inhibited cell proliferation of hypoxic tumor cells could compromise the effects of some anticancer drugs, since most drugs preferentially target rapidly proliferating cells¹¹⁸. Diffusion with oxygen into the spheroids may drive those cells to restart proliferating and become sensitive to some of the therapies.

We also demonstrated the increasingly glycolytic nature of heterogeneous spheroids that were co-cultured with endothelial cells or peritoneal WAT-derived SVF cells under hypoxia. This was greater than mathematically predicted, suggesting an interaction between the ovarian cancer cells and other cell types that result in an increase of lactate secretion, confirming that MOSE cells interacted either physically or via paracrine communication with other cell types⁹³.

Of note, the interaction between MOSE and SVF cells is greater than that between MOSE and MS1 cells, showing a big advantage for cancer cells to recruit and use SVF to improve their invasive phenotype. Obesity has been associated with the increase risk and/or mortality of many cancers^{126,127}. Central adiposity is also reported as a key risk factor in ovarian cancer¹²⁸. Stromal cells derived from visceral and obese adipose tissue support infiltration of inflammatory cells and support tumor growth and dissemination of ovarian cancers¹²⁹. SVF alone cannot survive as spheroids and their lactate secretion was very low after overnight incubation. However, SVF co-cultured spheroids excreted high amount of lactate. The level was even higher than the corresponding homogenous spheroids. This high amount of lactate production could cause the acidification of local microenvironment around the tumor edge and degradate ECM^{56,57}. Our results suggest that SVF will induce or support an invasive phenotype on our ovarian cancer model. This hypothesis was confirmed by the elevated proliferation rate and drug resistance of SVF co-cultured spheroids. SVF in the heterogeneous spheroids strongly

elevated growth rates for both MOSE-L and MOSE-FFL cells. SVF also promoted the resistance to cisplatin significantly in MOSE-L heterogeneous spheroids. Unexpectedly, MOSE-L/SVF heterogeneous spheroids were more sensitive to the paclitaxel anti-cancer treatment than homogeneous spheroids. This finding is important for selecting anti-drugs for treating the obese women with ovarian cancer, since using cisplatin could compromise the efficacy of chemotherapies. Although SVF did not show the same effect on MOSE-FFL heterogeneous spheroids on chemoresistance, it is possible that more stem-like MOSE-FFL cells response to the stromal cells differently. The relation between SVF and MOSE cells will be further investigated by using real stem cells that we isolated from our MOSE cells in the future.

The changes of glycolysis and cell growth rates of ovarian cancer spheroids under hypoxic environment were accompanied by the changes in protein markers. In this study, we measured E-cadherin and integrin $\alpha 5$ as these two proteins often have the changed expression during EMT process. E-cadherin plays important roles in cell adhesion, was initially believed to be a tumor suppressor^{130,131}. Recent research has shown that E-cadherin plays a more complicated role than just inhibiting the metastasis of tumor cells. Strong expression of E-cadherins was found in invasive ovarian tumors¹³². The cell-matrix adhesion molecule integrin $\alpha 5$ is remodeled during peritoneal anchoring to enhance spheroids disaggregation on ECM¹³³. Our results were consistent with those as described in above research^{132,133}. For instance, highly aggressive MOSE-FFL adherent cells were found to have an upregulated E-cadherin protein expression as compared with MOSE-L cells. A decreased E-cadherin expression and increased integrin $\alpha 5$ expression were also observed in 3D cells. SVF co-culture further increased the

integrin $\alpha 5$ expression compared to homogeneous spheroids. During the progression, ovarian tumor cells need to lose cell-cell adhesion ability to exfoliate from the primary tumor site, then up-regulate integrins to enhance spheroids to acquire the ability to adhere to and disaggregate on ECM, relevant for invasion of secondary sites. This confirms previous results in the lab showing the quick outgrowth of MOSE-FFL in both collagen and matrigel. It has been shown that E-cadherin expressing breast cancer cells that form compact spheroids have a higher metastatic potential¹³⁴. Our MOSE cells have a more aggressive phenotype in 3D cells but lose E-cadherin expression during progression via epigenetic silencing; the most aggressive MOSE-FFL re-express E-cadherin, consistent with the higher metastatic potential observed in breast cancer spheroids¹³⁴. MOSE spheroids could acquire the invasiveness through other molecules such as $\beta 1$ integrin and fibronectin to mediate the compact spheroid formation as demonstrated in a study using 6 human ovarian cancer cell lines¹³⁵, which need to be further investigated in the future since MOSE-FFL express high levels of fibronectin mRNA. The positive relationship found between spheroid formation and integrin $\alpha 5$ protein expression and invasive behavior, implies that integrin activation plays an important role in cancer cell invasiveness.

MOSE cell invasive potential was also assessed by invasiveness markers, MMP2 and osteopontin as well as stemness marker Oct4. MMPs and osteopontin are critical to tissue penetration by cancer cells, which facilitate cancer cell migration and metastasis. We found that active MMP2, osteopontin and Oct4 protein expression were upregulated in spheroids and under hypoxia. SVF co-culture also had an enhanced effects on active MMP2 protein level. Among the changed expression levels of osteopontin, we observed that the MMP-cleaved osteopontin fragment was highly upregulated in spheroids. Recent studies have demonstrated

that the MMP-cleaved osteopontin has increased activity in promoting both cell adhesion and migration compared to the full-length protein¹¹⁰. It is noteworthy that SVF co-cultured spheroids had a higher level MMP-2 expression than homogeneous spheroids. Research has showed that stromal cells express high levels of MMP-2 at the advancing tumor front¹³⁶, cancer cells can recruit the MMP-2 produced by surrounding stromal cells to increase their ability to degrade ECM and promote the metastasis. Together, our results provide evidence that spheroid formation and hypoxia increased the invasive and metastatic potential of ovarian cancer cells through the activation of corresponding proteins talked above.

The changes in cytoskeleton organization allow cancer cells to transit to a phenotype that easily invade into surrounding environment. The regulatory proteins, FAK and vinculin, that involve in the cytoskeleton organization were observed to change under different culture conditions in MOSE cells. We found that 3D cultured spheroids had the elevated FAK and vinculin protein expressions. SVF co-culture further increased these two protein's expression. Besides, vinculin expression showed a strong increase under hypoxic condition. FAK signaling had been shown to drive the motility and invasion of tumor cells through EMT¹³⁷ by activating Akt-mTOR pathway¹³⁸. Vinculin was reported to promote persistent protrusion, traction force generation to enable cell migration¹³⁹. Since our 3D cells have a greater invasiveness potential than 2D cells based on our discuss above, we predict that spheroids formation would contribute ovarian cancer spheroids invasiveness by disrupting the FAK signaling to promote EMT process and activation Akt pathway, also by promoting cell morph-dynamics. This hypothesis will be addressed in the future studies.

Chapter 5 Conclusions

We demonstrate the increasingly glycolytic nature of MOSE cells as they progress from a tumorigenic (MOSE-L) to a highly aggressive phenotype (MOSE-FFL), and changes in metabolism during ovarian cancer spheroid formation and SVF co-culture as well under different oxygen levels. A hypoxic environment as expected in the peritoneal cavity upregulated the glucose uptake and lactate secretion. Spheroid formation affects the cellular metabolism by increasing the lactate secretion to acidify local environments, modulating the expression of cell adhesion molecules to enhance cell motility and spheroids disaggregation, up-regulating invasiveness markers and stemness makers to promote ovarian cancer aggressive potential. Hypoxia and spheroid formation decrease ovarian cancer cells growth but increase the chemoresistance, which lead to the promotion of aggressiveness and metastasis potential of ovarian cancer. SVF co-cultured spheroids further increase the glycolytic shift of the heterogeneous of ovarian cancer spheroids, induces the aggressive phenotype by elevating the corresponding protein markers. The understanding of interaction between tumor microenvironment and ovarian cancer cells and identification of the protein markers and pathways that control the aggressiveness of spheroids under hypoxic tumor micro-environment in this study can help to find more accurate screening programs to support the early detection of ovarian cancer. Decreasing the glycolytic shift and suppression of the proteins/pathways may be used to inhibit aggressiveness or metastatic potential of ovarian cancer.

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