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Studies on Adipose Stem Cells and Ovarian Cancer Stem Cells

지방줄기세포 및 난소암줄기세포에 관한 연구

2015년 8월

서울대학교 대학원

농생명공학부 바이오모듈레이션

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ABSTRACT

The problems in ovarian cancer therapy are that ovarian cancer is diagnosed at an advanced stage and resistant to chemotherapy, leading to poor prognosis. Adipose stem cells (ASCs) and cancer stem cells (CSCs) can be driving forces acting on ovarian cancer progression and chemoresistance, respectively. The present study focused on the interaction between ASCs and ovarian cancer, and the role of 2) CSCs in ovarian cancer. First, ASCs regulate obesity, defined as abnormal or excessive fat accumulation, through the proliferation and adipogenic differentiation, and contribute tumor microenvironment supporting cancer cells. Although several epidemiologic studies have suggested evidence for the relationship between obesity and ovarian cancer, experimental evidence is still lack to verify it. Here, we suggested that ASCs affect ovarian cancer progression via paracrine factors. Among adipokines containing in the conditioned medium of ASCs, interleukin-6 (IL-6) strongly expressed and facilitated the migration and proliferation of ovarian cancer cells (SKOV3 and patient-derived cancer cells). IL-6 derived from ASCs activated the JAK2/STAT3 signaling pathway of ovarian cancer cells, leading to enhanced cancer progression. Taken together, ASCs as a part of tumor microenvironment facilitate the migration and proliferation of ovarian cancer cells via secretion of the paracrine factor. This result provides experimental evidence for the

relationship between obesity and ovarian cancer. Second, existence of CSCs with self-renewal and clonal initiating potential confers chemoresistance to ovarian cancer through the multiple way, such as drug efflux, detoxification (drug metabolism), and dormancy. Sphere formation induced enrichment of CSCs with high ALDH activity. CSCs exhibited expression of ABC transporter proteins (MDR1/ABCB1 and ABCG2), and were resistant to cisplatin compared to parental cells (attached-cultured cells). Oxidative stress caused by sphere formation induced PGC-1α expression (a key regulator of mitochondrial biogenesis and metabolism), resulting in the enhanced chemoresistance of sphere forming cells. When oxidative stress was exogenously exerted to parental cells, PGC-1α expression was observed. Overexpression of PGC-1α in parental cells led to the same response with sphere forming cells. Silencing of PGC-1α in sphere forming cells reduced the resistance to cisplatin. Collectively, PGC-1α induced by oxidative stress mediates chemoresistance in ovarian cancer. We first reported a new function of PGC-1α for the intermediate mechanism in chemoresistance. Therefore, targeting the microenvironmental effects of ASCs and chemoresistant properties of CSCs could elicit a better response to therapy and prognosis in ovarian cancer.

Keywords: Ovarian cancer, Adipose stem cells, Cancer stem cells, Tumor microenvironment, Chemoresistance, PGC-1α

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LIST OF ABBREVIATIONS

ABCB1: ATP-binding cassette sub-family B member 1

ABCG2: ATP-binding cassette sub-family G member 2

ALDH: Aldehyde dehydrogenase

ASC: Adipose stem cell

CDDP: Cisplatin

CM-ASC: Conditioned media of ASC

CSC: Cancer stem cell

IL-6: Interleukin 6

JAK2: Janus Kinase 2

MDR1: Multi-drug resistance protein 1

NAC: N-acetyl-cysteine

OXPHOS: Oxidative phosphorylation

PGC-1α: PPARγ coactivator 1 alpha

PPARγ: Peroxisome proliferator-activated receptor gamma

ROS: Reactive oxygen species

S-ASC: Subcutaneous adipose stem cell

STAT3: Signal transducer and activator of transcription 3

V-ASC: Visceral adipose stem cell

GENERAL INTRODUCTION

Ovarian cancer represents the fifth leading cause of death among gynecological malignancies in the Unites States (Vaughan et al., 2011). The incidence rate of ovarian cancer is also increasing among Korean women (Roberts, Dive, & Renehan, 2010). Although the incidence rate is relatively lower than other women cancers such as cervical and breast cancer, the survival rate of ovarian cancer patients has little changed since platinum-based therapy was introduced (Lumeng & Saltiel, 2011). The representative features of ovarian cancer are poor prognosis and survival rate, because ovarian cancer is diagnosed at an advanced stage.

1. Ovarian cancer and tumor microenvironment

Ovarian cancer cells commonly are disseminated into the peritoneum throughout the pelvic and abdominal cavity, and subsequently acquire chemoresistant properties (Thibault, Castells, Delord, & Couderc, 2014). Emerging evidence reveals the fact that secondary/metastatic tumors, present little genetic mutations compared to the primary tumors (Vaughan et al., 2011). Over the past decade, comprehensive genomic analyses including large-scale gene expression and DNA copy number variation have failed to find new oncogenic drivers in the common histotypes or to identify new drugable target in high-grade serous ovarian cancers (HGSC) (Gorringe et

al., 2010; Gorringe et al., 2007; Tothill et al., 2008). The Cancer Genome Atlas (TCGA) study using high throughput technologies on 489 HGSC also could not provide further information on the chemoresistance and recurrence (Cancer Genome Atlas Research, 2011). Focusing on the inherent genomic instability of ovarian cancer is shifting to targeting the tumor microenvironment, which comprises a large proportion of the cell mass of many ovarian cancers, and cancer stem cells contributing to chemoresistance.

2. Chemoresistance of ovarian cancer

The major cause of poor prognosis and low 5-year survival rate of patients with ovarian cancer is chemoresistance. Standard treatment for the advanced ovarian cancer patients is platinum/taxane-based chemotherapy following debulking surgery, but 50-70% of patients suffer recurrence of ovarian cancer within 18 months (Herzog, 2004). A consequence of conventional therapy can drive cancer stem cells (CSCs) to be enriched in a multiple cancers, including pancreatic (Mueller et al., 2009), colorectal (Dylla et al., 2008), lung (Levina, Marrangoni, DeMarco, Gorelik, & Lokshin, 2008), and breast cancers (X. Li et al., 2008). Ovarian cancers resistant to platinum-based chemoagents also have the enriched population of CSCs, implicating that CSCs may participate in chemoresistance (Abubaker et al., 2013).

Chapter I. Obesity-associated stem cells and cancer

1. Obesity

The prevalence of overweight or obesity is increasing in not only Western countries but Asian countries (W. J. Lee & Wang, 2005; Popkin, Adair, & Ng, 2012). Overweight or obesity is an abnormal or excessive body fat (or adipose tissue) accumulation caused by an imbalance between caloric intake and expenditure (Swinburn et al., 2011). As the report from World Health Organization (WHO), globally around 39% and 13% of adults (over 18-year-old) are overweight and obesity, respectively, in 2014. Criteria for the overweight or obesity are determined by the body mass index (BMI) based on the waist and height, which is recommended by the WHO. The WHO classifications of BMI are shown in Table 1.

Table 1. The WHO classifications based on the BMI
(Calle & Kaaks, 2004)

BMI (Kg/m^2)	Description	WHO Classification
< 18.5	Thin	Underweight
18.5 – 24.9	Normal	Normal range
25.0 – 29.9	Overweight	Overweight (Grade 1)
30.0 – 39.9	Obesity	Overweight (Grade 2)
≥ 40.0	Morbid obesity	Overweight (Grade 3)

2. The relationship between obesity and cancers

Obesity has been recognized as a major risk factor for several diseases, including cardiovascular diseases, metabolic syndrome, and cancers (Lumeng & Saltiel, 2011; Roberts et al., 2010). Recently, epidemiologic

studies have suggested that the obesity is significantly associated with an incidence or death risk of many cancers including liver, colon, endometrial, breast, pancreas and gallbladder cancers (Calle & Kaaks, 2004; Demark-Wahnefried et al., 2012). Mortality for many cancers is significantly increased in overweight or obese individuals in the Asia-Pacific region (Table 2) (Parr et al., 2010). Particularly, obesity plays an important role in the progression and metastasis in hormone-dependent cancers, such as breast, prostate and endometrial cancer (Bianchini, Kaaks, & Vainio, 2002; Gilbert & Slingerland, 2013; Schmandt, Iglesias, Co, & Lu, 2011; Yoo et al., 2001). However, an effect on the risk of epithelial ovarian cancer remains inconclusive. Cohort study reported by Lacey et al. suggested that overweight and obesity based on the BMI were not significantly associated with an incidence of ovarian cancer among postmenopausal women (Lacey et al., 2006). Fairfield et al. also found no evidence of an association between the BMI or weight change and ovarian cancer risk, which was conducted through replying submitted to a questionnaires (Fairfield et al., 2002). Unlikely these studies, the relationship between obesity and ovarian cancer is still suggesting through epidemiologic and experimental studies. Recent clinical evidence has suggested that obesity may be as a poor prognostic factor in ovarian cancer. Mortality of ovarian cancer is increased among obese women, and addition of BMI also is associated with the increased risk of recurrence (Olsen et al., 2007). It has been reported that

high BMI is associated with the increased risk in low-grade serous, endometrioid, and mucinous cancers, but high-grade serous cancers show null association with BMI (Olsen et al., 2013). The effect of obesity is different on the risk of ovarian cancer depending on the histological types. Based on experimental study, adipocytes contribute to ovarian cancer dissemination through providing fatty acids for supporting the rapid growth (Nieman et al., 2011). However, the experimental evidence is lack to verify the relationship between obesity and ovarian cancer. To elucidate the relationship between cancer and obesity/adipocytes, it needs to identify the fundamental cause forming obesity through excessive adipogenesis.

Table 2. Hazard Ratios (HR) for cancer mortality according to BMI in the Asia-Pacific region (Parr et al., 2010)

Cancer	BMI (Kg/m^2)		
	18.5 – 24.9 (Normal)	25.0 – 29.9 (Overweight)	30.0 – 60.0 (Obesity)
All cancers	1.00	1.06	1.11
Oesophagus	1.00	0.79	0.80
Stomach	1.00	1.05	1.04
Liver	1.00	1.06	1.10
Lung	1.00	0.68	0.83
Breast (female)	1.00	1.13	0.93
Ovary	1.00	1.46	2.62
Cervix	1.00	1.29	4.21
Prostate	1.00	1.41	1.45
Bladder	1.00	1.11	0.72
Pancreas	1.00	1.06	0.90
Kidney	1.00	1.42	1.59

3. Adipose stem cells

Adipose tissue consists of heterogeneous cell types including adipocytes,

macrophages, endothelial cells, and adipose stem cells (ASCs). ASCs present in stromal vascular fraction of adipose tissue have a multipotent capability to differentiate into adipogenic, chondrogenic, osteogenic, and myogenic lineages (Gwak et al., 2009; Iwen et al., 2014; Y. H. Lee, Mottillo, & Granneman, 2014; Noel et al., 2008; Zuk et al., 2002). ASCs contribute to excessive adiposity (overweight and obesity) through proliferation and adipogenesis to generate adipocytes, which could be regulated by diet and obesity in reverse (Joe, Yi, Even, Vogl, & Rossi, 2009; Zeve, Tang, & Graff, 2009). In animal experiment, obese mice with high-fat diet induce formation of adipocytes and activate physiological signals stimulating proliferation of ASCs (Rodeheffer, Birsoy, & Friedman, 2008). ASCs exhibit effects of cancer promoting and progression and are involved in formation of tumor microenvironment through differentiating into cancer-associated fibroblasts. Recent studies reveal that omentum ASCs facilitate proliferation, migration, and chemoresistance of ovarian cancer (Nowicka et al., 2013), and exosomes derived from ovarian cancer render ASCs acquire tumor-supporting myofibroblasts (Cho et al., 2011). However, the mechanism related to phenotypical changes of ovarian cancer is still more needed.

4. Adipose stem cells as a tumor microenvironment

Tumor microenvironment serves as an important factor to enhance chemoresistance and to facilitate cancer progression through the tumor-

stroma interaction (Correia & Bissell, 2012; Kansy et al., 2014; Karnoub et al., 2007). Mesenchymal stem cells (MSCs) contribute to generation of progenitor cells to participate in tumor microenvironment (Bhowmick, Neilson, & Moses, 2004; Chantrain, Feron, Marbaix, & DeClerck, 2008; Karnoub et al., 2007). Exposure to conditioned medium from breast, glioma, and pancreatic cancers stimulates differentiation of MSCs into cancer-associated myofibroblasts, facilitating construction of tumor microenvironment (Mishra et al., 2008). Similarly, ASCs migrating from white adipose tissue are locally recruited and infiltrated into tumor tissue, which is involved in the tumor microenvironment (Kidd et al., 2012; Y. Zhang et al., 2012). Circulating ASCs from the stromal vascular fraction of white adipose tissue are frequently observed in peripheral blood mononuclear cells (PBMCs) of the obese donors compared to the non-obese donors (Bellows, Zhang, Simmons, Khalsa, & Kolonin, 2011). ASCs are expanded in obese-mouse cancer model, and recruited into tumors from endogenous adipose tissue (Y. Zhang et al., 2012). ASCs recruited from adipose tissue can participate in tumor microenvironment through the supportive potentials.

Chapter II. Cancer stem cells and chemoresistance

1. Cancer stem cells

Cells within tumor tissue exhibit functional heterogeneity showing

different proliferative and differentiative activity (Heppner & Miller, 1983). Cancer stem cell (CSC) hypothesis is suggested as a model to account for the cellular mechanisms underlying tumor heterogeneity (Bonnet & Dick, 1997; Reya, Morrison, Clarke, & Weissman, 2001). CSCs referred to as tumor initiating cells or cancer initiating cells are a small subset of tumor cells having the self-renewal, clonal tumor initiation ability, and repopulation potential, which maintains tumor mass (Clarke & Fuller, 2006; Plaks, Kong, & Werb, 2015; Visvader & Lindeman, 2008). CSCs are suggested to be particular cells expressing specific surface markers and having functional characteristics of stem cells. CSCs can be enriched by sphere culture (3D or suspension culture) *in vitro* which mimics ascites in an advanced stage of ovarian carcinoma and tumor mass *in vivo* in an aspect of physiological gradients for nutrients, oxygen, catabolites, and pH due to the limitation in diffusion through multicellular layers (Bapat, Mali, Koppikar, & Kurrey, 2005; Chau, Ip, Mak, Lai, & Wong, 2013). In addition, the sphere culture condition is effective to conserve stem-like phenotypes of CSCs in primary ovarian cancer (Hu, McArthur, & Jaffe, 2010; Stewart et al., 2011).

2. Resistant features as markers for cancer stem cells in ovarian cancer

The significant clinical implication of CSCs is that CSCs have been shown to be resistant to chemotherapy and radiotherapy in cancer. The resistant features of CSCs to chemo-agents have been attributed to the

contribution of drug-resistant transporters and intracellular detoxifying enzymes, which efflux and metabolize drug (Alison, Lin, Lim, & Nicholson, 2012; O'Connor et al., 2014).

2-1. Aldehyde dehydrogenase (ALDH)

Aldehyde dehydrogenase (ALDH) is one of the most described markers for ovarian CSCs. ALDH is a family of $NAD(P)^+$-dependent enzymes, and can detoxify various aldehydes in cells. At first, a possibility of ALDH as a CSC marker was found in leukemia stem cells using ALDEFLUOR assay. To date, ALDH activity along with other surface markers, such as CD133, CD44 or/and CD177 (Burgos-Ojeda, Rueda, & Buckanovich, 2012), is used to identify CSCs in many cancers, including melanoma (Boonyaratanakornkit et al., 2010), colon (Carpentino et al., 2009), prostate (T. Li et al., 2010; van den Hoogen et al., 2010), and breast cancers (Marcato et al., 2011). ALDH-positive population of ovarian cancer cell lines and primary ovarian tumors has a potential to initiate tumors when engrafted in mice, whereas ALDH-negative population does not (Kryczek et al., 2012; Silva et al., 2011). In hematopoietic stem cells, ALDH1A1 and ALDH3A1 are involved in metabolizing and detoxifying cyclophosphamide (Sladek, Kollander, Sreerama, & Kiang, 2002), implicating increased ALDH activity is possible to enhance chemoresistance.

2-2. ATP-binding cassette (ABC) transporter

A further CSC marker is ATP-binding cassette (ABC) transporters

including MDR1 (ABCB1 or P-glycoprotein) and ABCG2 (BRCP), serving as drug-efflux pumps (Moitra, Lou, & Dean, 2011), which confer drug resistance in cancer. Blockage of ABC transporter using Verapamil (an inhibitor of MDR1/p-glycoprotein) sensitizes side population of squamous carcinoma cells (CSCs) to mitoxantrone (an anticancer drug) (Loebinger et al., 2008). In epithelial ovarian cancer, ABCA transporter gene expression is significantly correlated with poor outcome of patients (Hedditch et al., 2014), and inhibition of hedgehog signaling induces suppression of ABCG2 and MDR1, enhancing response to chemotherapeutic agents (Y. Chen, Bieber, & Teng, 2014).

In the present study, we attempted to approach the issue for ovarian cancer progression and chemoresistance in terms of ASCs and CSCs, respectively. ASCs creating adipose tissue through adipogenesis are involved in formation of tumor microenvironment, and ASCs themselves play a role as a factor to stimulate cancer development or progression. CSCs contribute to chemoresistance through pumping out and metabolizing drugs. Hereafter, we tried to describe how ASCs isolated from subcutaneous and visceral fat tissue influence ovarian cancer progression using cancer cell line and primary cells (ascites cells). Also, we tried to identify CSCs and to elucidate a key molecule to regulate cellular metabolism and chemoresistance in CSCs enriched by three dimensional culture (tumor

sphere formation) similar to status of *in vivo* tumor mass.

CHAPTER I.

Adipose stem cells from visceral and subcutaneous fat depots enhance growth and migration of ovarian cancer cells via activation of IL-6/JAK2/STAT3 pathway

Abstract

Adipose stem cells (ASCs), which are multipotent stem cells in adipose tissue, play an important regulatory role in cancer progression and metastasis by regulating systemic inflammation and tissue metabolism. Here, we found that conditioned medium (CM) of ASCs in visceral and subcutaneous fat depots (V- and S-ASCs) enhanced the migration of an ovarian cancer cell line, SKOV3, and the growth and migration of ascites cells from ovarian cancer patients. CM from ASCs activated IL-6/JAK2/STAT3 signaling pathway alongside the enhanced migration of SKOV3. Furthermore, the CM of ASCs enhanced the proliferation and migration of ascites cells. Utilization of a neutralizing antibody against secreted IL-6 blocked the activation of JAK2 and STAT3 in ovarian cancer cells cultured in the CM of ASCs, suppressing cell migration. Inhibition of JAK2 and STAT3 with small molecular weight inhibitors, WP1066 and TG101348, respectively, also suppressed the migration of the cancer cells. Anatomical differences in ASCs derived from subcutaneous or visceral fat depots did not affect the growth and invasiveness of the SKOV3 and ascites cells from the ovarian cancer patients. Collectively, these findings suggest that ASCs may regulate the progression of ovarian cancer, and possibly be a target for anticancer therapy.

Introduction

Ovarian cancer is a fatal disease that is commonly resistant to chemotherapeutic treatments, and is prone to subsequent relapse (Jemal et al., 2011). Although ovarian cancer is relatively less common among gynecologic cancers, it is responsible for highest mortality because patients are usually diagnosed at an advanced stage of the disease (Holschneider & Berek, 2000; Lowe et al., 2013). Ovarian cancer mainly disseminates into the peritoneal cavity and abdominal organs including the omentum via the physiological movement of peritoneal fluid (Lengyel, 2010; Naora, 2014). Given that the epidemiological data indicate 80% of patients with serous ovarian carcinoma show abdominal dissemination including the omentum, intra-abdominal fat is thought to act as a major regulator of ovarian cancer metastasis (Naora, 2014; Naora & Montell, 2005; Nieman et al., 2011). Moreover, fat tissue has been reported as a source of cytokines, which stimulate tumor growth and migration, and metabolites to meet the high-energy demands of tumors (Gilbert & Slingerland, 2013; Nieman et al., 2011). Therefore, understanding the molecular mechanisms by which fat depots regulate the phenotype of ovarian cancer cells may provide important clues for developing anticancer therapies for this deadly disease.

Fat tissue consists of various cell types including mature adipocytes, pre-adipocytes, immune cells, endothelial cells, and adipose stem cells

(ASCs) (Trayhurn & Beattie, 2001). ASCs resemble bone marrow mesenchymal stem cells, as indicated by their surface markers and their potential for differentiation into mesenchymal lineages. Accumulating evidence suggests that ASCs as well as other cells such as adipocytes, endothelial cells, and macrophages in fat tissue can be major sources of cytokines and chemokines (Kilroy et al., 2007; Trayhurn, 2005; Wang, Crisostomo, Herring, Meldrum, & Meldrum, 2006). For instance, ASCs have been shown to produce and secrete specific growth factors and pro-inflammatory cytokines such as interleukin-6, hematopoietic colony-stimulating factors (CSFs), and macrophage colony-stimulating factor (M-CSF), which promote cell proliferation, vascularization, and angiogenesis in endometrial, breast, and prostate cancers (Gehmert et al., 2011; Karnoub et al., 2007; Klopp et al., 2012; Prantl et al., 2010). Thus, ASCs may be linked to the progression of cancer by forming a more attractive microenvironment for tumor growth. However, the relationship between ASCs and ovarian cancer and the molecular mechanisms behind any interaction between these cells have not been established. In this study, we show that ASCs are involved in promoting the growth and migration of ovarian cancer cells via the activation of IL-6/JAK2/STAT3 pathway.

Materials and Methods

Isolation of $CD45^-/CD31^-$ ASCs

All protocols were reviewed and approved by the Seoul National University Hospital Institutional Review Board. Human subcutaneous and visceral adipose tissues were surgically obtained from four patients with benign urologic or gynecologic diseases. Adipose tissues were washed three times with cold phosphate-buffered saline (PBS) and dissociated (37℃, 1 h) with gentle agitation every 10 min in collagenase type IA solution (*Clostridium histolyticum*; Sigma-Aldrich, St. Louis, MO). The cells were centrifuged (500 × *g*, 4 min), supernatant was removed, and the stromal vascular fraction (SVF) was collected as a pellet. The SVF was treated with Ammonium-Chloride-Potassium lysing buffer (Lonza, Muenchsteinerstrasse, Switzerland) at room temperature for 2 min, and then centrifuged (500 × *g*, 4 min). After removal of the supernatant, the cell pellet was diluted with a magnetic-activated cell sorting (MACS) buffer (MiltenyiBiotec, Teterow, Germany). To avoid contamination with hematopoietic stem cells and endothelial cells, the cells were incubated (room temperature, 15 min) with anti-CD45 and anti-CD31 antibodies attached to microbeads (BD Biosciences, San Jose, CA). MACS then separated the CD45- and CD31-positive cells from the SVF. The column-passed cells were collected on ice. $CD45^-$ and $CD31^-$ ASCs were cultured for three days without medium change (Figure 1A).

Culture of ASCs and ovarian cancer cells

The human ovarian cancer cell line SKOV3 was purchased from the American Type Culture Collection (ATCC, Rockville, MD). ASCs were cultured in DMEM/F12 (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; Life Technologies), 100 U/ml penicillin, and 100 g/ml streptomycin (Life Technologies), as previously described (Kilroy et al., 2007). We cultured SKOV3 cells in the same culture medium as that of ASCs to minimize the bias from different culture medium. The cells were maintained at 37℃ in a humidified atmosphere of 5% CO_2.

Isolation and culture of ascites cells from ovarian cancer patients

Ascites aspirated from two patients with serous and clear ovarian carcinoma at the advanced stages (IIIC) was diluted with an equal volume of PBS and centrifuged (4℃, 1,400 × *g*, 30 min). For the removal of red blood cells, the cell suspension in PBS was gently overlaid onto Ficoll-Paque^{TM}-PREMIUM, and centrifuged (4℃, 1,400 × *g*, 30 min). The cell layer was washed (three times with PBS), centrifuged (500 × g, 4 min), re-suspended in DMEM/F12 supplemented with 10% FBS, 100 U/ml penicillin, and 100 g/ml streptomycin, and maintained at 37℃ in a humidified atmosphere of 5% CO_2. To avoid contamination of other cells, the isolated cells were sub-cultured for at least five passages before their use in subsequent experiments

(Supplementary Figure 1).

Immunophenotype of ASCs

Cultured ASCs were analyzed for the expression of specific surface markers using flow cytometry. The ASCs were detached using 0.05% trypsin-EDTA (Life Technologies), and centrifuged (500 × *g*, 4 min). At least 1×10^5 cells were incubated (20 min, 4℃ in the dark) with fluorescence-labeled antibodies or matching isotype control. After the cells were washed, propidium iodide was added to identify the dead cells. The labeled cells were isolated by a FACS Canto II flow cytometer, and analyzed using BD FACS Diva™ software (BD Biosciences). The antibodies used in this study were phycoerythrin (PE)-conjugated mouse anti-CD45, -CD90, -CD73, and -CD166, and fluorescein isothiocyanate (FITC)-conjugated mouse anti-HLA-DA, -CD31, -CD34, and -CD105. Isotype controls were PE-conjugated mouse IgG1κ and FITC-conjugated mouse IgG1κ.

In vitro differentiation of ASCs into adipogenic, chondrogenic, and osteogenic lineage

To determine their differentiation potential, ASCs were seeded onto a 12-well plate (1×10^5 cells/well) and cultured in an induction medium (described in this paragraph). For adipogenic differentiation, subcutaneous and visceral ASCs (S- and V-ASCs) were incubated in adipogenic induction

medium, consisting of DMEM/F12 (Life Technologies), 0.5 mM isobutylmethylxanthine (Sigma-Aldrich), 50 μM indomethacin (Sigma-Aldrich), 5 mM dexamethasone (Sigma-Aldrich), 10% FBS, 100 U/ml penicillin, and 100 g/ml streptomycin. The medium was changed every other day and the cells were cultured for 3 weeks. Oil Red O staining was performed for visualization of lipid droplets. For chondrogenic differentiation, S- and V-ASCs (1×10^6 cells) were cultured in chondrogenic induction medium, consisting of high glucose DMEM (HyClone, Logan, UT), containing 500 ng/ml BMP-6 (R&D Systems, Minneapolis, MN), 10 ng/ml TGF-β3 (R&D Systems), 100 nM dexamethasone, 50 μg/ml ascorbate-2-phosphate (Sigma-Aldrich), 40 μg/ml proline (Sigma-Aldrich), 100 μg/ml pyruvate (Sigma-Aldrich), and 1X ITS liquid media (Sigma-Aldrich), 10% FBS, 100 U/ml penicillin, and 100 g/ml streptomycin. After three weeks, deposition of sulfated glycosaminoglycans was detected with Toluidine Blue. For osteogenic differentiation, the cells were cultured in osteogenic induction medium, which consisted of DMEM/F12 supplemented with 1 nM dexamethasone, 10 mM β-glycerolphosphate (Sigma-Aldrich), 50 μM ascorbate-2-phosphate, 10% FBS, 100 U/ml penicillin, and 100 g/ml streptomycin. After three weeks, calcium accumulation was visualized with Alizarin Red S.

Proliferation assay

Conditioned medium (CM) was collected from 2×10^3 cells/cm^2 of ASCs cultured for 48 h. SKOV3 cells were seeded onto 96-well plates to determine their proliferative ability in ASC-CM after 48, 72, and 96 h of culture. Ascites cells were cultured in ASC-CM for 7 days. The cultured cells were treated (37℃, 3 h) with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 50 μl; Amresco-inc, Solon, OH). After incubation, MTT was removed and solubilized with dimethyl sulfoxide (DMSO; 100 μl) at room temperature for 30 min. The absorbance was measured spectrophotometrically at 540 nm.

Wound healing assay

SKOV3 cells were cultured in 6-well plates for 24 h. After the complete medium was changed to CM of ASCs or control medium, the cell layer was scraped with a pipette tip to create a wound. The wound closure distance (in an arbitrary unit) was measured using ImageJ software.

Boyden chamber assay

To assess the influence of ASCs on the migratory ability of SKOV3, the Boyden chamber assay (transparent PET membrane with 8 μm pore size, BD Biosciences) was used. SKOV3 and ascites cells were seeded onto the inserts, which were transferred to ASC-CM or control medium. After 24 h (48 h for ascites cells), the migratory cells were fixed with 4%

formaldehyde, and stained with 0.5% crystal violet. The migrated cells were counted with the ImageJ software.

Depletion of secreted IL-6

Culture medium (control medium) and ASC-CM were incubated with 500 ng/mL of a goat polyclonal anti-rh IL-6 (R&D Systems, Minneapolis, MN) or a normal goat IgG control (R&D Systems) on a shaker at 4℃ for 3 h. The media were used in migration assays as described above.

Western blotting

Cultured SKOV3 and ascetic cells were harvested and lysed with protein extraction buffer (0.5 M NaCl, 0.5 M Tris-HCl, 50 mM EDTA, 50 mM EGTA, 10% triton X-100, 1 mg sodium deoxycholate, 1 mM Na_3VO_4, 1mM phenylmethylsulfonyl fluoride, EDTA-free protease inhibitor, and distilled water). Proteins (20 μg) were separated on a 6% SDS-gel and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and then incubated (overnight, 4℃) with primary antibodies specific for total and phosphorylated JAK2 and STAT3. After washing three times with TBST, the membrane was incubated with peroxidase-conjugated secondary antibody and visualized using a Western blot detection kit (Ab Frontier, South Korea).

Statistical analysis

Data were presented as mean ± SEM of triplicate for each replicate of four ASC samples (n = 4). Normality of our data were confirmed by the Shapiro-Wilk's test ($P > 0.05$) in advance of the parametric analyses. One-way ANOVA and, when appropriate, Student's *t*-test were used for statistical analyses. Significant differences among experimental groups were analyzed by Scheffe's post hoc test. All analyses were conducted using IBM SPSS statistics 20. P values of < 0.05 were considered statistically significant.

Results

ASCs isolated from subcutaneous and visceral fat depots share similar features to mesenchymal stem cells

The isolated ASCs from subcutaneous and visceral fat exhibited the morphology of mesenchymal stem cells with fibroblast-like shape (Figure 1B). To determine the differentiation ability of S- and V-ASCs, we performed the adipogenic, osteogenic, and chondrogenic differentiation assays. S- and V-ASCs formed lipid droplets and sulfated glycosaminoglycans, and accumulated calcium in the cytoplasm under the adipogenic, chondrogenic, and osteogenic differentiation conditions, respectively (Figure 1C). There were no differences in morphology and differentiation ability between S- and V-ASCs. However, colony-forming

ability was higher for S-ASCs than V-ASCs (Figure 1D). S- and V-ASCs expressed CD73, CD90, CD105, and CD166, but did not express a hematopoietic cell marker (CD45), an endothelial cell marker (CD31), CD34, and HLA-DR (Figure 2). These results show that ASCs, which are negatively selected for CD45 and CD31, and mesenchymal stem cells, have similar characteristics.

ASC-CM enhance the migratory ability of ovarian cancer cells with no difference between CM of V-ASC and S-ASC

Proliferation of SKOV3 cells was not altered by the presence of S- and V-ASC-CM when compared to control medium ($P > 0.05$; Figure 3A). However, SKOV3 cells migrated more readily across the membrane of the insert in S- and V-ASC-CM than in the control medium in the Boyden chamber assay (Figure 3B), and the wound healing ability of SKOV3 cells was also increased in ASC-CM relative to control (Figure 3C). Taken together, these results suggest that paracrine factors secreted from S- and V-ASCs may regulate the migration of ovarian cancer cells.

Of the several factors including IL-6, TNF-α, adiponectin, and leptin, only IL-6 enhances the migration of ovarian cancer cells.

To determine the cancer-promoting effects of ASCs, we screened the mRNA expression of several adipokines including IL-6, TNF-α, adiponectin, and

leptin. As shown in Figure 4A, IL-6 mRNA was highly expressed in S- and V-ASCs, but those of the other adipokines were either marginal or not detectable. The concentration of secretory IL-6 protein was higher in ASC-CM than in the CM of adipocytes and control medium ($P < 0.05$, Figure 4B). However, there was no difference between S- and V-ASCs with respect to their effects on the relative cell growth and migration of cancer cells. To determine whether IL-6 present in ASC-CM influences the migratory ability of SKOV3 cells, we added a neutralizing antibody specific for IL-6 to its CM. The anti-IL-6 antibody decreased the number of migratory cells present at the bottom of Boyden chamber inserts (Figure 4C) in spite of there being no difference between S- and V-ASC-CM ($P > 0.05$, S- vs. V-ASC-CM). These data suggest that IL-6 secreted from ASCs may play an important role in the migration of ovarian cancer cells.

JAK2/STAT3 signaling is activated by IL-6 secreted from ASCs in ovarian cancer cells

We evaluated the activation of the JAK2/STAT3 pathway in ovarian cancer cells in response to IL-6 secreted from ASCs. Levels of phosphorylated JAK2 and STAT3 in SKOV3 cells, but not total JAK2 and STAT3, increased in 30 min, and then started to decrease in 1 h (Figure 5A). To confirm that IL-6 secreted from ASCs affects the JAK2/STAT3 signaling in SKOV3 cells, we added an anti-IL-6 neutralizing antibody to ASC-CM. The addition of

IL-6 to control medium activated JAK2 and STAT3 in SKOV3 cells as did the ASC-CM, and JAK2/STAT3 activation was attenuated by the anti-IL-6 neutralizing antibody (Figure 5B). To confirm the relationship between JAK2/STAT3 and the migration of ovarian cancer cells, SKOV3 cells were treated with a JAK2/STAT3 inhibitor (WP1066) and a JAK2 inhibitor (TG101348). Both treatments were found to reduce the number of migratory cells (Figure 5C). Inhibition of endogenous STAT3 using siRNA specific for STAT3 also reduced the migration of SKOV3 cells (Supplementary Figure 2). These results support the finding that IL-6 secreted from ASCs may promote migration of ovarian cancer cells through the activation of the JAK2/STAT3 signaling pathway.

IL-6/JAK2/STAT3 signaling pathway activated by ASCs also enhances proliferation and migration of ascites cells isolated from ovarian cancer patients

In contrast to that observed for the SKOV3 cells, the relative growth of ascites cells increased significantly in ASC-CM as compared to the cells in control medium (Figure 6A). However, similar to the SKOV3 cells, ASC-CM increased the migration of ascites cells, which was related to IL-6/JAK2/STAT3 signaling pathway (Figure 6B). The relationship between IL-6 and the migratory phenotype of ascites cells was shown by small molecular inhibitors of JAK2 and STAT3 (WP1066 and TG101348,

respectively). The migration of ascites cells was inhibited when either inhibitor was used (Figure 6C). Therefore, ASCs enhance the proliferation and migration of ascites cells via IL-6/JAK2/STAT3 pathway.

Discussion

In the present study, we demonstrated the cancer-promoting role of ASCs and their potential to contribute the tumor microenvironment. IL-6 from ASCs enhanced the migration of an ovarian cancer cell line (SKOV3) and the relative growth and migration of ascites cells. The effect of ASCs was closely linked to the activation of the JAK2/STAT3 signaling pathway in ovarian cancer cells. These findings strongly suggest that ASCs could affect the aggressive phenotype of ovarian cancer through secreting a paracrine factor.

Epidemiological and experimental studies have shown that ASCs, depending upon the individual's fat distribution, are distinct with respect to their genome-wide mRNA expression profile, proliferative activity, and differentiation potentials (Baglioni et al., 2012; Perrini et al., 2013). Genome-wide expression profiles of primary preadipocytes from abdominal subcutaneous, mesenteric, and omental fat depots are also distinct, while their morphological features are not different (Tchkonia et al., 2007). However, ASCs obtained from subcutaneous, visceral, and omental adipose

tissue from the same subject share the typical markers of mesenchymal stem cells, and there was no significant differences in ultrastructural features and the pattern of cytokine secretion (Baglioni et al., 2012; Kipps, Tan, & Kaye, 2013). We isolated two types of ASCs from subcutaneous and visceral fat depots of the same subject to investigate the effect of different depot sites of ASCs (S- and V-ASCs) on ovarian cancer cells. In line with previous studies, colony-forming ability was higher in S-ASCs than in V-ASCs, but significant differences in phenotypic character and in secretion of cytokines were not observed between S- and V-ASCs. In contrast, the cancer-promoting stimulation of ASCs on ovarian cancer cells was not dependent on fat depot site. In addition, there was no significant difference in IL-6 concentration between S- and V-ASC-CM. These results suggest that the growth and migration of ovarian cancer cells could not be linked to the anatomical origin or distribution of ASCs. However, a possible involvement of other inflammatory cytokines/chemokines could not be ruled out, given that our investigation focused solely on secreted IL-6. Another possible reason for the apparent discrepancy between our findings and previously published data may be related to the use of cellular models derived from subjects with different body mass index (BMI), sex, or other diseases.

ASCs have the features of multipotent mesenchymal stem cells (Zuk et al., 2002). Mesenchymal stem cells provide tumor cells with a functionally and structurally supportive environment through the establishment of the

pre-metastatic niche, causing the induction of metastatic phenotypes (Coffelt et al., 2009; Lis et al., 2011; Spaeth, Klopp, Dembinski, Andreeff, & Marini, 2008). The same may be true of ASCs, which are known to promote the progression of prostate cancer, and stimulate migration and invasion of estrogen receptor-negative breast cancer (Prantl et al., 2010; Walter, Liang, Ghosh, Hornsby, & Li, 2009). Moreover, endometrial cancer proliferation is increased by co-culture with omental ASCs (Klopp et al., 2012). Recently, the effect of ASCs on ovarian cancer cells has been reported (Nowicka et al., 2013). Nowicka et al. suggested that ASCs derived from omental fat tissue contributes to the proliferation and migration of ovarian cancer cell lines, including SKOV3 and A2780, through the metabolic support of ASCs to ovarian cancer cells. Consistent with this study, our findings indicate that ASCs derived from subcutaneous and visceral fat contributed to the increased migration of ascites cells isolated from ovarian cancer patients as well as of an ovarian cancer cell line. Interestingly, ASCs contribute to enhanced proliferation of only patient-derived ascites cells, but not SKOV3. SKOV3 is frequently used as an *in vitro* model instead of primary tumor cells, but Domcke et al. reported that SKOV3 has an overall genomic difference from the high grade serous ovarian cancer (Domcke, Sinha, Levine, Sander, & Schultz, 2013). For this reason, there could be a proliferative difference between SKOV3 and ascites cells.

It is known that ASCs secrete many cytokines or chemokines (Kilroy et al., 2007). We found that the mRNA expression of IL-6 was the highest among several cytokines. The IL-6 cytokine family is involved in many biological responses such as inflammation, immune reaction, and oncogenesis by regulating growth and differentiation (Lutticken et al., 1994). The binding of ligand to its common receptor subunit gp130 activates the JAK/STAT pathway, and in tumor cells, activation of STAT3 plays a central role in cell migration (Hirano, Ishihara, & Hibi, 2000). In a previous study, the IL-6 receptor (IL-6R) was highly expressed in SKOV3 cells and exogenous IL-6 stimulated STAT3 activation and enhanced migration. STAT3 is activated through direct activation of EGFR or IL-6R or indirect induction of the IL-6R pathway in high-grade ovarian carcinomas (Colomiere et al., 2009). Consistent with this previous study, our study indicated that IL-6-induced ovarian cancer cell migration and motility was hampered by blocking IL-6R with an IL-6 antibody or applying specific inhibitors of STAT3 or JAK2, suggesting that IL-6 could play a key role in proliferation and migration of ovarian cancer cells. We identified that ASCs express IL-6 mRNA and secrete IL-6 protein without any extrinsic stimulation. Based on these results, we found that the enhanced migration and proliferation of ovarian cancer cells is, at least in part, due to the activation of the JAK2/STAT3 pathway by ASCs-derived IL-6. JAK2 is involved in progression and drug-response of ovarian cancer. In our

previous study, next-generation sequencing data showed that JAK/STAT signaling pathways including JAK2 were amplified in primary and metastatic tumor clusters (J. Y. Lee et al., 2015). Other studies also reported that JAK2-STAT3 is significantly activated in high-grade ovarian carcinomas compared to normal ovaries and benign tumors, which is involved in cancer progression and EMT (Colomiere et al., 2009), and efficacy of cisplatin is dependent on JAK2 activity (Song, Sondak, Barber, Reid, & Lin, 2004). In addition, CA125 used as an ovarian cancer marker can bind JAK2 and activates STAT3, suggesting the importance of JAK2 in the pathogenesis of CA125-expressing cancers (Lakshmanan et al., 2012). In line with the previous studies, JAK2 activated by ASCs promoted STAT3 phosphorylation, leading to enhanced migration of ovarian cancer cells, and inhibition of JAK2 resulted in the decrease of migration. Therefore, targeting JAK2 using several inhibitors could be an important therapeutic strategy to mitigate dissemination of ovarian cancer.

Overall, using primary ovarian cancer cells (ascites cells), as well as a cancer cell line, we provided direct experimental evidence that IL-6 secreted from ASCs activates the JAK2/STAT3 pathway and contributes to ovarian cancer progression and metastasis. Targeting both ovarian cancer cells and supportive stem cells in adipose tissue could be a new therapeutic approach for ovarian cancer therapy.

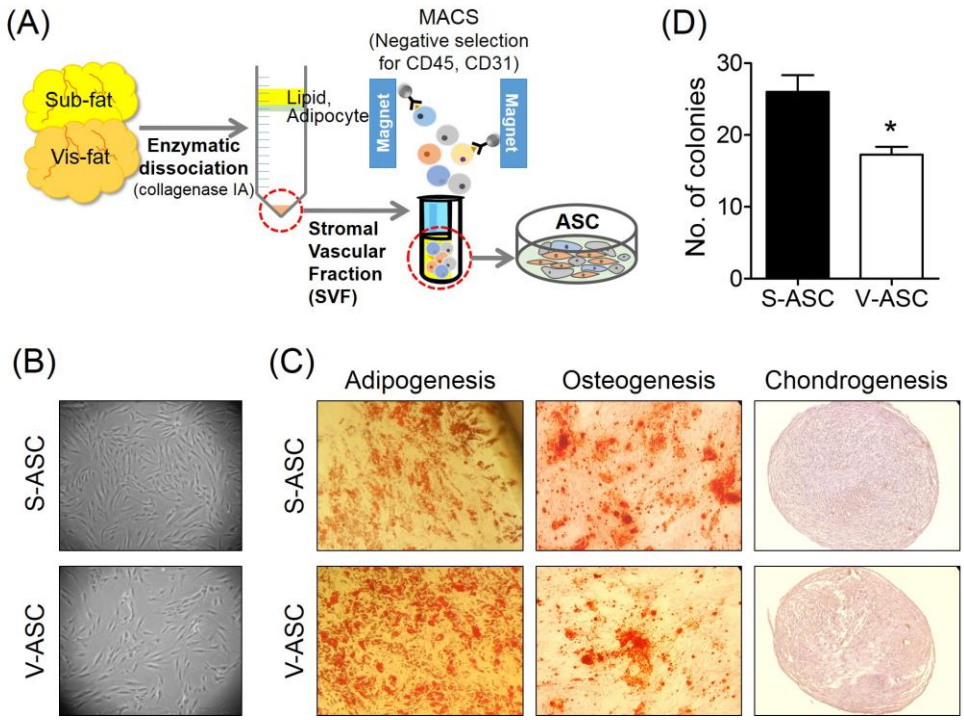


Figure 1. ASCs isolated from human subcutaneous and visceral fat have similar traits to mesenchymal stem cells. (A) The stromal vascular fraction (SVF) was isolated from subcutaneous and visceral fat tissue. To remove endothelial and hematopoietic cells from the SVF, CD31- and CD45-negative ASCs were isolated by magnetic-activated cell sorting (MACS). The isolated ASCs were cultured for 5–7 days. (B) Morphologies of S- and V-ASCs were evaluated by an inverted phase-contrast microscope (40X objective) at passage 2. (C) S- and V-ASCs were able to differentiate into adipogenic, osteogenic, and chondrogenic lineages under induction conditions. (D) One hundred cells of S- and V-ASCs were cultured on a 100 mm-plate for 7 days, and the number of clones was counted after staining

with 0.5% crystal violet, The representative data were presented as mean ± SEM of triplicate for each replicate of four samples ($^{*}P < 0.05$, S-ASC vs. V-ASC; Student's *t*-test).

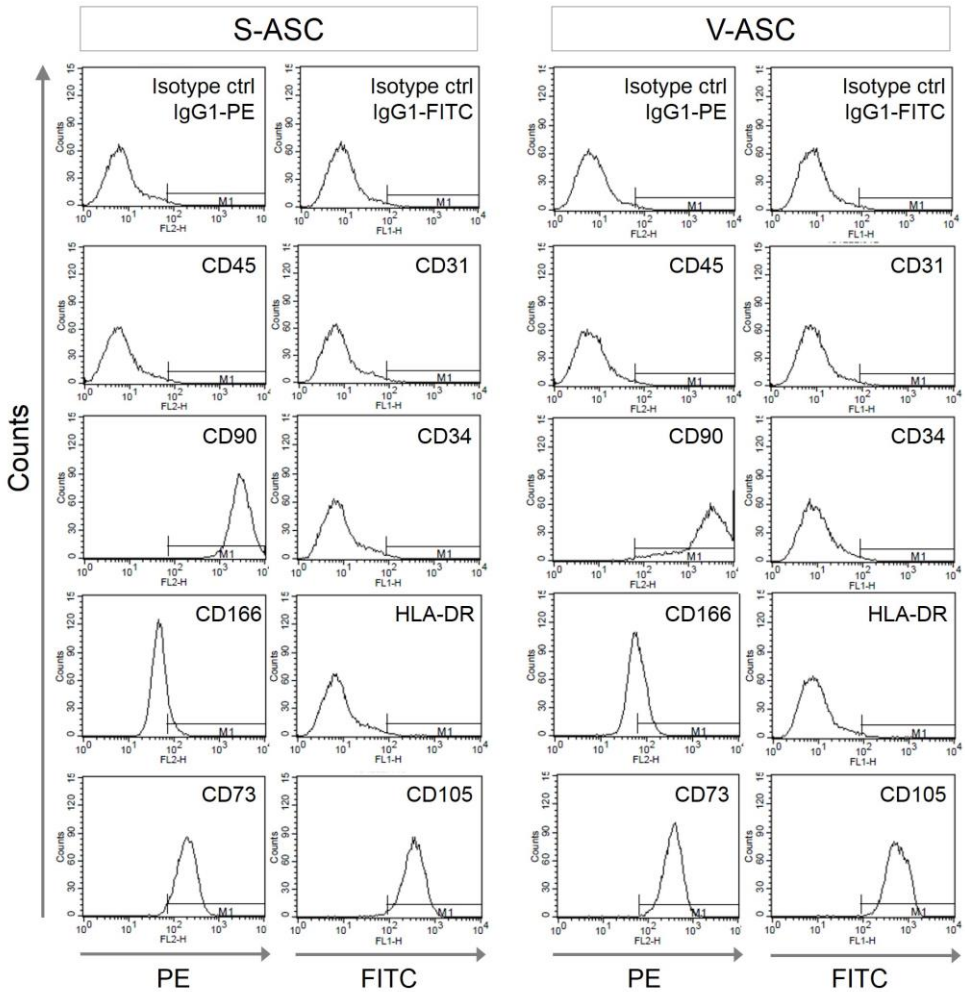


Figure 2. S- and V-ASCs express specific surface markers shared with mesenchymal stem cells. S- and V- ASCs were immunotypically labeled with CD45, CD73, CD90, and CD166 antibodies conjugated to R-phycoerythrin (PE) and with CD31, CD34, CD105, and HLA-DR antibodies conjugated to fluorescein isothiocyanate (FITC) at passage 3. The labeled ASCs were analyzed using flow cytometry. IgG1κ-conjugated to PE and to FITC were used as Isotype controls. Both S- and V-ASCs expressed CD90,

CD166, CD73, and CD105, but did not express CD45, CD31, CD34, and HLA-DR.

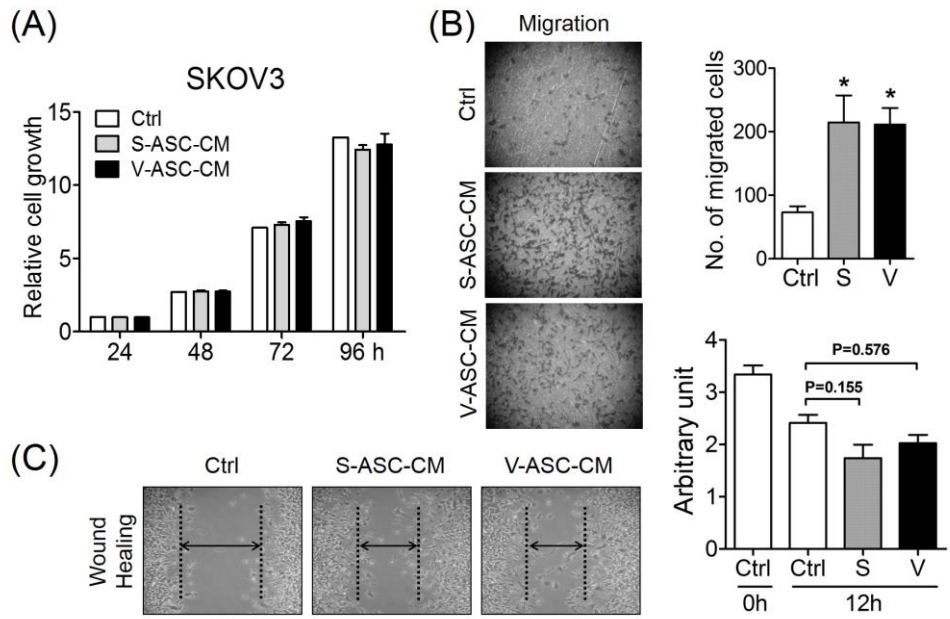


Figure 3. Conditioned media from S- and V-ASCs promote migration of ovarian cancer cells. (A) SKOV3 cells were cultured in conditioned media (CM) of S- and V-ASC for 24 and 48 h. Relative cell growth of SKOV3 cells was determined by the MTT assay. (B) Migration of the SKOV3 cells was determined by the Boyden chamber assay. Migratory cells on the membrane of an insert were stained with 0.5% crystal violet, and counted ($^{*}P < 0.05$ vs. control; one-way ANOVA with Scheffe's post hoc test). (C) Wound healing ability of SKOV3 cells was verified by measuring wound closure distance in 12 h. The representative data were presented as mean ± SEM of triplicate for each replicate of four samples.

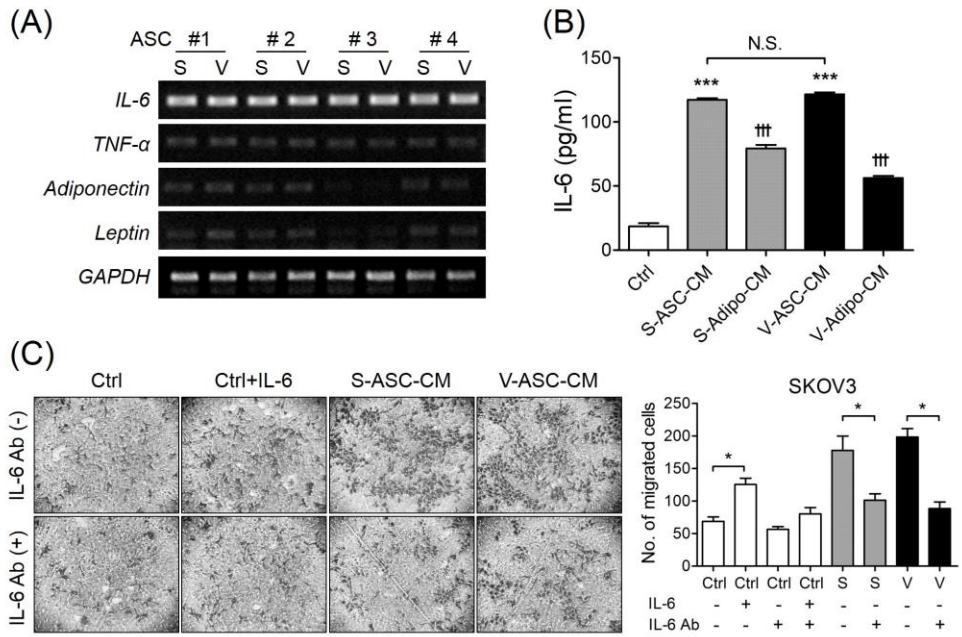


Figure 4. IL-6 secreted from S- and V-ASCs enhances migration of ovarian cancer cells. (A) mRNA expression of adipokines including IL-6, TNF-α, adiponectin, and leptin was measured by RT-PCR. GAPDH was used as a reference gene. (B) ELISA assay was conducted to detect secretory IL-6 protein present in the control medium, S- and V-ASC-CM, and subcutaneous and visceral differentiated adipocytes (S- and V-adipo-CM). Complete medium was used as a control. (C) SKOV3 cells were cultured in S- and V-ASC-CM with or without anti-IL-6 neutralizing antibody (500 ng/ml; IL-6 Ab). Migration ability was determined by the Boyden chamber assay. Migratory cells on the bottom of the inserts were stained with 0.5% crystal violet, and counted. Control medium with recombinant human IL-6 (50 ng/ml) was used as a positive control. All data

were presented as mean ± SEM of triplicate for each replicate of four samples ($^{*}P < 0.05$; $^{***}P < 0.001$, ASC-CM vs. control; $^{\dagger\dagger\dagger}P < 0.001$, adipo-CM vs. control; one-way ANOVA with Scheffe's post hoc test).

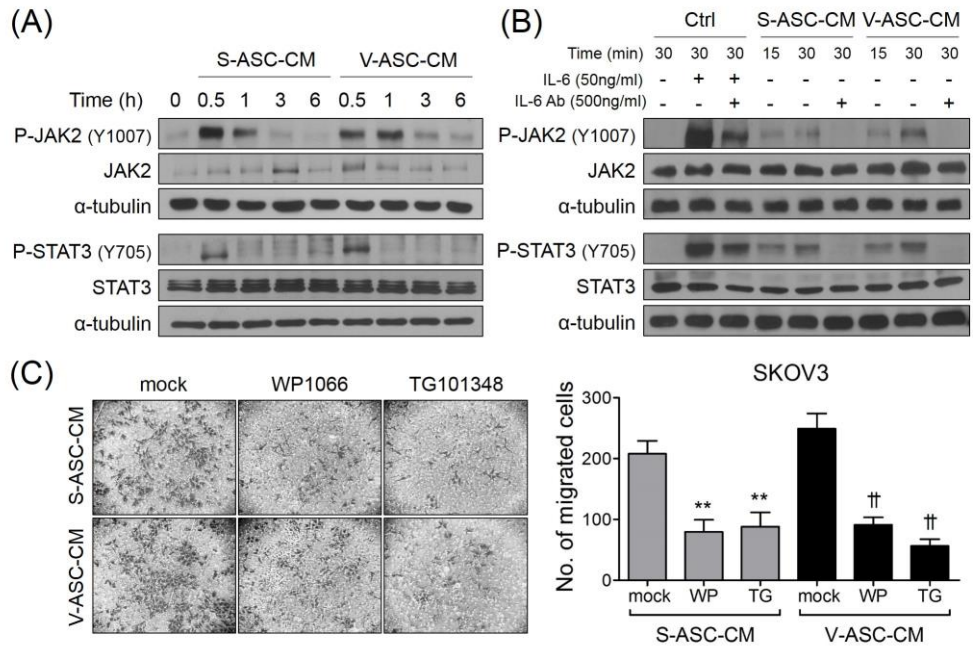


Figure 5. Activation of JAK2 and STAT3 in response to IL-6 from ASCs increases migration of ovarian cancer cells. (A) Protein expression levels of total JAK2, phosphorylated JAK2 (Y1007), total STAT3 and phosphorylated STAT3 (Y705) were determined by Western blot analyses. (B) After depletion of IL-6 using anti-IL-6 antibody (500 ng/ml; IL-6 Ab), total JAK2, phosphorylated JAK2, STAT3 and phosphorylated STAT3 were analyzed by Western blot. Control medium with recombinant human IL-6 (50 ng/ml) was used as a positive control. Alpha-tubulin was used as a loading control. (C) After SKOV3 cells were incubated in S- and V-ASC-CM with the addition of mock (DMSO), 2 μM WP1066 (an inhibitor for JAK2 and STAT3) or 1 μM TG101348 (an inhibitor for JAK2) for 12 h, the Boyden chamber assay was conducted. Migratory cells were stained with

0.5% crystal violet, and counted on the bottom of the inserts. The representative data were presented as mean ± SEM of triplicate for each replicate of four samples ($^{**}P < 0.01$, WP and TG in S-ASC-CM vs. mock; $^{††}P < 0.01$, WP and TG in V-ASC-CM vs. mock; one-way ANOVA with Scheffe's post hoc test).

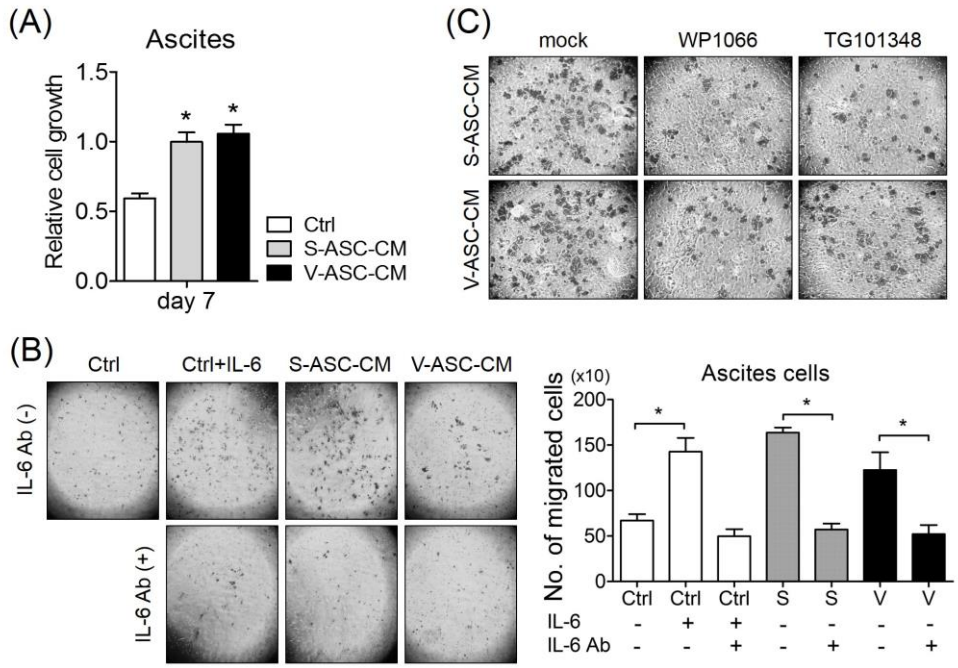
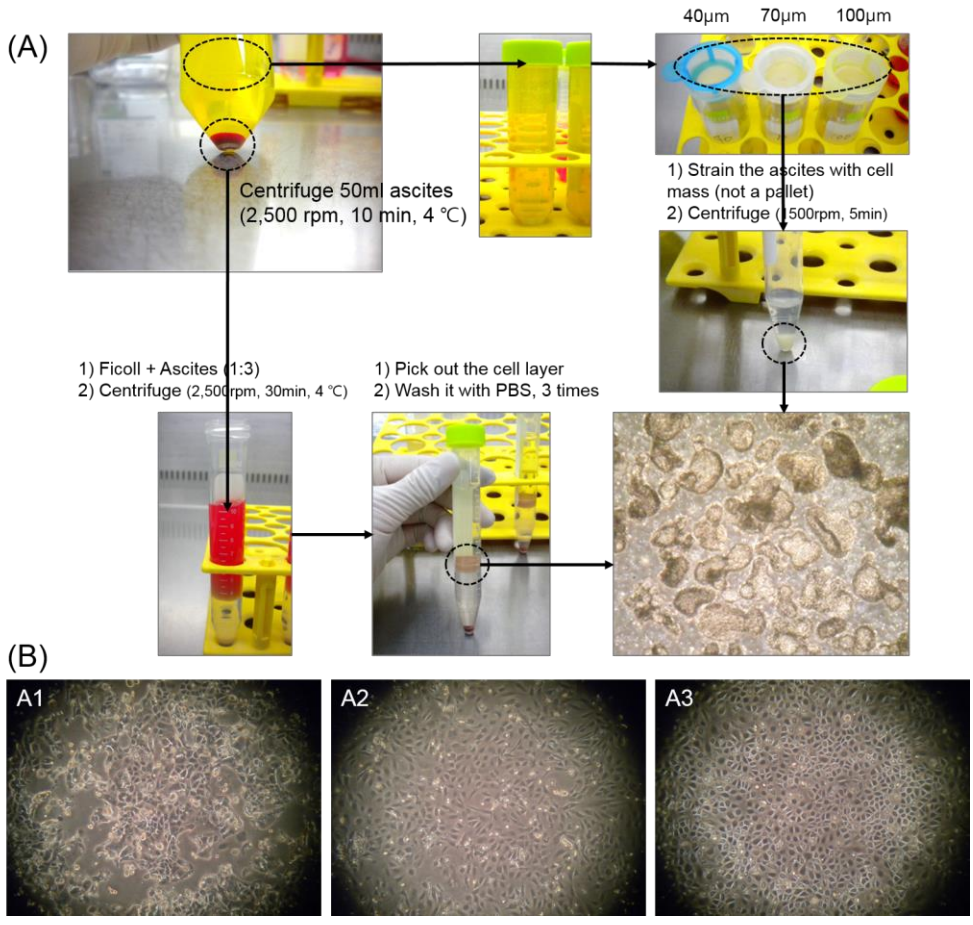
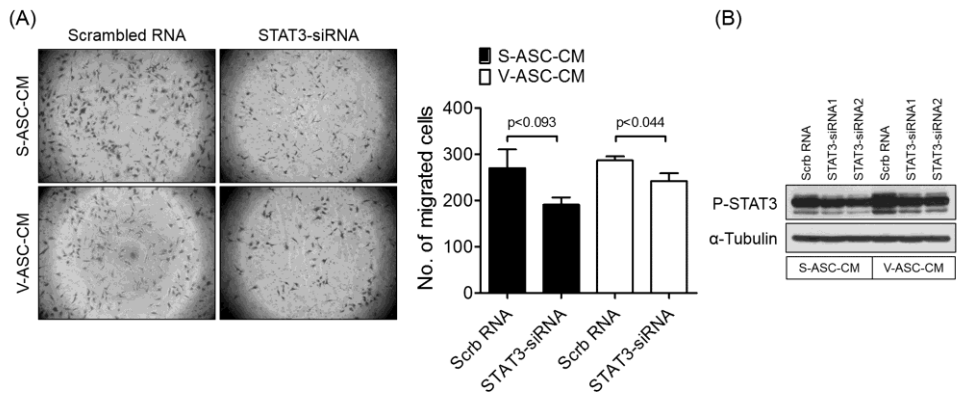


Figure 6. ASCs enhance proliferation and migration of ovarian cancer cells isolated from ascites of ovarian cancer patients through activation of IL-6/JAK2/STAT3 pathway. (A) Ascites cells were cultured in S- and V-ASC-CM for 7 days. Proliferation of ascites cells was determined by the MTT assay. Statistical significance was determined from each triplicate experiment of two ascites samples. (B) Ascites cells were cultured in ASC-CM with or without anti-IL-6 neutralizing antibody (500 ng/ml; IL-6 Ab), and in control medium with rhIL-6 (50 ng/ml) and/or anti-IL-6 neutralizing antibody. After culture for 24 h, migratory cells were stained with 0.5% crystal violet, and counted. (C) JAK2 and STAT3 inhibitors (2 μM WP1066 or 1 μM TG101348) were treated on ascites cells cultured in ASC-CM for 24 h. Migration was determined by the Boyden chamber assay. The

representative data were shown as mean ± SEM of triplicate for each replicate of four samples ($^{*}P < 0.05$ vs. control; one-way ANOVA with Scheffe's post hoc test).



Supplementary Figure 1. Isolation and culture of patient-derived cancer cells from ascites of ovarian cancer patients.



Supplementary Figure 2. Silencing of STAT3 using siRNA reduces migration of SKOV3. SKOV3 was transfected with scrambled RNA (Scrb RNA; 100 nM) and siRNA for STAT3 (STAT3-siRNA; 100 nM), and cultured in S- and V-ASC-CM for migration assay. (A) Migratory cells were stained with 0.5% crystal violet and counted. (B) P-STAT3 expression was inhibited after silencing of STAT3 using siRNA. The representative data were shown as mean ± SEM of triplicate for each replicate of four samples (scrb RNA vs. STAT3-siRNA; Student's t-test).

CHAPTER II.

PGC-1α under oxidative stress mediates chemoresistance of sphere forming cells in ovarian cancer

Abstract

Cancer undergoes metabolic alterations, which are the bioenergetic shift from mitochondria to glycolysis as a primary source of energy and anabolic support. Despite the enhanced aerobic glycolysis, tumor cells still generate significant adenosine triphosphate (ATP) through the mitochondrial respiration. Here, we found that PGC-1α, which is a key molecule to regulate mitochondrial biogenesis and metabolism, enhances chemoresistance in response to generation of reactive oxygen species (ROS) under the culture condition for sphere formation. Sphere forming cells exhibited stem-like phenotypes with a high activity of aldehyde dehydrogenase (ALDH) and expression of stemness-related genes, and showed the drug-resistant phenotype. Sphere forming cells maintained the high level of ROS, and subsequently induced antioxidant gene expression. Scavenging ROS using N-acetyl-cysteine reduced ALDH-positive population and inhibited proliferation of sphere forming cells. PGC-1α was activated in response to ROS generation, resulting in the altered mitochondrial biogenesis and structure of sphere forming cells. The drug-resistant phenotype was observed in both sphere forming cells and PGC-1α-overexpressed parent cells, but not in parental cells. Knock-down of PGC-1α using siRNA, however, sensitized sphere forming cells to the treatment of cisplatin. Our findings first suggest that PGC-1α induced by ROS

mediates drug resistance in ovarian cancer cells, and provide a possibility as a new therapeutic target to overcome chemoresistance in ovarian cancer.

Introduction

Tumors undergo metabolic reprogramming to meet the increased energetic and anabolic demands (Ward & Thompson, 2012). It has been established that cancer cells typically rely on aerobic glycolysis, so called Warburg effect, because they were considered to have defects in mitochondrial function (Vander Heiden, Cantley, & Thompson, 2009; Warburg, 1956). Tumor cells, however, still generate significant adenosine triphosphate (ATP) through the mitochondrial oxidation of fatty acids and amino acids when glucose becomes depleted. The major roles of mitochondria are production of ATP and intermediate metabolites in cells. Carbon-containing molecules, including pyruvate generated from glycolysis, amino acids, and fatty acid, move into TCA cycle in mitochondrial matrix, where they are involved in generation of reducing coenzymes such as NADH and $FADH_2$ (S. E. Weinberg & Chandel, 2015). Intermediates as well as NADH and $FADH_2$ are generated from the metabolism of carbon-containing molecules in TCA cycle, which can contribute to produce macromolecules to use building-blocks in cancer cell (S. E. Weinberg & Chandel, 2015). This means that cancer cells display functional mitochondria, and usefully utilize mitochondria as a source of energy and anabolic materials.

Reactive oxygen species (ROS) as a byproduct of mitochondrial

metabolism function as a second messenger in the transduction of extracellular signals to control cellular proliferation and cell cycle progression. Altered metabolism of tumor causes an increase of ROS, and sustains ROS at higher levels than normal tissue (F. Weinberg & Chandel, 2009). The increased ROS level drives cancer cells to develop adaptive system against oxidative stress (Sabharwal & Schumacker, 2014). As a detoxifying mechanism, PGC-1α, which is a major regulator of mitochondrial biogenesis and respiratory function, is required for the induction of ROS-detoxifying enzymes, and contributes to the reduction of ROS generation derived from mitochondrial metabolism (Lin, Handschin, & Spiegelman, 2005; Scarpulla, 2011; St-Pierre et al., 2006). Under the normal conditions, PGC-1α is sustained at low level, but it is induced in response to bioenergetic demands or metabolic alterations (Lin et al., 2005). Recently, it is reporting that PGC-1α has oncogenic properties in cancer cells (LeBleu et al., 2014; Martinez-Outschoorn, Pavlides, Sotgia, & Lisanti, 2011; Mayevsky, 2009; Vazquez et al., 2013). However, the role of PGC-1α and chemoresistance is not fully understood in ovarian cancer. In the present study, we hypothesized that PGC-1α induced by ROS generation enhances mitochondrial biogenesis and structural changes, resulting in phenotypical alteration of ovarian cancer cells.

Materials and Methods

Cell culture of ovarian cancer cells, PA-1

PA-1 cells (ovarian cancer cell line) were purchased from American Type Culture Collection, and cultured in DMEM/F12 (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; Life Technologies), 1% penicillin and streptomycin (Life Technologies). PA-1 cells were maintained at 37 ℃ in humidified atmosphere of 5% CO_2.

Sphere formation of ovarian cancer cells

PA-1 cells (1,000 cells/cm^2) were plated onto poly-2-hydroxyethyl methacrylate (poly-HEMA; Sigma Aldrich, St. Louis, MO) coated plate, and cultured in DMEM/F12 containing human recombinant EGF (20 ng/ml; Life Technologies), bFGF (20 ng/ml; Life Technologies), human recombinant insulin (5 μg/ml; Life Technologies), B27 (0.1x; Life Technologies), and penicillin and streptomycin (1%; Life Technologies). Tumor sphere cells were maintained at 37 ℃ in humidified atmosphere of 5% CO_2 for two weeks.

Measurement of ALDH activity

Cancer stem-like cells with a high ALDH activity were identified from tumor sphere and parental cells (1 x 10^6 cells) using ALDEFLUOR® assay

kit (StemCell Technologies, Vancouver, BC). Dissociated tumor sphere and parental cells were suspended in ALDEFLUOR assay buffer containing ALDH substrate without/with 50 mM DEAB (specific inhibitor for ALDH) as a negative control, and incubated for 30 min at 37 ℃. ALDH-positivity was analyzed by BD FACS Canto II flow cytometer (BD Biosciences, NorthRyde, Australia).

Measurement of intracellular ROS levels in ovarian cancer cells

To measure ROS levels in ovarian cancer cells, dihydroethidium (DHE; Sigma Aldrich, St. Louis, MO) and 6-carboxy-2,7-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma Aldrich) were used for detecting superoxide anion and hydrogen peroxide, respectively. Cultured cells were dissociated with 0.5% trypsin-EDTA and incubated in culture medium with DHE (5 μM for 10 min) or DCFH-DA (10 μM for 30 min with vortexing every 10 min) at 37 ℃ in the dark. Relative fluorescence intensity of ethidium (ETH, oxidized form of DHE by superoxide) or DCF (oxidized form of DCFH by hydrogen peroxide) was measured by BD FACS Canto II flow cytometer (BD Biosciences).

Total RNA isolation and quantitative real time-PCR (qRT-PCR)

Total RNA was extracted from parental and tumor sphere cells with TRIzol reagent (Invitrogen). Complementary DNA (cDNA) was synthesized from 1

μg of total RNA using superscript III first-strand synthesis system with oligo(dT)$_{20}$ primers (Invitrogen). The synthesized cDNA was diluted with nuclease free water (five-fold), and mixed with Master Mix SYBR Green I dye (Bio-Rad, Hercules, CA). Relative mRNA level was determined by CFX96 Real-Time PCR Detection System (Bio-Rad), according to the manufacturer's protocol. Amplification conditions were followed for 35 cycles at 94 ℃ for 30 s, 60 ℃ for 30 s, and 72 ℃ for 30 s. The relative gene expression levels were quantified using the $2^{-\Delta\Delta Ct}$ method and normalized to the C_t value of the reference genes, β-actin and GAPDH.

Western blotting

Tumor sphere and parental cells were harvested and lysed with protein extraction buffer (0.5 M NaCl, 0.5 M Tris-HCl, 50 mM EDTA, 50 mM EGTA, 10% triton X-100, 1 mg sodium deoxycholate, 1 mM Na_3VO_4, 1mM phenylmethylsulfonyl fluoride, EDTA-free protease inhibitor, and distilled water). Proteins (20 μg) were separated on 6 - 10% SDS-PAGE, and transferred onto a nitrocellular membrane. After blocked with 5% skim milk solution in tris-buffered saline with 0.1% Tween 20, the membranes were incubated with primary antibodies specific for MDR1 (Cell Signaling Technology, Beverly, MA), ABCG2 (Santa Cruz Biotechnology, Santa cruz, CA), total OXPHOS complexes (Abcam, Cambridge, MA), and PGC-1α (Calbiochem, Darmstadt, Germany). Signals were visualized using a

chemiluminescence detection kit (AbFrontier, Seoul, Korea).

Relative cell viability using MTT assay

To measure cell viability after treated with cisplatin, cultured cells (parental and tumor sphere cells) were incubated with 2 mg/ml of MTT dissolved in phosphate buffered saline (PBS) solution at 37 ℃ in the dark. After incubation for 3 h, MTT was removed, and dimethyl sulfoxide (DMSO) was added to dissolve formazan in the live cells. Aliquots (200 μl) of DMSO solution were transferred in 96-well plates, and absorbance was recorded at 540 nm using a spectrophotometer (Labsystem Multiskan, Helsinki, Finland)

Measurement of apoptosis

Tumor sphere cells were cultured for two weeks and treated with various concentrations of CDDP for 48 h. Drug-treated cells were dissociated with 0.05% Trypsin-EDTA, and centrifuged at 500 x g for 4 min. The cell pellet was suspended in PBS and stained with annexin V-PI apoptosis detection kit (BD Bioscience Pharmingen, San Jose, CA). Apoptotic cells were analyzed by BD FACS Canto II flow cytometer using the CellQuest analysis program (BD Biosciences, NorthRyde, Australia).

Immunocytochemistry for the detection of PGC-1α in tumor spheres

After culture for two weeks, tumor sphere samples were fixed with 4% formaldehyde in 4 ℃ overnight. Fixed samples were washed with PBS for 30 min (three times every 10 min) followed by dehydration through a graded ethanol of 25, 50, 70, 90, and 100% for 10 min in each step. Paraffin-embedded preparations of tumor sphere samples were sectioned at 7 μm thickness by using a microtome (HM340E, Microm). Sections were dried at 40 ℃ overnight, and dewaxed with Citri-Solv (Fisher BioSciences). After rehydrated with a graded ethanol of 100, 70, 50, and 25%, and PBS for 10 min in each step, samples were blocked in PBS containing 0.1% Tween-20 (0.1% PBS-T) and 3% normal goat serum (Vector Laboratories, Burlingame, CA) at room temperature for 1 h. Sectioned samples were incubated in 0.1% PBS-T containing 3% normal goat serum and primary antibody (anti-PGC-1α mouse antibody, 1:200; anti-ABCG2 mouse antibody, 1:500) at 4 ℃ overnight. After washed in 0.1% PBS-T for 60 min (three times every 20 min), samples were incubated in 0.1% PBS-T containing secondary antibody (anti-mouse IgG conjugated with AlexaFluor488, 1:250) for 4 ℃ overnight. Samples were washed with 0.1% PBS-T for 60 min (three times every 20 min), and mounted with mounting medium (Vectashield with DAPI, H-1200, Vector Laboratories). Fluorescent images were captured using a LSM 700 confocal microscope (Zeiss, Germany).

Statistical analysis

Data were presented as mean ± SEM of replicate experiments. Student's t-test was used for statistical analyses. Significant difference between experimental groups was analyzed by Scheffe's post hoc test. All analyses were conducted using IBM SPSS statistics 20.

Results

Tumor sphere formation increases cancer stem cell population and exhibits enhanced drug-resistance in ovarian cancer cells.

Tumor sphere culture condition enriched cancer stem-like cells with a high ALDH activity. Ovarian tumor sphere cells exhibited higher activity of ALDH than parental cells (Figure 1A). Serial subculture of tumor sphere enriched ALDH-positive population (Figure 1A). Expressions of mRNA for two subtypes of ALDH (ALDH1A and 2) and stemness-related genes, including Nanog, Sox2, and Bmi1, were increased in tumor sphere cells relative to parental cells (Figure 1B and 1C). To confirm drug-resistance of tumor sphere cells to cisplatin (CDDP), we treated parental and sphere cells with serial concentrations of CDDP. Tumor sphere cells showed a higher IC_{50} value of CDDP than parental cells (Figure 2A). Apoptotic cells were significantly decreased in tumor sphere cells (Figure 2B). Drug-resistance related proteins, such as multi-drug resistance protein 1 (MDR1) and ATP-binding cassette sub-family G member 2 (ABCG2), were highly expressed in tumor sphere cells (Figure 2C). These results suggest that tumor sphere formation enriches stem-like cells in ovarian cancer cells, and enhances drug-resistance.

Tumor sphere formation stimulates ROS generation, related to stem-

like phenotype of ovarian cancer cells.

Tumor sphere formation stimulated generation of reactive oxygen species (ROS). Hydrogen peroxide was increased, while superoxide was decreased in tumor sphere cells relative to parental cells (Figure 3A). Tumor sphere cells showed relatively high expression of antioxidant genes in response to ROS (Figure 3B). Removal of ROS using N-acetyl-cisteine (NAC) alleviated ALDH activity in tumor sphere cells (Figure 3C). Scavenging ROS by NAC revealed a decrease of size (diameter) and the number of tumor spheres, while NAC did not affect to parental cells (Figure 3D and 3E). These findings indicate that intracellular ROS generation caused by tumor sphere formation is involved in phenotypical changes of cancer stem-like cells.

Intracellular ROS generated in tumor spheres lead to PGC-1α expression.

We assumed ROS generation induces PGC-1α expression to promote detoxifying system. Quantitative PCR analyses revealed significant up-regulation of genes associated with mitochondrial biogenesis (PGC-1α, -1β, and NRF1) and oxidative phosphorylation (OXPHOS; SDHA, SDHD, and COX4I) (Figure 4B). We focused on PGC-1α, which regulates mitochondrial biogenesis and metabolism. Tumor sphere formation led to enhanced expressions of PGC-1α and OXPHOS complex II, III, and IV

(Figure 4C and 4D). We observed localization of PGC-1α and mitochondrial structure within tumor sphere. Immunocytochemistry data showed that PGC-1α expression was localized in the central part of tumor sphere (Figure 4E). Tumor sphere formation also led to alteration of mitochondrial structure. Tubular structure (fusion) was shown in parental cells, while fragmented structure (fission) was shown in sphere cells (Figure 4F). We thought that PGC-1α expression within tumor spheres is caused by ROS generation. To confirm whether H_2O_2 induces PGC-1α expression, we added serial concentrations of H_2O_2 to parental cells. Consistent with an increase of exogenous H_2O_2, PGC-1α expression was increased in parental cells (Figure 5A). Removal of ROS in tumor spheres caused the decrease of PGC-1α and OXPHOS complexes expression (Figure 5B and 5C). More interestingly, inhibition of ROS generation down-regulated drug resistance-related proteins in tumor spheres (Figure 5D). In addition, pretreatment of NAC to tumor spheres showed the increase of CDDP-induced cell death (Figure 5E). From these results, we suggest that ROS caused by sphere formation lead to induction of PGC-1α, resulting in mitochondrial biogenesis and structural changes, and inhibition of ROS sensitizes tumor spheres to CDDP-induced cell death through down-regulation of PGC-1α and drug resistance-related proteins.

PGC-1α mediates drug-resistance in tumor sphere cells

To whether PGC-1α is involved in drug-resistance, we overexpressed PGC-1α in parental cells. We confirmed overexpression of PGC-1α using Western blotting following transfected with pcDNA-PGC-1α in parental cells (Figure 6A). Mitochondrial activity of PGC-1α overexpressed cells was increased as much as tumor sphere cells compare to parental cells (Figure 6B). To investigate that overexpression of PGC-1α enhances cancer stem-like properties, we performed ALDEFLUOR assay and determined drug-resistance using MTT assay and AV-PI staining. The ALDH activities of both tumor sphere and PGC-1α overexpressed cells revealed higher tendency than parental cells (Figure 6C). Overexpression of PGC-1α induced MDR1 and ABCG2 expression (Figure 6D). To determine drug-resistance, we treated parental cells and PGC-1α overexpressed cells with various concentrations of CDDP (0, 1, 2, 5, and 10 μM) for 48 h. PGC-1α overexpressed cells showed higher IC_{50} value than parental cells (Figure 6E). Apoptotic cells were also significantly lower in PGC-1α overexpressed cells than parental cells following treatment of CDDP (Figure 6F). On the other hand, silencing PGC-1α drove tumor sphere cells to be vulnerable to CDDP. These results indicate that PGC-1α mediates chemoresistance in ovarian cancer cells.

Discussion

It has been considered that cancer cells dominantly utilize glycolysis to meet the metabolic demand due to mitochondrial defects (Warburg, 1956). However, emerging evidence is showing that cancer cells have functional mitochondria, which are involved in tumorigenesis. Here, we provide the evidence that PGC-1α induced by ROS mediated chemoresistance in ovarian cancer cells. Sphere forming cells show enhanced ALDH activity and expression of several stemness-related genes, such as Nanog, Sox2, and Bmi1, and sphere formation stimulates ROS generation leading to expression of detoxifying enzymes along with PGC-1α expression. In sphere forming cells, induction of PGC-1α results in mitochondrial biogenesis and structural changes (from fusion to fission), and causes chemoresistance through the expression of ABC transporters, MDR1 (ABCB1) and ABCG2. Suppression of PGC-1α through scavenging ROS sensitizes tumor spheres to CDDP resulting from alleviating ALDH activity and drug resistance-protein levels.

Cancer stem cells exhibit stem-like properties implicated in the tumorigenesis, aggressive, metastatic, and chemoresistant phenotype (Visvader & Lindeman, 2008). Culture condition for sphere formation is considered as an efficient method for enrichment or isolation of cancer stem cells (Hashimoto et al., 2014; Huang et al., 2011; Yu et al., 2008; S. Zhang et al., 2008). In addition, three dimensional (3D) culture through the tumor sphere formation is representative method to mimic in vivo tumor

microenvironment. According to our findings, tumor sphere formation mimicking *in vivo* tumor mass enables ovarian cancer cells to acquire stem-like properties such as high ALDH activity and drug-resistance. Interestingly, 2D (attached culture) and 3D (sphere formation) culture conditions display the opposite response to NAC treatment (Figure 3D). This means that tumor microenvironmental effect should be regarded as an important factor influencing cancer phenotypes. Even though it is known that NAC has a cancer prevention effect rather than anticancer effect, recent research reveals that ROS scavengers such as NAC, Tiron, and Trolox, inhibit tumorigenic initiating cells through the impairment of cell cycle progression in glioblastoma (Monticone et al., 2014). In general, intracellular ROS as a second messenger stimulate cell proliferation and regulate the determination of differentiation in stem cells (Zhou, Shao, & Spitz, 2014). Our results show that 3D culture condition encourages ROS generation different from 2D culture, where ROS play a role in stimulating proliferation of tumor cells. Inhibition of ROS decreases the size and number of tumor spheres concomitant with the reduction of ALDH activity. In addition, up-regulation of antioxidant genes is observed in sphere forming cells accompanying ROS elevation in tumor spheres. This result may affect to the fact that superoxide is decreased and hydrogen peroxide is increased, because superoxide dismutase 2 (SOD2) may act in the process to convert superoxide to hydrogen peroxide in mitochondria. It is reported that SOD2 expression is

correlated with chemoresistance in lymphoma (Tome et al., 2012), basal-like breast carcinoma (Kumar et al., 2014), and lung adenocarcinoma (P. M. Chen, Cheng, Wu, Chen, & Lee, 2015). In line with previous data, our findings implicate that acquirement of chemoresistance or stem-like phenotypes is attributed to expression of detoxifying enzymes induced by ROS generation in sphere forming cells. Thus, removal of ROS could be a strategy to target proliferative cancer stem cells in ovarian cancer.

Recent studies have shown that pharmacological inhibition of ROS scavengers in glioblastoma stem cells decreases clonogenicity, resulting in radiosensitization (Diehn et al., 2009), and tumor sphere cells from hepatocellular carcinoma are arrested at the G0/G1 phase and lower ROS production compared to their parental cells (Hashimoto et al., 2014). In contrast, our findings exhibit that ROS are maintained at higher level in sphere forming cells than parental cells, even though detoxifying genes are expressed. It seems that maintaining relatively high level of ROS is caused by hypoxic condition within sphere, because hypoxia generates ROS which are promote angiogenesis, proliferation, and systemic metastatic capacity in cancer (Heddleston, Li, McLendon, Hjelmeland, & Rich, 2009; Selvendiran et al., 2009).

Mitochondrial metabolism is rising as a potential therapeutic target in cancers. It is important to determine how much cancers rely on mitochondrial metabolism. PGC-1α plays a central role in regulating the

transcriptional control of mitochondrial metabolism and biogenesis (Scarpulla, 2011; Wu et al., 1999). Recent reports have suggested that PGC-1 family is relevant to oncogenic properties, such as metastasis and chemoresistance of cancers. PGC-1α and mitochondrial transcription factor A (TFAM) are increased in high grade serous ovarian cancers with highly chemoresistant feature (Gabrielson, Bjorklund, Carlson, & Shoshan, 2014). Circulating breast cancer cells exhibits the enhanced mitochondrial biogenesis and respiration through PGC-1α expression, resulting in the increase of metastasis in breast cancer cells (LeBleu et al., 2014). In glioma CSC, an increased reliance of OXPHOS with a marked shift away from aerobic glycolysis is observed (Oliva, Moellering, Gillespie, & Griguer, 2011). In this study, when we induce to form tumor spheres, ROS inside spheres stimulate PGC-1α expression, leading to the increase of OXPHOS-related genes and mitochondrial complexes II, III, and IV. However, mitochondrial fission is shown in sphere forming cells. Even though it seems to be irrational that both mitochondrial biogenesis and fission are simultaneously increased, it may be possible that cancer cells stimulate biogenesis to compensate inefficient energy metabolism caused by mitochondrial fission under low oxygen condition.

We first provide important evidence that PGC-1α induced by oxidative stress mediates chemoresistance in ovarian cancer. Oxidative stress by low oxygen concentration inside tumor sphere increases mitochondrial

biogenesis and fission mediated by PGC-1α, resulting in the increase of CSCs with ALDH activity and chemoresistance in ovarian cancer. Many previous studies have focused on the increase of mitochondria related to caspase-dependent death pathways in cancer. However, the present findings provide the opposite functions of mitochondria increased by PGC-1α in terms of cancer cell survival. Although more studies are required to elucidate the detailed molecular mechanisms, these findings propose that ovarian cancer cells have functional mitochondria and PGC-1α can affect to determine chemoresistant phenotype in ovarian cancer cells.

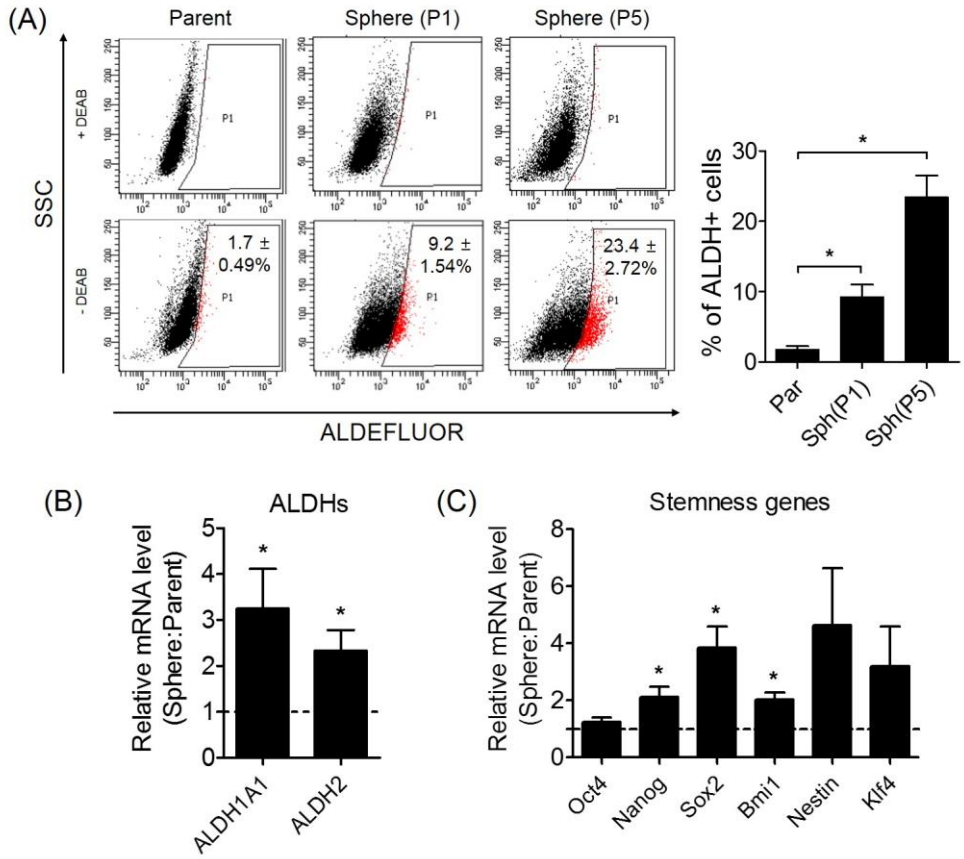


Figure 1. Tumor sphere formation enriches stem-like population in ovarian cancer cells. (A) Tumor sphere formation increased the activity of aldehyde dehydrogenase (ALDH; a cancer stem cell marker) which was detected by ALDEFLUOR (par, parental cells; sph, sphere cells). (B, C) Tumor sphere formation increased mRNA expression of *ALDHs* and stemness-related genes (*Oct4*, *Nanog*, *Sox2*, *Bmi1*, *Nestin*, and *Klf4*). Relative mRNA expression was evaluated by quantitative real-time PCR (qRT-PCR) and normalized to parental cells. All data were presented as the mean ± SEM of replicate experiments ($^{*}P < 0.05$, sphere vs. parent; one-

way ANOVA with Scheffe's post hoc test in figure 1A; Student's t-test in figure 1B and 1C).

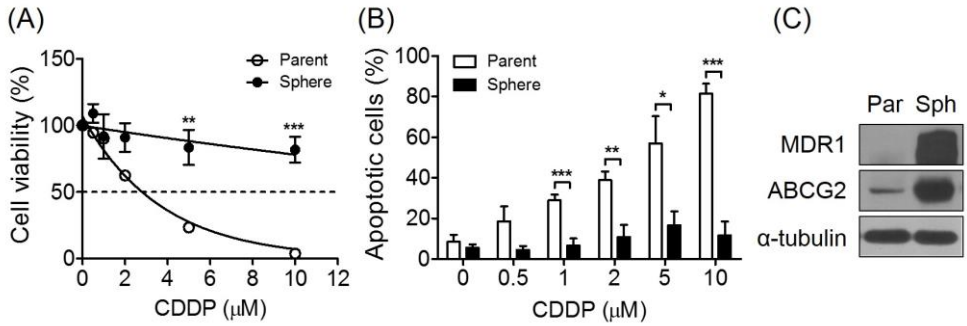


Figure 2. Tumor sphere formation exhibits enhanced drug-resistance in ovarian cancer cells. The indicated concentrations of cisplatin (CDDP) were treated in tumor sphere and parental cells for 48 h. (A) Tumor sphere cells showed the increase of IC_{50} values compared to parental cells. Cell viability was determined by MTT assay. (B) Apoptotic cells were significantly decreased in tumor sphere cells. This was determined by flow cytometry following annexin V and propidium iodide staining. (C) Expression of drug resistance-related proteins, such as MDR1 and ABCG2, was enhanced in tumor sphere cells. All data were presented as the mean ± SEM of replicate experiments ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, sphere vs. parent; Student's t-test).

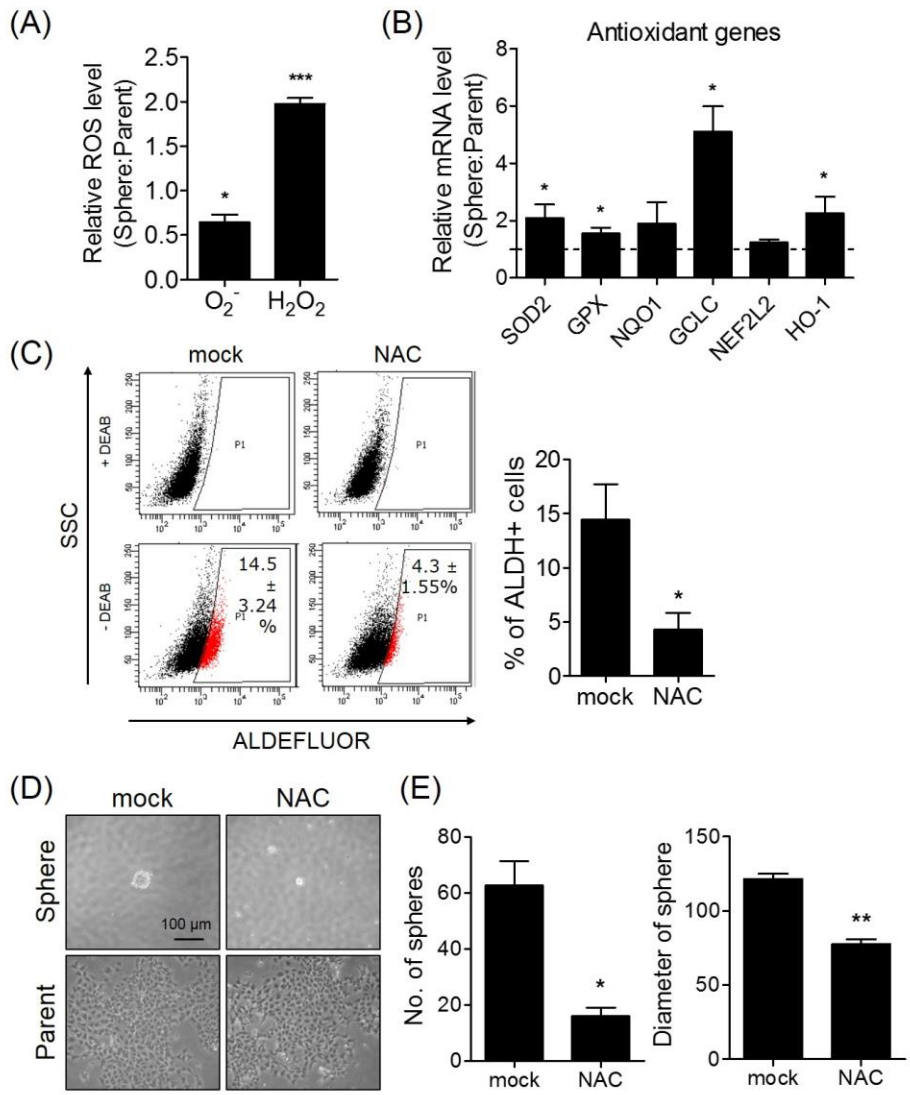


Figure 3. Tumor sphere formation causes an increase of intracellular ROS level accompanying stem-like phenotypical changes. (A) Hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) were relatively higher in tumor sphere than parental cells. Relative H_2O_2 and O_2^- level were detected using flow cytometry after DCFH-DA and DHE staining, respectively. (B) Tumor sphere showed higher expression of several antioxidant genes relative to

parental cells. The relative mRNA expression was determined by qRT-PCR. (C) ALDH-positive population was detected in NAC-treated and non-treated tumor sphere cells (10 mM for 4 days). Treating NAC alleviated ALDH activity in tumor sphere cells. ALDEFLUOR assay kit was used to assess a proportion of ALDH activity. (D) Tumor sphere and parental cells were cultured with/without 10 mM NAC for 4 days. Treating NAC decreased size of tumor spheres, while did not affect to parental cells. The morphology of tumor sphere and parental cells was determined by phase-contrast microscopy (Scale bar, 100 μm). (E) Diameter and the number of tumor spheres were significantly decreased in NAC-treated tumor spheres. Arbitrary unit was used for measuring the diameter of tumor spheres using ImageJ software. All data were presented as the mean ± SEM of replicate experiments ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; sphere vs. parent in Figure 3A and 3B; NAC-treated vs. mock in Figure 3C and 3E; Student's *t*-test).

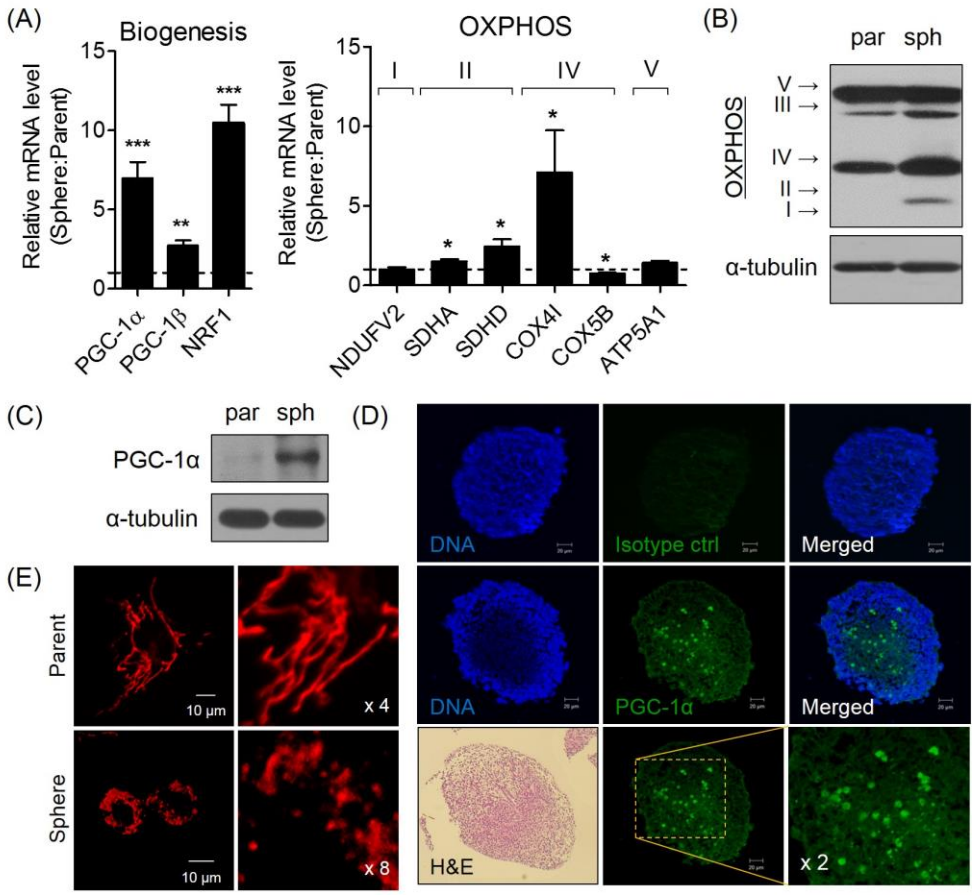


Figure 4. Tumor sphere formation induces PGC-1α expression resulting in mitochondrial changes. (A) Relative mRNA expression of genes related to mitochondrial biogenesis and oxidative phosphorylation (OXPHOS) was increased in tumor sphere cells. Values were normalized to parental cells. (B) Tumor sphere formation induced PGC-1α expression, verified by Western blotting (par, parental cells; sph, spheres). (C) Increased expression of OXPHOS complex II, III, and IV were observed in tumor sphere cells. (D) PGC-1α expression was localized in the center of tumor spheres. Immunocytochemistry was performed to detect PGC-1α within tumor

spheres. Blue and Green signals mean DAPI (nucleus) and PGC-1α, respectively. Isotype of IgG was used as a control. (E) Parental cells showed tubular structure of mitochondria, while sphere cells showed fragmented structure. For fluorescent labeling of mitochondria, pDsRed-Mito vector was introduced to parental and sphere cells. Mitochondrial morphology was observed using an LSM confocal microscope. All data were presented as the mean ± SEM of replicate experiments ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, sphere vs. parent, Student's *t*-test in figure 4A).

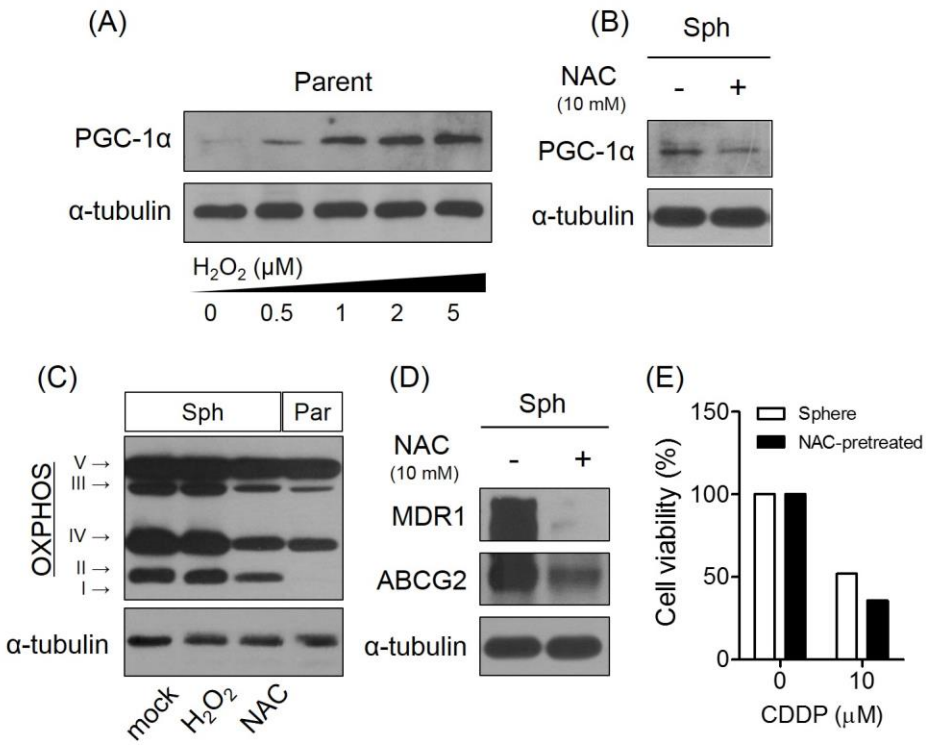


Figure 5. Scavenging ROS sensitizes tumor sphere cells to CDDP through the decrease of PGC-1α. (A) Exogenous addition of ROS caused PGC-1α expression in parental cells. (B, C) Inhibition of ROS generation using NAC (10 mM for 4 days) showed down-regulation of PGC-1α expression in sphere cells, leading to the decrease of mitochondrial mass (complex proteins). (D) Scavenging ROS in tumor spheres down-regulated expression of MDR1 and ABCG2 proteins. (E) NAC-pretreated sphere cells revealed the increased cell death.

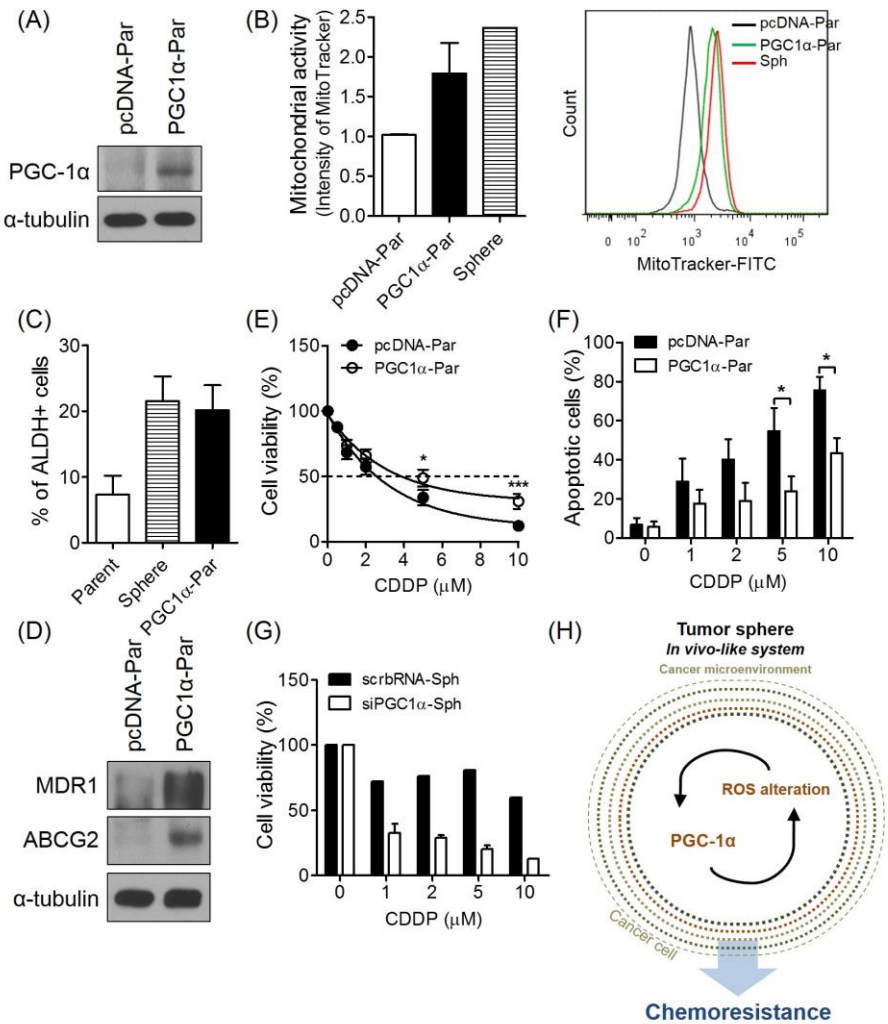
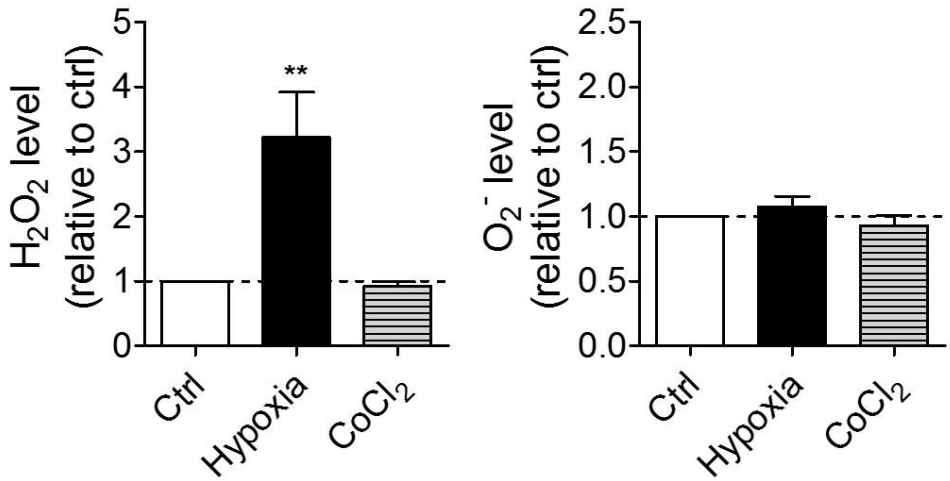


Figure 6. PGC-1α is involved in enhanced chemoresistance in ovarian cancer cells. (A) Parental cells were transfected with pcDNA-PGC1α vector, and the transformants were selected by treatment of 200 μg/ml Zeocin for three weeks. Overexpression of PGC-1α was confirmed by Western blotting. (B) Overexpressed PGC-1α in parental cells increased mitochondrial activity as much as of tumor sphere cells. Mitochondrial activity in tumor sphere and parental cells was measured with 300 nM MitoTracker Green

FM dye, and analyzed by flow cytometry (par, parental cells; sph, sphere; PGC1α-par, PGC-1α overexpressed parental cells). (C) Overexpressed PGC-1α parental cells showed tendency toward increased ALDH-positive population. (D) Overexpression of PGC-1α drove the parental cells to enhance drug resistance-related proteins. (E) Parental and PGC-1α-overexpressed cells were treated with serial concentrations of CDDP for 48 h. PGC-1α overexpressed cells became relatively more resistant to CDDP than parental cells. (F) Apoptosis was decreased in PGC-1α overexpressed cells. (G) Silencing PGC-1α in tumor sphere (siPGC-1α sphere) sensitized cell death to CDDP. Small interference RNA (50 nM) specific for PGC-1α was transfected to parental cells, and the cells were cultured 2 weeks for forming spheres. CDDP was treated for 2 days to sphere and siRNA-transfected sphere cells. (H) Summary of the present study. Data were presented as the mean ± SEM of replicate experiments ($^{*}P < 0.05$, $^{***}P < 0.001$, PGC-1α overexpressed cells vs. parent, Student's t-test in figure 6E and 6F).



Supplementary Figure 1. Hypoxia induces ROS generation in parental cells.

GENERAL CONCLUSION

In the dissertation, I tried to focus on the roles of ASCs in ovarian cancer progression and CSCs related to chemoresistance of ovarian cancer.

In Chapter I, this study showed the cancer-progression role of ASCs as a component to contribute to tumor microenvironment. ASCs were isolated from subcutaneous and visceral fats to identify phenotypical differences at the level of undifferentiated stem cells. At the level of differentiated adipocytes, anatomical origin of fats from which they are originated confers many differences between subcutaneous and visceral adipose tissue. Unexpectedly, we could not observe phenotypical difference between S- and V-ASCs to promote cancer progression. In order to cancer-promoting effects of ASCs, conditioned medium of ASCs (ASC-CM) was exposed to ovarian cancer cell line (SKOV3) and primary cancer cells (ascites cells). A particular factor from ASCs enhanced the relative proliferation and migration of SKOV3 and ascites cells. Among several adipokines, including IL-6, TNF-α, adiponectin, and leptin, IL-6 highly expressed in both S- and V-ASCs was related to the stimulating effects. IL-6 contained in ASC-CM was closely linked to activation of JAK2/STAT3 signaling pathway in ovarian cancer cells. These findings suggest that ASCs can influence the phenotypic change of ovarian cancer through secreting particular paracrine

factors. This implies that ASCs themselves serve as tumor microenvironment supporting particular factors to cancer, even though not fully differentiated to adipocytes or cancer-associated fibroblasts.

In chapter II, this study first showed that PGC-1α, a master regulator of mitochondrial biogenesis and metabolism, induced by the oxidative stress enhances chemoresistance of sphere forming cells in ovarian cancer. Sphere forming culture (3D culture) enriched cancer stem cells with high ALDH activity. Sphere forming cells had chemoresistant features, such as higher IC_{50} value than parental cells and expression of ABC transporter proteins. Although ABC transporters perform drug efflux, they cannot solely cause chemoresistance. Blockage of ABC transporters and simultaneously interruption of metabolizing/detoxifying chemotherapeutic drugs would be more effective. So, our focus moved to cancer metabolism. From our findings, there was PGC-1α in the center of the phenomena. We recognized that PGC-1α expression via ROS generation mediates chemoresistance in ovarian cancer cells. Forming spheres retained at low oxygen concentration due to limited diffusion drove ROS generation along with induction of antioxidant/detoxifying enzymes. Inhibition of ROS could not induce PGC-1α expression, resulting in alleviation of chemoresistance to CDDP treatment. Therefore, this indicated that ROS-mediated PGC-1α regulates stem-like properties and chemoresistance of cancer stem cells. However,

detailed mechanisms whether or how PGC-1α is related to ABC transporter proteins or metabolizes CDDP within cells are not uncovered. Further studies are necessary to understand more detailed.

These two studies show that ASCs participate in stimulating the growth and migration and CSCs contribute to enhancing chemoresistance in ovarian cancer cells. To date, researchers have focused on the inherent features of only cancer cells regardless of the interaction with other cells or their circumstance. From these studies, we suggest that targeting not only cancer cell itself, but also microenvironment and condition making/enriching cancer stem cells will be a more effective way for improving the prognosis of patients with ovarian cancer.

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국문 초록

난소암은 생존율이 낮은 부인암으로 약 75%의 경우 3기 이상의 진행성 난소암으로 진단되고, 초기 치료 후 80%의 환자에서 재발한다. 이는 효과적인 조기 발견 방법의 부재와 항암제에 대한 내성에 의한 것으로 지속적인 연구에도 불구하고 지난 10년간 예후의 뚜렷한 향상이 없었다. 2012년까지 10년간 전체 암의 5년 생존율은 증가하였으나, 난소암의 5년 생존율은 고작 3.2% 증가하였으며, 매년 난소암 환자의 50% 이상이 2 - 5년 내에 재발하며, 새롭게 진단받는 환자의 수는 지속적으로 증가하는 추세이다. 최근 암의 전이와 재발 및 약물 저항성은 종양 조직 주변의 미세환경과 종양 내부에 유발세포 또는 암줄기세포의 존재를 원인으로 보고 있다. 본 연구에서는 주변 미세환경을 구성하는 요소로서 지방조직을 생성하는 지방줄기세포(adipose stem cell)와 종양조직 내 암줄기세포(cancer stem cell)가 난소암의 진행 및 약물 저항성에 작용하는 역할과 그 기전을 알아보고자 한다.

비만은 심혈관 질환과 대사성 증후군을 포함한 여러 질병에 영향을 미치는 것으로 보고 되고 있다. 최근 암과 관련한 연구들이 보고되고 있으며, 그 중 호르몬 의존적인 암인 유방암과 자궁내막암에서 발생률과 전이 증가에 관한 상관관계 연구가 많이 진행되어 왔다. 난소암의 경우, 비만과 난소암 환자의 전체 생존율 및 암 발생률에 관한 역학적 연구가 보고되고 있지만 그 관계에 대한 정확한 결론은 얻어내지 못하고 있으며,

실험적 근거도 부족한 실정이다. 본 연구에서는 비만을 지방조직의 과다한 축적에 의한 것으로써 이를 생성해 내는 지방줄기세포에 그 원인을 두고 난소암의 진행에 어떠한 영향을 미치는지 알아보았다. 지방줄기세포는 개복을 요하는 환자의 피하 및 내장지방으로부터 분리하였고, 조혈줄기세포(hematopoietic stem cell)와 내피세포의 오염을 최소화하기 위해 자성 활성 세포 분류법(MACS)을 이용하여 CD31과 CD45 발현 세포를 제거하였다. 분리된 피하 및 내장지방줄기세포는 중간엽 유래 세포인 골세포(osteocyte), 연골세포(chondrocyte), 지방세포(adipocyte) 계열로의 분화가 가능했고, 중간엽줄기세포(mesenchymal stem cell)와 유사한 패턴으로 표면항원단백질을 발현하였다. 종양미세환경을 조성하는 요소로써 지방줄기세포가 난소암에 미치는 역할을 규명하기 위하여 지방줄기세포 배양액(conditioned medium)에 난소암 세포를 배양하였다. 그 결과 난소암 세포주(SKOV3)의 증식에는 영향을 주지 않았던 반면 난소암 세포의 이동(migration)은 통계적으로 유의하게 증가시켰다. 지방줄기세포의 배양액 중 어떤 요소들이 영향을 미쳤는지 알아보기 위해 역전사 중합효소연쇄반응(RT-PCR)을 이용하여 사이토카인(cytokine)의 발현을 조사하였다. 그 결과 여러 종류의 지방 유래 사이토카인 즉 아디포카인(adipokine) 중 IL-6 유전자의 발현이 높게 나타났으며, 효소면역측정법(ELISA)을 이용하여 피하 및 내장지방줄기세포의 배양액 속에서 분비 단백질 형태의 IL-6를 감지할 수 있었다. IL-6는 JAK2/STAT3 신호전달체계를 활성화 시키는 것으로 알려져 있어 난소암 세포주에서 이를 확인해 본 결과 지방줄기세포

의 배양액에 노출되면 30분 이내에 JAK2와 STAT3가 활성화 되었다. IL-6 중화항체를 이용하여 분비 단백질을 억제 했을 때, 난소암 세포의 JAK2/STAT3 활성화가 감소되었고, 이로 인하여 세포의 이동 또한 감소되었다. JAK2와 STAT3 억제제인 WP1066과 TG101348를 통해 위와 같은 효과를 보임을 입증하였다. 난소암 세포주뿐만 아니라 난소암 환자의 복수로부터 일차암세포(primary cancer cell)를 분리하여 앞선 과정을 확인해 본 결과 난소암 세포주와 유사하게 IL-6를 매개로 한 세포의 이동이 증가함을 확인하였지만, 난소암 세포주와는 다르게 지방줄기세포의 배양액에 노출된 경우 세포의 증식이 증가하는 결과를 보여주었다. 따라서 지방줄기세포는 종양 미세환경의 일부로 작용하여 측분비인자(paracrine factor)를 통해 난소암 세포의 증식과 이동을 촉진시킨다고 할 수 있으며, 이 결과는 간접적으로 비만과 난소암의 관계에 관한 실험적인 증거를 제시하는 연구가 될 수 있을 것이다. 또한 완전히 분화된 지방세포와는 달리 미분화상태인 지방줄기세포의 해부학적 차이(피하 또는 내장지방)는 통계적으로 유의한 영향을 미치지 않았다.

자기복제능(self-renewal)과 종양 유발 가능성(clonal initiating potential)을 가지는 암줄기세포는 약물유출(drug efflux), 해독과정(detoxification), 휴면상태의 유도(dormancy)등의 방법들을 통해 난소암이 약물에 대한 저항성을 갖게 한다. 본 연구에서는 3차원 배양 방법 중 하나인 구형성배양(sphere formation culture)을 통해 aldehyde dehydrogenase(ALDH; 난소암줄기세포 표지자)의 활성화가 높은 암줄기세포를 증가시키고, 이 세포가 어

떠한 기전을 통해 약물저항성을 갖게 되는지 알아보았다. 구형성배양법은 높은 ALDH 활성화를 가지는 세포 즉 난소암줄기세포의 증가를 유도하였고, 이는 항암제 cisplatin에 의한 세포사멸의 감소와 ABC transporter인 MDR1과 ABCG2의 발현 증가를 통해 약물저항성을 가진다는 것을 확인하였다. 구형성배양법은 영양소나 산소의 투과 또는 산도(pH)에 있어서 실제 종양조직과 유사한 환경을 만들어준다. 3차원 배양 시 구 내부의 산소 투과도는 감소하여 저산소상태(hypoxia)가 되고 이는 활성산소종(reactive oxygen species)을 증가시키는 원인으로 보고되고 있다. 본 연구에서도 구형성배양 시 내부 활성산소종 중 과산화수소의 발생이 증가 하는 것을 확인하였고, 이와 함께 항산화 효소(antioxidant enzyme)들의 발현도 증가함을 확인하였다. 활성산소종에 의한 산화적 스트레스는 PGC-1α의 발현을 유도하는 것으로 알려져 있으며, 이는 항산화 효소들의 발현에 있어서 필수적이다. 본 연구에서도 구형성배양 시 나타난 산화적 스트레스에 의하여 PGC-1α의 발현이 유도되었고, 이로 인해 미토콘드리아 생합성 및 에너지 대사 관계 효소들의 유전자 발현이 증가하였다. N-acetyl-cysteine을 이용하여 활성산소종을 제거하였을 때 ALDH의 활성화 감소와 함께 PGC-1α의 발현이 감소함을 확인하였다. PGC-1α의 약물저항성 관계 여부를 확인하기 위해 모세포에 PGC-1α의 과발현을 유도하였을 때 ALDH 활성이 증가하였고, 약물저항성 관계 단백질인 ABC transporter의 발현이 증가하였다. 반대로 구형성세포에 siRNA를 이용하여 PGC-1α의 발현을 억제시킨 결과 항암제 cisplatin에 대한 저항성이 감소되었다. 따

라서 구형성배양은 실제 종양조직과 유사한 환경을 조성함으로써 종양유발세포를 증가시키고, 여기에 산화적 스트레스로 인한 PGC-1α의 발현 증가가 약물에 대한 저항성을 증가시킨다고 결론 지을 수 있다. 난소암에서 PGC-1α의 약물저항성 관계여부는 기존에 보고된 바 없으며, PGC-1α의 발현을 통해 난소암 세포는 증식을 위한 에너지 생산에 있어서 와버그 효과(Warburg effect)에 의한 포도당 대사(glucose metabolism)에만 의존하는 것이 아니라 미토콘드리아 대사 또한 이용할 것이라 추측해볼 수 있다.

기존의 연구는 난소암 그 자체를 표적으로 하여 유전자 수준의 돌연변이(genetic mutation)와 발암촉진유전자(oncogenic driver)를 찾는데 집중하였다. 그러나 The Cancer Genome Atlas (TCGA) 연구를 통해 일차적 난소암과 전이된 조직 사이에서의 유전자 수준에서의 돌연변이는 p53을 제외하고 거의 없음을 확인할 수 있었다. 이는 효과적인 난소암 치료제의 개발을 위해서는 난소암 그 자체를 표적으로 할뿐만 아니라 종양 외부에 존재하는 주변 미세환경과 종양 내부 환경(산소 및 영양소 투과의 제한)에 의한 암줄기세포의 발생을 고려해야 한다는 것을 의미한다. 따라서 본 연구에서 제시된 종양미세환경으로써 지방줄기세포와 종양 내부 환경에 의해 증가된 암줄기세포를 표적으로 한다면 난소암 치료에 대한 반응성을 증가시킬 수 있을 것이고 이로써 좋은 예후를 기대할 수 있을 것이다.