T.C.
DOKUZ EYLUL UNIVERSITY
IZMIR INTERNATIONAL
BIOMEDICINE AND GENOME
INSTITUTE

EXOSOME-MEDIATED INDUCTION IN TUMORIGENESIS OF OVARIAN TUMOR MICROENVIRONMENT CELLS

GİZEM YILMAZ

MOLECULAR BIOLOGY AND GENETICS

MASTER OF SCIENCE THESIS

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Dokuz Eylül University İzmir International Biomedicine and Genome Institute
Department of Genomics and Molecular Biotechnology,
Molecular Biology and Genetics graduate program Master of Science student
Gizem YILMAZ has successfully completed her Master of Science thesis titled
'EXOSOME-MEDIATED INDUCTION IN TUMORIGENESIS OF OVARIAN TUMOR MICROENVIRONMENT CELLS' on the date of
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ABBREVIATIONS

A2780: Human Primary Ovarian Carcinoma Cell Line

A2780cis: Cisplatin Resistant A2780 Cells

abm: Applied Biological Materials

Alix: ALG-2-interacting protein X

ATP: Adenosine Triphosphate

BCA: Bicinchoninic Acid

BIOMER: Biotechnology and Bioengineering Research and Application Center

BSA: Bovine Serum Albumin

CA125: Cancer Antigen 125

CAFs: Cancer-Associated Fibroblasts

CAMs: Cell Adhesion Molecules

CASH: Cancer and Steroid Hormone

CDE: Caveolin-dependent Endocytosis

CFDA: 5(6)-carboxyfluorescein diacetate

CFSE: Carboxyfluorescein Succinimidyl Ester

CME: Clathrin-mediated Endocytosis

CT: Computed Tomography

DCs: Dendritic Cells

DiD, DiIC18(5): 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt

DiI, DiIC18(3): 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate

ECACC: European Collection of Authenticated Cell Cultures

ECM: Extracellular Matrix

EGFR: Epithelial Growth Factor Receptor

EIPA: 5-(N-Ethyl-N-isopropyl)amiloride

EMT: Epithelial Mesenchymal Transition

EOC: Epithelial Ovarian Cancer

ER: Endoplasmic Reticulum

ESCRT: Endosomal Sorting Complexes Required for Transport

EVs: Extracellular Vesicles

FAK: Focal Adhesion Kinase

FBS: Fatal Bovine Serum

FIGO: The International Federation of Gynecology and Obstetrics

GTP: Guanosine Triphosphate

HGSOC: High-grade serous ovarian cancer

HRT: Hormone Replacement Therapy

HSP70: Heat Shock Protein 70

IgSF: Immunoglobulin Superfamily Members

IL-6: Interleukin-6

IL-8: Interleukin-8

ILVs: Intraluminal Vesicles

MßCD: Methyl-ß-cyclodextrin

MAPK: Mitogen-Activated Protein Kinase

MeT-5A: Mesothelial Cells

miRNA: MicroRNA

MMPs: Matrix Metalloproteinases

MRI: Magnetic Resonance Imaging

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MU16: Mucin 16

MVBs: Multivesicular Bodies

Na+/H+ exchanger, NHE: Sodium/proton exchanger or sodium/hydrogen exchanger

OSE: Ovarian Surface Epithelium Cell

PBS: Phosphate Buffer Saline

PFA: Paraformaldehyde

PKH26: Paul Karl Horan 26

PKH67: Paul Karl Horan 67

PS: Penicilin-Streptomicin

qPCR: Quantitative Polymerase Chain Reaction

RH: Relative Humidity

RhB, R18: Rhodamine B

ROS: Reactive Oxygen Species

RT: Room Temperature

SDS: Sodium Dodecyl Sulfate

TCGA: The Cancer Genome Atlas

TEVs: Tumor-Derived Extracellular Vesicles

TNF-α: Tumor Necrosis Factor-α

TNM: Tumor, Node, Metastasis

TSG101: Tumor Susceptibility Gene 101 Protein

VEGF: Vascular Endothelial Growth Factor

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EXOSOME-MEDIATED INDUCTION IN TUMORIGENESIS OF OVARIAN TUMOR MICROENVIRONMENT CELLS

Gizem Yılmaz, İzmir International Biomedicine and Genome Institute, Dokuz Eylul University Health Campus, Balcova 35340 - Izmir / TURKEY

ABSTRACT

Ovarian cancer, seventh most extensive reason of cancer demise in women, has a low incidence of 3% among gynecological diseases. It is substantial to explain the carcinogenesis mechanism of ovarian cancer because of difficulties in diagnosis at early phase, prognosis at late stages, and low survival rate after surgery and chemotherapy. The purpose of this research is to demonstrate the induction of tumorigenesis in tumor microenvironment cells after uptake of oncogenic signal carrying ovarian cancer-related exosomes.

Exosomes were isolated from cisplatin-resistant A2780 cells by ultrafiltration and differential centrifugation. Primary ovarian cancer (A2780), ovarian surface epithelial (OSE) and mesothelial (MeT-5A) cells were used as recipients. Isolated exosomes were characterized by western blotting and particle size analysis. Internalization of PKH26-labeled exosomes into the recipient cells was examined by confocal microscopy. Uptake mechanisms of exosomes were detected by using inhibitors via flow cytometry. The effects on the differentiation of EMT, ROS production, invasion, migration and proliferation rates were examined following exosome uptake. Isolated exosomes ranging from 30-150 nm in diameter, contained exosome marker proteins Alix and Tsg101, except ER marker; calnexin. PKH26 labeled-exosomes were internalized by recipient cells in 6 hours by different endocytic mechanisms. ROS production, invasion and migration rates increased in A2780 and OSE cells except in MeT-5A cells. EMT and proliferation were induced following exosome uptake.

In conclusion, the carcinogenesis mechanism of recipient cells in the primary and secondary tumor microenvironment of ovarian cancer was induced after the uptake of ovarian cancer-released exosomes.

Key Words: ovarian cancer, exosome, tumor microenvironment, invasion, migration

YUMURTALIK TÜMÖR MİKROÇEVRESİNDEKİ HÜCRELERDE TÜMOR OLUŞUMU VE GELİŞİMİNİN EKSOZOM ARACILIĞIYLA UYARILMASI

Gizem Yılmaz, İzmir Uluslararası Biyotıp ve Genom Enstitüsü, Dokuz Eylül Üniversitesi Sağlık Yerleşkesi, Balçova 35340 - İzmir / TÜRKİYE

ÖZET

Jinekolojik hastalıklar arasında 3% oranı ile düşük insidanslı olmasına rağmen ovaryum kanseri, kadınlarda kansere bağlı ölümlerde yedinci sırada yer almaktadır. Erken dönemde diyagnozunun zor olması, hastalığın son evrelerinde teşhis edilebilmesi, ameliyat ve kemoterapi sonrası sağkalım oranının düşük olması sebebiyle ovaryum kanserinin karsinojenez mekanizmasının açıklanması oldukça önemlidir. Bu çalışmada kanser hücrelerinden salınan onkojenik sinyal taşıyan eksozomların, tümör mikroçevredeki hücreler tarafından alınmasıyla tümörigenezin uyarıldığını göstermek amaçlanmıştır.

Cisplatin dirençli A2780 hücrelerinden salınan eksozomlar ultrafiltrasyon ve diferansiyel santrifüjleme yöntemi ile izole edildi. Primer yumurtalık kanseri (A2780), ovaryum yüzey epitelleri (OSE) ve mezotelyel (MeT-5A) hücreler hedef olarak kullanıldı. İzole edilen eksozomlar, immunoblot analizi ve nanopartikül boyut ölçümüyle karakterize edildi. PKH26 ile işaretlenen eksozomların hedef hücrelere alınması konfokal mikroskobuyla gözlenirken hücre içine alınım yolları inhibitörler kullanılarak akış sitometrisiyle belirlendi. Eksozom alınımıyla ROS üretiminin farklılaşması, migrasyon, invazyon ve proliferasyon hızları ile EMT üzerindeki etkileri incelenmiştir. Boyutu 30-150 nm arasında bulunan eksozomlar, eksozom proteinleri olan Alix ve Tsg101 içerirken ER belirteci olan calnexini içermemektedir. PKH26 ile işaretlenen eksozomlar hedef hücrelere farklı endosomal yolları kullanarak 6 saatte girmektedir. Eksozom alınımından sonra ROS üretimi, migrasyon ve invazyon hızları MeT-5A hücreleri hariç, A2780 ve OSE hücrelerinde artmıştır. EMT ve proliferasyon hızları da etkilenmiştir.

Sonuç olarak, yumurtalık kanserinin primer ve sekonder tümör mikroçevresindeki hedef hücrelerin karsinojenez mekanizması, yumurtalık kanserindeki eksozomların alınmasıyla uyarılmıştır.

Anahtar Kelimeler: yumurtalık kanseri, eksozom, tümör mikroçevresi, migrasyon, invazyon

1. INTRODUCTION AND AIM

Ovarian cancer, one of the most substantial problems among women, also leads to numerous-deaths due to absence of disease-specific clinical symptoms and lack of effective screening techniques. In addition, it has a complex characteristic because of being heterogenous disease subdivided into at least five different subtypes. This heterogeneity results in indication of different symptoms between each patient so that targeted therapy for ovarian cancer also needs to be patient-specific. Furthermore, this specificity can cause challenges against controlling disease for other patients therefore, new researches are developing for understanding the factors especially cell-cell communication effecting progression of this disease from primary tissue to metastasis.

Cell-cell communication provides cancer cells to induce the differentiation of neighbor cells and spread of cancer to the cells located in tumor microenvironment. Thus, tumor microenvironment is very essential for cancer progression due to supplying the required environment for metastasis and consisting of diverse cells according to different stages of the tumor. In the initial stages of the tumor; primary region where the tumor is first formed, especially in the cancer types formed by differentiation of epithelial cells, it consists of epithelial and myoepithelial cells. However, in later stages in which tumor reaches to secondary region, its microenvironment comprises of diversified kinds of cells as; fibroblasts, adipocytes endothelial, epithelial and immune cells.

The communication between tumor microenvironment and cancer cells is achieved both by direct contact with cell adhesion factors and by secreted paracrine factors consisting of cytokine and pro-angiogenic-like-released proteins, nucleic acids and extracellular vesicles. In order to provide this interaction, cell-cell communication key mediators; exosomes in size 30-150 nm, including endocytic cargos such as microRNA (miRNA), mRNA, DNA fragments, and proteins released from cancer cells, allow reprogramming of the tumor microenvironment. Furthermore, they provide the transfer of pro-tumorigenic conditions after uptake into less metastatic character or non-chemotherapeutic-drug-resistant cancer cells, normal epithelium, mesothelial, fibroblast, adipose and mesenchymal stem cells or endothelial cells. In recent

studies and proofs suggest cancer derived exosomes take part in tumor growth, tumorigenesis, tumor immune escape, angiogenesis; also, especially in drug resistance and metastasis.

To indicate the internalization of exosomes into the recipient neighbor cells, we first isolate nanoparticles from drug-resistant ovarian cancer A2780cis cell line by different ultrafiltration and ultracentrifugation. The characterization of these nanoparticles was carried out via western blotting and size measurement analysis. The "exosome" character of these nanoparticles was defined after observing exosomal markers Alix and Tsg101 and measurement of size between reference range 30-150 nm. Then, exosomes were labelled with PKH26 to indicate its internalization by fluorescent imaging into recipient primary ovarian cancer A2780 cells, ovarian surface epithelial OSE cells and mesothelial MeT-5A cells. To state the uptake mechanism of different cells, recipient cells cultured with PKH-26 labelled exosomes were analyzed by flow cytometry in the attendence of different inhibitors. The target location of PKH- 26 labelled exosomes in recipient cells were observed by confocal microscopy. Finally, the differentiation in the oncogenic signal mechanisms such as; migration, invasion and proliferation rates, reactive oxygen species (ROS) generation and epithelial-mesenchymal transition (EMT) were examined.

The results we achieved after this research can be evaluated as; the proof of internalization of drug-resistant ovarian cancer-released exosomes into the primary and secondary ovarian tumor microenvironment cells by utilizing different exosome uptake mechanisms. Furthermore, with this internalization; cancer progression can be induced with exosome mediation on oncogenic signal mechanisms consisting of migration, invasion, ROS, EMT and proliferation.

H1: We hypothesize that cell-cell communication key mediators; exosomes isolated from drug-resistance ovarian cancer can be uptaken into various cells located in primary and secondary tumor microenvironment of ovarian cancer via utilizing different endocytic exosome uptake pathways.

H2: We also hypothesize that drug-resistant ovarian cancer released exosomes can mediate tumorigenesis by inducing differentiation in oncogenic signal mechanisms including; ROS, migration, invasion, epithelial-mesenchymal transition and proliferation.

2. GENERAL INFORMATION

2.1. Ovarian Cancer

Nowadays, the researchers are focusing to find developments about the diagnosis and the treatment of cancer, also known as one of the most crucial health problems and the most common diseases of our age. Because of causing high mortality among the world, cancer is also described as a major worldwide burden [1]. Ovarian cancer is one of the most substantial global problem due to causing numerous lethal gynecological malignancies in women. It is the seventh most extensive reason of cancer demise in women, although it has a low incidence of among gynecological diseases [2, 3]. In gynecologic cancers, ovarian cancer finds itself at third place in the ranking after uterine and cervical cancer [4]. Apart from that, highest mortality rate and worst prognosis also belong to ovarian cancer [5]. When compared to breast cancer, the prevalence is lower but opposite to this, lethality of ovarian cancer is three times higher [6].

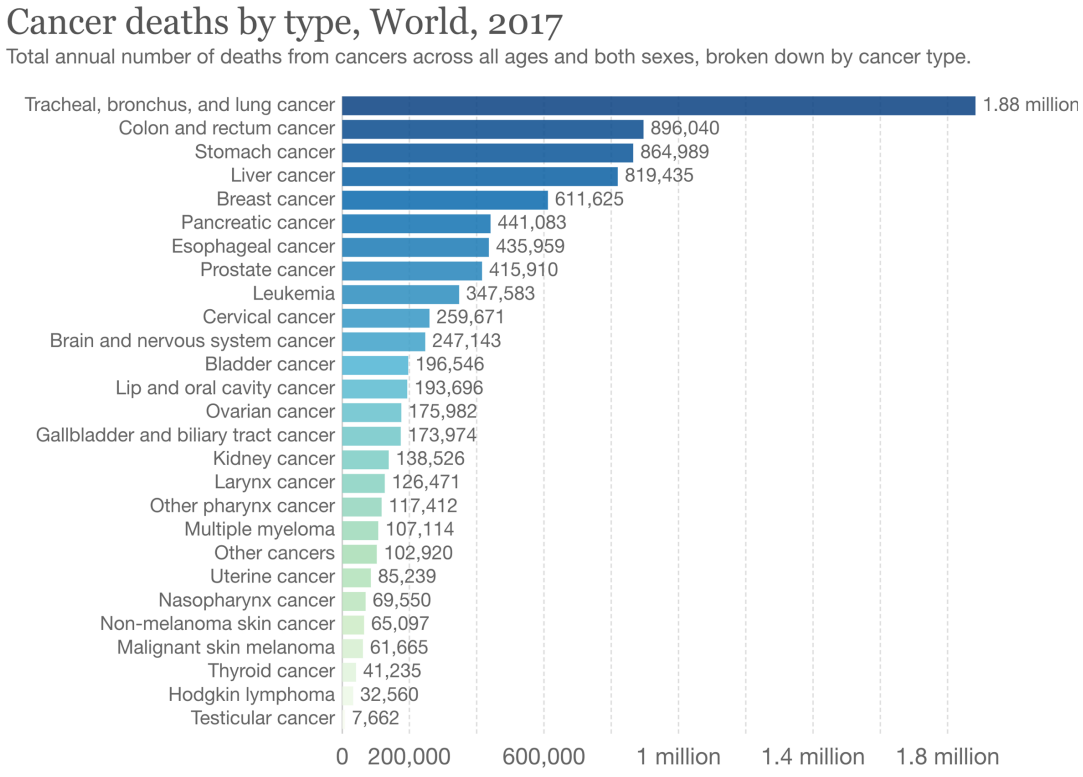


Figure 2.1. Total annual number of deaths in 2017 from cancers according to type among world [7].

According to the researches and statistics, it was estimated that almost 22.240 diagnosed novel ovarian cancer cases and 14.070 ovarian cancer caused deaths in United States of America during 2018 [8, 9]. The priority of the recent studies is to early detect and improve the prevention of ovarian cancer due to 5-year of survival rate is roundly 93% when the patient diagnosed at the local stage [10]. However, absence of disease-specific clinical symptoms, secretly growing tumor and insufficient effective screening tools procure the diagnosis of ovarian cancer at advanced stage which results in mortality by a majority. Hence, this cancer has gained another name called silent-killer [6, 11, 12].

As mentioned before, it is hard to recognize the symptoms of ovarian cancer at early stages since there are no visible symptoms about the disease [13]. Unfortunately, sometimes these symptoms can be evaluated mistakenly as minor illnesses due to imitating the common issues also for digestive and stomach problems [14]. Therefore, women can recognize the symptoms when the cancer passes the boundary of the ovaries and spreads to the pelvic or abdominal organs and lymph nodes. Moreover, cancer mass-related pressure on these organs provides to uncover the inevitable symptoms [15]. Common symptoms of ovarian cancer after early stage can be aligned as; abdominal bloating, pelvic and back pain, irregular menstruation, vaginal bleeding problems during sexual intercourse or post menopause, nausea, diarrhea, loss of appetite, fatigue and urinary issues [16].

2.1.1. Types of Ovarian Cancer

Ovarian Cancer can vary into more 30 different types, classified and determined by the type of the cell where cancer starts. In addition, it subdivides into heterogenous group of malignant types differentiated by diverse conditions. Risk factors, prognosis of the cancer, treatment, pathological grade and especially site of the origin contribute to create this heterogeneous malignant groups [17, 18].

Three mainly common cell types that form cancerous ovarian tumors can be aligned as; surface epithelium cells, stromal cells and germ cells. Surface epithelium tumors are formed after covering of the outer lining of the ovaries by the cancerous cells. Epithelial malignancies are highly aggressive when compared to non-epithelial cancers including sex-cord stromal cells

and germ cell. In all ovarian cancers, these 2 subtypes of non-epithelial cancers encompass for only 2% and 3% respectively. Furthermore, non-epithelial ovarian cancers also cover ovarian sarcoma and small cell carcinoma. When germ cells form tumors, they are impelled to form eggs in reproductive system. Stromal cells are responsible for hormone releasing and communication between different parts of the ovaries. Thus, they form tumors originally at connective tissue cell kinds such as; Sertoli either Leydig cells and granulosa cells [19].

2.1.1.1. Epithelial Ovarian Cancer

Epithelial ovarian cancer (EOC) cells are the most dangerous and widespread type of cancer forming ovarian cells due to accounting approximately 90% of ovarian cancer. Tumor cell histology separated epithelial cells in different phenotypes such as; serous (also known as fallopian-tube like epithelium) formed 52%, endometrioid (also called endometrium-like epithelium) formed 10% and clear cell (gestational endometrium epithelium) around 6% or mucinous (colonic or endocervix epithelium) around 6% mainly [10, 20]. There are also small groups of epithelium cancer called urinogenital tract epithelium also known as Brenner or transitional tumors [21]. Furthermore, based on clinicopathologic factors and epithelial malignant cancers are classified into 2 main groups known as type I and type II. When type I and type II epithelial malignancies compared to each other, genetic instability is noted as their uppermost distinguishing molecular factor [18].

Type I of the epithelial cancers is usually defined as unilateral, huge and cystic tumors when they were diagnosed with indolent behavior. The development of this type of epithelial cancer is presumed to evolve from extraovarian benign lesions which are also embedded in the ovaries. Afterwards, benign lesions gain metastatic characteristic due to undergoing various mutations. This metastatic transformation leads to formation of low-grade serous carcinoma, endometrioid or clear cell carcinomas and lowest grade mucinous carcinomas originating from various benign foci such as; fallopian tubes (endosalpingiosis), endometrial tissue (endometriosis) and tuboperitoneal junctions inside ovaries, respectively. (Figure 2.2) In general, type I of the epithelial ovarian cancer are frequently at early stage and low grade, their progression develops slowly and in an indolent way additionally. When compared to type II cancers, they are related to the 10% of fatal cases from ovarium cancer [18].

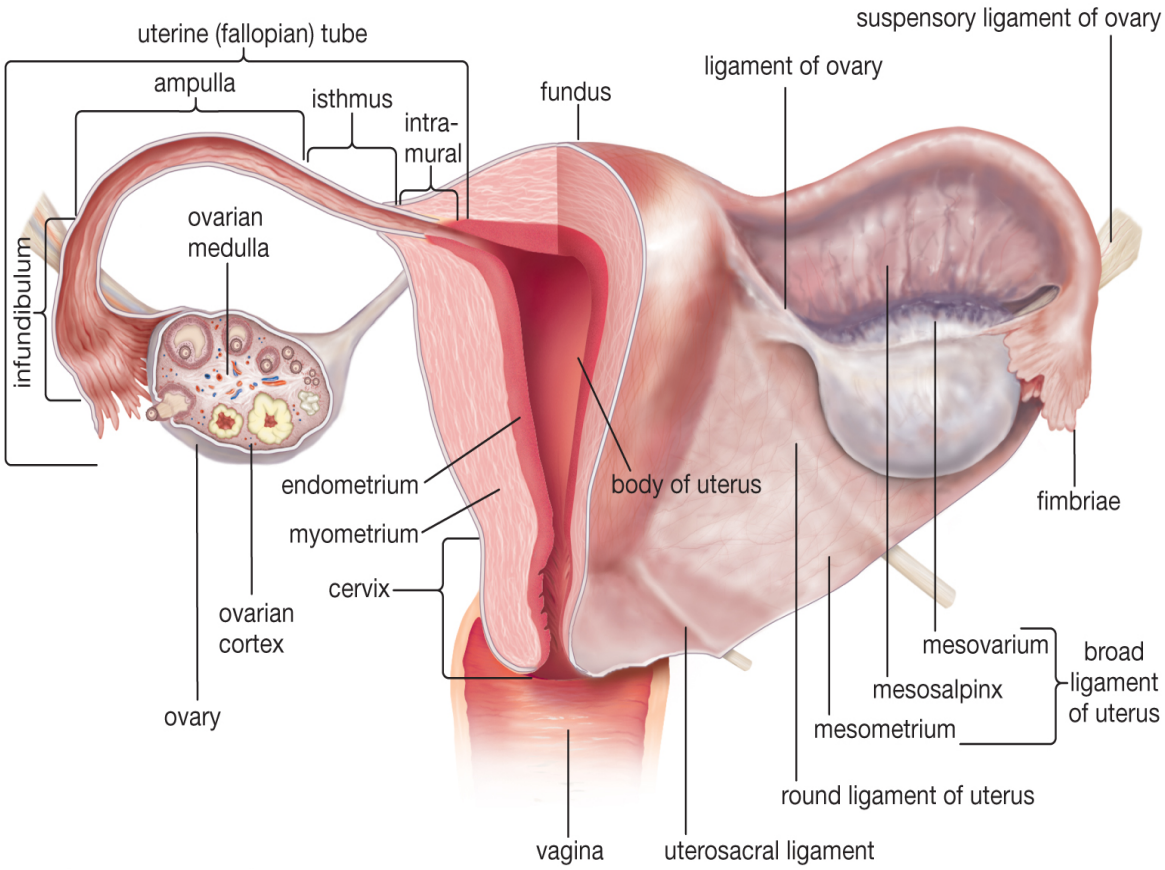


Figure 2.2. The anatomy of the female reproductive system [22].

Unlike type I epithelial cancers, type II of epithelial cancers is evaluated as high grade and almost all the time late or advanced stage. Progression of type II develops rapidly and aggressively. The aggressive behavior of type II epithelial cancer tends to involve both ovaries and following metastasis leads to poor prognosis and low survival rate. Fallopian tube fimbriae carcinomas are the sources for type II ovarian cancers to progress their spread especially to ovaries following peritoneum [18, 23, 24]. The spread of type II ovarian cancers into peritoneum appears as spacious extraovarian ailment and ascites, which commonly present in women prognosed with these cancers. Type II includes not only the most widespread subtype of epithelial cancer; high grade serous carcinoma but also contains undifferentiated and normal carcinomas [18]. When the molecular level of both type of tumors were examined; it can be detected that tumors evaluated as type I are often related with BRAF and KRAS whereas, tumors evaluated as type II are related with p53 mutations [25, 26].

2.1.2. Risk Factors of Ovarian Cancer

Ovarian cancer leads to one of the highest mortality rates among world as mentioned before. In addition, the differentiation in tissue or molecular biomarkers are also unpredictable and undetectable at early stage of ovarian cancer because of most of this type of cancer appears at advanced stage [27]. However, if the differentiation of these biomarkers were detectible and/or predictable, another factor; the relative inaccessibility of the ovary in the body would lead to challenges to detect the women under increased or high risk [28]. When these factors and mortality of ovarian cancer are considered, the risk factors of ovarian cancer play an essential role for developing new treatment approaches and preventing the complications causing high rate mortality [2]. The commonly known risk factors to adjust the potential ovarian cancer patients are aligned in table hereinafter. (Table 2.1)

Table 2.1. The commonly known risk factors of Epithelial Ovarian Cancer [2].

Factors		**Protective**	**Predisposing**	**Controversial**
Demographic	Age		✓	
Reproductive	Menstrual-related factors		✓	
	Age of menarche and menopause			✓
	Parity	✓		
	Pregnancy characteristics			✓
	Higher age of childbirth	✓		
Gynecologic	Pelvic inflammatory disease			✓
	Endometriosis		✓	
Hormonal	Contraceptive methods	✓		
	Hormone Replacement Therapy			✓
	Infertility treatments			✓
Genetic	Family history		✓	
	BRCA mutations		✓	
	Lynch Syndrome		✓	
Lifestyle	Nutrition and Diet			✓
	Obesity and physical activity			✓
	Alcohol, caffeine and cigarettes			✓
Other	Lactation	✓		
	Lower socioeconomic status		✓	

The risk factors of ovarian cancer seen in Table 2.1 are classified into categories as; primarily hereditary, reproductive, inflammatory, geographic, surgical, dietary and hormonal. One of the most important and critical risk factors; hereditary, also defined as familial background mostly increase ovarian cancer risk from first-degree relatives [29, 30]. Hereditary-affected ovarian cancer was first assigned and documented in 1866 with the familial background study covering ovarian and breast cancer about the wife of the scientist; Pierre Paul Broca [31]. Women who have ovarian cancer relatives in first and second degree are more likely to be at risk for being ovarian cancer when confronted to women with no ovarian cancer related family background. In addition, after the examination of CASH (Cancer and Steroid Hormone) study data in 1980s, it is elicited that family background including either ovarian or breast cancer enhance the risk of both types of cancer occurring in first-degree relatives [32, 33, 34, 35]. The Cancer Genome Atlas (TCGA) carried out an exhaustive analysis in 2011 for detecting number of genes mutated significantly in one of the exceedingly mutated ovarian cancer types; high-grade serous ovarian cancer (HGSOC). According to the results, approximately 96% mutated p53 was the most remarkably gene for HGSOC. Apart from p53, BRCA 1/2 were also the most essential genes have a role in progression of many HGSOC, regardless of the germline status [36]. Lynch syndrome is also evaluated as one of the most critical predisposing risk factors because of being responsible for 10-15% of inherited ovarian cancer progression [37]. It is an autosomal dominant syndrome seen in 6-8% in individuals with the Lynch syndrome family background in their lifetime [38]. Most cases of ovarian cancer related to Lynch syndrome are involved in non-mucinous type and approximately 82-84% of these cases are staged at level I or II [37]. Mutations in MLH1 and MSH2 are the most extensive ones observed in individuals [39]. In addition, Lynch syndrome is comprised from hereditary mutations mostly mismatch repair genes like; MSH2, MSH6, PMS2 and MLH1 [40].

Outside of hereditary; age is among the most predisposing risk elements that stimulates ovarian cancer to develop especially in postmenopausal period [41, 42]. Ovarian cancer demonstrates rising incidence mostly marked in the women over the age of 65 [43]. Although the previous researches supported the idea that the average age of ovarian cancer diagnosis was between ages 50-79 [42, 44, 45]; the connection of age-to-ovarian cancer outcome preserves its mystery due to the correlation between younger age and improved outcome of ovarian cancer in some researches [44, 46, 47] and statement of age cannot be considered as an independent

prognostic factor in other studies [48]. In addition, for ovarian cancer older age factor is also related to lower survival rate and diagnosis of more highly advanced staged disease [42, 49]. The lower survival rate is seen mostly in women-with older age when compared to younger women; since the less aggressively treatment is applied to them [50]. Age is not only essential for developing ovarian cancer, but also age-related to menarche, menopause and childbirth are critical factors. As mentioned before, age is a controversial factor also in menarche and menopause since some cases encourage the relation between ovarian cancer outcome incidence and the beginning of menarche [51, 52] unlike, other studies indicate no relation between these elements and risk in ovarian cancer [53, 54, 55, 56, 57]. However, risk of ovarian cancer diminished in the women with older-aged pregnancy when the various clinical patients observed. This reduced risk is also connected with the number of pregnancies these women had [58, 59, 60]. Besides, according to another study the risk in ovarian cancer can be decreased as much as 10% when the first childbirth age rose for each 5 years [61].

Except age, reproductive factors can be mentioned as; menstrual-related factors and parity. Menstrual-related factors are mostly consisting of menstrual periods and ovulation cycles. Ovarian cancer risk and ovulation cycles share a relationship inversely according to plenty researches [62, 63]. Thus, this opinion promotes the theory called “incessant ovulation” explained as; continuously ovulation can provide the development in the incidence of ovarian cancer due to harming the epithelium part of the ovaries. Hence, any agent that lends to the decrease in ovulation may own preventive influence against formation of ovarian cancer [64]. Unlike ovulation frequency, pregnancy is evaluated as a preserving factor versus ovarian cancer [57, 59, 60, 63]. Also, according to the reported research, the increase in the number of giving births is related to coherent decline in the jeopardy of invasive ovarian, germ-cell, stromal and epithelial cancer formation [61]. Moreover, another research mentioned that less offensive disease can be related to protective effect of pregnancy when compared to advanced level of ovarian cancer [49].

When gynecologic or inflammatory factors examined; endometriosis and ovarian cysts play an exactly predisposing role in the ovarian cancer, but pelvic inflammatory disease is controversial when compared to others [65, 66]. When pelvic inflammatory disease is taken into consideration, researchers separated into two groups; the group which enounces the

essential contribution of inflammation to the increase in ovarian cancer risk [67] and another group suggesting that not only inflammation is sufficient for ovarian cancer onset but also other mechanisms and ovulation are required to promote ovarian cancer [68]. Otherwise; apart from these controversial opinions, reduction in the risk of ovarian cancer (significantly in some types) can be observed in the women with the operation called tubal ligation [69, 70, 71, 72]. According to the comprehensive cohort research, 20% decreased risk in high-grade serous carcinoma was related to tubal ligation [73]. In addition; among women with tubal ligation, the decline in the risk of endometrioid cancer, clear cell cancer, invasive mucinous and invasive serous cancer is observed approximately around 52%, 42%, 32% and 19%, respectively [69, 72].

Hormonal factors including oral contraceptive methods, hormone replacement therapy (HRT) and infertility treatments affect the ovarian cancer development in different ways. For instance, according to results in numerous studies indicates utilizing oral contraceptive methods provides a reduction of risk in each type of ovarian cancer [59, 74, 75, 76]. On the other hand, HRT and infertility treatments are regarded as controversial factors in the promotion of ovarian cancer. However, they are mostly predisposing factors because of application of estrogenic procedure in a long time [77] and including ovulation inducing drugs. [78] These procedures and drugs may be mentioned to induce the increasing risk of ovarian cancer onset.

Finally, lifestyle factors consisting of obesity, smoking, physical activity, alcohol and caffeine consumption are controversial factors to increase the risk in ovarian cancer. Intercalarily, period of breastfeeding enters into prevent the increment in the risk of ovarian cancer onset. Based on case-study results, the reduction of cancer risk can reach to 22% and above depending on the duration of the breastfeeding [79].

2.1.3. Stages of Ovarian Cancer

On 26 July 1954 founded non-governmental organism called The International Federation of Gynecology and Obstetrics (FIGO), also known as its French acronym Fédération Internationale de Gynécologie et d'Obstétrique in common. This organization exemplifies obstetricians and gynecologists all around the world with the aim of encouraging women to be

decent, following developments in scientific researches and improving application canonicals in gynecology and/or obstetrics. This organization also creates a FIGO staging system to classify the stages of ovarian cancer [80]. On October 7, 2012; The Committee of FIGO was met up in Rome to renew the staging system after the recent developments. New changes and criteria in the classification of ovarian cancer can be observed below. (Table 2.2)

Table 2.2. Ovarian cancer staging system according to 2014 FIGO and corresponding to Tumor, Node, Metastasis (TNM) values [81].

Stage I. Tumor confined to ovaries or fallopian tube(s)
T1-N0-M0
IA: tumor limited to one ovary (capsule intact) or fallopian tube; no tumor on ovarian or fallopian tube surface; no malignant cells in the ascites or peritoneal washings
T1a-N0-M0
IB: tumor limited to both ovaries (capsules intact) or fallopian tubes; no tumor on ovarian or fallopian tube surface; no malignant cells in the ascites or peritoneal washings
T1b-N0-M0
IC: tumor limited to one or both ovaries or fallopian tubes, with any of the following:
IC1: surgical spill
T1c1-N0-M0
IC2: capsule ruptured before surgery or tumor on ovarian or fallopian tube surface
T1c2-N0-M0
IC3: malignant cells in the ascites or peritoneal washings
T1c3-N0-M0

Stage II. Tumor involves one or both ovaries or fallopian tubes with pelvic extension (below pelvic brim) or primary peritoneal cancer
T2-N0-M0
IIA: extension and/or implants on uterus and/or fallopian tubes and/or ovaries
T2a-N0-M0
IIB: extension to other pelvic intraperitoneal tissues
T2b-N0-M0

Stage III. Tumor involves one or both ovaries or fallopian tubes, or primary peritoneal cancer, with cytologically or histologically confirmed spread to the peritoneum outside the pelvis and/or metastasis to the retroperitoneal lymph nodes
T1/T2-N1-M0
IIIA1: positive retroperitoneal lymph nodes only (cytologically or histologically proven):
IIIA1(i) Metastasis up to 10 mm in greatest dimension
IIIA1(ii) Metastasis more than 10 mm in greatest dimension
IIIA2: microscopic extrapelvic (above the pelvic brim) peritoneal involvement with or without positive retroperitoneal lymph nodes
T3a2-N0/N1-M0
IIIB: macroscopic peritoneal metastasis beyond the pelvis up to 2 cm in greatest dimension, with or without metastasis to the retroperitoneal lymph nodes
T3b-N0/N1-M0
IIIC: macroscopic peritoneal metastasis beyond the pelvis more than 2 cm in greatest dimension, with or without metastasis to the retroperitoneal lymph nodes (includes extension of tumor to capsule of liver and spleen without parenchymal involvement of either organ)
T3c-N0/N1-M0

Stage IV. Distant metastasis excluding peritoneal metastases
Stage IVA: pleural effusion with positive cytology
Stage IVB: parenchymal metastases and metastases to extra-abdominal organs (including inguinal lymph nodes and lymph nodes outside of the abdominal cavity)
Any T, any N, M1

According to the Table 2.2, the changes can be detected and actual classification can be observed clearly. It was defined as; Stage I ovarian or fallopian tube cancer can be developed in a surrounded boundary area up to the peritoneal fluid, tubes in fallopian and finally ovaries. In addition, Stage IC can be determined when the conditions at tumor rupture as; existence of

malignant ovarian cancer cells within peritoneal fluid or ascites and envelopment of surface via ovarian tumor cells are provided. Also, peritoneal cancer cannot be observed at stage I.

Minor and heterogenous group which causing less then 10% among ovarian cancers, is included in Stage II. Stage II can be also characterized as metastasis or spread of cancer cells to extraovarian organs. Curable tumors also known as, non-metastasized but spreading through the neighbor organs, form the large scale of ovarian cancer staging class; Stage II. Stage II also contains tumors spread to the pelvic peritoneal, while leaving out the sigmoid colon metastasis located above the pelvic brim. Therefore, stage IIC classified in the staging report in 1988, was eliminated in the renewed list since IIB stage referred pelvic extension [82]. In addition, the committee stated that subclassification of Stage IIB into IIB1 and IIB2 was not attributed on the evidence such as; macroscopic and microscopic observation of metastases surrounding pelvic peritoneal. Hence, when adjuvant chemotherapeutic treatment of Stage II classified tumors was taken into consideration, these small categories of Stage II were evaluated unnecessary [80].

HGSOCs covered most of the ovarian cancers, exist generally with the tremendous preponderance 84% especially in subclass of Stage III known as Stage IIIC [83]. Furthermore, due to their characteristics, these carcinoma tumors prefer to metastasize along pelvic and abdominal peritoneum, which are contained in peritoneal surfaces, also with the inclusion of mesentery, diaphragm, paracolic gutters, omental superficies and peritoneal superficies of the large and small intestine; liver and spleen, respectively. Less than 10% of epithelial ovarian cancers spread past the pelvis, through the solely involvement of retroperitoneal lymph node [84, 85, 86, 87]. According to the researches, this type of ovarian cancer tumors can be prognosed better compared to the ones with the involvement of abdominal peritoneal [88, 89, 90]. This renewed staging list revised the Stage III diagnosed patients and also appointed a new subclass called Stage IIIA1 according to its retroperitoneal lymph nodes dissemination to the retroperitoneal lymph nodes, but not including the intraperitoneal spreading. Furthermore, subdivision of Stage IIIA1 continued even without the retrospective data which promote the metastasis size quantification for IIIA1 and new substages called IIIA1(i) and IIIA1(ii) were joined to the group. IIIA1(i) is valid when the largest size of the tumor metastasis up to 10 mm where the largest size of the tumor metastasis more than 10 mm for IIIA1(ii). However,

cytologically or histologically evidences are required for indicating the retroperitoneal lymph node involvement [81].

In conclusion, Stage IV of the FIGO list which seen in 12% to 21% of patients, is described according to parenchymal metastasis of tumors through the distant organs such as; liver or spleen and extra-abdominal organs [83]. It is also highly recommended that the differentiation between Stage IIIC (including tumor dissemination from omentum to liver or spleen) and Stage IVB (including isolated parenchymal metastasis) must be taken into consideration.

2.1.4. Treatment of Ovarian Cancer

Ovarian cancer generally has a poor prognosis because of diagnosed at the later stage as a high-grade disease. It is very essential to prognose ovarian cancer at early stage which is evaluated as; Stage IA, IB and IC according to FIGO Staging system (Table 2.2), since 70-90% five-year survival rate can be accomplished [91]. However, early stage ovarian cancer prognosis requires effective screening strategies, which are not available yet [92]. On the other hand, advanced stage ovarian cancer can be described when the tumors disseminate into the widespread area of peritonea and the ascites show up surrounding peritoneal cavity. Even if the diagnosis was carried out for this level, it can be too late for the patient due to challenging against metastasis. In other words, 35-45% five-year survival rate can be accomplished against metastasis [93]. Patients suspected of being cancer are diagnosed via complete physical examination including breast, pelvic and rectovaginal examination [94]. Furthermore, blood test consisting of diagnostic biomarker mucin 16 (MU16) which is commonly known as cancer antigen 125 (CA125), is done to detect the levels of cancer biomarker presence [95]. Nevertheless, CA125 blood test is not adequate to determine the cancer presence when is applied alone so that gynecologic oncologists benefit from radiographic imaging such as; transvaginal ultrasonography, pelvic ultrasound, magnetic resonance imaging (MRI) and computed tomography (CT) scan to observe the dissemination of cancer [96]. The screening strategy is evaluated as the followings above since CA125 levels are not only increased in ovarian cancer cases but also in conditions containing infections, benign ovarian cysts, uterine fibroids and liver diseases [97, 98]. Information about the tumor histology can be detected after

removing the tumor mass via laparoscopic surgery [99]. When histology and data about size, level of the mass and location especially from transvaginal ultrasonography are combined, tumor diagnosis is completed.

Early-stage ovarian cancer can be treated firstly by adequate surgical staging and tumor removal by gynecologic oncologist. In addition, carboplatin and paclitaxel combined adjuvant chemotherapy is also applied to patient after operation to prevent any risk of recurrence [100]. For advanced stage ovarian cancer, tumor debulking surgery is recommended to conduct by gynecologic oncologist due to achieving more successful results in removing as many tumors from abdomen of the patient [101]. Unlike early-stage type, advanced stage cancer is applied neoadjuvant and adjuvant chemotherapy mostly with the composition of taxanes as; docetaxel or paclitaxel and platinums as; cisplatin and carboplatin [92]. Response of the patients to the treatment are examined with the aid of imaging techniques and CA125 levels [14]. Whether the recrudesce of the disease is observed in 6 months, it can be mentioned that the disease is chemoresistant. Whether the recrudesce of the disease is observed in 12 months, disease can be evaluated as chemosensitive [102]. Apart from these; new targeted therapies against ovarian cancer are developed to understand the factors especially cell-cell communication effecting progression of this disease from primary tissue to metastasis.

2.2. Molecular Mechanism of Ovarian Carcinogenesis and Tumor Microenvironment

Cancer progression begins in the primary region of the tumor microenvironment; ovarium and develops in the secondary region of the tumor micro environment; peritoneum and omentum. To develop cancer from benign form (usually early stage) to the more malignant form (advanced stage) requires EMT to stimulate cells for metastasis. The cells located in this tumor microenvironment go through some morphological and molecular changes. Furthermore, these cells acquire some mesenchymal traits where they slip their epithelial features due to expression changes in epithelial and mesenchymal markers. Transcriptionally and post-transcriptionally regulated E-cadherin expression can be suppressed when the Snail, Slug, ZEB-1 and ZEB-2 are present in the environment [103]. In other words, upregulation of Slug, Snail and other transcription factors provoke loss in EMT hallmark E-cadherin expression [104]. In ovarian cancer SKOV-3 cells; enhancement in EMT-related tumorigenicity, invasiveness and

motility by the Slug or Snail expression ectopically is demonstrated [105]. When the expression of E-cadherin decrease, it stimulates an increase in the expression of α5-integrin which connected to β1-integrin to form fibronectin receptor in the end. This stimulation of α5-integrin occurs via epithelial growth factor receptor (EGFR)/focal adhesion kinase (FAK)/mitogen-activated protein kinase (MAPK) pathway. Moreover, benefition of increment in the fibronectin receptor expression have been discovered in a study. According to the study, it assists spreaded ovarian cancer cells to conjoin to the mesothelial cell-secreted fibronectins covers the peritoneum and omentum linings in the secondary tumor microenvironment [106]. Thus, loss in expression of E-cadherin is essential for enabling spill of ovarian cancer cells to reach at the remote metastatic site for reattachment. (Figure 2.4)

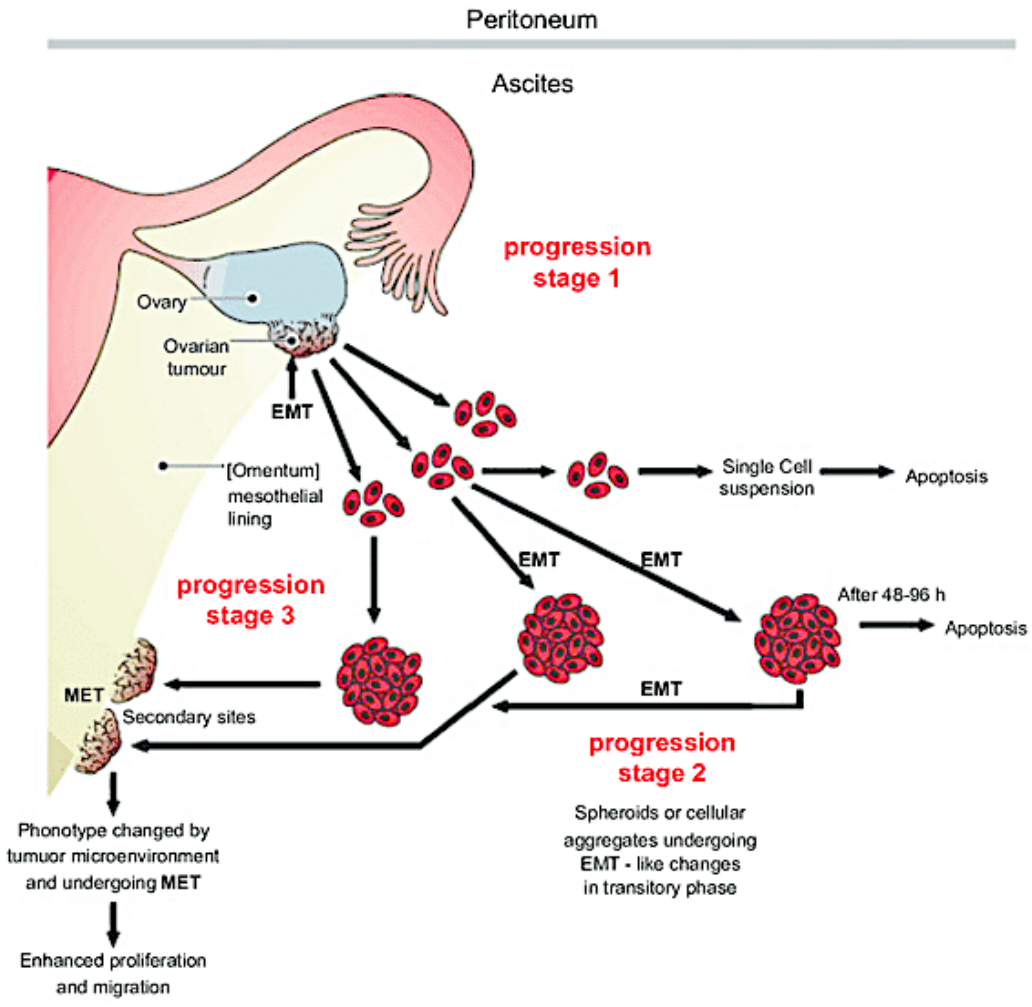


Figure 2.3. Ovarian cancer progression model affected by cellular carcinogenesis mechanisms [107].

Cytokines and plenty of growth factors including Tumor Necrosis Factor-α (TNF-α), vascular endothelial growth factor (VEGF) Interleukin-6 (IL-6) and Interleukin-8 (IL-8) are secreted by spheroids and mesothelial cells surrounding these cancer cells to form peritoneum ascites. Ascite formation observed in ovarian cancer ensure cancer cells to spread to metastatic peritoneal cavities. In the ascites covering peritoneal cavity, ovarian cancer cells are existing in the spheroid shapes or as single cells. These properties gain ovarian cancer cells to improve the ability to resist at anoikis and adopt the cancer stem cell characteristics [108]. Therefore, single cells and spheroids in the metastatic cavity become resistant to chemotherapy due to sharing similarities with cancer stem cells. Spheroids located in the metastatic cavity, include high levels of E-cadherin and EpCAM but depressed amount of CD44, vimentin and matrix metalloproteinases (MMPs) expressions [109]. Apart from cancer cells, non-cancerous cells as; cancer-associated fibroblasts (CAFs), platelets, immune, mesothelial and mesenchymal stem cells are involved in tumor microenvironment to provide niche to cancer progression and assisting cancer cells [110].

Apart from these, mucin expression is only observed in the region commonly known as ovarian surface epithelium which is also comprised of epithelial-mesenchymal phenotype [21]. Mucin mechanisms are not enlightened well but they can be evaluated as the targeted agents to prevent ovarian cancer or treat it. Furthermore, normal surface epithelium and benign ovarian tumors generates less quantity of mucins compared to epithelial ovarian carcinomas. Overexpression of from MUC1 to MUC4, MUC5AC and MUC16 is indicated in epithelial ovarian cancer [111, 112]. During disease progression and development, anomalous differentiation in mucin expression (also known as mucin switching) results in the spread of tumors, metastasis. (Figure 2.4) It also participates reprogramming of cell signaling of ovarian cancer cells, cell-cell or cell-matrix attachment differentiation, spheroid formation, immune suppression, communication with mesothelial cells to installation of the secondary tumor in the tumor microenvironment.

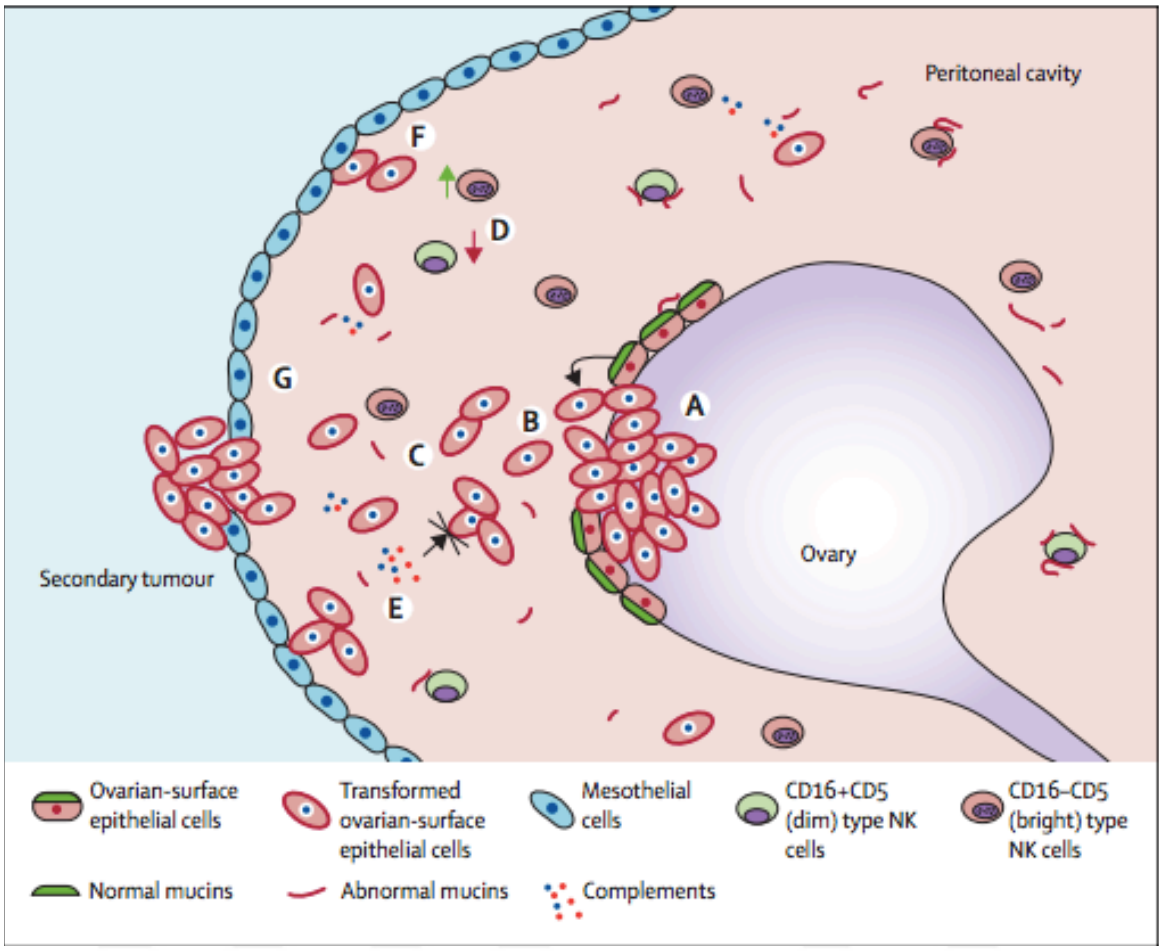


Figure 2.4. Ovarian tumor progression from epithelial cells in primary microenvironment to mesothelial cells in the second microenvironment [93].

2.2.1. Tumor Microenvironment Agents

To maintain the tumor invasion and metastasis, cell-cell communication in the tumor microenvironment is requiring due to prepare the suitable niche for ovarian cancer progression [113]. Tumor microenvironment includes signaling molecules and spacious range of cell types both malignant and non-malignant such as; fibroblasts, macrophages, adipocytes, cancer, endothelial, stem and immune cells. It also consists of extracellular matrix (ECM) which contributes to migration, cell adhesion, settlement of cancer and metastasis [114]. Signaling molecules which contribute to simplify the tumor progression with the enhanced mechanisms; immune system suppression, cancer initiation, angiogenesis and metastasis by exchanging through the cells located in tumor microenvironment [115, 116]. To maintain tumor

development, interaction between cell-cell and cell-substratum of the created tumor microenvironment is essential. Therefore, cell adhesion molecules (CAMs) and extracellular vesicles especially; exosomes (cargos in tumor microenvironment) providing communication between cells and tumor microenvironment might be mentioned like the key mediators of cancer progression in the niche [113, 115, 117, 118].

2.2.1.1. Cell Adhesion Molecules

Cell adhesion molecules are commonly classified to 5 main groups such as; selectins, integrins, mucins, cadherins and nectins one of the immunoglobulin superfamily members (IgSF) [119, 120]. Furthermore, each molecule has different role in cell adhesion. Selectins, members of IgSF and cadherins are responsible for cell-cell adhesion whereas; integrins are essential for ECM binding. Binding of ECM and integrin adhesion is requisite for cancer progression by activating oncogenic signaling pathways results in tumor dissemination [121]. In ovarian stromal and cancer cells express mostly αv and β1 subunits of integrin family [122]. Other adhesion molecules selectins, which are vascular and intervene the physiological responses including hemostasis, inflammation and immunity [123]. Apart from that, selectins are also important for stimulating tumor progression by facilitating cancer cells to communicate with endothelial cells, leukocytes, platelets.

When peritoneal metastasis induced, cells undergo EMT as mentioned above because of gaining mesenchymal traits rather than epithelial characteristics via altering the expression of specific markers [124]. To gain this characteristic, compact cell-cell attachment, cuboidal shape and polarities of cancer cells are began to lost. Main key mediator of cell-cell adhesion and one of the commonly known epithelial marker E-cadherin, also takes part in tumor suppression [125]. While the cells lose their expression of epithelial marker mainly E-cadherin; expression of N-cadherin increases which leads to decrease in cell-cell adhesion through adherent junctions among cancer cells and gain the ability of interaction with the normal cells located in tumor microenvironment to cancer cells [103].

2.2.1.2. Extracellular Vesicles

Another important factors for tumor progression in tumor microenvironment are secreted from numerous types of cell in body including both cancer and normal cells, also commonly known with the name of extracellular vesicles (EVs). They supply the intercellular communication thanks to their structure of being a package containing data consisting of mRNAs, microRNAs, DNA fragments of donor cells (transported to target cells), lipids, oncopeptides, oncoproteins and membrane receptors to initiate deep alterations in the tumor micro environment [126]. Subclasses of EVs are mentioned as; ectosomes, apoptotic bodies and exosomes [127]. Apart from their responsibility in cell-cell interaction, they are defined as "sine qua non" component of cancer development by effecting various mechanisms covered pre-metastatic niche formation, inflammation, chronic disease development and formation of organotropism of various types of tumors [128].

Recent evidences indicate that especially tumor-derived extracellular vesicles (TEVs), are main factors of triggering initiation, progression and metastasis mechanisms of diverse cancer types containing ovarian [129], colorectal [130], prostate [131] and breast cancers [132]. When the findings in the literature examined, tumor microenvironment is affected mostly from cell-cell communication key mediators known as exosomes as a subtype of extracellular vesicles [133].

2.3. Exosomes

Exosomes, referred as nanoparticles with the size between 30-150 nm and derived from late endosomes originated from multivesicular bodies (MVBs) [129]. Exosomes are enriched in various types of biomolecules as; nucleic acids, proteins, lipids, cell surface receptors and miRNAs that can be transposed between cells to provide intercellular communication both in systematic and paracrine way [134]. Biogenesis of exosomes arises from endocytic pathway by starting with invagination of endosomal limiting membranes [127, 135, 136]. It also induces the generation of intraluminal vesicles (ILVs) included within the endosomes. The formed compartments are defined as MVBs and their extracellular-released combination with plasma membrane comes out as exosomes. Coordinated endeavor of protein networks located in cell is

necessary to generate exosomes. Among the proteins; (1) tetraspanins, one of transmembrane proteins stimulating the cell skews to facilitate vesicle formation; (2) lipid-modifying enzymes like sphingomyelinase, which produces ceramides in order to encourage the generation of vesicles; Rab GTPase proteins, the controller of endosomal trafficking and endosomal sorting complexes required for transport (ESCRT), including in manifold protein complexes which are regulators of ILV formation [137].

When they observed via electron microscopy, exosomes represent an ordinary "cup-shape" or circular morphology [138]. They can be contained within physiological fluids such as; saliva [139, 140], blood and plasma [141, 142], breast milk [143], urine [144] and amniotic [145], seminal [146] and cerebral fluids [147]. They can also be secreted under the pathological and physiological conditions from diverse cell types consisting of dendritic cells (DCs) [148], platelets, reticulocytes [149], B and T lymphocytes [150, 151], neurons [152], macrophages [153], fibroblasts [154]; mast [155], epithelial [156] and stem cells [157]. They are determined as a cargo with the ability of transporting molecules which have role in transformation of normal cells into cancerous structure with the formation of premetastatic niche. They also take part in intercellular communications between stromal and cancer cells. Apart from that, the proofs suggest the cancer derived exosomes have role in tumor growth, tumorigenesis, tumor immune escape, angiogenesis and especially metastasis and drug resistance [158]. According to the researches, cancer patients' blood includes more exosomes compared to healthy human blood [159]. Various kinds of exosomes released from both normal and cancerous cells, are contained which in the plasma of the cancer patients. These various kinds of exosomes released from different cells forms the heterogeneity in the exosomal population size between 30-150 nm [160]. Also, the isolation of exosomes from the plasma of the cancer patients requires different methods including not only the standard techniques as ultracentrifugation but also the contemporary ones as size exclusion chromatography. [161, 162] For characterization of exosomes, main markers expressed from exosomes as; tumor susceptibility gene 101 protein (TSG101), members of tetraspanin, ALG- 2-interacting protein X (Alix) and heat shock protein 70 (HSP70) are mainly targeted to examine [163].

2.3.1. Uptake Mechanisms of Exosomes

Exosomes are internalized into the recipient cells via diverse endocytic pathways, consisting of clathrin-dependent endocytosis and clathrin-independent mechanisms including phagocytosis, caveolin-mediated endocytosis, lipid-raft mediated internalization and micropinocytosis, in general. (Figure 2.5) Exosome uptake can be observed directly following the labelling of exosomes with fluorescent lipid membrane dyes such as; Paul Karl Horan 26 (PKH26) [164, 165, 166], Paul Karl Horan 67 (PKH67) [167, 168, 169], 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI, DiIC18(3)) [170], 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (DiD, DiIC18(5)) [171] and rhodamine B (RhB, R18) [172]. Apart from these, exosomes can also be labelled with membrane permeable chemical compounds like 5(6)-carboxyfluorescein diacetate (CFDA) and carboxyfluorescein succinimidyl ester (CFSE) which results in internalization of exosomes into recipient cells can be analyzed by confocal microscopy and flow cytometry [173, 174].

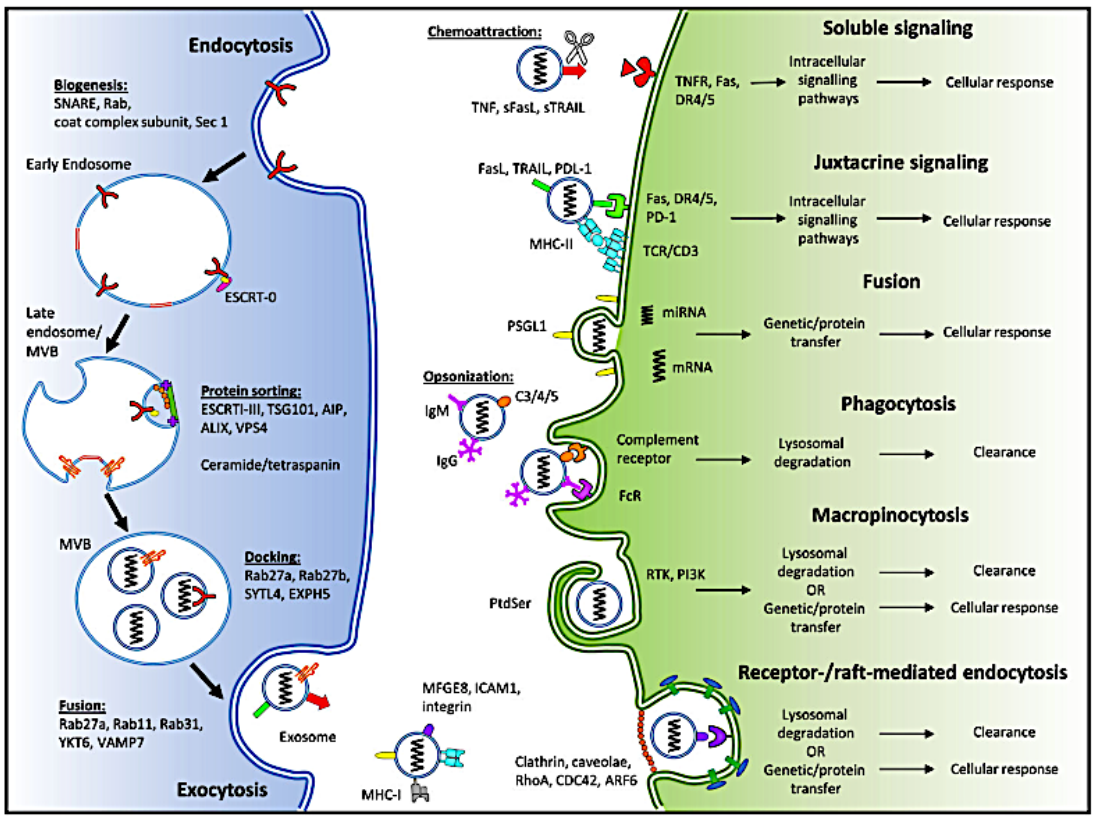


Figure 2.5. Cellular response, biogenesis and uptake of exosomes in schematic representation [175].

In literature, proofs recommend the uptake of EVs, especially exosomes, into the endosomal compartments by generally following the endocytosis pathway. Endocytosis can be defined as a hypernym for a set of molecular uptake mechanisms such as; phagocytosis, micropinocytosis and clathrin-mediated endocytosis (CME) [176]. To elucidate the endocytic course which is responsible for exosome internalization, some inhibitors are utilized to block particular pathways. Cytochalasin D, one of the inhibitors of exosome uptake, type of a metabolite which depolymerizes the actin filament network [177, 178]. Therefore, functional cytoskeleton is necessary for exosome internalization. In a dose dependent manner, treatment of Cytochalasin D in different cells reduces the internalization of exosomes but not entirely arrests [164, 167, 168, 170, 172, 174]. In addition, with the support of the previous information, internalization of exosomes can be stated as energy-dependent processes since multiple researches also suggest that the capacity of exosome uptake is reduced intensely at the time cells incubated at 4°C [170, 174, 179]. Microtubule depolymerization also effects negatively exosome uptake [167]. By utilizing nocodazole, one of the inhibitors of endocytosis by depolymerization of microtubules with free tubulin binding and arresting the incorporation of free tubulins to microtubules [180].

Cellular uptake of molecules included in CME, occurs through onward and consecutive installation of clathrin-coated vesicles containing various ligands and their transmembrane proteins. The vesicles with a clathrin coating can strategically able to deform the membrane that fully collapses into bud of the vesicular and maturates. The following intracellular vesicle uncovers the clathrin-coat combines with the endosome (accommodation of the vesicle contents) [181]. Chlorpromazine prohibits the genesis of these clathrin-coated cavities at the membrane which results in the inhibition of CME and decrease in exosome uptake [182] mostly indicated in recipient phagocytic [168] and ovarian cancer cells [174]. Dynamin2, one of the GTPases, is collected into nascent clathrin-coated cavities and generates a collar-like structures at the flange of this invaginated cavities [183, 184]. Hydrolysis of guanosine triphosphate (GTP) intercedes conformational change in Dynamin2 which also mediates the membrane split and clathrin-coated vesicle extrication [185]. To block Dynamin2, its specific inhibitor called Dynasore is utilized which results in the prohibition in internalization of approximately all exosomes in phagocytic cells [186, 187].

Apart from CME, in recent years the studies indicate plenty of another clathrin-independent endocytic mechanisms which are present in eukaryotic cells as caveolin-dependent endocytosis (CDE) [176]. Tiny-cavern-like invaginations are called caveolae which are glycolipid rafts' subdomains and consisting of sphingolipids, caveolins and cholesterol. Thus, cholesterol attenuation inhibits the CDE mechanism involving lipid rafts [188]. Furthermore, cholesterol decreasing elements like Filipin-III [167, 172], Methyl-ß-cyclodextrin (MßCD) [167, 168, 174] and simvastatin [167] reduce the internalization of the exosomes in recipient cells which are pre-treated with them. Dynamin2 mentioned in CME is also essential for CDE due to its activity facilitates caveolar endocytic vesicles to be assembled and expanded [176, 189]. Genistein, inhibitor of tyrosine kinase, procures the two significant mechanisms for CDE; (1) corruption of actin network and (2) placement of Dynamin2 into the plasma membrane [190]. In a study, the genistein used as an inhibitor of EV internalization by CDE in a dose dependent manner [191]. In various cells including A549 and HCT116 studied in a research, exosome uptake does not reduce dramatically, however decrease in EV uptake into the HeLa and COLO205 cells was indicated [191, 192].

Macropinocytosis, type of endocytic internalization mechanism, is responsible for genesis of the ruffles of invaginated membrane. Then these ruffles are compressed into the intracellular partition. This mechanism shares similarities with phagocytosis but it also differs from phagocytosis by not in the need of straight communication with the uptaken compound [176]. Sodium/proton exchanger or sodium/hydrogen exchanger (Na+/H+ exchanger, NHE) is necessary to maintain macropinocytosis [193]. The blockage applied to the Na+/H+ exchanger by 5-(N-Ethyl-N-isopropyl)amiloride (EIPA) can result into the inhibition of exosome internalization in ovarian cancer cells and macrophages [168, 174]. Phagocytosis, a process to engulf an unfamiliar or opsonized compound, can also be benefit to uptake exosomes in the recipient cells. For driving phagocytosis, also an energy-requiring mechanism, well-checked actin cytoskeleton rearrangement is necessary [194]. Therefore, to block this mechanism actin polymerization inhibitor Cytochalasin D can be employed [195].

2.3.2. Change in Cellular Mechanisms after Exosome Uptake

Exosomes attend in a diverse of processes included in which normal physiological and pathological. Normal physiological processes can be mainly mentioned as; differentiation of stem cells [196], autophagy [197], angiogenesis and regeneration of tissues [198], coagulation of blood [199], immunomodulation and acquired or innate immunity [200, 201], reproductive biology [202], embryo implantation [203] and pregnancy [204]. Moreover, EVs have been committed as the new novel intermediaries of cell-cell internalization through the physiology and regular development of the nervous system. They are also essential to regenerate the normal neurons. [205, 206] When the pathological processes are considered, progression of various diseases consisting of cancer [207] and neurodegenerative diseases [208] can be stimulated. EVs are the main underlying keys that mediates carcinogenic mechanisms also determined as "hallmarks of cancer" [209]. These stimulated hallmarks are cell migration, invasion, cell proliferation, EMT, inflammatory responses, immune suppression, angiogenesis and unfortunately metastasis. Viral pathogenesis can also be developed because of the resemblances in virion mounting and biogenesis of exosomes result in manipulation of host exosome pathway in order to assemble the structures of virions [210]. The studies encourage the opinion that numerous aspects on cancer progression is mediated by exosomes. Hence, EVs including exosomes, can be the optimum nominees for therapeutic agents or biomarkers of newly developing cancer treatment.

3. MATERIALS AND METHODS

3.1. Type of the Study

This study is an experimental type.

3.2. Time and Location of the Study

The experiments of this study were performed at Ayar Kayali Biopharmaceutic Technology and Bioanalysis Laboratory at İzmir Biomedicine and Genome Center between February 2018 - November 2019. The characterization of exosomes was analyzed by Yekta Günay at Biotechnology and Bioengineering Research and Application Center (BIOMER) in Izmir Institute of Biotechnology between September - October 2018. The flow cytometry and optical imaging were performed by Izmir International Biomedicine and Genome Center Core Facility members Xiaozhou Hu, Melek Üçüncü and Didem Çimtay, respectively.

3.3. The Universe and Sample of Research

There were no human primary samples used in this research.

3.4. Working Materials

We used human ovarian normal and cancer cell lines.

3.4.1. Cell Lines

We used normal human ovarian surface epithelial (OSE) cells purchased from Applied Biological Materials (abm-good) with the catalog number T4198. Then, OSE cells became immortalized by transfection of SV-40. Human primary ovarian carcinoma cell line (A2780) and cisplatin-resistant human primary ovarian carcinoma cell line (A2780cis) were purchased from European Collection of Authenticated Cell Cultures (ECACC) with the catalogue numbers 93112519 and 93112517, respectively. Mesothelial (MeT-5A) cells were obtained from

Senturk Functional Cancer Genomics Laboratory in Izmir International Biomedicine and Genome Center.

3.4.2. Equipments

10 mL Open-Top Thickwall Polycarbonate Tube (Cat. No. 355630, Beckmann Coulter), Amicon® Ultra-15 15 ml- 100 KDa Cutoff (Cat. No. UFC910024, Merck), New Brunswick™ U410 -86 °C Refrigerator (Eppendorf), Optima™ L-100 XP Ultracentrifuge (Beckmann Coulter), Safe 2020 Class II Biological Safety Cabinets (Thermo Scientific™), In-VitroCell ES NU-5800 CO_2 Incubator (NuAire), Laser Scanning Microscope (LSM) 880 with Airyscan (ZEISS), Zetasizer Nano ZS (Particulate Systems), Varioskan® Flash Spectral Scanning Multimode Microplate Reader (Thermo Electron Corporation), LSR Fortessa™ (Becton, Dickinson and Company (BD) Biosciences), NB 9 Water Bath (Nüve), SimpliAmp™ Thermal Cycler (Applied Biosystems), 7500 Fast Real Time PCR System (Applied Biosystems), NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific™), Centrifuge 5810 R (Eppendorf), Chemiluminescence System (Vilber Lourmat)

3.4.3. Kits, Antibodies and Inhibitors

PKH26 Red Fluorescent Cell Linker Kit for General Cell Membrane Labeling (PKH26GL, Sigma Aldrich), Pierce™ BCA Protein Assay Kit (Cat. No. 23225, Thermo Scientific™), DCDFA -Cellular Reactive Oxygen Species Detection Assay Kit (ab113851, abcam), Calcein AM Cell Viability Kit (Cat. No. 4892-010-K, Trevigen®), QCMTM 24-Well Fluorimetric Cell Migration Assay (Cat. No. ECM 509, Chemicon® International), QCMTM 24-Well Collagen-Based Cell Invasion Assay (Cat. No. ECM 552, Chemicon® International), ATP Bioluminescence Assay Kit CLS II (Cat. No. 11 699 695 001, Roche), Recombinant Anti-TSG101 antibody (ab125011, abcam), Anti-ALIX antibody (ab117600, abcam), Anti-Calnexin antibody (ab22595, abcam), Anti-GM130 antibody - cis-Golgi Marker (ab31561, abcam), Anti-LAMP1 antibody (ab25245, abcam), Anti-TGN46 antibody (ab2809, abcam), Recombinant Anti-LMAN1 antibody (ab125006, abcam), Anti-EEA1 antibody (ab50313, abcam), Anti-RAB7 antibody (ab50533, abcam), Anti-F-actin antibody (ab205, abcam), Genistein (Cat. No. G6649-5MG, Sigma-Aldrich), Dynasore (ab120192, abcam), Filipin III (F4767-5MG, Sigma-

Aldrich), 5-(N-Ethyl-N-isopropyl) amiloride (A3085-25MG, Sigma-Aldrich), Cytochalasin D (C8273-1MG, Sigma Aldrich), Nocadozole (ab120630, abcam), Chlorpromazine (C8138-5G, Sigma Aldrich)

3.5. Variables of the Study

Our study's variable is drug-resistance exosomes.

3.6. Data Collection Tools

3.6.1. Cell Culture

Human ovarian carcinoma cell line A2780, immortalized human ovarium surface epithelial cell line OSE-SV40, human mesothelium cell line MeT-5A and cisplatin resistant human ovarian carcinoma cell line A2780cis were cultured in flasks including complete RPMI 1640 Medium supplemented with 10% (v/v) FBS (Fetal Bovine Serum) and 100 units/ml Penicillin/Streptomycin (PS) (1%) antibiotics. Then the cells were incubated in an adequately humidified atmosphere (Relative Humidity (RH)=90-95%) containing Nuaire Incubator with the temperature at 37°C and 5% CO_2.

3.6.2. Exosome Isolation

When the confluency of the A2780cis cells reached to 70-80 %, growth medium of the cells were discarded and the cells were washed with 1X Phosphate Buffered Saline (PBS). The new complete RPMI 1640 Medium with the supplementation of 10 % (v/v) exosome-depleted FBS and 1% PSA was added to the flasks and cultivated for 48 hours. Exosome-depleted FBS was concocted via ultracentrifugation at 100.000 x g for 70 minutes. The pellets were discarded and the supernatant was the exosome-depleted FBS.

After 48 hours, the growth medium in the flasks were collected and centrifuged at 800 x g for 10 minutes to get rid of death cells. After centrifugation step, supernatants were filtrated through 0.22 μm filter and added to 100 kDa MWCO tubes and centrifuged at 3300 x g for 15

minutes. The upper phase collected above the filter in 100 kDa tubes were collected and this upper phase was ultracentrifuged at 100.000 x g for 70 minutes. The exosome pellets were observed in the ultracentrifuge tubes. Exosome pellets were washed with PBS twice and ultracentrifuged at the same procedure. Washed exosome pellets were stored at -20 °C and dissolved in 100 µl PBS before using in the further experiments.

3.6.3. Exosome Characterization

3.6.3.1. Western Blotting

Exosome pellet dissolved in 100 µl PBS was mixed with 5X Lysis Buffer and sonicated for 30 seconds. After sonication, exosome solution was incubated on the ice for 15 minutes and mixed with 4X Laemmli Buffer. Exosome samples were incubated at 90 °C for 10 minutes and loaded into SDS-PAGE. 10% SDS-PAGE gel was prepared and added to the Western Blotting tank. After polymerization of 10% gel, prepared stacking gel was also added on the 10% gel. The comb of the wells was put on the stacking gel and the prepared SDS-PAGE gel was kept at room temperature (RT). Before loading the samples, the comb was taken out from the gel. Then the samples were loaded on the gel and the system was run at 100 V first, then the voltage level was increased to 120 V until the protein bands separated from each other clearly. After running step, the proteins were transferred to the nitrocellulose membranes. For protein transfer, 1X Transfer Buffer was prepared and the all the materials used in sandwich method were interacted with transfer buffer. Sandwich method for protein transfer can be explained as follows; the cassette for transfer was opened and black surface of the cassette was placed at the bottom part. Transfer buffer interacted-sponges were laid on the black surface and blotting papers were also placed on the sponge. The SDS-PAGE gel was taken from the running tank and placed on the transfer cassette on the blotting papers. Then, nitrocellulose membrane was placed on the gel and the blotting papers and sponge were laid respectively as mentioned previously. The cassette was closed and located in the transfer tank. The transfer buffer filled the transfer tank and the tank was placed on the magnetic stirrer in the +4 °C refrigerator. The magnetic fish and ice pack were also placed in the tank. The system was run at 250 mA for 2 hours.

After the transfer, the gel and the membrane were dyed with Coomassie blue and Ponceau S to control the transfer of the proteins. Then, the membranes were blocked with 5% Bovine Serum Albumin (BSA) for 1 hour and incubated with primary antibodies (Alix, Tsg101 and Calnexin) for another 1 hour. After incubation, the membranes were washed with 1X TBST for 3 times and secondary antibodies were added to membranes. The membranes were incubated with secondary antibodies for 1 hour and the bands were observed with the usage of ECL Imaging Machine.

3.6.3.2. ZetaSizer Size Measurement

The dissolved exosome pellets were diluted 1/1000 with PBS and added into the plastic cuvettes of ZetaSizer. Then the size measurement analysis was performed at Zetasizer Machine and the measurements were recorded at every 60 seconds.

3.6.4. Exosome Labeling

For labeling exosomes, PKH26 Dying Kit was used by implying the protocol inside the kit. Exosome suspension was mixed with 900 µl Diluent C. PKH26 dye was also mixed with Diluent C. PKH26 dye added into the exosome suspension was incubated for 5 minutes and the reaction was stopped with 2 ml exosome-depleted RPMI 1640 Medium. Approximately 4 ml total suspension was ultracentrifuged at 100.000 x g for 70 minutes. Then, this labeled exosome pellet was washed with PBS twice via ultracentrifugation. The labeled exosome was dissolved in 100 µl for using in further experiments.

3.6.5. Cell Lysate Preparation

70-80% confluent cells were treated with RPMI Medium including exosome depleted FBS and 100 µl exosomes suspension. After 24 and 48 hours, the cells were scrapped from petri dishes and washed with cold PBS, and the prepared cell pellet was stored at -20 ° C. Samples were mixed with 5X Lysis Buffer and sonicated for 30 seconds. After sonication, samples were incubated on the ice for 15 minutes and centrifuged at 13.000 x g for 15 minutes. Then, the samples were mixed with 4X Laemmli Buffer and the protein levels were measured by

Bicinchoninic Acid (BCA) Protein Assay. Samples containing 50 μg protein were mixed with 4X Laemmli buffer for loading in the 8-10% SDS-PAGE gel.

3.6.6. Protein Concentration Measurement

BCA Assay Kit was used for determining the protein concentration of the samples. The working solution mixture including 90 A solution and 1 B solution inside was added to the sample solutions. The sample mixtures were added in the 96 well-plate and incubated for 30 minutes at 37 ° C. Then, aluminum folio covered-96 well-plate was waited a while to cool down after incubation. The BCA Standard graph was used to detect the protein concentration in the samples. The absorbances of the samples were measured at 562 nm by Multiscan Go Spectrophotometer.

3.6.7. Uptake of Exosomes into Recipient Cells

A2780, OSE and MeT-5A cells were seeded in the 6-well plates including 150.000 cells per well. After overnight incubation, the cells can adhere to the surface of the plate. Then, the cells were washed with PBS followed by the addition of completed exosome-depleted RPMI 1640 Medium. After 6 hours of exosome treatment to the recipient cells, the uptake of the exosomes was observed by confocal microscope.

3.6.8. MTT Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay was carried out to measure the cell viability of A2780cis cells. The A2780cis cells were seeded in the 96-well plates with the density of 7500 cells/well. After overnight incubation at 37 °C, A2780cis growth mediums were changed with the exosome-treated and non-exosome-treated A2780, OSE and MeT-5A growth mediums, respectively. After 24 and 48 hours of medium change process, MTT was also applied to the cell containing wells at 37 °C for 4 hours. The absorbances of the wells were measured at 540 nm by Multiscan Go Spectrophotometer.

3.6.9. Cell Viability Assay

A2780, OSE and MeT-5A cells were seeded in the black 96-well plates including 7500 cells per well. After overnight incubation, the inhibitors determined at Table 3.1 were applied to recipient cells with the detected concentrations. After 24 hours, growth mediums were removed and 1X Calcein AM DW Buffer was added 100 µl to the each well. Then, the buffer was discarded from the wells and 50 µl 1X Calcein AM DW Buffer and 50 µl 2X Calcein AM Working Solution were added to the wells recommended in the Cell Viability Kit. The plate was covered with aluminum and incubated in the cell incubator for 30 minutes. After incubation, the absorbances were read at Ex/Em=490/520 nm by Multiscan Go Spectrophotometer.

Table 3.1. Information about the mechanisms affected by inhibitors and the incubation times of the drugs.

Inhibitor	Uptake Mechanism	Incubation Time
Chlorpromazine	Clathrin-Mediated Endocytosis	30 min at 37° C and during analysis
Cytochalasin D	Phagocytosis by Actin Depolymerization	30 min at 37° C and during analysis
Dynasore	Clathrin-mediated and Caveole-dependent Endocytosis	30 min at 37° C and during analysis
5-(nethyl-n-isopropyl)-amiloride (EIPA)	Macropinocytosis	30 min at 37° C and during analysis
Filipin III	Caveole-dependent and Lipid Raft-mediated Endocytosis	48 hours at 37° C and during analysis
Genistein	Caveole-dependent Endocytosis	30 min at 37° C and during analysis
Nocadazole	Microtubule Depolymerization	Overnight at 37° C and during analysis

3.6.10. Flow Cytometry for Exosome Uptake with Inhibitors

A2780, OSE and MeT-5A cells were seeded in the 6-well plates including 150.000 cells per well. After overnight incubation, the adherent cells were washed with PBS and added completed exosome-depleted RPMI 1640 Medium. The inhibitors were added into the growth medium of the cells at the ideal dose of inhibitors detected at Table 4.2 after Cell Viability assay. The drugs were applied to cells according to their incubation time explained above at Table 3.1 and exosomes were added after their incubation time. Exosomes recommended 6 hours of incubation so that after 6 hours the cells were trypsinized and collected. Then, the cell suspensions were analyzed by flow cytometry analysis. The non-inhibitor used cells were determined as control cells.

3.6.11. Immunofluorescence Staining for Confocal Imaging

Approximately 70-80% confluent A2780, OSE and MeT-5A cells were seeded on the glass slides in the 6-well plates. Then, PKH26 labeled exosomes were applied to cells and incubated within well plates for 6 hours. After incubation, the cells were fixed for 15 minutes in 4% paraformaldehyde (PFA) at RT. Then, cells were permeabilized with PFA containing 0.1% Triton X-100 for 5 minutes at RT and washed with PBST 3 times for 5 minutes. After blocking cells with 5% BSA in PBST, primary antibodies were added into the cells for 1 hour incubation at RT. After primary antibody incubation, the slides were washed 3 times with PBST for 5 minutes while shaking. The target organelles were dyed with AlexaFluor 488 conjugated secondary antibodies seen in Table 3.2 and the slides were washed 3 times with PBST for 5 minutes while shaking. The last dying step was the DAPI staining of the nucleus of the cells for 5 minutes at RT. Finally, the slides were turned upside down to mount on microscope slide in the mounting medium which prevents photobleaching. The colocalization of the cells were examined in 3-dimensional imaging by confocal microscope.

Table 3.2. The markers of the targeted organelles of the exosome after their uptake.

Target Organel	Molecules
Endoplasmic Reticulum (ER)	calnexin
ER-golgi interface	LMAN1
Golgi	GM130
Trans-golgi network	TGN46
Early Stage Endosome	EEA1
Late Stage Endosome	Rab7
Lysosome	LAMP-1
Cytoskeleton and Microtubule	ß-tubulin

3.6.12. Change of Cell Mechanisms in Recipient Cells after Exosome Uptake

3.6.12.1. Intracellular Reactive Oxygen Species (ROS) Measurement

A2780, OSE and MeT-5A cells were seeded in the clear bottom, black 96-well plates including 25.000 cells per well and incubated overnight for allowing cells to adhere. After 24 hours, the growth mediums were discarded and 100 µl of 1X Buffer was applied to the wells. The buffer was also removed and 100 µl 25 µM of DCDFA dye solution was added to each well. After dye addition, the aluminum covered well-plate was incubated at 37 ° C for 45 minutes. Then, DCDFA dye solution was discarded and each cell was washed with 100 µl of 1X Buffer. The exosomes diluted in 10% FBS Supplement Buffer were applied to cells. For control cells, only 10% FBS Supplement Buffer was added. Finally, after 3 and 6 hours of incubation, the fluorometric measurement analysis was performed at Ex/Em= 485/535 nm with Multiscan Go Spectrophotometer.

3.6.12.2. Invasion Assay with Boyden Chamber

The 500 µl Serum Free Medium was added to the wells in 24 well-plate before placing the invasion chamber plate. To the upper filter of the collagen-coated polycarbonate membrane-based chamber; 250.000 recipient cells in 250 µl and 100 µl exosome suspensions were added. After 48 hours of incubation, invasive cells degraded the collagen and passed through the polycarbonate membrane for adhering the bottom part of the membrane. These cells were incubated with 225 µl Cell Detachment Solution inside the kit at 37 ° C for 30 minutes to separate them from the bottom part of the membrane. During this incubation, the invasion chamber plate was tilted carefully several times for extracting the bottom surface adherent-cells. Cells after completely suspended in the Cell Detachment Solution, were dyed with 75 µl Lysis Buffer/Dye Solution for 15 minutes at RT. (CyQuant GR Dye was diluted in 4X Lysis Buffer.) Then 200 µl of sample mixture was added into the flat-bottom black 96-well plate to measure the invasion rates with a fluorometric analysis using 480/520 filters. Non-exosome treated cells were assumed as control cells.

3.6.12.3. Migration Assay with Boyden Chamber

The 500 µl Serum Free Medium was added to the wells in 24 well-plate before placing the migration chamber plate. After 48 hours of incubation, metastatic cells passed through the polycarbonate membrane for adhering the bottom surface of the membrane. These cells were incubated with 225 µl Cell Detachment Solution at 37 ° C for 30 minutes to separate them from the bottom surface of the membrane. During this incubation, the migration chamber plate was tilted carefully several times for extracting the bottom surface adherent-cells. Cells after completely suspended in the Cell Detachment Solution, were dyed with 75 µl Lysis Buffer/Dye Solution for 15 minutes at RT. (CyQuant GR Dye was diluted in 4X Lysis Buffer.) Then 200 µl of sample mixture was added into the flat-bottom black 96-well plate to measure the migration rates with a fluorometric analysis using 480/520 filters. Non-exosome treated cells were assumed as control cells.

3.6.12.4. Epithelial-Mesenchymal and Mesothelial-Mesenchymal Transition Analysis

The cell lysates were prepared as above in "Cell Lysate Preparation" part and western blotting analysis was performed as explained previously. The primary antibodies used to detect the difference in these mechanisms were stated in the Table 3.3 below.

Table 3.3. The epithelial and mesenchymal marker for detecting the changes in EMT mechanism in recipient cells.

Epithelial-Mesenchymal Markers

Epithelial	**Mesenchymal**
E-cadherin	Vimentin
EpCAM	ZEB1
	Slug

3.6.12.5. Proliferation Assay

Recipient A2780, OSE and MeT-5A cells with the confluency of 70% were treated with exosomes for 24 and 48 hours. Cells were counted and prepared as 10^6 cells/ml. Then, these cell samples were incubated with boiled Lysis Buffer at 100° C for 5 minutes and centrifuged at 3000 x g for 3 minutes. The supernatant was mixed with the luciferase agent as recommended in the protocol of ATP Bioluminescence Analysis Kit. The luminometric measurement of the cells were performed by Multiscan Go Spectrophotometer. The concentration of the ATP levels was related to the proliferation rates of the cells.

For detecting the proliferation rate of A2780cis cells after treating with the growth mediums of recipient A2780, OSE and MeT-5A cells; the recipient cells were seeded and after 24 hours for adherence, the recipient cells were treated with exosomes. Then, the exosome treated growth mediums were collected both after 24 hours and 48 hours to apply into pre-

seeded A2780cis cells. In this way, the A2780cis cells were cultured with exosome-treated growth mediums for another 24 and 48 hours. In conclusion, the luminometric analysis was performed after the procedure mentioned in the previous paragraph.

3.6.13. RNA Isolation

Pre-seeded and exosome-treated recipient A2780, OSE and MeT-5A cells were trypsinized and counted via hemocytometer. 3-5 x 10^6 cells were suspended in 1 ml of Trizol and the suspension was homogenized with needle. Then, 200 µl chloroform added to the homogenized cell suspension and mixed with pipetting. The chloroform added-tubes were incubated at RT for 15 minutes and centrifuged at 12.000 x g for 20 minutes at the temperature 4° C. Supernatant was transferred to the new tube and the pellet was discarded. The supernatant was added 500 µl isopropanol, following the suspension was mixed via pipetting. For obtaining better pellet, the tubes were incubated at -80 ° C overnight.

After incubation, the samples were centrifuged at 12.000 x g for 20 minutes at 4 ° C. Then, the supernatant was discarded and the pellets were dissolved in 1 ml 70% ethanol. Another centrifugation step at 12.000 x g for 10 minutes at 4 ° C was performed. Finally, the supernatant was discarded once more and the pellet was airdried approximately 15 minutes. Thereafter, the samples were prepared to RNA measurement in Nanodrop by adding 50 µl RNase and DNase free sterile water. Finally, the RNA concentrations were analyzed and A260/280 and A230/280 values were checked.

3.6.14. cDNA Synthesis

When the Nanodrop analysis was finished, the RNA concentration for each cell was calculated. The cDNAs were synthesized according to the protein concentration up to 2 µg. The samples were prepared according to the cDNA protocol and the table below. (Table 3.4)

Table 3.4. The information about the reagents were required in cDNA synthesis of the recipient cells.

Reagents	Volumes in 20 µl reaction
10X Reverse Transcriptase Buffer	2 µl
25X dNTP mix (100 mM)	0.8 µl
10X Random Primers	2 µl
RNase Inhibitor (20U/100 µl) (40U/50 µl)	0.5 µl
RNA Sample	2 µg
Reverse Transcriptase	1 µl
Nuclease-Free Water	Up to 20 µl

The samples were prepared according to the data given in Table 3.4 for 20 µl reaction, the tubes were spinned and mixed by vortex. At final, they were placed in the PCR machine and the program was downloaded to run the cDNA synthesis.

3.6.15. Quantitative Polymerase Chain Reaction (qPCR)

After synthesis of cDNAs, samples for qPCR analysis were prepared as shown in the Table 3.5 below.

Table 3.5. The information about the reagents required for preparing qPCR samples.

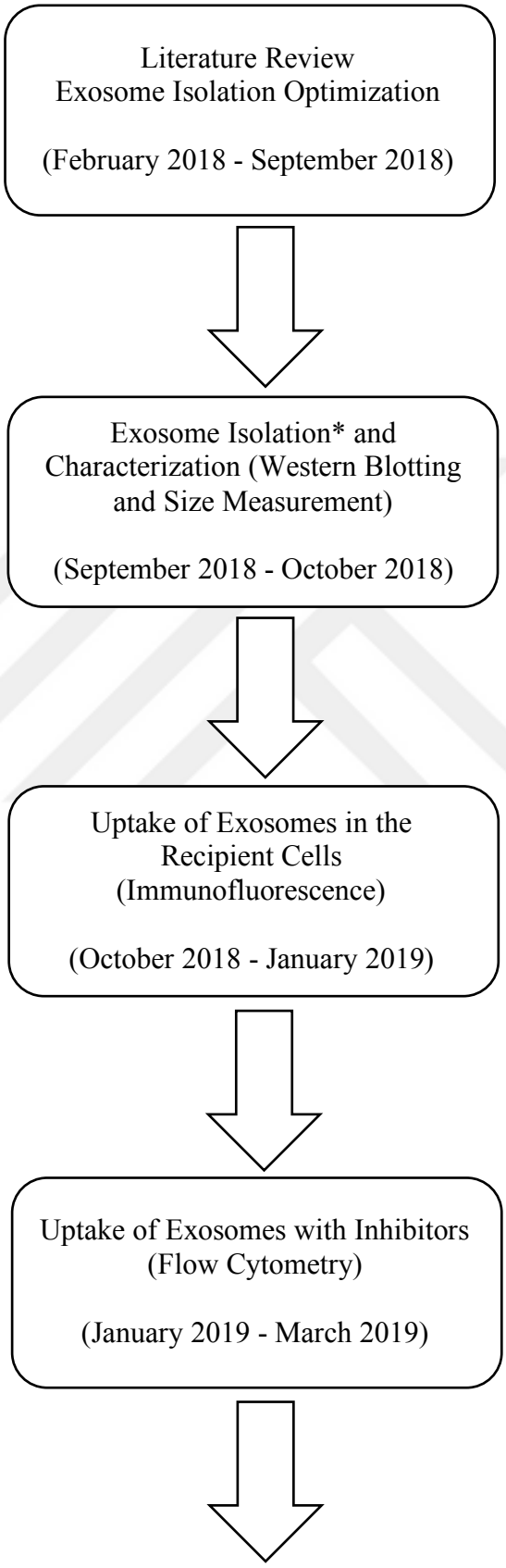
Reagents	Volumes in 40 µl reaction (3 trials x 10 µl)
2X Syber Green Mix	20 µl
Forward and Reverse Primer Mix (Final Concentration=0.5 µM)	4 µl
PCR Grade Water	12 µl
cDNA	4 µl (diluted 1:2 with PCR Grade Water)

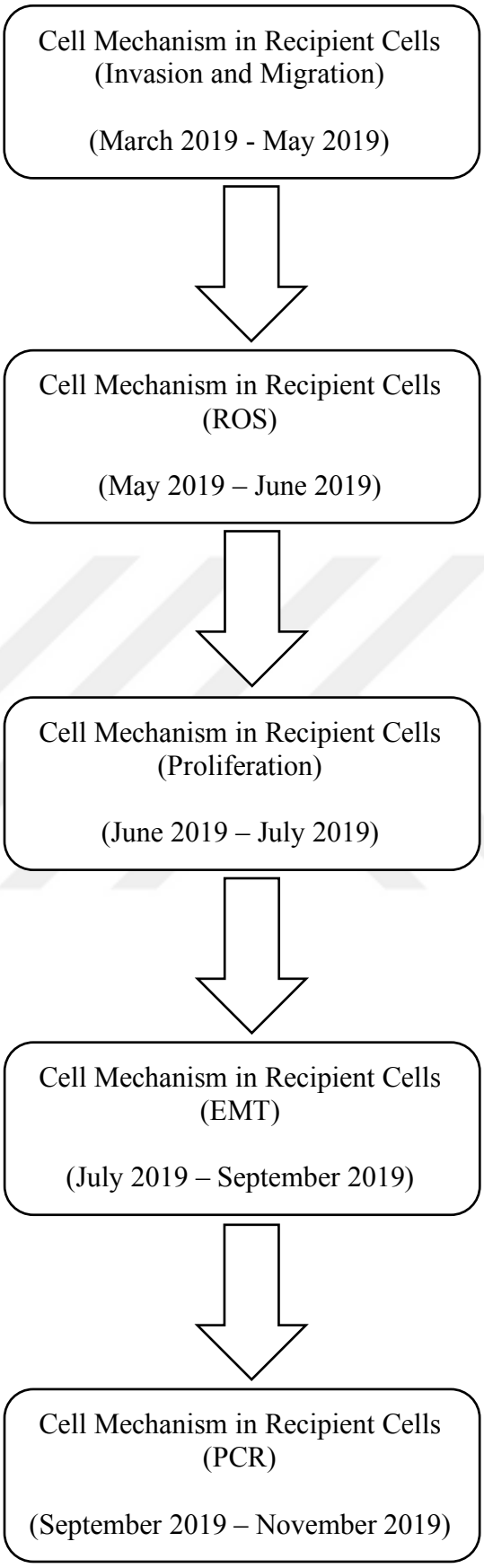
The samples were prepared according to the Table 3.5 and mixed well. The tubes were spinned carefully and 10 µl of samples was loaded in each well of 3 trials. The well-plate was covered with the transparent paper and the PCR method was set at the computer. The analysis was run about 3 hours. (Table 3.6)

Table 3.6. The experimental setup for qPCR analysis.

Experimental Setup	
Reaction volume per well	10 µl
Holding Stage	95 °C-20 sec-100%
Cycling Stage	**Step 1:** 95 °C-15 sec-100% **Step 2:** 60 °C-1 min-100%
Number of Cycles	40
Melting Curve Stage	**Step 1:** 95 °C-10 sec-100% **Step 2:** 60 °C-1 min-100% **Step 3:** 95 °C-15 sec-1% **Step 4:** 60 °C-15 sec-100%

3.7. Research Plan





*: Performed until the project completed between the dates February 2018-November 2019.

3.8. Data Evaluation

To evaluate statistical significance, Student's t test was employed and the $P<0.05$ values were taken into consideration.

3.9. Limitations of the Study

There were no limitations to handle during the study.

4. RESULTS

Characterization of nanoparticles isolated from drug-resistant ovarian cancer A2780cis cells as exosomes

Isolated nanoparticles that are released from drug-resistant ovarian cancer A2780cis cells, were used in characterization steps as; western blotting and size measurement analysis. According to western blotting results, the bands of Tsg101 (47 kDa) and Alix (97 kDa) proteins were observed. (Figure 4.1) These bands are the proof of A2780cis nanoparticles expressing Tsg101 and Alix proteins, also known as exosomal markers. The negative control for this analysis was the Calnexin (67 kDa) protein that is also known as an endoplasmic reticulum marker and as we expected; there were no bands observed. According to this result, the pellet was pure and had no contamination so that the nanoparticles can be determined as exosomes.

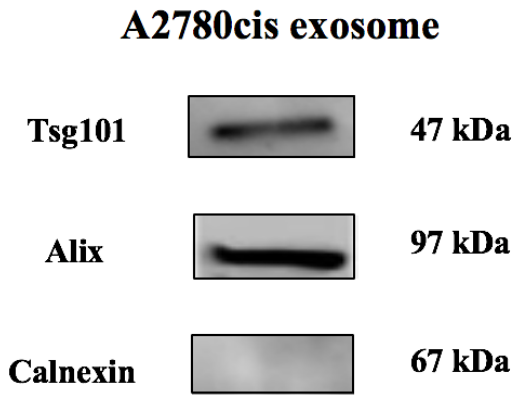


Figure 4.1. Western blotting analysis results for nanoparticles isolated from drug-resistant ovarian cancer A2780cis cells. Positive results for Tsg101(47 kDa) and Alix (97 kDa) markers. Negative result for Calnexin (67 kDa) marker.

Apart from western blotting analysis, nanoparticle size measurement analysis was also performed by Zetasizer. The diameter of the nanoparticles isolated from A2780cis cells was detected between 130-140 nm. (Figure 4.2) According to this result in Figure 4.2, the size of the nanoparticles isolated from A2780cis cells concur with the exosome size reference range, known as 30-150 nm. This is also another proof that supports to determine the nanoparticles isolated from A2780cis cells as exosomes.

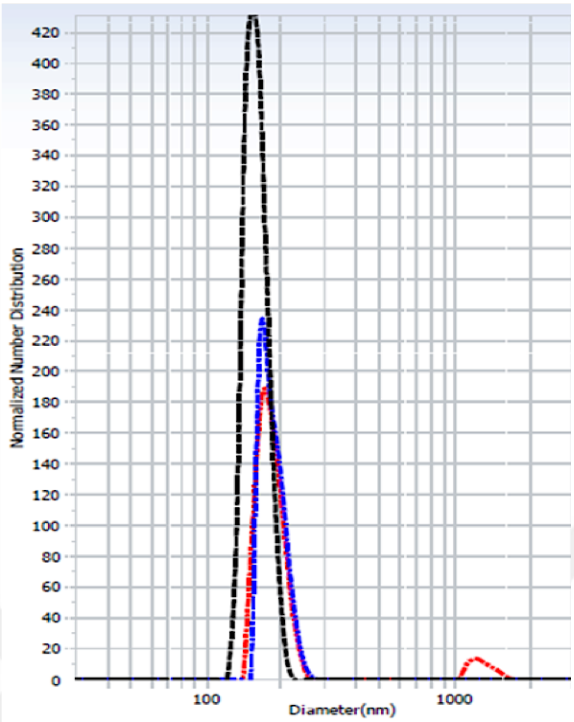


Figure 4.2. Size measurement of nanoparticles isolated from A2780cis cells by ZetaSizer. The diameter of the nanoparticles is between 130-140 nm.

Uptake of exosomes isolated from drug-resistant ovarian cancer A2780cis cells by the. recipient cells

After proving the nanoparticles as exosomes, the following step is to demonstrate the uptake of exosomes into the recipient cells; primary ovarian cancer A2780 cells, ovarian surface epithelial OSE cells and mesothelial MeT-5A cells. The exosomes were labelled with PKH26 (red) dye where the nucleus of recipient cells was labelled with DAPI (blue) dye. The uptake of the exosomes was observed into the recipient cells A2780, OSE and Met-5A cells respectively after 6 hours by confocal microscope with magnification 20X. (Figure 4.3)

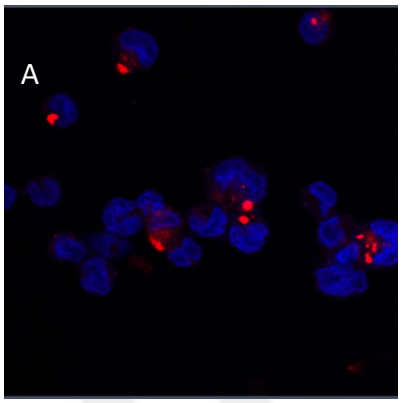
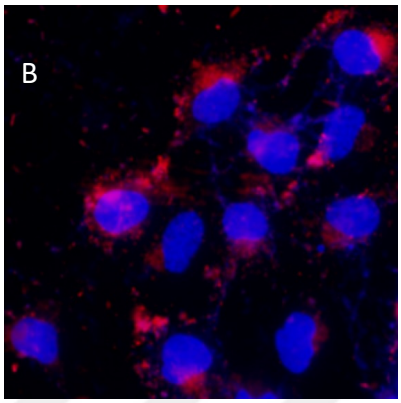
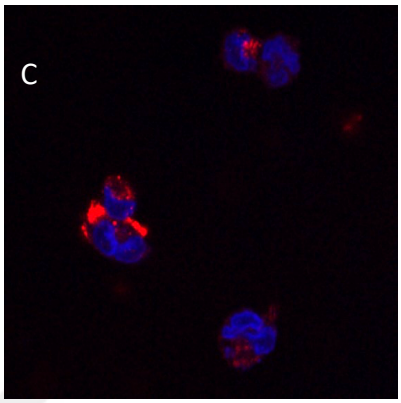
A2780 (Nucleus + Exosomes)	OSE (Nucleus + Exosomes)	MeT-5A (Nucleus + Exosomes)
		

Figure 4.3. Uptake of PKH26 labelled exosomes into recipient A2780 (A), OSE (B) and MeT-5A (C) cells after 6 hours by confocal microscopy at 20X magnification.

As observed in figure above, the exosomes were located near the nucleus of the A2780, OSE and MeT-5A cells. Therefore, it was demonstrated that the recipient cells of our study can uptake isolated A2780cis exosomes after 6 hours of incubation.

Detecting the uptake mechanism of A2780cis released exosomes in recipient cells by using inhibitors

To detect the uptake mechanism of the exosomes into the recipient cells of this study, some inhibitors were used to block the certain uptake mechanisms of exosomes. First, the dose of the inhibitors used in the recipient cells was detected by cytotoxicity studies with cell viability analysis. For each inhibitor, different concentrations of inhibitors were prepared as below and added to the cells at 96 well-plate. (Table 4.1)

Table 4.1. Information and the concentration about the inhibitors used in cytotoxicity analysis.

Inhibitor	**Uptake Mechanism**	**Concentration**
Chlorpromazine	Clathrin-Mediated Endocytosis	1, 5, 10, 25, 50 μM
Cytochalasin D	Phagocytosis by Actin Depolymerization	0.5, 1, 5 μg/ml
Dynasore	Clathrin-mediated and Caveole-dependent Endocytosis	1, 10, 25, 50 μM
EIPA	Macropinocytosis	10, 25, 50, 100 μM
Filipin III	Caveole-dependent and Lipid Raft-mediated Endocytosis	1.25, 2.5, 5 ve 10 μM
Genistein	Caveole-dependent Endocytosis	1, 50, 100, 200 μM
Nocadazole	Microtubule Depolymerization	40, 75, 100 ng/mL

After the analysis, suitable doses of the inhibitors were chosen by evaluating their effects on the cell viability of the recipient cells. When the suitable doses of the inhibitors were applied to the cells, cell viability should be ensured at least 70-80%. The differentiation in the cell viability of the recipient cells were observed after the analysis. (Figure 4.4) The fluorometric analysis was performed with the excitation filter at 490 nm and emission filter at 520 nm.

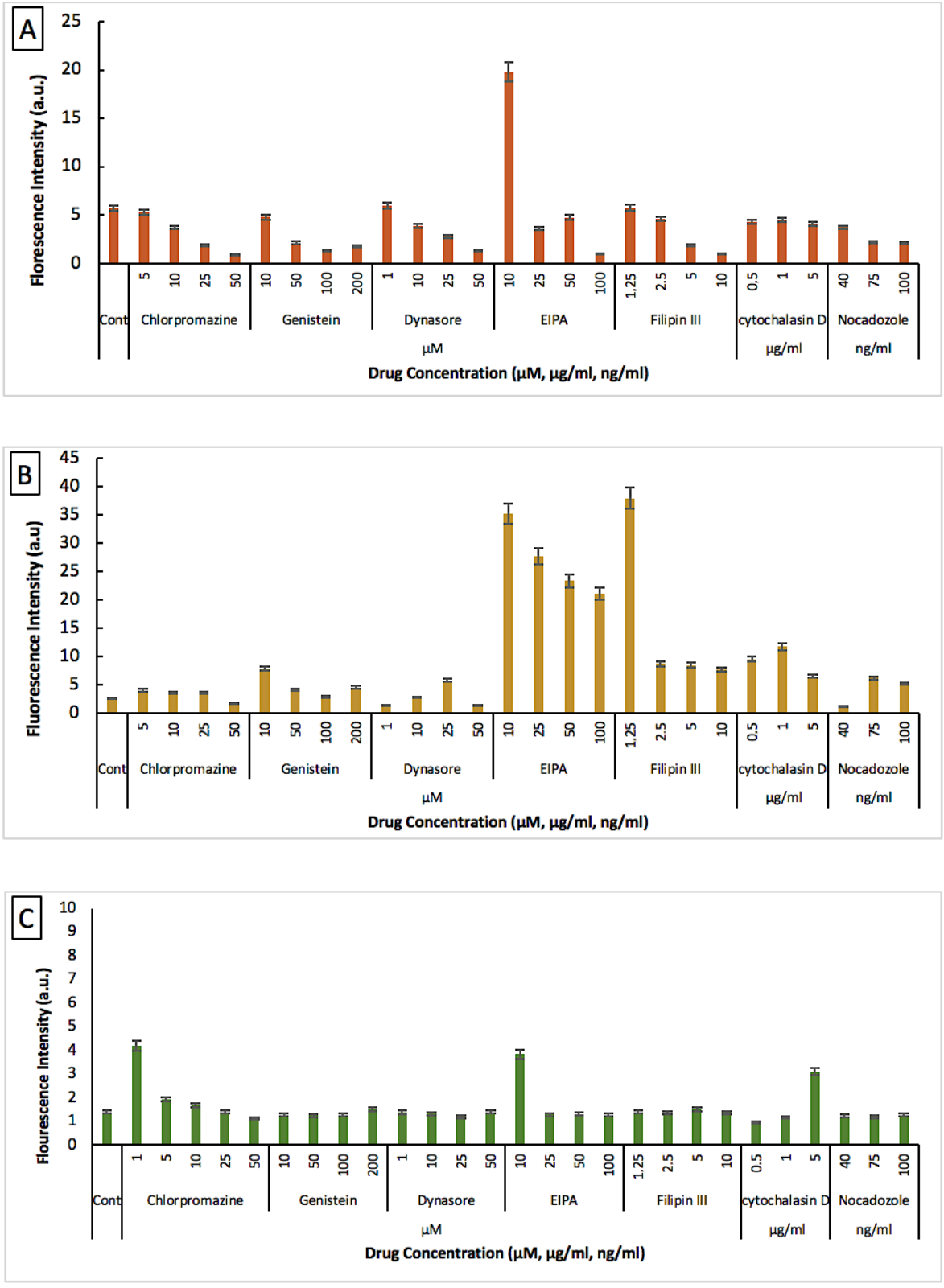


Figure 4.4. Differentiation in cell viability of the recipient A2780 (A), OSE (B) and MeT-5A (C) cells with the presence of drugs that inhibit exosome uptake mechanisms.

After fluorometric analysis, the cell viabilities were examined and the best concentration of inhibitors were preferred when the cell viability decreased 30% at most. The ideal concentration of the inhibitors providing this condition were stated and applied to recipient cells. (Table 4.2)

Table 4.2. Detected suitable concentration of the inhibitors to apply to each recipient cell lines.

Inhibitors	A2780	OSE	MeT-5A
Chlorpromazine	5 µM	5 µM	5 µM
Cytochalasin D	5 µg/ml	5 µg/ml	5 µg/ml
Dynasore	10 µM	10 µM	10 µM
EIPA	50 µM	50 µM	50 µM
Filipin	2.5 µM	2.5 µM	2.5 µM
Genistein	10 µM	10 µM	10 µM
Nocadozole	40 ng/ml	75 ng/ml	75 ng/ml

The concentrations shown above at Table 4.2 were applied to the recipient cells and the exosome uptake for each cell was analyzed with Flow Cytometry analysis. In each condition, the exosome uptake inhibition was observed and the most effective inhibitor was determined. (Figure 4.5) According to the results in Figure 4.5, for each recipient cell, the exosome uptake mechanism can be indicated. When the results compared according to the cell lines, the uptake mechanisms of exosomes were also different for each cell.

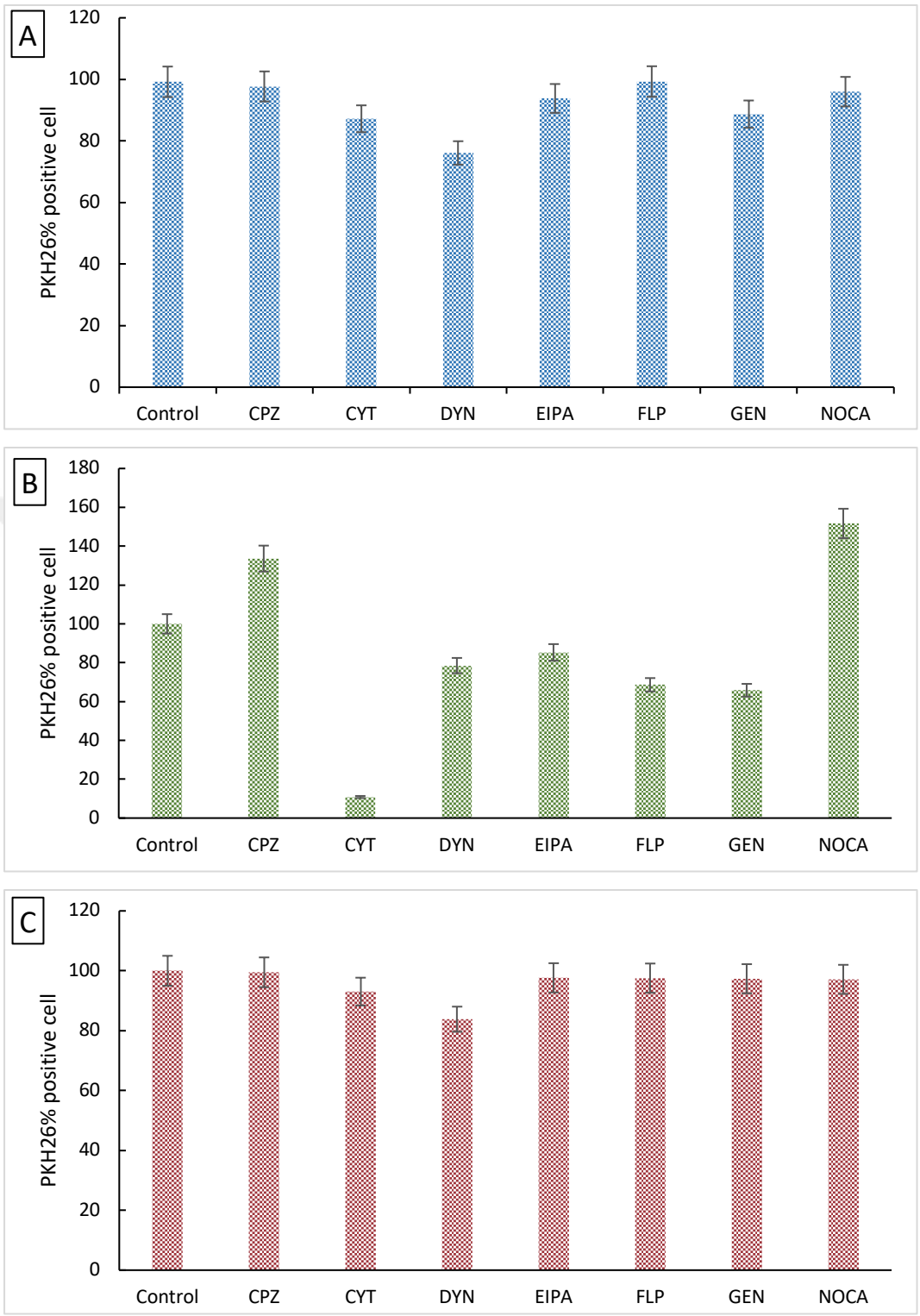


Figure 4.5. Detecting the uptake mechanism of the PKH26 labelled A2780cis exosomes into the recipient A2780 (A), OSE (B) and MeT-5A (C) cells via flow cytometry by using inhibitors (CPZ, chlorpromazine; CYT, cytochalasin D; DYN, dynasore; EIPA, 5-(n-ethyl-n-isopropyl)-amiloride; FLP, Filipin; GEN, genistein; NOCA, nocadozole).

When the results in Figure 4.5 were examined, A2780cis exosomes preferred mostly dynasore, cytochalasin-D and genistein inhibited uptake mechanisms respectively while entering into the A2780 cells. In dynasore, cytochalasin-D and genistein results, the exosome uptake reduced to 76.1%, 87.2% and 88.7% when they compared to control. In other words, it can be evaluated that A2780 cells uptake A2780cis exosomes by clathrin-mediated and caveole-dependent endocytosis, phagocytosis by actin depolymerization and caveole-dependent endocytosis mainly. Apart from A2780 cells, OSE cells utilized different uptake mechanisms. Exosome uptake mostly reduced when cytochalasin-D, genistein, filipin, dynasore and EIPA respectively to 11%, 66%, 69%, 79% and 85% when they compared to control. Therefore, OSE cells make use of mainly phagocytosis by actin depolymerization; then caveole-dependent endocytosis, caveole-dependent and lipid raft-mediated endocytosis, clathrin-mediated and caveole-dependent endocytosis and macropinocytosis. Finally, MeT-5A cells cannot uptake exosomes effectively when dynasore inhibitor applied. Exosome uptake decreased to 84% under dynasore inhibition for MeT-5A cells. Therefore, MeT-5A cells utilize from clathrin-mediated and caveole-dependent endocytosis mechanism to uptake exosomes into the cell.

Detecting the target of the PKH26 labeled A2780cis-released exosomes in the recipient cells by confocal imaging

Following the uptake of exosomes in recipient cells, the targets of these exosomes in the recipient cells are required to be understood for the mechanism of tumorigenesis. Thus, the exosomes and the specific organelles were labelled with fluorescence dyes and the cells were observed via confocal microscopy. The locations of the exosomes were detected in detail by examining each organelle in different slides. To identify the exact locations of the exosomes, each sample was labelled with PKH26, DAPI and organelle-specific dye for showing the exosomes, nucleus and target organelle respectively under 63X magnification. As mentioned in the method part in Table 3.2, the organelle and pathway specific dyes were chosen and the targets of exosomes can be observed clearly for recipient A2780 (Figure 4.6), OSE (Figure 4.7) and Met-5A (Figure 4.8).

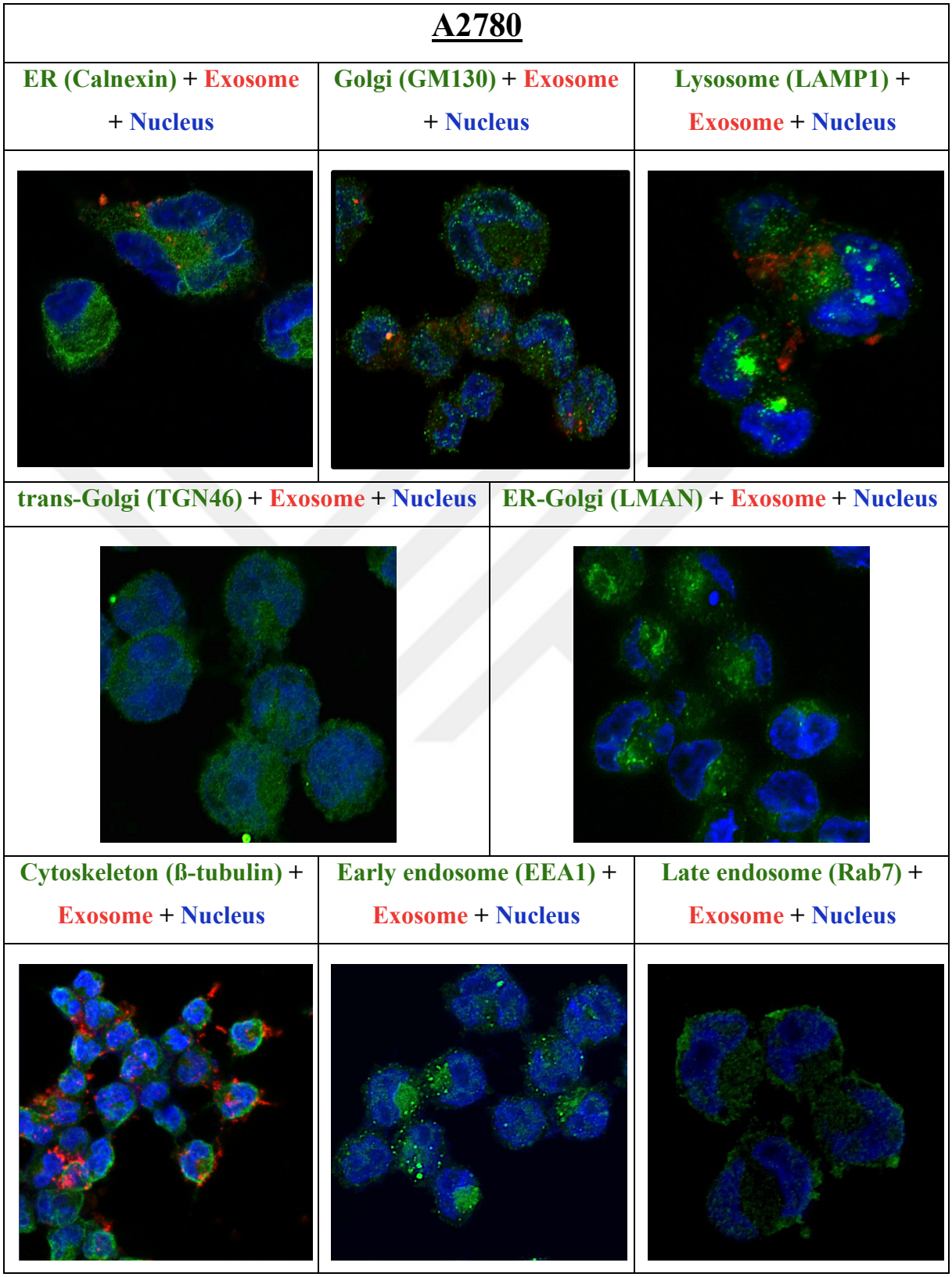


Figure 4.6. Targets of PKH26 labelled exosomes into recipient A2780 cells after 6 hours by confocal microscopy at 63X magnification.

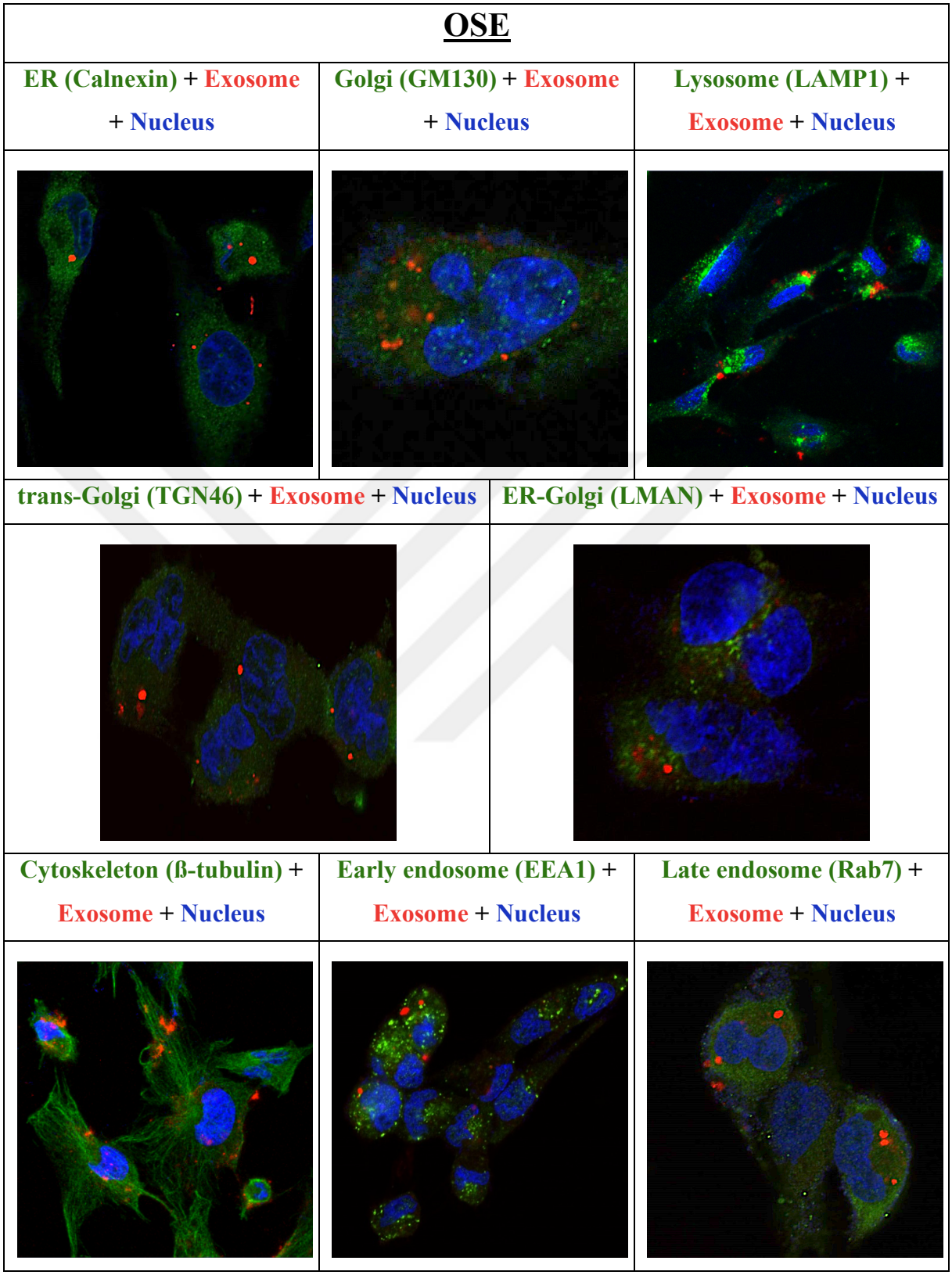


Figure 4.7. Targets of PKH26 labelled exosomes into recipient OSE cells after 6 hours by confocal microscopy at 63X magnification.

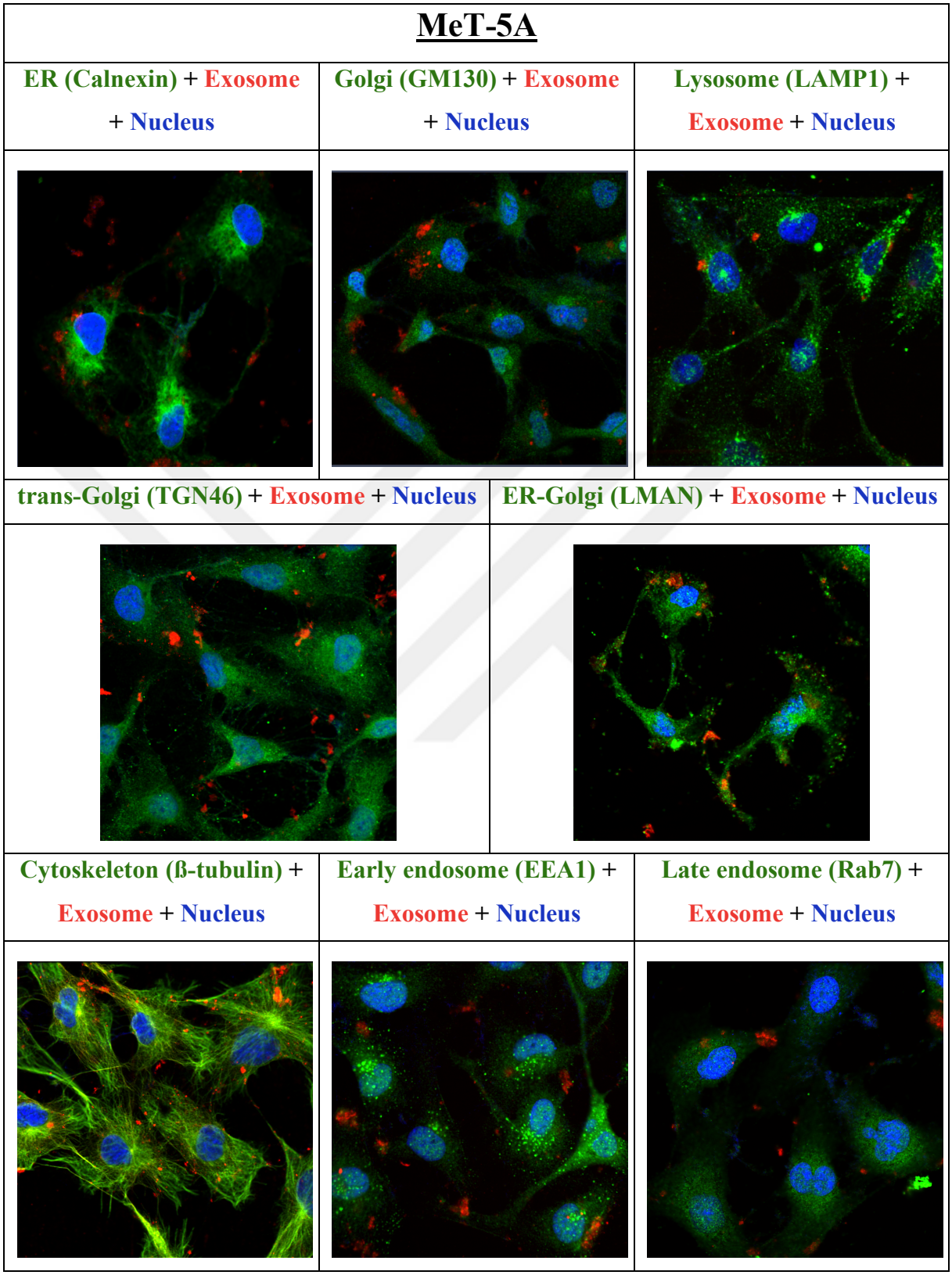


Figure 4.8. Targets of PKH26 labelled exosomes into recipient MeT-5A cells after 6 hours by confocal microscopy at 63X magnification.

As also shown in Figure 4.6 to Figure 4.8, A2780cis exosomes entered into recipient A2780, OSE and MeT-5A cells and were localized in various areas in the cells. In Figure 4.6, PKH26 labelled exosomes were uptaken by A2780 cells and localized in endoplasmic reticulum and Golgi apparatus especially. In addition, they were also localized in the lysosome and above the cytoskeleton. Therefore, it can be explained as; exosomes benefit from cytoskeleton to move towards these organelles. In Figure 4.7, OSE cells internalized PKH26 exosomes and these exosomes localized in different areas in the cells. It can be seen that PKH26 labelled exosomes were uptaken by early and late endosomes, also proved in flow cytometry analysis in Figure 4.5 Then, some of the exosomes located on ER-Golgi Network and trans-Golgi network. In addition, like in A2780 cells, exosomes were also localized in lysosome, ER and Golgi apparatus with the movement through cytoskeleton in OSE cells. Finally, as seen in Figure 4.8, PKH26 labelled exosome internalization and localization were also observed after confocal microscopy. As proven in flow cytometry analysis in Figure 4.5,
MeT-5A cells were benefit from endocytosis to internalize exosomes. This proof was also encouraged by confocal images including the localization of exosomes in early and late endosomes. As also results in OSE and A2780 cells, the exosomes were also localized in ER, Golgi apparatus and lysosome by moving through cytoskeleton. Both flow cytometry and confocal imaging results support each other for using endocytosis to uptake exosomes inside recipient cells.

Differentiation in ROS levels after A2780cis exosome uptake into recipient cells

After observing the targets of exosomes in cells, the changing mechanisms by exosome uptake were examined. First, changes in the ROS levels in the recipient cells were investigated and measured by fluorometric analysis at 485/528 nm. ROS levels were detected after 3 and 6 hours of exosome incubation. (Figure 4.9 and Figure 4.10) The levels were compared according to the controls as seen in Figure 4.9 and 4.10.

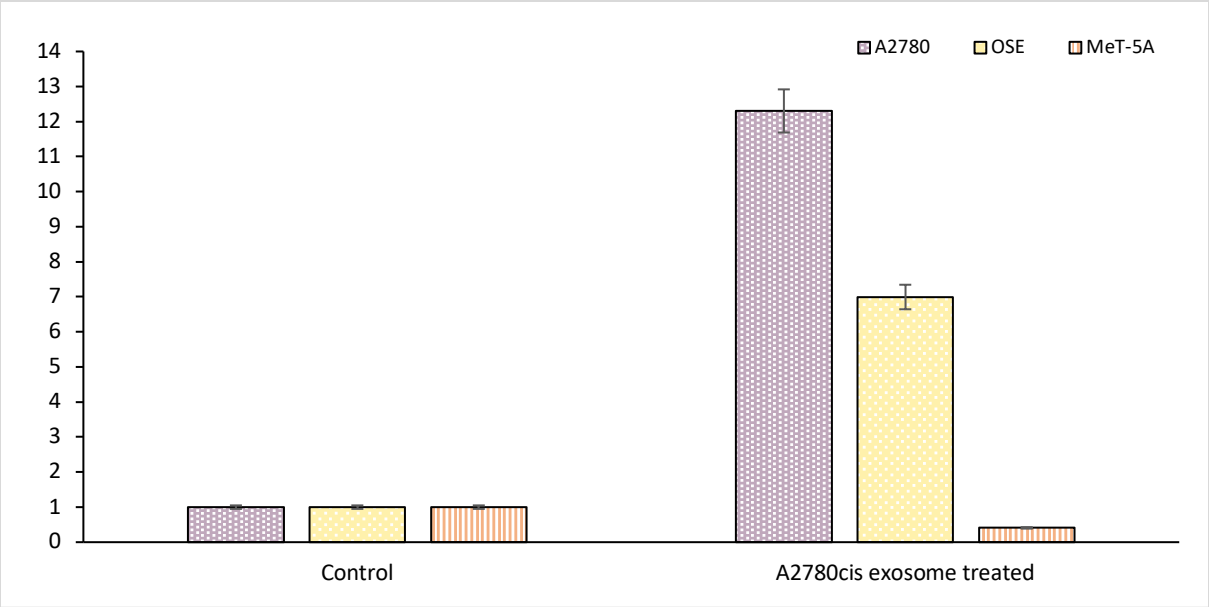


Figure 4.9. Detection of the difference in reactive oxygen species levels of the recipient A2780, OSE and MeT-5A cells respectively after treated with A2780cis exosomes for 3 hours. Fluorometric analysis at 485/528 nm.

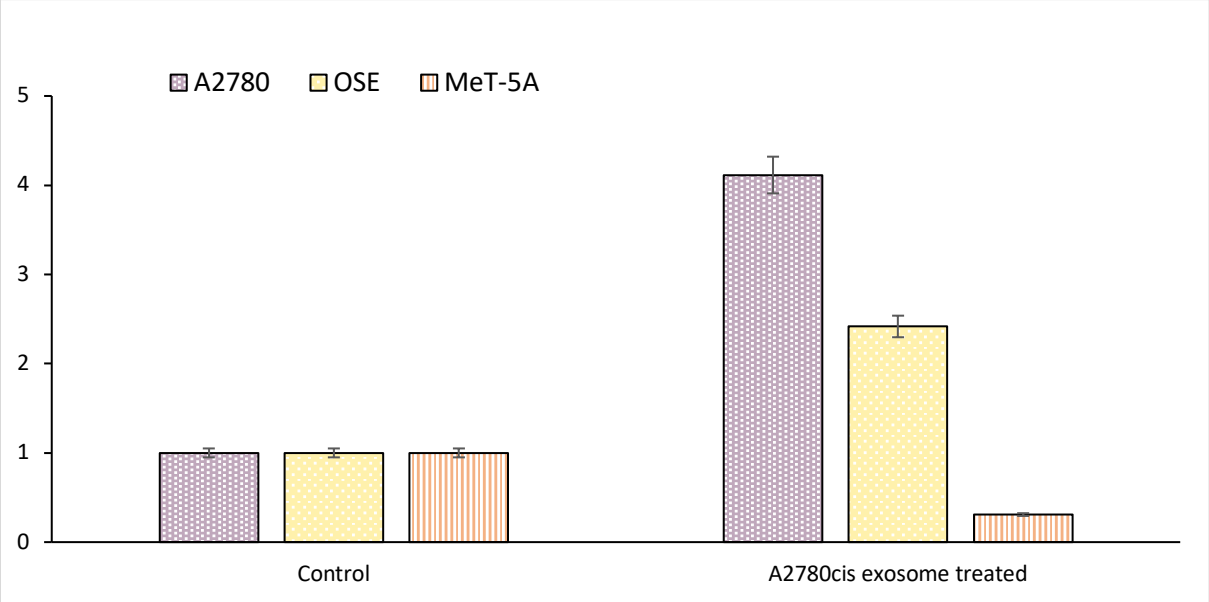


Figure 4.10. Detection of the difference in reactive oxygen species levels of the recipient A2780, OSE and MeT-5A cells respectively after treated with A2780cis exosomes for 6 hours. Fluorometric analysis at 485/528 nm.

When the results in Figure 4.9 were observed, it was detected that ROS levels in A2780 cells after exosome treatment increased 12.3 times when compared to control A2780 cells in 3 hours. Moreover, ROS levels in OSE cells after exosome treatment increased 3.9 times when compared to control OSE cells in 3 hours. However, the results were different for MeT-5A cells because when compared to control cells, ROS levels in exosome-treated MeT-5A cells reduced 0.4 times after 3 hours of incubation. After that, when results in Figure 4.10 were observed, it was seen that the increase and decrease of ROS levels were like cells in 3 hours. After 6 hours, in exosome-treated A2780 cells ROS levels were higher 4.1 times. In exosome-treated OSE cells, ROS levels got higher 2.4 times when according to control OSE cells after 6 hours. On the other hand, like after 3 hours of exosome treatment, ROS levels of MeT-5A cells decreased 0.3 times after 6 hours of exosome treatment.

Changes in invasion and migration rates after A2780cis exosome uptake into recipient cells

After detecting the changes in ROS levels of recipient cells, the next cell mechanisms were selected as invasion and migration rates. Detection of invasion rates in recipient A2780, OSE and Met-5A cells were detected via fluorometric analysis at 480/520 nm after 48 hours of exosome treatment. (Figure 4.11)

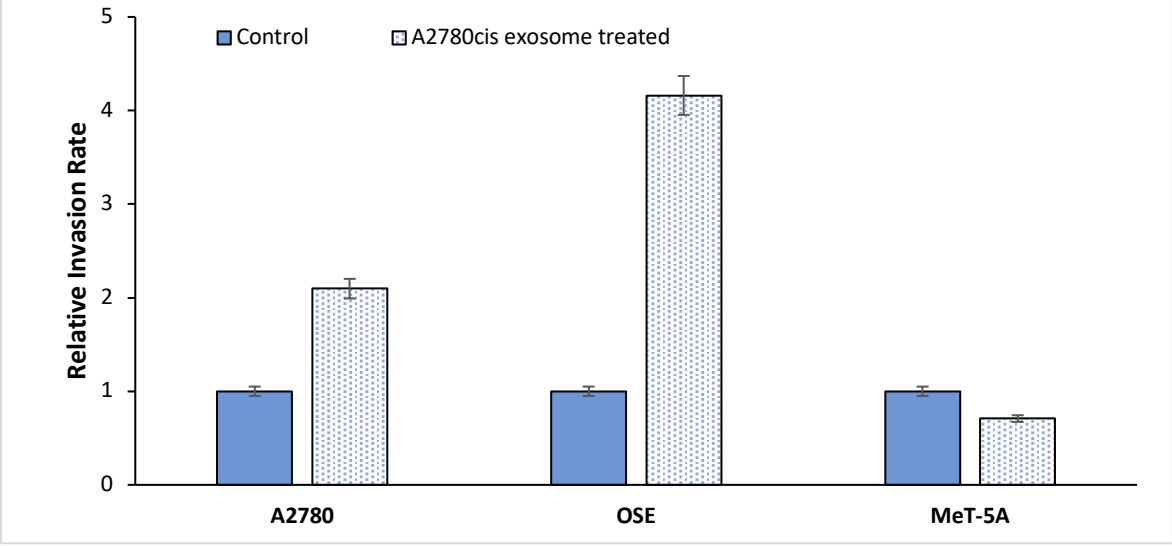


Figure 4.11. Change in relative invasion rate of the recipient A2780, OSE and MeT-5A cells respectively after treated with A2780cis exosomes for 48 hours. Fluorometric analysis at 480/520 nm.

As seen in Figure 4.11, relative invasion rates increased in A2780 and OSE cells 2.1 and 4.1 times after 48 hours of A2780cis exosome treatment when compared to control cells. However, the result was opposite for MeT-5A cells. After 48 hours of A2780cis exosome treatment, when compared to control cells relative invasion rate of MeT-5A cells decreased 0.7 times.

After detecting the changes in relative invasion rates of recipient cells, relative migration rates were also investigated. Detection of migration rates in recipient A2780, OSE and Met-5A cells were detected via fluorometric analysis at 480/520 nm after 48 hours of exosome treatment. (Figure 4.12)

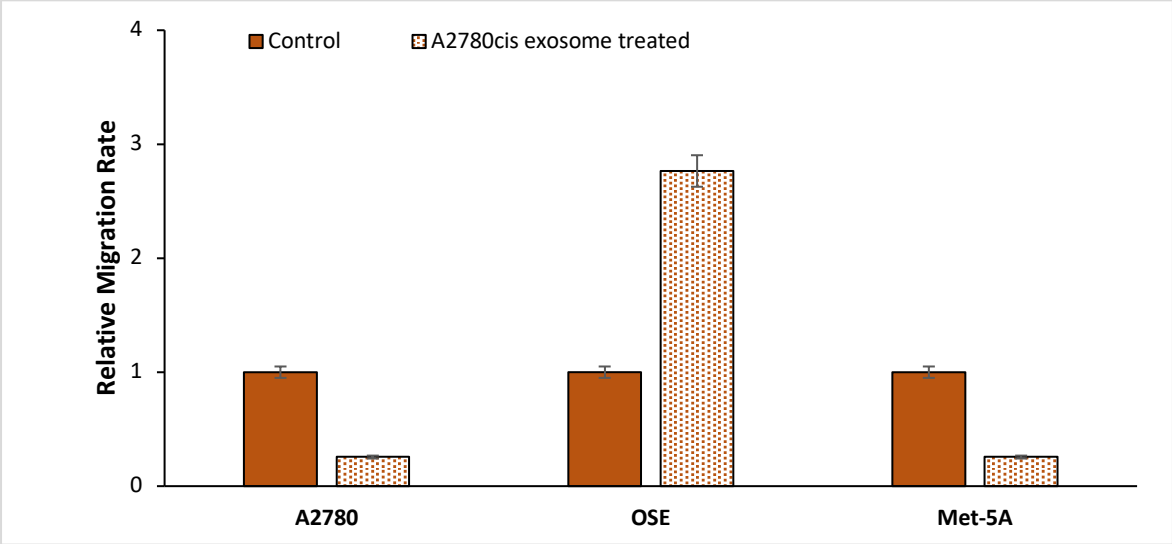


Figure 4.12. Change in relative migration rate of the recipient A2780, OSE and MeT-5A cells respectively after treated with A2780cis exosomes for 48 hours. Fluorometric analysis at 480/520 nm.

As shown in Figure 4.12, relative migration rates decreased in both A2780 and MeT-5A cells 0.3 times after 48 hours of A2780cis exosome treatment when compared to control cells. However, the result was different for OSE cells due to increase in relative migration rate. After 48 hours of A2780cis exosome treatment, when compared to control cells relative migration rate of OSE cells increased 2.8 times. According to the results in Figure 4.11 and Figure 4.12, it can be enounced that exosome uptake affects migration and invasion rates in recipient cells.

Alteration in the proliferation rates after A2780cis exosome treatment into recipient cells

After detecting the changes in invasion and migration rates of recipient cells, cell proliferation rates were targeted to research. Observation of changes in proliferation rates in recipient A2780, OSE and Met-5A cells were detected by ATP levels measurement via luminometric analysis after 24 and 48 hours of exosome treatment. (Figure 4.13)

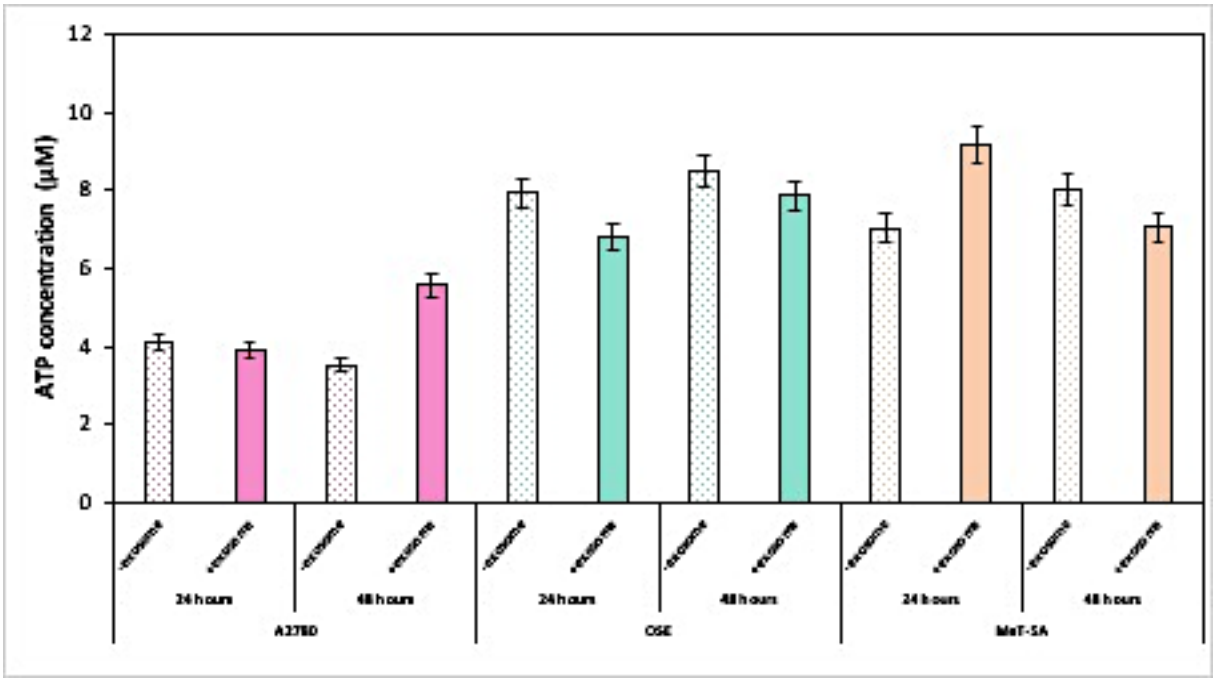


Figure 4.13. Luminometric measurement of ATP levels for observing the change in proliferation rate of the recipient A2780, OSE and MeT-5A cells respectively after treated with A2780cis exosomes for 24 and 48 hours.

ATP concentrations of the recipient cells, as results of ATP bioluminescence analysis were directly proportional to cell proliferation rate. As shown in Figure 4.13, after exosome treatment in A2780 cells, change in proliferation rate was not observed in 24 hours but ATP concentration increased after 48 hours. However, ATP concentrations in OSE cells decreased both after 24 and 48 hours of exosome treatment. For MeT-5A cells, while the proliferation rates got higher after 24 hours of exosome treatment; decreased after 48 hours of exosome treatment.

Apart from recipient cells, A2780cis cells were also examined to detect whether the growth mediums of exosome-treated recipient cells affect the proliferation rate of A2780cis cells. Therefore, luminometric measurement of 24 and 48 hours ATP levels in A2780cis cells were analyzed after the cells were cultured exosome-treated recipient A2780, OSE and MeT-5A cells. A2780cis cells were cultured with the 24 hour- (Figure 4.14) and 48 hour- (Figure 4.15) growth mediums of three recipient cells.

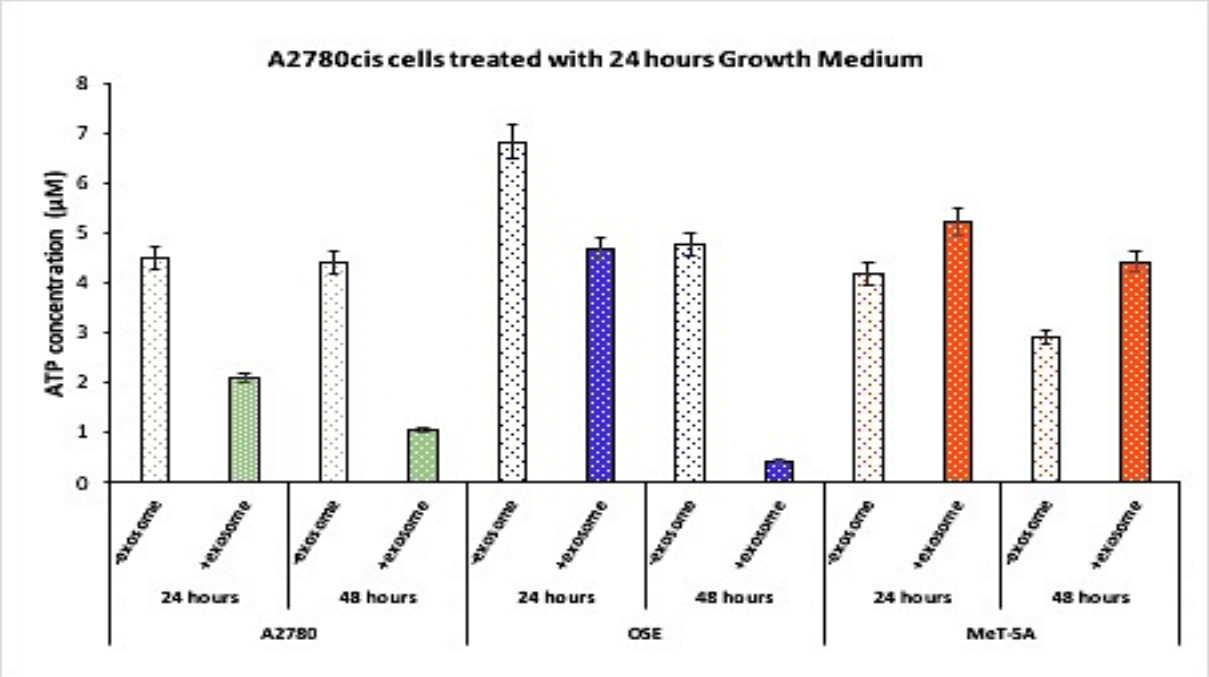


Figure 4.14. Luminometric measurement of ATP levels for observing the change in proliferation rate of the recipient A2780cis cells after treated with A2780cis exosome-treated growth mediums of recipient A2780, OSE and MeT-5A cells for 24 hours.

The proliferation rate of A2780cis cells were changed after the cells were cultured with 24 hour-growth medium of recipient A2780, OSE and MeT-5A cells. As shown in Figure 4.14, the luminometric measurement of ATP levels were analyzed both after 24 and 48 hours. As mentioned before, proliferation rate and ATP levels are directly proportional. Therefore, proliferation rate of A2780cis cells cultured in A2780 and OSE mediums decreased both in 24 and 48 hours due to reduction in ATP levels. However, MeT-5A cells provide A2780cis cells to proliferate highly according to the rise in ATP levels both in 24 and 48 hours.

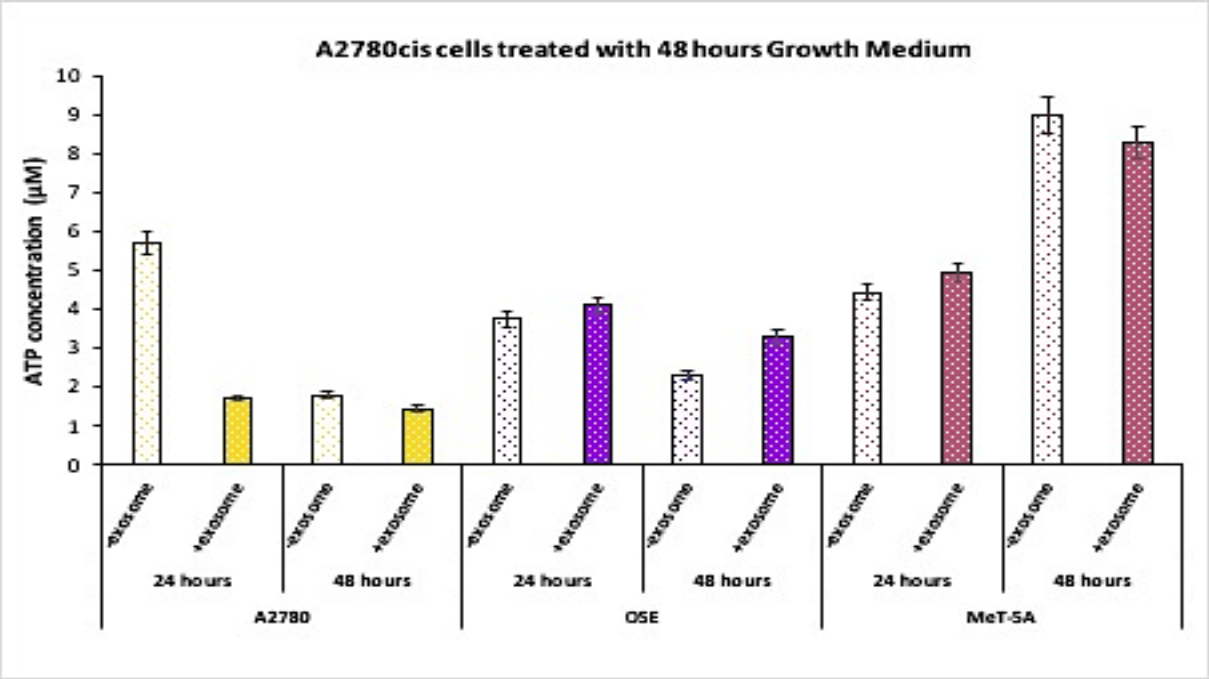


Figure 4.15. Luminometric measurement of ATP levels for observing the change in proliferation rate of the recipient A2780cis cells after treated with A2780cis exosome-treated growth mediums of recipient A2780, OSE and MeT-5A cells for 48 hours.

After treated with 48-hour growth medium of recipient cells, the proliferation rate of A2780cis cells were affected different than 24-hour growth medium of recipient cells. The results were not altered for A2780-growth medium treatment. The ATP levels of A2780cis cells were both reduced at 24 and 48 hours after 48-hour growth medium treatment. Unlike previous results for OSE cells, the proliferation rate of A2780cis cells were increased. When the results of MeT-5A-growth medium treated A2780cis cells were observed, the proliferation rate switched from increase to decrease in 24 and 48 hours respectively.

Differentiation of EMT mechanisms of recipient cells after exosome treatment

Finally, EMT mechanism of the recipient cells were taken into consideration to evaluate the changes in cells after exosome uptake. To detect these changes, western blotting analysis was performed and some certain epithelial, mesothelial and mesenchymal markers were observed. (Figure 4.16-18) Epithelial markers were E-cadherin (97 kDa), also a mesothelial marker, and EpCAM (35 kDa), where mesenchymal markers were Vimentin (54 kDa), ZEB1 (124 kDa) and Slug (30 kDa). Western blotting analysis were performed for recipient A2780 (Figure 4.16), OSE (Figure 4.17) and MeT-5A (Figure 4.18) cells after treating with exosomes for 24 and 48 hours.

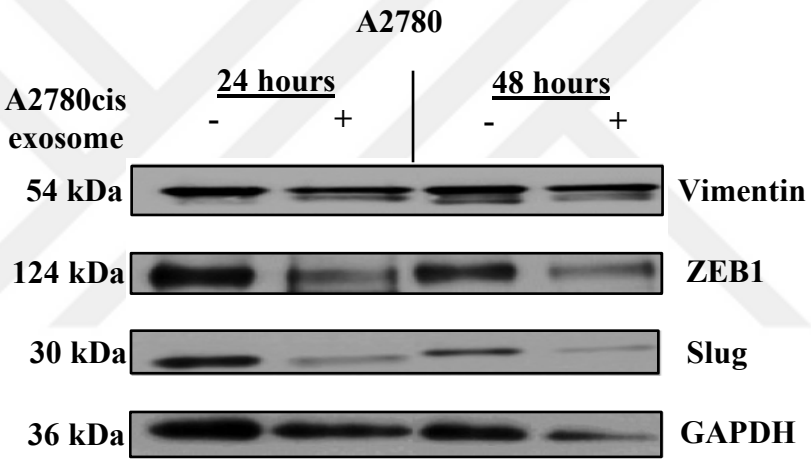


Figure 4.16. Western blotting analysis for observing the alteration in epithelial-mesenchymal markers of the recipient A2780 cells after treated with A2780cis exosomes for 24 and 48 hours.

When the results in Figure 4.16 were observed, all mesenchymal markers Vimentin (54 kDa), ZEB1 (124 kDa) and Slug (30 kDa) decreased both in 24 and 48 hours of exosome-treated A2780 cells when compared to control A2780 cells. However, epithelial markers E-cadherin (97 kDa) and EpCAM (35 kDa) were not observed in both 24 and 48 hours exosome treatment conditions (results were not shown.) GAPDH (36 kDa) control marker was also observed in all conditions of A2780 cells.

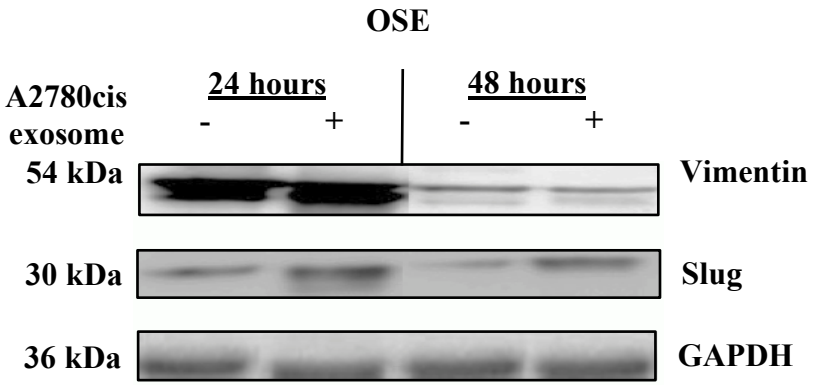


Figure 4.17. Western blotting analysis for observing the alteration in epithelial-mesenchymal markers of the recipient OSE cells after treated with A2780cis exosomes for 24 and 48 hours.

When the results of OSE cells in Figure 4.17 were analyzed, mesenchymal markers Vimentin (54 kDa) and Slug (30 kDa) were observed in OSE cells. In 24 hours of exosome treatment, OSE cells expressed Vimentin protein highly but in 48 hours of exosome treatment, this expression reduced. Slug expression increased both in 24 and 48 hours exosome-treated OSE cells when compared to control OSE cells. There were no expression of epithelial markers E-cadherin (97 kDa) and EpCAM (35 kDa) (results were not shown). GAPDH (36 kDa) control marker was also expressed in all conditions of OSE cells.

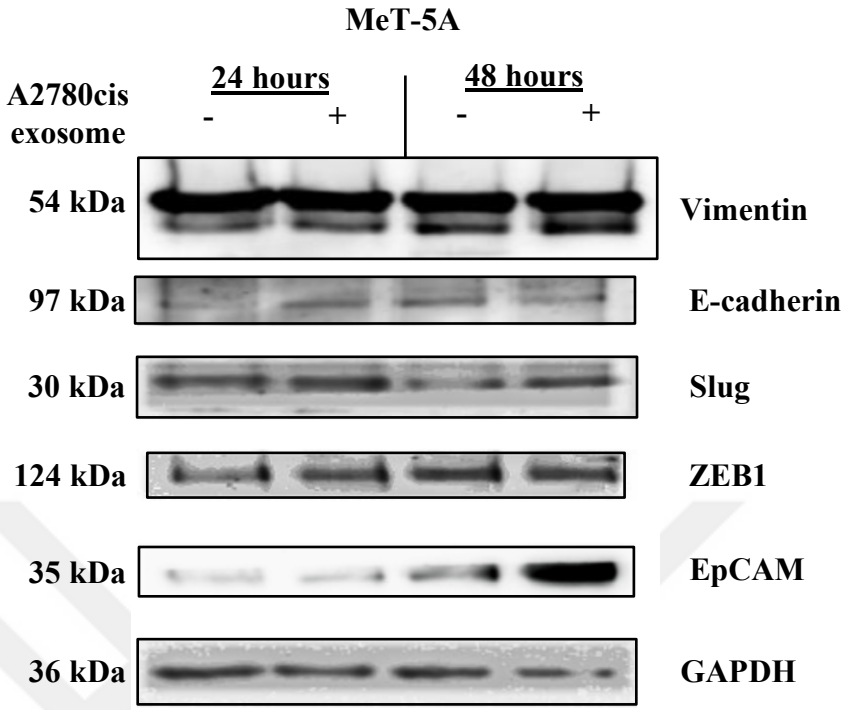


Figure 4.18. Western blotting analysis for observing the alteration in epithelial-mesenchymal markers of the recipient MeT-5A cells after treated with A2780cis exosomes for 24 and 48 hours.

In Figure 4.18, change in epithelial and mesenchymal markers expressed from MeT-5A cells was shown. The expression of all mesenchymal markers Vimentin (54 kDa), ZEB1 (124 kDa) and Slug (30 kDa) was observed. There were no changes in the expression of Vimentin protein in all conditions of MeT-5A cells. However, both Slug and ZEB1 protein expression increased in 24 and 48 hours exosome-treated MeT-5A cells when compared to control MeT-5A cells. Epithelial markers EpCAM (35 kDa) and E-cadherin (97 kDa) were also observed in western blotting analysis for MeT-5A cells. There were no changes in the expression of E-cadherin protein in both control and 24 and 48 hours exosome-treated MeT-5A cells. On the other hand, epithelial marker EpCAM was highly expressed after 48 hours of exosome treatment. Finally, when all the results were taken into consideration, it can be assumed that A2780 and OSE cells had epithelial-mesenchymal changes where MeT-5A cells had mesothelial-mesenchymal change.

To verify the Western Blotting results, protein expressions of the recipient cells were also analyzed by qPCR. The same epithelial-mesenchymal markers were taken into consideration to control. After RNA isolation and cDNA synthesis, qPCR was set up and the protein expressions of recipient A2780 (Figure 4.19), OSE (Figure 4.20) and MeT-5A (Figure 4.21) were observed.

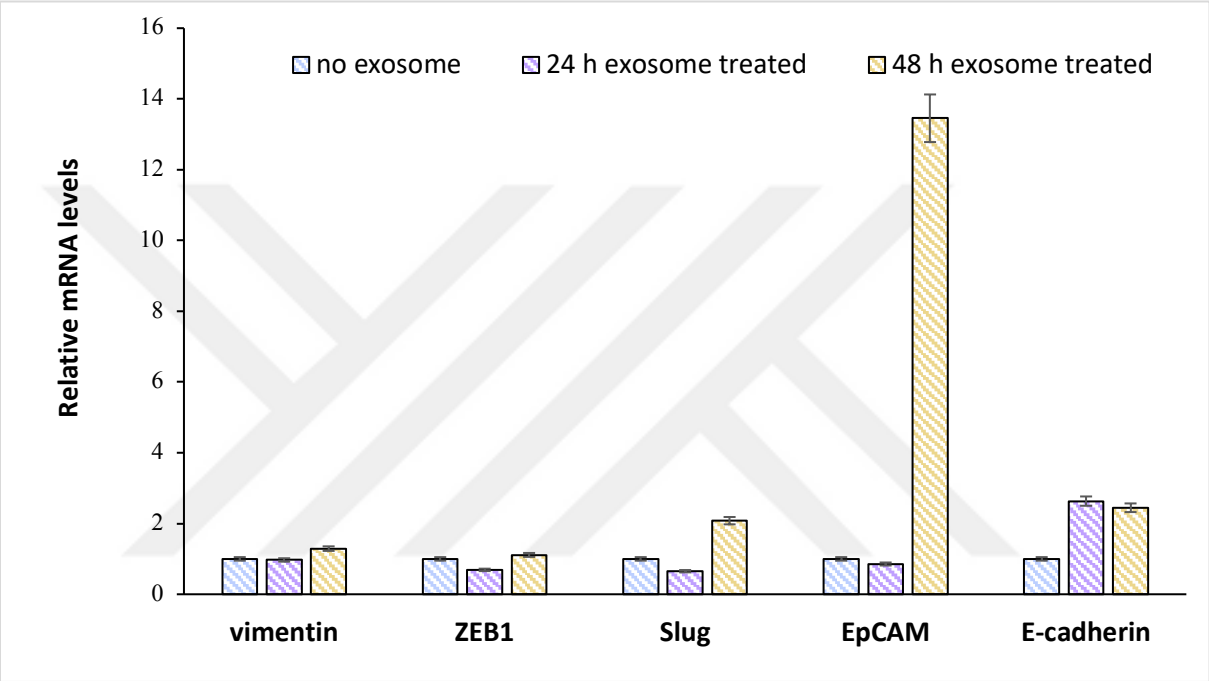


Figure 4.19. qPCR analysis for observing the change in the expression levels of epithelial-mesenchymal markers of the recipient A2780 cells after treated with A2780cis exosomes for 24 and 48 hours.

For A2780 cells, qPCR results indicated approximately same results as western blotting analysis in Figure 4.16. As we observed in western blotting results, there were no slightly changes in vimentin and ZEB1 proteins but Slug expression increased in 48 hours. Although EpCAM and E-cadherin proteins were not observed after western blotting, the protein expressions increased highly in both 24 and 48 hours for A2780 cells in Figure 4.19.

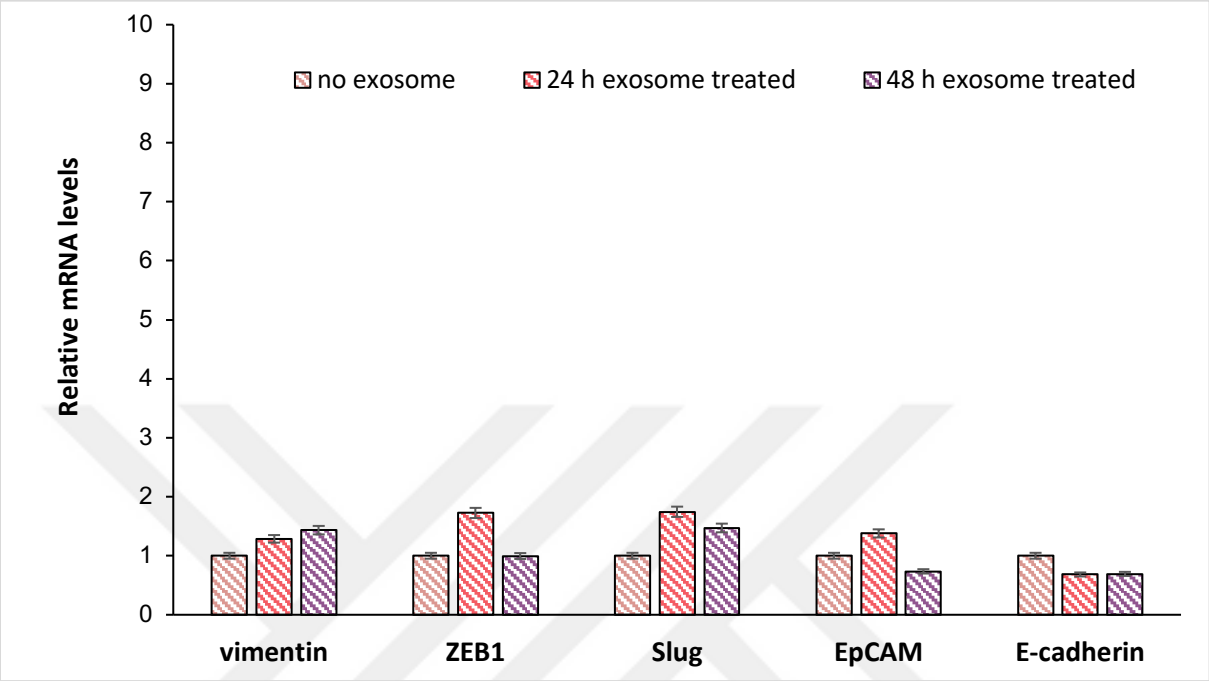


Figure 4.20. qPCR analysis for observing the change in the expression levels of epithelial-mesenchymal markers of the recipient OSE cells after treated with A2780cis exosomes for 24 and 48 hours.

The qPCR results of OSE cells demonstrated the expression levels of the target proteins were approximately like controls. Like in A2780 cells, EpCAM and E-cadherin expressions were also observed after qPCR analysis, therewithal ZEB1 expressions were also examined. While Slug, EpCAM and ZEB1 protein expressions increased in 24 hours, the protein levels decreased after 48 hours. E-cadherin levels decreased both in 24 and 48 hours unlike vimentin expressions according to the results in Figure 4.20. More protein expressions were detected after qPCR analysis when compared to western blotting results in Figure 4.17.

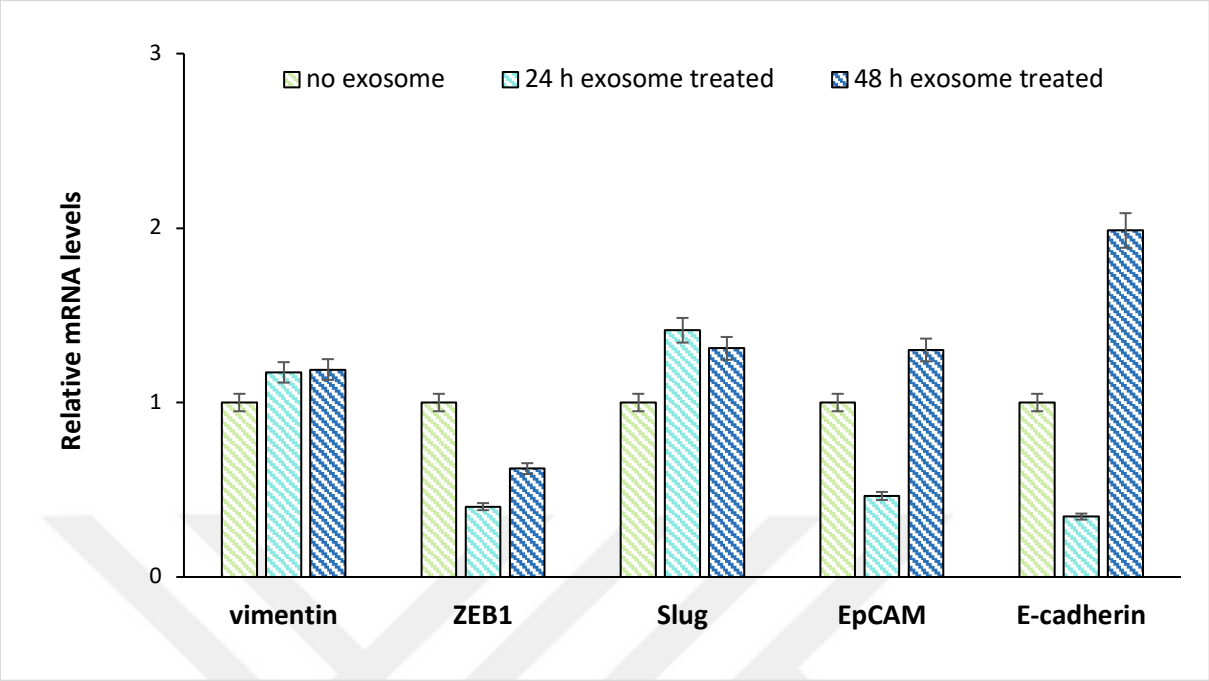


Figure 4.21. qPCR analysis for observing the change in the expression levels of epithelial-mesenchymal markers of the recipient MeT-5A cells after treated with A2780cis exosomes for 24 and 48 hours.

For MeT-5A cells, the relative mRNA levels indicated that each target protein was also expressed after exosome treatment both in 24 and 48 hours. After exosome treatment, ZEB1 protein expression decreased slightly. E-cadherin and EpCAM expression increased in high level similarly observed after western blotting. When considering Vimentin and Slug, the mRNA levels increased somehow but western blotting results were not be capable of showing this increasing difference in Figure 4.18. Finally, when the all results were evaluated, the qPCR results for each cell promote the western blotting results for EMT markers on a large scale.

5. DISCUSSION

Epithelial ovarian cancer (EOC) is the seventh most widespread cancer and the major global problem due to causing numerous lethal gynecological malignancies among women. In addition, tumor growth confidentially due to lack of disease-specific clinical symptoms especially in FIGO stages I-II, and absence of efficient screening instruments bring along the ovarian cancer diagnosis at advanced stage FIGO stages III-IV which is concluded in numerous mortalities. Thus, "silent-killer" nickname is given to this cancer. Due to its heterogenic character, ovarian cancer creates challenges while treating the patient. To discover the suitable therapy for each patient is approximately impossible since the heterogeneity may lead to different stimulated oncogenic mechanisms for everyone. Therefore, recently investigated and developed therapy should be specific to each ovarian cancer diagnosed patient.

By the time the new therapy is discovered by scientists, known treatments such as; surgery, radiotherapy, chemotherapy, are applied to patients which bring another leading problem: drug resistant cancerous cells after adjuvant chemotherapy. These newly drug-resistant cancerous cells innovate its metastatic niche which is formed by primary and secondary tumor microenvironment. Hence, this innovation procures the cancerous cells to gain another property which causes invading and metastasing aggressively. To deliver the signals from drug-resistant cancer cells in tumor microenvironment require cell-cell communication providing agents; which are also recently studied as hot topic; exosomes. Key mediators of cell-cell interaction especially inducing angiogenesis, tumor progression, metastasis and drug-resistance, also reprogram tumor microenvironment by transferring pro-tumorigenic conditions to the cells located in this microenvironment. Therefore, in this research, we focalized on the oncogenic mechanisms induced by the drug-resistant ovarian cancer cell (A2780cis) released exosomes. Also, the isolation and characterization of A2780cis-released nanoparticles, their uptake into recipient cells (A2780, OSE, MeT-5A) with the mechanism they benefit from, their intercellular trafficking and oncogenic mechanisms differentiated after uptake are also studied in detail.

Characterization of nanoparticles is the major step in this study. The requiring conditions for defining them as “exosomes” according to their size and proteins consisting within must be achieved. Size measurement analysis verified the size of nanoparticles that we isolated are in the reference range, also reported in literature; 30-150 nm [160]. In addition, mostly mentioned and specific markers Alix and TSG101 are also present in our nanoparticles [163], except calnexin absence (the endoplasmic reticulum marker) proves the pure isolation and separation of exosomes from other vesicles consisting within the A2780cis cell growth mediums. To illustrate, average peak examined after Nanoparticle Tracking Analysis (NTA) in PC3 cells-secreted exosomes is between 110-120 nm [211]. Also, mentioned in the “2.3 Exosomes” part, cancerous cells released exosomes are greater in size when compared to normal cell released ones. Isolated exosomes generated from breast cancer cells and multidrug resistant chronic myeloid leukaemia cell lines, RH460 and K562Dox are larger in size between 70-200 nm. Mentioned breast cancer cells are also including the exosomal markers but no calnexin expression [212, 213].

Then to visualize the uptake of PKH-26 labelled exosomes by recipient cells, flow cytometry and confocal imaging were performed. According to the literature, internalization of exosomes has shown in various types of cells. For instance, exosomes released from ovarian cancer cells have uptaken into identical cells [174] and mesenchymal stem, macrophages, mesothelial and endothelial cells driven from adipose tissue in the tumor microenvironment, respectively [214, 215, 216, 217, 218].

Cancer progression is induced by feeding from oncogenic signals and various types of cell-cell communications in tumor microenvironment. Both primary (ovarium) and secondary (peritoneum and omentum) regions of tumor microenvironment contribute to effectuate the ideal pre-metastatic niche. Not only cancerous cells but also the normal cells mainly including immune, epithelial, mesenchymal and mesothelial cells are the significant contents of tumor microenvironment to continue oncogenic signal traffic. According to our results, after 3 hours, the internalization of PKH-26 labelled A2780cis exosomes is examined into the pre-exosome-treated recipient A2780, OSE and MeT-5A cells. When the information in the literature was checked, the data and the proofs also have been suggested the uptake of cancer cell-derived exosomes into the recipient stromal [219], cancer [220] and epithelial [213] cells in the tumor

microenvironment. Also, ovarian cancer SKOV-3 cells released exosomes were internalized into the identical cells after incubation for 30 minutes [174]. After the observation of exosome internalization, the mechanism that the recipient cells utilized became the new target to be explained. According to the studies, exosomes are internalized into the recipient cells via the mechanisms such as; endocytosis, macropinocytosis and phagocytosis [175]. When we chose the inhibitors to detect the uptake mechanisms, previous studies were our guidance. We used chlorpromazine, Cytochalasin-D, nocodazole, genistein, Filipin-III, EIPA and Dynasore to block the specific uptake pathways. Our results showed similarities with the literature data. The recipient A2780 and MeT-5A cells also utilized mostly the clathrin-mediated and caveole-dependent endocytosis based on energy-dependent process [167, 174, 191, 220]. However, OSE cells benefit from phagocytosis with actin depolymerization just like macrophage cells [168, 221]. Inhibition of A2780cis exosomes into recipient A2780 and MeT-5A cells decreased slightly when compared to other mechanisms. Therefore, contribution of phagocytic mechanism on exosome internalization is low, almost nothing. Effects of inhibitors on uptake mechanisms depend on the type of the cell, so that the results for exact enlightenment of exosome uptake mechanisms may not be adequate. Nevertheless, the results of our study can contribute to upcoming studies.

To elucidate the mechanisms that exosomes contribute to, the target organelles in the recipient cells can also be appraised as key answers. Type of the cell was not only effective to specify the uptake mechanism of exosomes, but also stimulate cells to undergo different signaling pathways to directly bring exosomes up to specific organelles. Whence, confocal imaging experiments were carried out and results supported the exosome uptake mechanism through endocytosis and phagocytosis according to evidences demonstrated localized PKH-26 labelled exosomes into the endosomes and phagosomes. However, the localization of exosomes and their intracellular targets were not studied much so the data including previous studies is restricted. A research about the intercellular trafficking of exosomes indicated initially endosomes and phagosomes, then phagolysosomes and endoplasmic reticulum are the targets of K562 and HEK293 cells [168, 222]. Apart from that PC12 cell derived exosomes were also internalized into cells by endocytic pathway and transported into lysosomes [179]. Exosome uptake into identical cells utilized from the contribution of three different pathways according to results from dynamic imaging. The movement can be occurred through actin filaments,

straightly cell peripheries to perinuclear region and only at perinuclear region because of exosome stuck in the large vesicles [171].

In conclusion, the cellular mechanism alterations after internalization of A2780cis exosomes were studied in our research. As given information in the "2.2. Molecular Mechanism of Ovarian Carcinogenesis and Tumor Microenvironment", exosomes participate reprogramming of cellular signaling in tumor microenvironment and the cells located in that area. In addition, "cargo" structure can be advantageous for exosomes due to transport various ingredients consisting of mainly nucleic acids, proteins and lipids in order to stimulate the differentiation in various carcinogenic pathways. Invasion, cell proliferation, migration, drug resistance, EMT and ROS generation are one of the carcinogenic mechanisms induced with the aid of exosomes. They are also key mediators to generate the pre-metastatic niche to develop cancer progression properly. Like exosomes, ROS contribute to both physiological and pathological conditions, depending on its concentration. When ROS concentration is at low level, the cell will be in the cellular signaling regulation process [223]. Oppositely, when ROS concentration is high, it will be pathological process for cells such as; aging and cancer. ROS levels or generation can be altered by microvesicles or signaling pathways included in ROS metabolism can regulate them [224]. Induction of ROS generation has been demonstrated in breast epithelial after breast cancer exosome uptake [213]. According to our study, for A2780 and OSE cells ROS generation and invasion were stimulated intensely after exosome internalization in the primary tumor microenvironment region. When the results were observed proliferation increased for A2780 cells whereas, migration increased for OSE cells. Several studies have been indicated the cancerous cell-derived exosomes stimulate mesothelial cell to initiate invasion mechanism and metastasis [216, 218, 225, 226]. However, in our study, these mechanisms were slightly affected in MeT-5A cells. Furthermore, cells require EMT while differentiate into metastatic cancerous cells to gain mesenchymal characteristics. Our results represent different EMT analysis results for each recipient cell. Therefore, in overall, our study indicates exosomes derived from drug-resistant ovarian cancer cells induce carcinogenesis mechanisms of various types of recipient cells in a different way. Therefore, exosome-mediated carcinogenesis mechanisms can depend on the cell type that internalized exosomes. In conclusion, our research can be expressed as a study that sheds light into a long dark tunnel which also requires supportive studies to develop a prevention or treatment.

6. CONCLUSION AND FUTURE ASPECTS

The characterization of the isolated nanoparticles from A2780cis cells is proved as "exosomes" according to the size measurement and western blotting results. The exosomes are taken into the recipient cells in 6 hours however the uptake mechanisms depend on the type of the cells such as; (1) for primary ovarian cancer A2780 and mesothelial MeT-5A cells, the mechanism is mostly clathrin-mediated and caveole-dependent endocytosis; (2) for ovarian epithelial OSE cells, it is mostly phagocytosis with actin depolymerization. Furthermore, uptake of exosomes into the recipient cells resulted in the changes in; ROS generation, EMT, invasion, migration and proliferation rates. To state the exact results, experiments based on the specific mechanisms and their association with oncogenic signaling pathways should be investigated in detail. Therefore, our studies about exosome-mediated tumorigenesis and differentiation on the oncogenic signal mechanisms of the recipient cells are still in progression.

In conclusion, the enlightenment of the communication between the drug resistant cancer cells and recipient cells located in primary and secondary tumor microenvironment is essential for the development of the new targets for prevention and treatment of ovarian cancer.

7. REFERENCES

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8. APPENDIX

8.1. Curriculum Vitae

ÖZGEÇMİŞ

Adı Soyadı: Gizem YILMAZ

TC Kimlik No / Pasaport No:	15251431428
Doğum Yılı:	1993
Yazışma Adresi:	Yalı Mahallesi, 6500/1 Sokak, Park Yaşam Sitesi, No: 5 Blok: F D:43 K:17 35550 Karşıyaka/İZMİR
Telefon:	05549884835
Faks:	-
e-posta:	yilmazngizem@gmail.com

EĞİTİM BİLGİLERİ

Ülke	Üniversite	Fakülte/Enstitü	Öğrenim Alanı	Derece	Mezuniyet Yılı
TR	Dokuz Eylül Üniversitesi	İzmir Uluslararası Biyotıp ve Genom Enstitüsü	Moleküler Biyoloji ve Genetik	Yüksek Lisans	2020
TR	Yeditepe Üniversitesi	Mühendis ve Mimarlık Fakültesi	Genetik ve Biyomühendislik	Lisans	2017

AKADEMİK/MESLEKTE DENEYİM

Kurum/Kuruluş	Ülke	Şehir	Bölüm/Birim	Görev Türü	Görev Dönemi

UZMANLIK ALANLARI

Uzmanlık Alanları
Cell Culture DNA and RNA Isolation from blood and cell Exosome Isolation from plant and cell Exosome Characterization Western Blotting PCR Techniques Cytotoxicity Analysis (Cell Viability, MTT) ROS Analysis Invasion and Migration Rate Analysis Fluorometric and Luminometric Analysis Immunofluoroscence (Confocal and Fluorescent Imaging) Flow Cytometry

GÖREV ALDIĞI PROJELER

Projenin Adı	Kurum	Proje Dönemi	Projedeki Görevi
Ovaryum Kanser Hücrelerinden Salınan Eksozomların Primer Ovaryum Kanser Hücreleri ile Sağlıklı Epitel ve Mezotel Hücreler Tarafından Alınım Yolları ve Hedef Hücrelerdeki Karsinojenez Mekanizmasına Etkisi	Dokuz Eylül Üniversitesi BAP	2019-2020	Araştırmacı
Ovaryum Kanser Hücrelerinden Salınan Eksozomların Primer Ovaryum Kanser Hücreleri ile Sağlıklı Epitel ve Mezotel Hücreler Tarafından Alınım Yolları ve Hedef Hücrelerdeki Karsinojenez Mekanizmasına Etkisi	TÜBİTAK	2017-2020	Araştırmacı

ULUSAL ve ULUSLARARASI KONGRE/SEMPOZYUM/KONFERANS BİLDİRİLERİ

ULUSAL
Yilmaz, G., Tavsan, Z., Kursunluoglu, G., Cagatay, E., Ayar Kayali, H. "İlaç dirençli ovaryum kanser hücrelerinden salınan eksozomların primer ve sekonder tümor mikroçevredeki hücreler tarafından alınımı ve etkileri" Onkolojide İz Bırakanlar Zirvesi 2019, 14-17 November 2019, Antalya. (Sözlü Bildiri)
ULUSLARARASI

KATILDIĞI VE SERTİFİKA ALDIĞI KONFERANS/SEMPOZYUM VE EĞİTİMLER

Therapeutic mAb Engineering and Production Workshop, 11 Kasım 2017, İzmir Uluslararası Biyotıp ve Genom Merkezi, İzmir.
New Frontiers in Life Sciences, 10 Kasım 2017, İzmir Uluslararası Biyotıp ve Genom Merkezi, İzmir.
Deney Hayvanları Kullanımı Sertifikası Kursu, 9-18 Ekim 2017, Dokuz Eylül Üniversitesi, İzmir.
X Annual Congress of the European Proteomics Association, 21-25 Haziran 2016, Acıbadem Üniversitesi, İstanbul.

DİĞER AKADEMİK FAALİYETLER

Son Bir Yılda Uluslararası İndekslere Kayıtlı Makale/Derleme İçin Yapılan Danışmanlık Sayısı			
Son Bir Yılda Projeler İçin Yapılan Danışmanlık Sayısı			
Yayınlara Alınan Toplam Atıf Sayısı			
Danışmanlık Yapılan Öğrenci Sayısı		**Tamamlanan**	**Devam Eden**
	Yüksek Lisans		
	Doktora		
	Uzmanlık		
Diğer Faaliyetler (Eser/görev/faaliyet/ sorumluluk/olay/üyelik vb.)			

ÖDÜLLER

	Ödülün Adı	**Alındığı Kuruluş**	**Yılı**
☐			

YAYINLARI

SCI, SSCI, AHCI indekslerine giren dergilerde yayınlanan makaleler

Diğer dergilerde yayınlanan makaleler

Hakemli konferans/sempozyumların bildiri kitaplarında yer alan yayınlar
