The Use of CRISPR/dCas9 Engineered MSCs to Prevent Cisplatin-induced Cellular Damage in Human Granulosa Cells

by

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A Dissertation Submitted to the
Graduate School of Health Sciences

in Partial Fulfillment of the Requirements for

the Degree of

Master of Science

in

Reproductive Biology

KOÇ
ÜNİVERSİTESİ

August 09, 2022

The Use of CRISPR/dCas9 Engineered MSCs to Prevent Cisplatin-induced Cellular Damage in Human Granulosa Cells

Koç University

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To all those who contributed to my success

ABSTRACT

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Mehmet Yunus ÇOMAR

Master of Science in Reproductive Biology

August 09, 2022

Chemotherapy-induced ovarian failure (COF) can cause ovarian dysfunction diseases in which the ovaries temporarily or permanently stop functioning due to chemotherapy. Low estrogen serum levels and amenorrhea are infertility signs may contribute the clinical appearance of COF. Cisplatin is a platinum-based anticancer chemotherapeutic drug that has a gonadotoxic effect on all ovarian cells. Stem cells, especially mesenchymal stem cells (MSCs) obtained from different adult tissues, have been tried as an adjunct therapy for COF. CRISPR genome editing technology is the most broadly used gene editing system, and dCas9 (dead Cas9) is a targeted CRISPR activation tool that lacks the nuclease effect. A synergistic activation mediator (SAM) is a type of CRISPR/dCas9 activation system composed of the dCas9-VP64 fusion protein integrated with a modified sgRNA, including two MS2 aptamers. MS2 binding protein is fused with two other activator domains, p65 and HSF1, and this fusion protein (MS2-p65-HSF1) interacts with MS2 aptamers. Exosomes are extracellular vesicles that range between 20–200 nm and can be isolated from biological fluids. They contain mRNA, long non-coding RNA, microRNAs, proteins, and lipids. Exosomes play an essential role in intercellular communication, in delivering gene and protein expression signals of cells. The aim of this research is to produce dCas9 genome edited MSCs and their exosomes and to test whether these cells are more resistant or chemotherapeutic, cisplatin, or not. Cisplatin-resistant mesenchymal stem cells were obtained by activating the CD99 gene with the SAM activator system. Then, exosomes were obtained from normal and resistant mesenchymal stem cells separately. Later, spheroids were formed with these cells. Co-cultures of MSCs were established with the experimental treatment groups at with hGRC1 (human granulosa cell line) cells in the insert using the Transwell aid. After the co-cultures were established, the cells were exposed to cisplatin. After that, hGRC1 cells were harvested at 24, 48, 72, and 96 hours, respectively. Firstly, a cell viability assay was performed on the collected cells. Then, by qPCR

the expression of inflammatory or apoptotic markers as IL-10, IL-1Ra, Bax, and Bcl-2 genes was analyzed. As a result, it was observed that there were more viable cells in all experimental groups compared to the control group, and inflammatory and apoptosis values decreased in each hour group. And it was also discovered that resistant cells have provided a more effective protection than the non-resistant cells. The group that provides the most significant protection compared to all other experimental groups is the group that uses resistant mesenchymal stem cells and exosomes isolated from these cells, and this finding is important as it can be interpreted that the use of resistant mesenchymal cells with their exosomes can be an alternative adjunct therapy to protect ovarian granulosa cells during the use of cisplatin like chemotherapeutics for cancer.

Keywords: Mesenchymal Stem Cells, Cisplatin, CRISPR/dCas9, Exosomes, Cancer, Infertility

ÖZETÇE

Sisplatin ile İndüklenen İnsan Granüloza Hücrelerinde ki Hücre Hasarını Önlemek için CRISPR/dCas9 Tasarlanmış MSC'lerin Kullanımı

Mehmet Yunus ÇOMAR

Üreme Biyolojisi Yüksek Lisans

Ağustos 09, 2022

Kemoterapiye bağlı yumurtalık hasarı, kadınlarda kemoterapi alan kadınlarda yumurtalıkların işlevini geçici veya kalıcı olarak yitirebildiği bir durumdur ve yumurtalık disfonksiyonunda düşük östrojen serum seviyesi, amenore ve infertilite gibi belirtiler görülebilir . Cisplatin, tüm yumurtalık hücreleri üzerinde gonadotoksik etkisi olan platin bazlı bir antikanser kemoterapötik ilaçtır. Kök hücreler, özellikle yetişkin bir kök hücre olan mezenkimal kök hücreler kemoterapiye bağlı yumurtalık hasarı için yardımcı bir tedavi olarak denenmektedir. CRISPR genom düzenleme teknolojisi, en yaygın olarak kullanılan gen düzenleme sistemidir ve nükleaz etkisinden yoksun dCas9 (ölü Cas9), bir CRISPR aktivasyon aracıdır. Bir sinerjistik aktivasyon aracısı (SAM), iki MS2 aptameri dahil olmak üzere modifiye edilmiş bir sgRNA ile entegre edilmiş dCas9-VP64 füzyon proteininden oluşan bir CRISPR/dCas9 aktivasyon sistemidir. MS2 bağlayıcı proteini, diğer iki aktivatör alanı olan p65 ve HSF1 ile birleştirilir ve bu füzyon proteini (MS2-p65-HSF1), MS2 aptamerleri ile etkileşime girer. Bu şekilde daha yüksek bir gen aktivasyonu sağlanmış olur. Eksozomlar, 20-200 nm arasında değişen ve biyolojik sıvılardan izole edilebilen hücre dışı veziküllerdir. Eksozomlar mRNA, uzun kodlamayan RNA, mikroRNA'lar, proteinler ve lipitler içerirler. Eksozomlar, hücreler arası iletişimde, hücrelerin gen ve protein ekspresyon sinyallerini iletmede önemli bir rol oynarlar. Bu çalışmanın amacı, dCas9 ile genomu düzenlenmiş MSC'leri sisplatine reve eksozomlarını üreterek ve bu hücrelerin daha dirençli veya kemoterapötik, sisplatin olup olmadığını test etmektir. Bu çalışmanın amacı CRISPR/ dCas9 genom düzenleme aracı ile MSC hücrelerini sisplatine karşı dirençli hale getirmek ve ardından bu hücreleri ve bu hücrelerden elde edilmiş eksozomları kemoterapiye bağlı yumurtalık hasarını korumaya yönelik kullanmaktır.SAM aktivatör sistemi kullanılırak CD99 geni aktive edildi sisplatine karişı dirençli mezenkimal kök hücreler elde edildi. Ardından normal ve dirençli mezenkimal kök hücrelerden ayrı ayrı eksozomlar izole edildi. Daha sonra bu hücrelerden sferoidler elde edildi. “Transwell”

kültür tabaklarının alt kısmında deneysel tedavi grupları ve üst kısmında(insert) hGRC1 (insan granuloza hücre dizisi) hücreleri kullanılarak ko-kültürler oluşturuldu. Ko-kültürler oluşturulduktan sonra hücreler sisplatine maruz bırakıldı. hGRC1 hücreleri sırasıyla 24, 48, 72 ve 96 saat sonra toplandı. İlk olarak, toplanan hücreler de canlılık testi yapıldı. Daha sonra qPCR ile inflamasyon ve apoptoz belirteçleri olan IL-10, IL-1Ra, Bax ve Bcl-2 genlerinin ifadeleri analiz edildi. Sonuç olarak, tüm deney gruplarında kontrol grubuna göre daha fazla canlı hücre olduğu, her saat grubunda inflamatuar ve apoptoz değerlerinin düştüğü gözlemlendi. Ayrıca dirençli hücrelerin, dirençli olmayan hücrelere göre daha etkili bir koruma sağladığı görüldü. Diğer deney gruplarına göre önemli ölçüde daha fazla koruma sağlayan grup, dirençli mezenkimal kök hücreleri ve bu hücrelerden izole edilen eksozomların birlikte kullanıldığı grup olmuştur. Bu sonuçlar sisplatin gibi kemoterapötiklerle dirençli hale getirilmiş mezenkimal kök hücrelerin ve onların eksozomlarının kullanımının granuloza hücrelerini koruyabileceğini göstermesi açısından önemlidir.

Anahtar Kelimeler: Mezenkimal Kök Hücreler, İnfertilite ,CRISPR/dCas9, Eksozomlar, Kanser

ACKNOWLEDGMENTS

First of all, I would like to thank everyone who contributed even the smallest part to this thesis. I would like to colossally thank my advisor, Dr. Serçin Karahüseyinoğlu, for providing me with this wonderful working environment and allowing me to work on my thesis. I learned from her the importance of applying theory to practice, being in control of the smallest detail of things you read, presenting ideas and projects as much as making them, writing them down and visualizing them, and explaining something as if you were explaining it to someone who doesn't know.

I want to thank everyone who works in KUTTAM, where I shared happiness, concerns, ideas, and materials during my master's degree, especially Arda, Canan, Ebru, and Alihan.

I want to thank my old friends, Ali, Kerem, Serkan, Eke, Kerim, Fatih, Uğur, Burak, Yasin, and Ebru, for being with me in activities outside of work.

I am thankful to thank Jhan, who has been with me since the beginning of high school, for discussing every idea, answering my technical and research questions, and always being there for me.

Many thanks to my lab partners Gizem Söyler, Aylin, İpek, Ariana, and Yağmur. I want to give huge thanks to Gizem Söyler, for all the techniques she taught me and for always being my companion. I would also like to thank the Kayabölen family for everything. Alişan and Gizem answered my questions without getting bored, gave good suggestions for my problems and future, and made me love to run.

I am very grateful to my family, who have brought me to this day, who have never spared me their financial and moral support, who have accepted me under all circumstances and stood by me. I would like to thank my grandfather and mother, who always supported me in my adventure in Istanbul. I am grateful to my father, who calls me every day and asks if there is anything I need and stands by me with all his support without opposing my decisions. I would also like to thank my brother, who never lost his love. Finally, I would like to thank my mother, who always loved me and stood by me, who did not withhold her love and attention from me even when she was battling cancer, and who made an effort to talk to me even during the most intense chemotherapy times.

My most enormous thanks and gratitude to Ferzan, the person who has been with me since 2018, who was with me even in my most difficult, most hopeless, and worst times, and whom I love most to share my love, happiness, and success. We had unlimited adventures with her, visited dozens of countries and cities, and became food and coffee gourmets together. She did not spare me her support in every field, was always by my side, gave advice that improved me, and became the person who impacted my life most. I'm sure I wouldn't be the person I am now without her. I am sure the pages would not be enough if I wrote here what we experienced, tasted, shared, and changed together. Once again, I would like to thank Ferzan for everything.

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ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
Akt/PKB	Protein kinase B
ATM	Ataxia-telangiectasia-mutated
BAX	B-cell lymphoma 2 -associated X protein
BCL-2	B-cell lymphoma 2
BMP	Bone morphogenetic protein
BRAF	V-raf murine sarcoma viral oncogene homolog B1
BRCA	Breast cancer gene
BRIP	BRCA1 Interacting Protein 1
CAR	Chimeric Antigen Receptor
Cas9	CRISPR Associated Protein 9
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
COF	Chemotherapy-Induced Ovarian Failure
CTG	Cell Titer Go
CTLA-4	Cytotoxic T-Lymphocyte Associated Protein 4
DAZ	Deleted in Azoospermia
DMEM	Dulbecco's Modified Eagle Medium
DPBS	Dulbecco's phosphate-buffered saline
DSB	Double Strand Break
EDTA	Ethylenediamine tetraacetic acid
EMT	Epithelial-mesenchymal transition
EPCAM	Epithelial cellular adhesion molecule
EPD	Eukaryotic Promoter Database
ESC	Embryonic stem cell
Ex	Exosome
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FMR1	Fragile X Mental Retardation 1
FSH	Follicle Stimulating Hormone
FSH-R	Follicle Stimulating Hormone Receptor
GALT	Galactose-1-Phosphate Uridylyltransferase
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
gRNA	guide RNA
HDR	Homology-directed repair
hGRC1	Immortalized human granulosa cell line
HRP	Horse Radish Peroxidase
IC50	Half-maximal inhibitory concentration

IL	Interleukin
ILV	Intraluminal vesicles
iPSC	induced Pluripotent stem cells
LB	Lysogeny broth
LH	Luteinizing hormone
LH-R	Luteinizing hormone Receptor
MAPK	Mitogen-activated protein kinase
MLH	MutL homolog
MOI	Multiplicity of Infection
MSC	Mesenchymal stem cell
MSH	MutS homolog
NHEJ	Non-homologous end-joining
NOA	Non-obstructive azoospermia
NR5A1	Nuclear Receptor Subfamily 5 Group A Member 1
OA	Obstructive azoospermia
P/S	Penicillin-Streptomycin
PALB	Partner and Localizer of BRCA2
PAM	Protospacer adjacent motif
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PI3-kinase	Phosphoinositide 3-kinase
PID	Pelvic inflammatory disease
pRb	Retinoblastoma
R	Resistant
SAM	Synergistic activation mediator
SDS	Sodium dodecyl sulfate
SDS–PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Scanning electron microscope
Sp	Spheroid
TALEN	Transcription Activator-Like Effector Nuclease
TGF-β	Transforming growth factor beta
TIL	Tumor-Infiltrating Lymphocytes
tracrRNA	trans-activating CRISPR RNA
TSS	Transcription start site
ZFN	Zinc-Finger Nucleas

1

CHAPTER 1: INTRODUCTION

1.1 Cancer

Each cancer cell is initially a normal body cell. There are approximately 37.2 trillion cells in the human body. Cancer occurs when one of these cells or a group of cells differentiates. Human normal cells grow, divide (proliferate), and when they get old or damaged, they die, and new cells take their place. Still, cancer cells multiply uncontrollably and spread throughout the body(metastasis)[1]. Cancer is seen in many different types and tissues/organs such as bone, brain, bladder, breast, kidney, lung, lymphoma, ovary, stomach, etc. Cancer cells can form malignant tumors, and in many types of cancers, malignant tumors are solid, but in some cancer types, such as blood cancer, cancer can occur without being solid. Examples of malignant tumors are adenomas, fibroids, hemangiomas, lipomas, meningiomas, myomas, neuromas, osteochondromas, papilloma, etc[2].

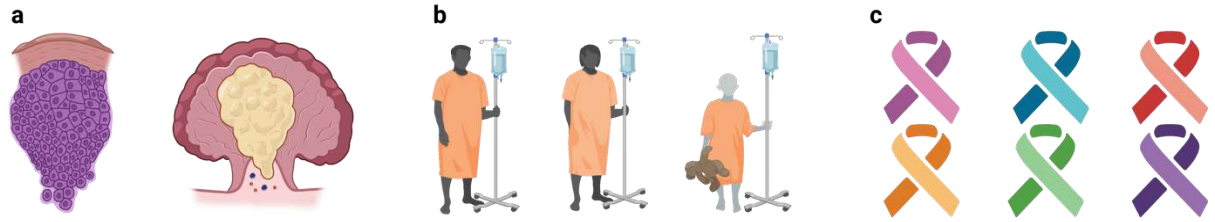

Figure 1. Cancer. *a.Tumors in the tissues b.Cancer patients of different gender and ages c.Different types of cancer symbols (Generated via Biorender.com)*

1.1.1 Cancer Mechanism

The reasons for getting cancer are not sure, yet some reasons are shown for it, which include genetic disorders, unhealthy food habits, stress, tobacco/alcohol, exposure to radiation, toxins, inflammations, etc[1]. Once cancer cells begin to spread, they begin to invade tissues and/or organs and destroy them; that's why cancer is the second most deadly disease in the world; millions of people have been diagnosed with new cancer every year, and hundreds of thousands of people die from cancer[3]. Somatic mutations are one of the biggest reasons that activate the formation of cancer cells. For example, sequencing analyses of human melanomas revealed mutations in the BRAF gene in 40% of these cells, which affect the structure of the B-Raf protein. Somatic mutations mainly affect growth factor genes, and the most common of these is the MAP-kinase pathway[4]. In some tumor arrays, hyperactivity was detected in the phosphoinositide 3-kinase signaling pathway similar to the MAP-kinase pathway; somatic mutations with phosphoinositide 3-kinase (PI3-kinase) affected the PI3-kinase signaling pathway, including the Akt/PKB signal transduction pathway[5,6]. In addition to the hyper-activating signal pathways of cancer cell growth, there are also changes in tumor suppressor genes and proteins that prevent growth and send cells to death. The two most important tumor suppressor proteins, retinoblastoma (pRb) and P53 control the growth and apoptosis programming of cells[7]. Damage/change in these two proteins enables cancer cells to grow and escape apoptosis. Telomeres are simply involved in essential functions such as chromatin organization, chromosome replication, and cell proliferation in healthy mammalian cells. Telomeres shorten with each replication, and cells with completely shortened telomeres get old and die. However, cancer cells can maintain the activity of their telomeres. Therefore, their telomeres do not shorten, or they develop a special recombinant to lengthen the chromosome ends, so that cell division does not stop like healthy cells. Cancer cells can continue to multiply uncontrollably. Many cancer cells are of epithelial origin, but cancer cells must escape from this epithelial origin in order to spread to other tissues and grow the tumor structure. Carcinomas do this by utilizing the epithelial-mesenchymal transition (EMT) mechanism, thereby disrupting the mechanisms that bind the carcinomas around, spreading, and growing in the environment[7]. Cancer cells also avoid many antiproliferative pathways, the best known of which is Transforming growth factor beta (TGF-β)[8–10]. Studies have shown that cancer cells avoid these antiproliferative signaling

pathways in a more complex way than simply turning them off. Cancer cells move away from these antiproliferative signaling pathways by activating the system called epithelial-mesenchymal transition (EMT).

1.1.1.1 Inflammation and Apoptosis

In cancer treatments, especially in chemotherapy, inflammatory and apoptosis values in somatic cells in the body increase due to the damage caused by chemotherapy. Interleukins (ILs), the cytokines, are secreted by different cells in the body. In research, interleukins with pro- and anti-inflammatory properties are also used as inflammation markers. Some of the most common types of interleukin used to measure cell inflammation values are IL-10 and IL-1Ra[11–13]. As the inflammation value increases, the expression values of IL-10 and IL-1Ra also increase. Apoptosis is the programmed cell death. In particular, cells with irreparable DNA damage die by apoptosis. Chemotherapy drugs also cause irreparable DNA damage by making multiple DSBs on DNA. Hence, cancer and other cells go into apoptosis. In chemotherapy, apoptosis levels are incredibly high, especially in the rapidly proliferating cells. There is more than one marker that can measure apoptosis. One of them is the Bax/Bcl-2 pair. Bax/Bcl-2 are two different genes that work as inhibitors of each other. Bcl-2 is an anti-apoptotic protein that binds to Bax protein and inhibits Bax protein. Bax is an apoptotic protein; that is, when the Bax gene is activated, the cell goes into apoptosis. If Bax expression is more than Bcl-2 in a cell, it indicates that the cell has gone into apoptosis. If Bcl-2 expression is high, the cell is not apoptosis. Therefore, Bax and Bcl-2 are used as apoptosis markers[11,14,15].

1.1.2 Gynecological Cancers

Cancer type is named according to the region of cancer in the human body. If the cancer is in the female reproductive organs, they are called as gynecological cancer. The main gynecological cancers are cervical, ovarian, vaginal, fallopian tube, vulvar, and endometrial. Although the incidence of many gynecological cancers increases significantly after the age of 50, a substantial proportion of women of reproductive age are diagnosed with gynecological cancer. According to the cancer statistics report collected between 2012 and 2016, the ratio of women aged 45 and

younger who were diagnosed with new uterine, ovarian, and cervical cancers is respectively 5%, 12%, and 36.5%[16]. Gynecological cancers cause severe morbidity and mortality in the United States. Based on the 2020 data, 113520 new gynecological cancers have been diagnosed in the United States, and these cancers are estimated to cause 33620 deaths[17].

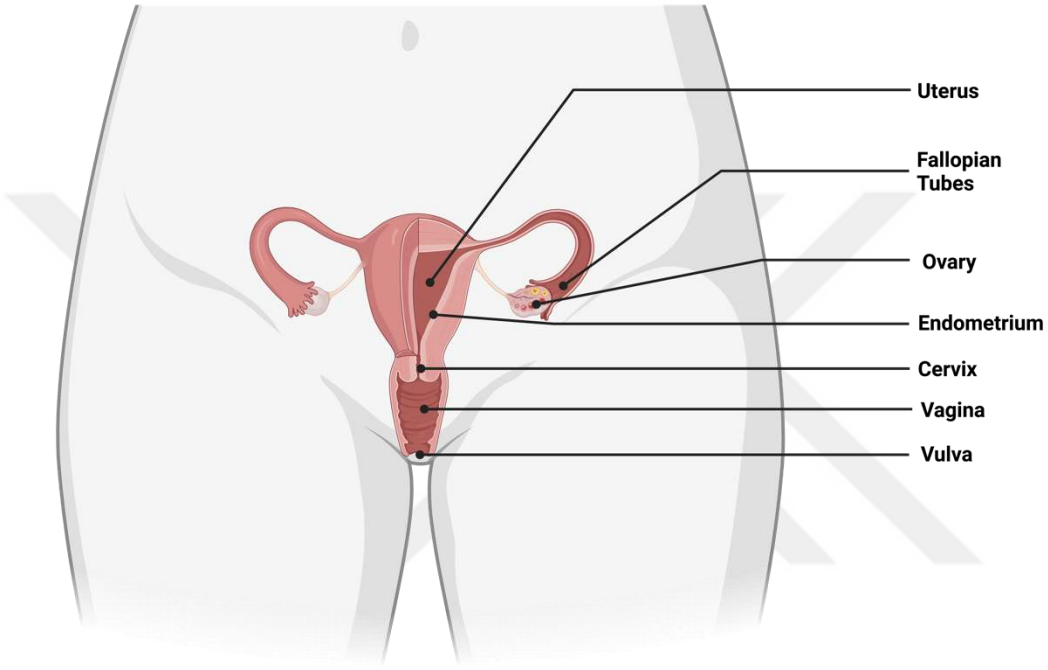

Figure 2. Female Reproductive Organs. *Female reproductive organs where gynecological cancers occur. (Generated via Biorender.com)*

1.1.2.1 Ovarian cancer

Ovarian cancer is a type of gynecological cancer in which cancer begins in the ovaries. The main symptoms of ovarian cancer are vaginal bleeding that is not during the menstrual cycle, pain and pain in the pelvic area, and abdominal and back pain. Diagnosis of ovarian cancer can be made by ultrasound, computed tomography, lower gastrointestinal series, intravenous pyelogram, or biopsy. Ovarian cancer is one of the most common types of cancer in women. 1.2% of the general female population will face ovarian cancer once in their lifetime[18]. In 2019, 19571 new cases of ovarian cancer were diagnosed in the United States, and 13445 women died of ovarian cancer. These numbers are that in 2020, 21750 new ovarian cancers were diagnosed, while 13940 women died from this cancer[17,19]. Ovarian cancer is the gynecological cancer causing the most deaths in

the United States. There are different varieties of ovarian cancer tumor types. The most seen one is high-grade serous carcinoma, nearly 70% of ovarian tumors. About 25% of ovarian cancers due to genetic causes are pathogenic mutations in the BRCA1 and BRCA2 genes[16,20,21]. BRCA1 (breast cancer gene 1) and BRCA2 (breast cancer gene 2) genes are a subfamily of the ataxia-telangiectasia-mutated (ATM)-mediated DNA damage signaling pathway and play a significant role in damaged DNA repair. In some sources, these genes are referred to as tumor suppressor genes because especially breast and ovarian cancers may develop due to mutations in these genes. Mutations in these genes in women cause not only ovarian cancer but also affect lowered ovarian reserve and reproductive aging. In addition, cancer incidence at an early age is higher in women with the pathogen variant of these genes compared to those without this variant. Genes with other pathogenic mutations that cause ovarian cancer are BRIP1, PALB2, RAD51C, RAD15D, and also mutations in Lynch syndrome genes (MLH1, MSH2, MSH6, PMS2, EPCAM) can cause ovarian cancer[17,19,21].

1.1.3 Treatment

Various cancer treatment methods vary according to the type of cancer, the extent and extent of cancer, and the patient's condition. There is no definitive and single treatment for cancer. Cancer treatment options are hormone therapy, chemotherapy, immunotherapy, photodynamic therapy, radiotherapy, and surgical operations[1]. With the development of technology, new treatment options are emerging, or existing treatments are developing. In some cancer patients, more than one type of treatment can be applied together to maximize the treatment[22].

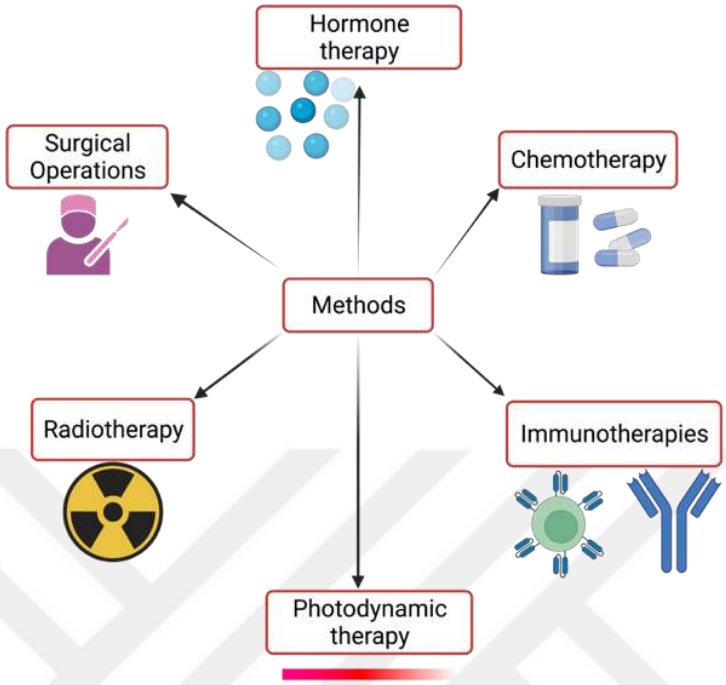

Figure 3. Cancer Treatment. *The most common cancer treatments: Hormone therapy, Chemotherapy, Immunotherapies, Photodynamic therapy, Radiotherapy, and Surgical Operations. (Generated via Biorender.com)*

1.1.3.1 Hormone Therapy

Hormone therapy is applied as a treatment for some types of cancer and is mainly applied in prostate cancer in men and breast cancer in women[23,24]. In addition to treatment, hormone therapy is used to reduce or protect symptoms in men with prostate cancer who cannot undergo surgery or radiotherapy[23]. Hormone therapy is divided into two wide groups, as it stops hormone production in the body and interferes with how hormones act. Hormone therapy is mainly used in combination with other cancer treatment methods. Hormone therapy in combination with other treatments methods:

i) To shrink the tumor when used before surgery and chemo/radiotherapy;

ii) To prevent recurrence of cancer after the main treatment;

iii) To destroy cancer cells that have spread to other parts of the body

is used[23,25–27]. Like other cancer treatment methods, hormone therapy, also have side effects. It can show different side effects in men and women. The most common and common side effects in men are hot flashes, loss of interest in sex, weakened bones, diarrhea, nausea, enlarged breasts, and fatigue. In women, besides the side effects seen in men, vaginal dryness and menstrual cycle are also different (if they have not entered menopause)[23,25–27].

1.1.3.2 Chemotherapy

Chemotherapy is a common, easy to find, and cost-friendly cancer treatment method that uses chemical drugs to kill or destroy cancer cells. There are various types of chemotherapeutic drugs, and they are used in treatments alone or sometimes combined[22,28]. Chemotherapy can be taken in different ways. The most common forms are intravenous and oral chemotherapy. On the other hand, besides chemotherapy killing cancer cells, it has significant side effects on normal cells, tissues, and organs. The side effects of chemotherapy changes from person to person and the type of drug administered. In general, the side effects of chemotherapy are hair loss, vomiting, diarrhea, nausea, fever, a sore mouth, fatigue, constipation, easy bruising, and infertility[29].

1.1.3.3 Photodynamic Therapy

Photodynamic therapy is a cancer treatment method applied using light. Basically, in photodynamic therapies, drugs that sensitize cancer cells to photos are given first. These drugs make cancer cells photosensitive. After the cancer cells are sensitized to light with a drug, when they are exposed to the light of a specific wavelength (which varies according to the drug) for a certain period of time, photosensitizers produce oxygen radicals, and these radicals cause the cells to destroy[30,31]. In addition, photodynamic therapy inhibits the growth of tumors by damaging the blood vessels that the tumors feed on, and some photodynamic therapies can direct tumor cells to immune cells from the other part of the body, triggering them to attack cancer cells[30]. Photodynamic therapies are locally applied treatments. In today's technology, photodynamic therapies cannot affect cancer cells located in the deep part of the body, so this type of treatment is used to treat cancer cells closer to the surface or in the skin. Photodynamic therapy commonly causes burns, swelling, pain, and scarring in the area where it is applied. In addition, it also causes side effects such as redness, itching, cough, loss of appetite, and difficulty breathing[32].

1.1.3.4 Radiotherapy

Radiotherapy (also called radiation therapy) is a form of cancer treatment. Radiotherapy uses high doses of radiation to kill cancer cells or shrink tumors. Radiation damages the DNA of cancer cells, cancer cells with too much DNA damage cannot repair the DNA damage, and they stop dividing and die[33,34]. Sudden death does not appear in cancer cells after radiotherapy because these multiple DNA damages and cell death due to these damages take time. That's why these treatments can take weeks. There are various types of radiotherapy. i)External Beam Radiation Therapy: In this type of radiotherapy, a device targets the area of the tumor and sends high radiation to that area from many directions[35]. This form of treatment treats locally; for example, if the tumor is in the breast, the device only targets the chest area and sends radiation to that area.

ii)Internal Radiation Therapy: In this type of radiation therapy, a radiation source is placed inside the body, unlike external beam radiation. The placed source is in solid or liquid form. If done with a solid radiation source, this treatment is called brachytherapy. In brachytherapy, a solid radiation source is placed in or around the tumor[35]. This treatment is also applied locally; only a radiation source is placed where the tumor is. If a liquid radiation source is used instead of a solid, this radiation therapy is called systemic therapy. In systemic treatment, liquid radiation is taken into the body by the intravenous route, swallowing, or direct injection, and then this radiation circulates throughout the body, searching for and destroying cancer cells[36,37]. Radiation therapy is often used in combination cancer treatments, that is, in combination with other types of cancer treatment. The most common forms of cancer treatment in which radiation is used are chemotherapy and surgery. Radiation is used to reduce the size of the tumor before these treatments are applied or to kill the cancer cells entirely afterward. Common side effects of radiotherapy include hair loss, nausea, weakness, headache, dry, peeling, and itchy skin, difficulty swallowing, shortness of breath, diarrhea, and infertility[38].

1.1.3.5 Surgical Operations

Surgical cancer treatment is removing cancer from the body by surgeons under operation. There are many types of surgery, and they vary according to the patient's condition, the type of cancer,

the tissue, the amount of tumor, and the tissue to be taken. Cancer surgeries can be open or minimally invasive surgery. In open surgeries, a large incision is made in the patient to remove the tumor, some healthy tissues around the tumor, and, if necessary, the surrounding lymph nodes. In minimally invasive surgery, the surgeon makes small incisions in the body; in one of these incisions, a camera called a laparoscope is used to view the tumor area, and through the other incision, the tumor is removed with special surgical instruments[39,40]. Surgery works well for solid tumors. Surgical operations are still more effective when combined with other cancer treatments. Before surgery, the tumor is shrunk through chemotherapy or radiotherapy, depending on the type of cancer so that the entire tumor can be removed more easily during surgery. Or, when these treatments are used after surgery, cancer cells that cannot be removed by surgery can be destroyed in the body. Side effects after surgery may vary depending on the condition of the surgery. There is also a risk of death in surgeries, depending on the region of the tumor, its spread, and its size. Pain is common after surgery, and as with any surgery, there is a risk of infection in cases where attention is not paid[39].

1.1.3.6 Immunotherapy

Immunotherapy is a type of biological therapy also used in cancer treatments. Biological treatments are treatment methods applied using living organisms or substances produced by these organisms. The use of immunotherapy in cancer treatments has increased in recent years, and these therapies have developed tremendously. Contrary to other treatments, these treatments are more target-based, and their non-target effects are less common than other treatment methods; thus, it is thought to be a more effective treatment method. The discovery and first application of cancer immunotherapy date back to ancient times until the end of the 19th century. William Bush Coley and Friedrich Fehleisen first discovered that the bacteria *Streptococcus pyogenes*, which causes common skin infections, spontaneously regress tumors[41]. Then William Bush Coley invented a cocktail of bacterial extracts called Coley toxins; this mixture consists of the bacteria *Serratia marcescens* and *Streptococcus pyogenes*[41,42]. William Bush Coley observed that by administering this cocktail mixture to the tumor and its surroundings, the tumors shrank and even disappeared in some patients. It is the first known cancer immunotherapy used in this history. Different kinds of immunotherapies are used in cancer treatments. These are i) Immune Check Point: Immune checkpoints are a part of the immune system, and their job is to prevent the body's immune

responses from activating enough to kill normal healthy cells[43]. These checkpoints are activated when proteins on the surface of T cells in the immune system recognize and bind to proteins in tumors or other cells. These proteins are called immune checkpoint proteins. When the immune checkpoint and partner proteins in the cells bind together, a signal is sent to the T cells, which may be an "off" signal, which can prevent the immune system from destroying cancer cells. These types of cancer-immunotherapy drugs are called immune checkpoint inhibitors. These drugs prevent immune checkpoint proteins and partner proteins from binding to each other, so the T cells cannot send the "off" signal, resulting in T cells destroying the cancer cells. The most widely used immune checkpoint inhibitor drug CTLA-4 acts on the checkpoint protein[44,45]. Besides the CTLA-4 protein, it is used against another checkpoint protein called PD-1[45]. ii) T-cell Transfer Therapy: T-cell therapy is a type of cancer immunotherapy that uses our own immune cells to better attack and destroy cancer cells. T-cell therapy is divided into two main topics: Tumor-Infiltrating Lymphocytes (TIL) and Chimeric Antigen Receptor - T (CAR-T) cell therapy. In both treatments, our own immune cells are collected, and these cells are grown and modified in a lab environment and returned to fight cancer again. In treating TIL, tumor-infiltrating lymphocytes, that is, these T-cells found in the tumor, as it is named, are used[46]. Doctors and researchers test these TIL lymphocytes in the lab to determine which T-cell tastes best for cancer cells. Then, they become an immune therapy by being together with selected lymphocytes and substances that increase the proliferation of these lymphocytes. Another type of T-cell Transfer Treatment is CAR-T treatment. CAR-T treatment is similar to TIL cell therapy, however, in CAR-T treatment, T cells taken from the patient are grown in a lab environment, and some genetic modifications are made before the patient is discharged. T cells are genetically modified in the lab environment to make chimeric antigen receptors (CAR)[47]. By making the T cell bind to specific proteins on the cancer cell's surface, this receptor enhances the ability of T cells to attack cancer cells. iii)Monoclonal Antibody Treatment: Monoclonal antibodies are immune system protein that antibodies can also create in the lab. Antibodies are produced by B cells in our immune system and are used to recognize and then destroy pathogens. Monoclonal antibodies made in the lab environment can also function like the antibodies produced in our body. In cancer treatments, these antibodies are used to mark cancer cells and to enable and accelerate immune cells to recognize cancer cells[48]. iv) Cytokines produced by white blood cells are immunomodulating agents which are sometimes used in cancer treatments. The most common cytokines used in cancer treatment are interferons and interleukins

(especially IL-2[49]). Cytokines are used in cancer treatment to increase the immune response and the number of immune cells[13]. Immunotherapy, as in other cancer treatments, also has side effects. The most common side effects are body itching, nausea, diarrhea, and extreme tiredness[48]. In addition, immunotherapies can cause an excessive immune reaction, and their sources can cause inflammation in different body parts. In addition to all these, CAR-T treatments may have the serious side effect that is not seen in other immunotherapies[47,48]. This side effect is called cytokine release syndrome. This syndrome is caused by genetically modified T cells or other immune cells reacting to these T cells, releasing large amounts of cytokines into the blood. These excessive amounts of cytokines cause high fever, sudden heart attack, low blood pressure, and difficulty breathing.

1.1.4 Cisplatin

Cisplatin is a platinum-based anticancer chemotherapeutic drug that is widely used in cancer treatments. It is also known as cisplatinum or cis-diamminedichloroplatinum (II) or CDDP. Cisplatin is the first drug compound that was discovered within the platinum metal group. In 1978, cisplatin was approved by Food and Drug Administration (FDA) for clinical usage in testicular and bladder cancer[50–52]. Cisplatin has been used in cancer treatments for more than 40 years, and it has a particular success rate for different cancer types treatments, such as head, neck, lung, breast, bladder, testicular, and ovarian cancers. The summary of the working mechanism of cisplatin is that after entering the cell, it becomes positively charged; due to this positive charge, cisplatin can interact with nucleophilic molecules, as well as DNA, RNA, and proteins[53]. Cytotoxicity is believed to be primarily due to its interaction with DNA, forming inter- and intra-strand adducts, inhibiting both RNA transcription and DNA replication, and leading to cell cycle arrest and apoptosis[53].

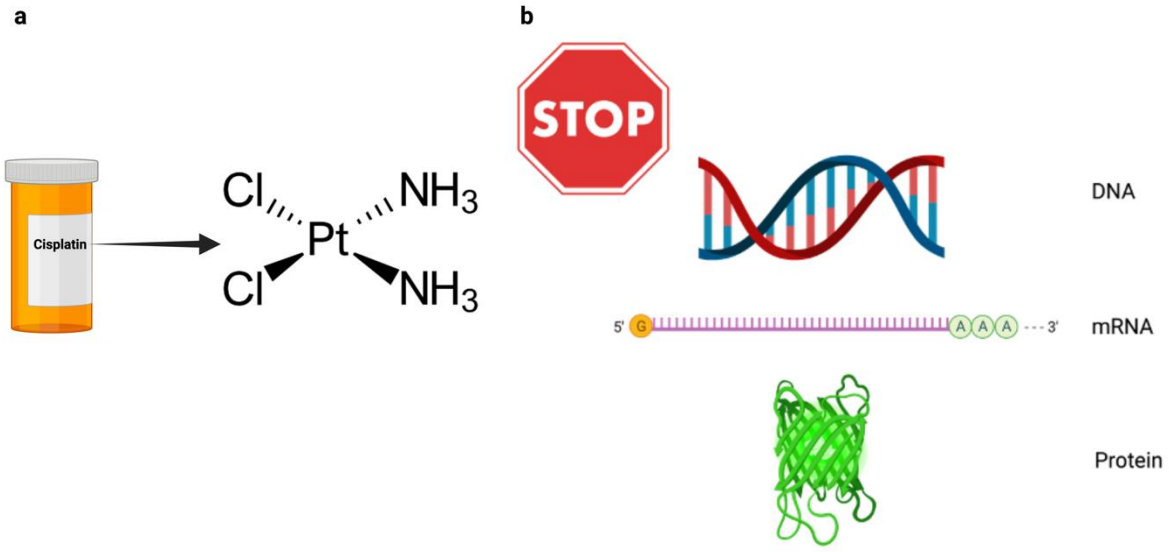

Figure 4. Cisplatin. *a. Cisplatin drug b. Cisplatin inhibits the division of DNA in cells and blocks mRNA and proteins. The most common cancer treatments. (Generated via Biorender.com)*

1.2 Infertility

According to the World Health Organization definition, infertility is a disease of the male or female reproductive system which causes a fail to get pregnant after 12 months of regular unprotected sexual intercourse. Infertility remains a relatively common global condition. It affects millions of people worldwide, so it strongly impacts communities. Approximately 48 million couples and 186 million individuals face infertility[54,55]. As a percentage, infertility affects between 8% and 12% of couples of reproductive age worldwide[56]. There are known and unknown causes of infertility, which vary according to gender and person.

1.2.1 Male Infertility

The general causes in men are as follows: idiopathic, varicocele, hypogonadism, infections, cryptorchidism, genetic, immunologic diseases, and systemic diseases. Azoospermia is simply the absence of sperm in the ejaculate and is one of the most common causes of male infertility[57]. Azoospermia is mainly divided into two groups: obstructive azoospermia and non-obstructive azoospermia. Non-obstructive azoospermia is one of the most severe forms of male infertility. In non-obstructive azoospermia (NOA) men, spermatogenesis fails, so there is no sperm in the

ejaculate. NOA may be caused by an intrinsic testicular disorder or insufficient gonadotropin production[58–60]. It is estimated that one in 100 men face NOA[61]. Obstructive azoospermia (OA) is another main type of azoospermia. As with NOA, there is no sperm in the ejaculate in obstructive azoospermia. Unlike NOA, the cause of OA is the obstruction of the testis or genital excurrent duct system[57,62,63]. 40% of all azoospermia patients have OA[57]. In addition, 6.1% to 13.6% of those who applied to the clinic for infertility were OA patients[64,65]. In most of the OA cases, in the reproductive canals, an obstruction is found in the rete testis, ductuli efferentes, epididymis, or vas deferens, and healthy normal spermatozoa may be found in the testis. One of the most common causes in OA patients is vasectomy, occlusion of the vasectomy ductus deferens channels, and their previous closure[57,62]. In addition, infection and genetic causes are among the common causes of OA. One of the causes of male infertility is chromosomal disorders, and the most common of these disorders is Klinefelter syndrome, with XXY chromosomes in men[60,66]. Physically, the testicular sizes of these patients are smaller than normal men and the functions of Leydig cells are commonly impaired in these men, so there is a low testosterone level[67]. In addition to chromosomal disorders in men, sometimes chromosomal damage causes infertility. The most common example of this situation is Y chromosome microdeletions[68]. Microdeletions on the long arm of the Y chromosome have been shown to cause infertility when they are in a gene region related to spermatogenesis[60,69]. The most common example seen in this situation is microdeletions in the region of the DAZ (Deleted in Azoospermia) gene on the Y chromosome. Deletions in this gene region cause abnormalities in spermatogenesis. The DAZ gene family is involved in the developmental stages of your gametogenesis. DAZ family proteins act in both the nucleus and cytoplasm of germ cells[70,71]. Therefore, deletions or mutations in this family directly affect the germ cells and cause infertility. Varicocele is a condition that occurs in most men who come to the clinic as infertile. According to clinical data, varicocele was found in 15% of all men and 35% of men who applied for infertility[72,73]. When we compared these data according to semen analysis, 11.7% of men with normal semen analysis and 25.4% of men with abnormal semen analysis were diagnosed with varicocele[74]. In short, varicocele is the swelling and enlargement of the veins in the scrotum[75–77]. As a result, the increased temperature of the scrotum also raises the temperature of the testicles. This increased temperature causes infertility by causing thermal damage to the nuclear DNA and proteins of spermatic tubules and Leydig cells[78,79]. Cryptorchidism, also known as undescended testicles, is one of the conditions that cause infertility. It is the condition that one

or both of the testicles do not descend into the scrotum and remain in the abdomen or groin[60,80]. Cryptorchidism has an incidence of 2% to 5% in newborn boys and is the most common congenital anomaly and three months after birth; this incidence rate spontaneously decreases to 1%[60,81,82]. The causes of cryptorchidism are multifactorial; the main reasons are endocrine regulation problems and genetic defects. Semen parameters are often abnormal in patients with cryptorchidism, and 2% to 9% of infertile men have a history of cryptorchidism[83].

Among other most common causes of infertility in men are cancer and cancer treatments. Cancer treatments cause temporary or permanent infertility in men. Infertility may occur due to surgery for cancers in the genital or pelvic organs. Especially in testicular cancers, definite infertility was observed after bilateral orchiectomy, while a decrease in sperm cell concentrations was observed after unilateral orchiectomy[84]. Chemotherapy, which is frequently used in cancer treatments, has a gonadotoxic effect, especially procarbazine and alkylating drugs, specifically Cyclophosphamide, which causes infertility[85–87]. In addition, Procarbazine and MOPP drugs, which are widely used in the treatment of Hodgkin lymphoma, cause infertility[88]. As in surgery, high radiation creates a gonadotoxic effect in radiotherapy taken in the pelvic and genital organ region. High radiation exposure to these areas causes infertility[85–87,89–91].

1.2.2 Female Infertility

The general causes in females are as follows: problems in ovulation and oocyte maturation, uterine or cervical abnormalities, endometriosis, genetic, early menopause, primary ovarian insufficiency, and cancer & its treatments. The prevalence of infertility in women is slightly higher than in men. While the prevalence of infertility is approximately 10% in men, this rate is 13% in women[92]. In addition, the prevalence of infertility in women increases proportionally with age. In studies conducted, 32-year-old women were diagnosed with 12% infertility, while 38-year-old women were diagnosed with infertility in 21%[93]. One of the biggest causes of infertility in women is anatomical disorders. These anatomical abnormalities include tuboperitoneal abnormalities, endometriosis, fibroids, and congenital uterine anomalies[94]. Approximately 25% to 35% of women who apply to the clinic for infertility have tuboperitoneal involvement[95,96]. Pelvic inflammatory disease (PID) is an infection in one or more of the upper reproductive organs, including the uterus, fallopian tubes, and ovaries[97,98]. PID is one of the most common causes of tubal damage. Studies show that 8% of women of reproductive age in the United States and 1.7% in the United Kingdom

are diagnosed with PID each year, and more than 1 million women in the United States are treated for PID each year[99]. Endometriosis is a condition in which endometrial-like tissue begins to grow in different locations, such as the ovaries and fallopian tubes[100]. Endometriosis is a common condition in women, affecting between 5% and 15% of women of reproductive age[94]. Endometriosis causes severe pelvic pain and infertility in women and can cause infertility in many different ways. Advanced pelvic endometriosis mechanically blocks the passage of oocytes or embryos to or from the uterine cavity by clogging the fallopian tube or ovaries[101]. Endometriomas may endanger the physiological innervation or blood supply of the ovarian cortex, in which case they affect follicle development and oocyte[102]. Another factor is that endometriomas secrete high levels of cytokines and growth factors, and these high-level secretions affect granulosa cells, triggering early follicle development causing infertility[102,103]. In women, uterine malformations cause infertility and recurrent pregnancy loss. The most common malformations in the female reproductive system are congenital uterine anomalies[94] and they can cause infertility and recurrent pregnancy loss[104,105]. In critical studies, congenital uterine anomalies constitute 6.7% of the general population, while this rate constitutes 7.3% in women with infertility and 16.7% in women with recurrent pregnancy loss[106]. The septate uterus, which is the most common malformation among these anomalies, has the most frequent infertility rate and 60% pregnancy loss and low fetal survival rates between 6% and 28%[104,105]. Ovulation disorders cause infertility in women, and approximately 25% of infertile women have ovulation disorders[107]. There may be different causes of ovulation disorders; amenorrhea, oligomenorrhea, and irregular cycles are the most common and known ones. Amenorrhea is the absence of a menstrual cycle, and oligomenorrhea is the infrequent menstrual cycle (cycle lengths more than 35 days)[108]. Since there is no menstrual cycle, ovulation cannot be achieved, and infertility occurs due to this. Common causes of amenorrhea and oligomenorrhea are hormonal disruptions[108]. In addition, the incidence of either of these two conditions is very high in patients with polycystic ovary syndrome (PCOS)[109,110]. Oocyte production disorders are also an important and common cause of female infertility. The most common disorders of oocyte production are amenorrhea and oligomenorrhea, which we mentioned above, as well as depletion of the follicle pool and aging of the ovarian follicle[98]. Depletion of the follicle pool and aging of the ovarian follicles affect the development and quality of the oocyte and cause infertility. Genetic factors, chromosomal anomalies, microdeletions, and duplications in women cause infertility by causing disorders in oogenesis, oocyte reserve, hormonal regulation,

and anatomical and functional development of reproductive organs[98,111]. Especially translocations of the X chromosome and intercalary deletions are associated with infertility. For example, in women with Turner syndrome (45, X0), the oocyte reserve is depleted early and becomes sterile[98]. These chromosomal abnormalities account for approximately 10% of POI cases[112]. In addition to POI, at least 5% of women experience recurrent pregnancy loss due to these chromosomal abnormalities[113,114]. Genes with known female reproductive pathogenic mutations are GALT, FSH-R, LH-R, FMR1, BMP15, and NR5A1[115–122]. A mutation in these genes can directly affect reproduction and cause infertility. In addition to these genes, a mutation in the ZP1 gene in the oocyte directly impairs oocyte function and causes infertility[123].

Cancer and its treatment methods can cause temporary or permanent infertility in a woman[98]. Permanent infertility occurs when reproductive organs or pelvic region tumors are surgically removed[124]. The size of the tumor and its location are the essential factors in determining this. In advanced cancers of the uterus, endometrium, cervix, and sometimes vagina, surgery to remove the uterus, called hysterectomy, is performed[125,126]. Permanent infertility occurs in women whose uterus is removed, as there will be no place for the embryo to attach and develop. In advanced ovarian cancers, it is an operation called oophorectomy to remove one or both ovaries. In addition to ovarian cancer, in some cases, oophorectomy can be performed for other gynecological cancers[127,128]. If oocyte freezing is not performed after bilateral oophorectomy, permanent infertility occurs. After removing one ovary, if hysterectomy is not performed simultaneously, oocyte ovulation and hormone production can continue, and pregnancy can occur naturally. In addition to cancer operations in the gynecological regions, the female reproductive system may be affected, and infertility may occur after the operations of tumors in the pelvic organs or brain / spinal cord. Cancers in the abdomen, pelvis, and spine regions and radiotherapy to be applied for treatment can also cause infertility[85,129]. Especially in gynecological cancers, radiotherapy to be applied directly to the female reproductive organs also causes a gonadotoxic effect on the ovarian follicle cells and oocytes, causing the death of these cells, resulting in infertility. In addition, even if the cancer is not directly in the reproductive organs, high radiation can be absorbed by the ovaries, and this absorbed radiation can also create a gonadotoxic effect[129,130]. High radiation exposure to the brain outside of these regions can cause infertility. High radiation can cause infertility by affecting the hypothalamus and pituitary region, damaging the production and release signals of the main hormones necessary for reproduction[129,130]. Hormone therapies for therapeutic

purposes in some cancer types cause temporary infertility because hormones that regulate oocyte development, ovulation, and menstrual cycle will be suppressed during the treatment, so they slow down or stop during the hormone therapy process[131,132]. Chemotherapy is the most commonly used treatment method in cancer treatments in women[133]. Chemotherapy represents the drugs used to prevent the growth of rapidly proliferating cells, namely cancer cells, in the body and kill them. However, it has the ability to multiply and divide rapidly into the cells in the ovaries, and chemotherapy also affects these cells. Chemotherapeutic drugs cause the death of oocyte and follicle cells by creating a gonadotoxic effect in the ovaries, resulting in infertility[130,133–135]. On the other hand, not all chemotherapy has such an effect; this is multifactorial, affecting the location and size of cancer, the type of chemotherapy drug (especially alkylating agents and platinum-based drugs have the most toxic effect on the ovaries), the dose, and the age of the patient[130,133]. When chemotherapy affects the cells in the ovaries in a toxic way, the amount of estrogen released from the ovaries also decreases, this situation can also disrupt the hormonal balance in women and cause early menopause in women, and therefore infertility occurs. One of the causes of primary ovarian insufficiency (POI) is that chemotherapy stops oocyte and estrogen release by affecting the ovaries and chemotherapy-induced POI can be temporary or permanent[136]. Infertility may be divided into two groups: primary and secondary infertility. Primary infertility is the one in which successful pregnancy never occurred, and in secondary infertility there is at least one successful pregnancy that has occurred.

1.2.3 Chemotherapy-Induced Ovarian Failure

Nowadays, more and more people are surviving cancer because of the development of cancer treatments. However, many patients experience side effects and other damage from cancer treatments for some time or throughout their lives after treatment. The biggest of these side effects in female cancer patients is the damage in the ovaries caused by chemotherapy. This damage causes ovarian dysfunction, which is referred to as chemotherapy-induced ovarian failure or chemotherapy-associated ovarian failure[94,137,138].

The effects of cancer and its treatment on the female reproductive system are more noticeable. In general, when women of reproductive age who receive and do not receive cancer treatment are compared, women with cancer treatment are less than 40% likely to become pregnant[139,140].

It has been observed that chemotherapeutic drugs can induce DNA damage in early ovarian follicles; premature activation of early ovarian follicles, induce apoptosis of follicles and oocytes and causes premature depletion of existing follicle accumulation in the ovaries. As a result of the damage done by chemotherapy to the ovaries, women have low estrogen levels, average serum FSH level above >40 IU/L, irreversible amenorrhoea, and infertility[137,141].

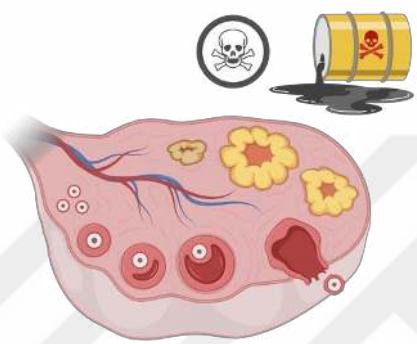

Figure 5. Chemotherapy-Induced Ovarian Failure *Chemotherapies have toxic effects on the ovaries. (Generated via Biorender.com)*

1.3 Stem cells

Stem cells are the source of differentiated/specialized cells, which are the cornerstones of tissues and organs in the embryonic, fetal or adult stages[142]. The essential feature of stem cells is that they can both differentiate and reproduce by renewing themselves at the same time[143,144]. Stem cells can be divided into five different groups according to their ability to differentiate; they can be totipotent, pluripotent, multipotent, oligopotent, and unipotent. Totipotent stem cells have the potential to differentiate to form a whole organism[145]. Pluripotent stem cells are cells that can form all other germ layers except embryonic tissues[145]. Multipotent stem cells can differentiate into specific cells and these stem cells are adult stem cells; the best example of these stem cells is mesenchymal stem cells[146]. Oligopotent stem cells can self-renew within a specific tissue and differentiate into two or more different cells. Finally, unipotent stem cells can self-renew and differentiate into only one particular cell type.

1.3.1 Mesenchymal Stem Cell

Mesenchymal stem cells (MSCs) were first described by Friedenstein and colleagues in 1966[147]. In bone marrow transplants into kidney capsules of mice, the researchers also demonstrated mesenchymal stem cells in the stromal matrix of the bone marrow. He revealed that these cells are a group of non-hematopoietic precursor cells in the bone marrow[147,148].

Mesenchymal stem cells (MSCs) have significant self-renewing properties[146,149] and can be obtained from various tissues/organs such as bone marrow, dental pulp, adipose tissue, umbilical cord, and placenta[150,151]. MSCs are also multipotent progenitor cells that can have multilineage potential to differentiate into different cell types such as adipocyte, chondrocyte, neuron, myocyte, and osteoblast[150,151].

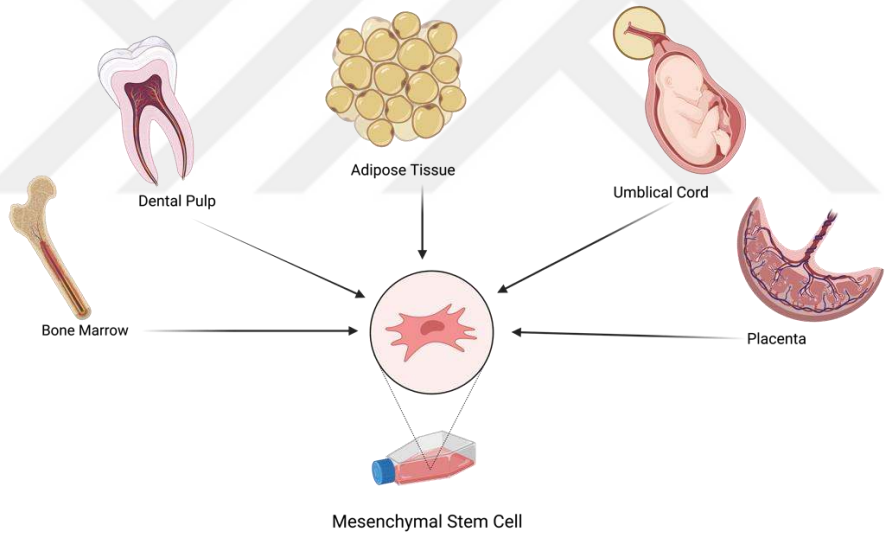

Figure 6. Sources of Mesenchymal Stem Cells*. Mesenchymal stem cells can be obtained from different sources such as Bone Marrow, Dental Pulp, Adipose Tissue, Umbilical Cord, and Placenta. (Generated via Biorender.com)*

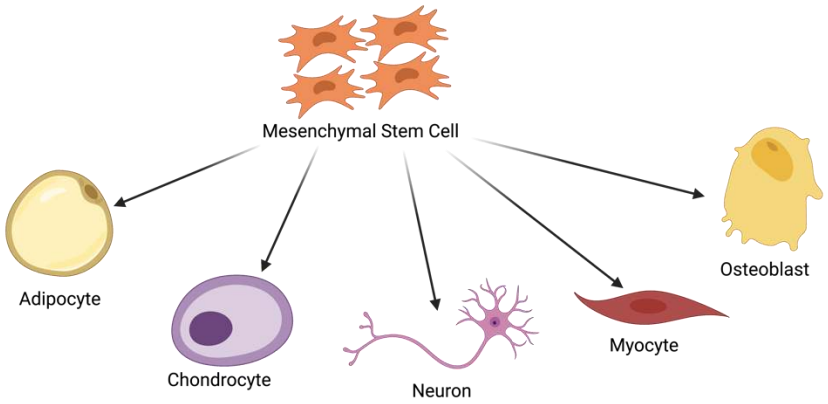

Figure 7. Mesenchymal Stem Cell Differentiation. *Mesenchymal stem cells are adult stem cells and can differentiate into different cells such as Adipocyte, Chondrocyte, Neuron, Myocyte, and Osteoblast. (Generated via Biorender.com)*

Mesenchymal stem cells have surface markers specific to them, which are positive for CD105, CD90, and CD73, negative for CD79a, CD45, CD34, CD19, CD14, CD11b, and HLA-DR[149].

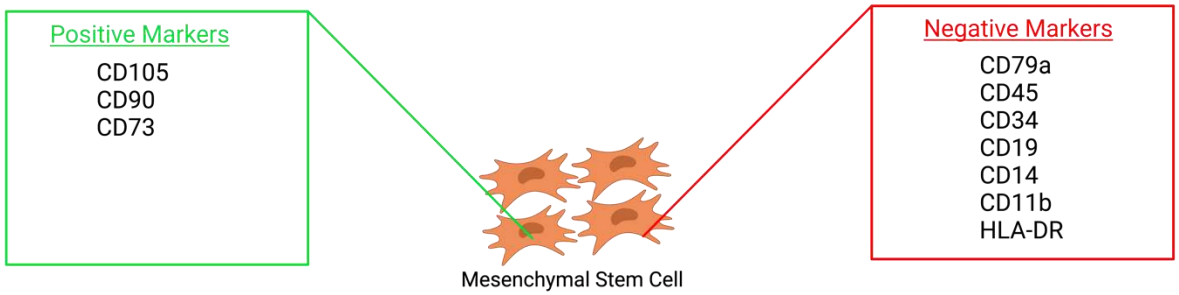

Figure 8. Surface Markers of Mesenchymal Stem Cells*. Mesenchymal stem cells have specific surface markers. They are positive for CD105, CD90, and CD73 while negative for CD79a, CD45, CD34, CD19, CD14, CD11b and HLA-DR. (Generated via Biorender.com)*

In regenerative medicine, Mesenchymal stem cells (MSCs) are promising and constitute one of the attractive stem cell sources, apart from induced pluripotent stem cells(iPSCs) and embryonic stem cells (ESCs)[152]. MSCs can be safely collected without significant ethical concerns and have low immunogenicity[150]. Thus, MSCs have been suggested as sufficient and safe cell sources for

stem cell therapies. MSCs can stimulate angiogenesis, prevent apoptosis and suppress inflammation. These properties make them an inviting option for developing clinical applications, including stem cell therapy and transplantation, due to their more minor ethical issues and almost no tumorigenic acitivity. MSCs have been successfully used in stem cell therapies, including ovarian regeneration[153], diabetes[12], cardiovascular diseases[154], and autoimmune diseases[155]. Even though numerous mechanisms remain unexplained, the immune modulation effects of MSCs make them potential promising candidates for stem cell therapy–based tissue repair and disease treatment, specifically for immune system abnormalities, including cancer & its treatment effects and autoimmune diseases.

1.3.2 3D Culture

Two-dimensional (2D) monolayer cell culture, used as a standard, is a general method applied for *in vitro* research. However, the natural environment of cells is not 2D, so there are changes in the gene expression and morphology of the cells in 2D cell cultures[156]. Researchers are developing Three-dimensional (3D) culture systems to prevent these changes and to mimic cells as if they were in their own environment. Complex cell-cell and cell-matrix interactions of cells in 3D cultures are closer to their natural habitat than in 2D cultures[157–159]. In addition, 3D cultures enable studies such as disease modelling, cell integration, and implantation which cannot be achieved efficiently in 2D cultures. 3D culture is most commonly used in drug trials[160]. In order to have the most effective and accurate results in the *in vitro* environment, before *in vivo* experiments, diseases are modelled in the most similar situations to *in vivo*, and drug trials are carried out in these models. One of the most used models in this context is Transwell and Matrigel co-cultures[157–159]. These products are used to culture at least two different cells together in the same environment in 3D, maximize the interaction between each other, and make it closest to *in vivo*. Then, these co-cultures enable various studies such as drug testing, wound healing, regenerative medicine, and intercellular communication. In addition, modelling of 3D structures such as ECM, solid tumor models, and the interaction between cells is most often used with 3D spheroids[161,162].

1.3.2.1 Spheroid Structure

Two-dimensional (2D) cell culture is a conventional culture condition, but this culture method is not well represented in the *in vivo* physiological environment. On the other hand three-dimensional

(3D) cell culture technique is more mimicked to *in vivo* physiological environment and cell-to-cell interactions[163]. There are several ways to make *in vitro* 3D cell culture, such as pellet culture, liquid overlay, hanging drop, spinner culture, rotating wall vessel, microfluidics, and magnetic levitation[164]. Spheroids are easy to manufacture and have low cost. Since they can be in a wide range of studies such as cell mechanics, cell interactions, tumor formations, and treatment, their use is very advantageous. In addition, some spheroid models may provide a disadvantage in long-term assays due to insufficient size or cell death dur to impaired feeding of the cells inside. Researchers aim to apply more effective stem cell treatments using 3D culture systems. Some studies show that MSC treatments using the 3D culture system have been a more effective treatment method than the 2D culture system[164–166].

1.4 Extracellular vesicles

Extracellular vesicles (EVs) were first introduced in the 1960s with the discovery of vesicles around cells in mammalian tissues, but they took the name "extracellular vesicles" in 2011[167–169]. EVs are extracellular structures with a lipid layer that migrate out of the cell after fusing their intraluminal vesicles (ILV) with the plasma membrane, and the release ILV form of 200nm or smaller vesicles constitute the most known ILVs are exosomes[170–172]. Although the exact contents of ILVs are not known, it is known that they contain abundant mRNA and microRNA[173]. EVs play a significantly central role in intercellular communication, and also regulate the physiological processes of cells[171]. They are structures are released under physiological conditions such as cell activation, senescence, and death signals. Some studies have also found that cells also use EVs to remove harmful substances[174].

Extracellular vesicles	Size (nm)
Exosomes	20-200
Microvesicles, Ectosomes	100-1000
Apoptotic bodies	100-5000

Table 1. Extracellular Vesicles.

1.4.1 Exosomes

In the last few years, the functions of exosomes have been broadly researched. Exosomes are essential messengers between cells for modifying genes or proteins in target cells. Many articles have been published on the biological effects of exosomes secreted by tumors and stem cells[171,172]. Exosomes are biological nanoparticles secreted by mammalian cells and subclasses of extracellular vesicles, which are 20–200 nm in diameter[171,172]. To characterize that the isolated vesicles are exosomes, it is necessary to show the exosome-specific tetraspanin markers (CD63, CD9, CD81, and CD82) and an exosome-negative marker (for example, Calnexin). In addition, it is necessary to show under the microscope that the size of the exosomes is between 50-200 nm and that they are also round or cup-shaped [171,172]. Although the exact content of exosomes is unknown, it has been determined that they contain mRNA, long non-coding RNA, microRNAs, proteins, and lipids[171,172,175]. Recently, exosomes obtained from mesenchymal stem cells have been used in treatment studies and have proven to be effective in studies. Exosomes obtained from mesenchymal stem cells have been observed to improve ovaries and increase the number of follicles in patients with chemotherapy-induced ovarian defects[15,176].

1.5 CRISPR System

A novel technology called "genome editing" appeared and has been broadly used in the studies of food technology, recombinant biology, and gene therapy in past decades. Genome editing is a new technology that builds upon engineering, biochemistry, and biology. Using these technologies, scientists have developed numerous artificial exo/endo nucleases. Zinc-Finger Nucleases (ZFNs) are one of the oldest in this technology. Basically, ZFNs compromise with a typical Cys2-His2 DNA-binding domain and FokI restriction endonuclease[177], which is a DNA cleavage part[178–182]. Transcription Activator-Like Effector Nucleases (TALENs) are the second gene editing technology naturally produced from *Xanthomonas* bacteria and the working mechanism of TALENs is that the DNA binding part of TALENs consists of approximately 30 to 35 amino acid repeating motifs and each of which recognizes a particular nucleotide[183–189]. Also, TALENs gene editing technology uses the same endonucleases with ZINCs, FokI endonuclease[177]. TALENs can

be programmed into target-specific DNA sequences by scrambling repetitive amino acid recognition motifs.

However, these methods were not very cost effective; they were also difficult to apply, and their application areas were not wide. A few years ago, scientists discovered a new gene editing technology. This low-cost, easy-to-apply, effective technology can adapt to many different fields of study. The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system is actually an adaptive immune system found in bacteria and archaea in nature[190,191]. Bacteria and archaea use this CRISPR system to protect themselves from viruses and phages. CRISPR/Cas9(CRISPR Associated Protein 9) system is detected in almost 40% of bacteria and 90% of archaea genomes[192]. The CRISPR locus is composed of a number of conserved repeating sequences spaced by different non-repeat sequences, called spacers. Invading foreign DNA is processed into small DNA fragments by Cas nuclease and then integrated into the CRISPR locus of host genomes as spacers in the CRISPR/Cas system.

In reaction to viruses and phages, spacers are applied for transcriptional templates to produce CRISPR RNA (crRNA), directing Cas nucleases to cut target DNA sequences by invading viruses and phages. There are a variety of different Cas protein types which contribute to DNA cleavage, crRNA biosynthesis, and spacers incorporation[193,194]. There are primarily three different CRISPR/Cas systems. These are Type I, Type II, and Type III, and these are generally divided according to the structure and properties of the Cas protein[194]. Type II is the most widely used CRISPR/Cas system. In the Type II system, only the Cas9 protein is needed, and this Cas9 protein should include an HNH nuclease and a RuvC-like nuclease domain[190]. In the CRISPR/Cas9 system, mature crRNA is merged with trans-activating CRISPR RNA (tracrRNA) to construct a tracrRNA-crRNA complex that leads Cas9 to a target site. tracrRNA is relatively complementary to crRNA and supply to crRNA maturation. A short Protospacer adjacent motif (PAM) sequence is a must-have in the target region for the CRISPR/Cas9 gene editing system. The PAM sequence is a short motif located in the target DNA, usually between 2 and 5 bp, located very close to the crRNA-targeted sequence[190].

After binding to the target site, the Cas9 protein creates a Double Strand Break (DSB) in the target site by creating a break respectively in the HNH and RuvC-like nuclease domains in the strand where crRNA joins in a single strand and the opposite strand[195].

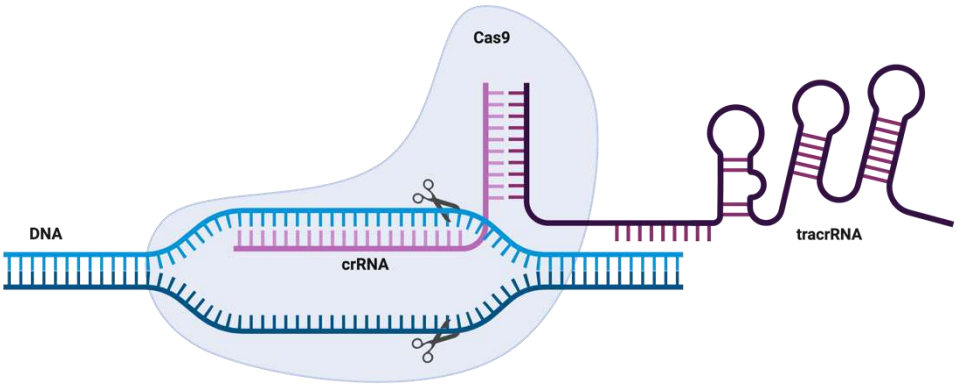

Figure 9. The CRISPR/Cas9 System. *Spacer sequence in the crRNA bind to Cas9 protein, and the tracrRNA forms to create effector complexes that recognize foreign DNA sequences by base pairing. (Generated via Biorender.com)*

For easy and broad application in CRISPR/Cas9 genome editing, the scientist developed a guide RNA (gRNA) which is a chimeric RNA including all the necessary crRNA and tracrRNA components. Numerous CRISPR/Cas9 types have been developed that recognize 20 to 24 nucleotide sequences that match the engineered gRNA and 2 to 4 nucleotide PAM sequences at target sites. Thus, CRISPR/Cas9 system can apparently target a particular DNA sequence with 22 to 29 nucleotides, most of which are unique in the genome[190,196–199].

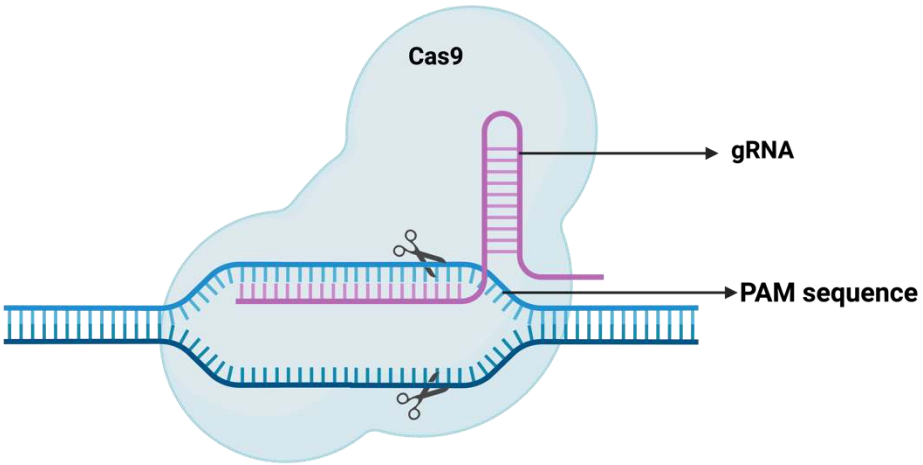

Figure 10. The CRISPR/Cas9 gRNA System. *gRNA mimics the crRNA and tracrRNA chimeric complex, allowing us to cut the target DNA we want with Cas9. (Generated via Biorender.com)*

Cas9 generates Double Strand Break (DSB), and that DSB will activate DNA repair processes which include non-homologous end-joining (NHEJ)-mediated error-prone DNA repair and homology-directed repair (HDR)-mediated defect-free DNA repair[195]. NHEJ-mediated DNA repair can quickly ligate DSB but create small indel mutations at target sites. These types of modifications can permit us to disrupt or eliminate the function of target genes or other genetic materials[195,200]. DSB can trigger HDR-mediated DNA repair, which is more complex than other DNA repair mechanisms such as NHEJ-mediated DNA repair. HDR-mediated error-free DNA repair needs a donor DNA sequence containing homology as the repair template[195,200].

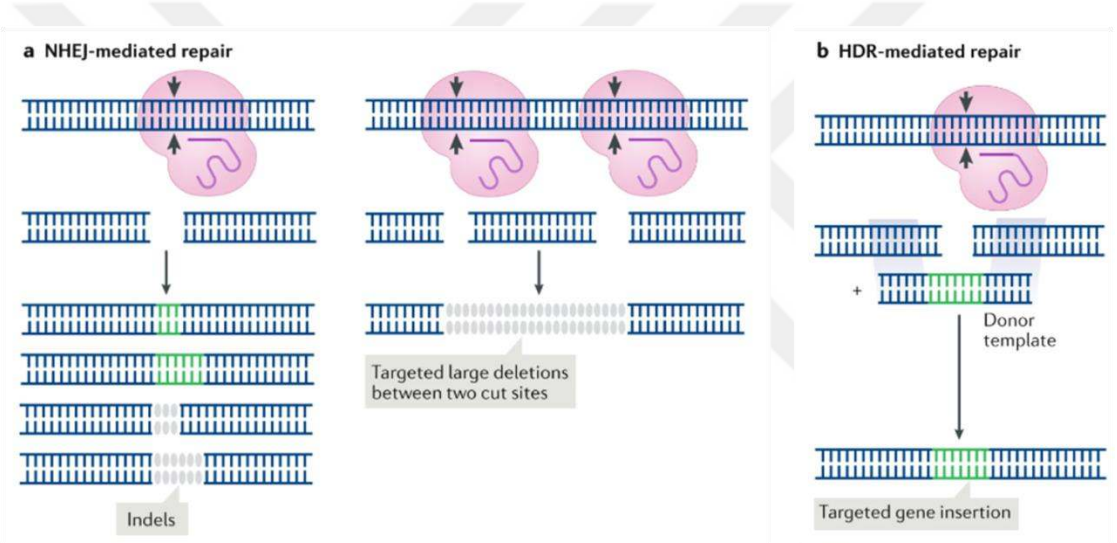

Figure 11. NHEJ and HDR-mediated Repair. *NHEJ mechanism can guide the collection of small insertions, and deletions come after repeated breakage and repair mechanisms. NHEJ mechanism is mainly used for knockouts. HDR, on the contrary, is applied for a stable modification. (Reprinted from [201])*

CRISPR/Cas genome editing system is currently used in many fields such as agriculture, animal husbandry, drug development, gene therapies, biofuel production, experimental animals, disease modeling, and many others due to its ease of use and low cost, and efficiency in applications[202,203]. The number of studies using the CRISPR/Cas system has increased exponentially day by day.

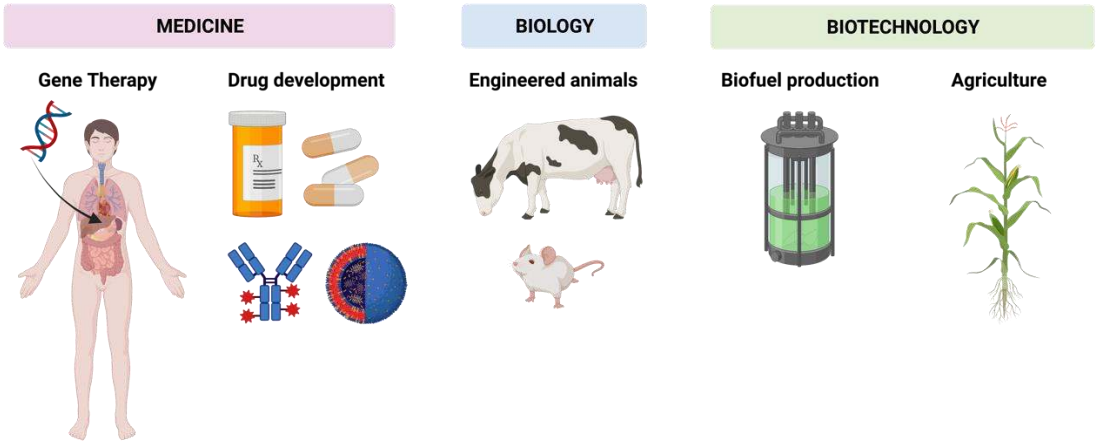

Figure 12. Application Areas of the CRISPR System. *The capacity to fast and specifically insert, extract, and also manipulate DNA sequences has fascinated the researchers' attention in a broad spectrum of biotechnology fields such as medicine, bioenergy, and agriculture studies. (Generated via Biorender.com)*

1.5.1 CRISPR/dCas9 System

Cas9 protein is composed of HNH and RuvC-like nuclease domains, which are two different endonuclease domains. HNH nuclease domain is responsible for cleavage of the target strand of DNA, which is complementary to the gRNA and the RuvC-like nuclease domain is responsible for cleavage of the non-target strand for leading the DSB at a particular site of genomes[195]. When both HNH and RuvC-like nuclease domains are deactivated by mutation, endonuclease dead Cas9 (dCas9) protein occurs and dCas9 protein has the same properties as natural Cas9 protein except for nuclease activity[204–206]. It has RNA-guided DNA-binding activity without cleavage activity. These properties allow dCas9 can be fused with effectors to intercede with site-specific genetic and epigenetic regulation without cleaving to target DNA[207].

In the CRISPR/dCas9 system, it is possible to establish an activation system by fusing activator transcription factors to the dCas9 protein[208]. These activators bind to enhancer domains of DNA and facilitate the uptake of RNA polymerases to increase transcription of the gene[209]. One of these activators is VP64 and the scientists found that VP64 binds to the dCas9 protein and transcriptionally upregulates a gene[209,210]. There are also other activators are available besides VP64 such as p65 and Rta but VP64 is widely used and more effective. There are additional extra activator systems to the dCas9-VP64 protein complex[205,208–210]. Using this method further

increases the effect of VP64, allowing us to increase further the activity of the gene we are targeting. The most used and popular ones are SAM and Tet1 systems.

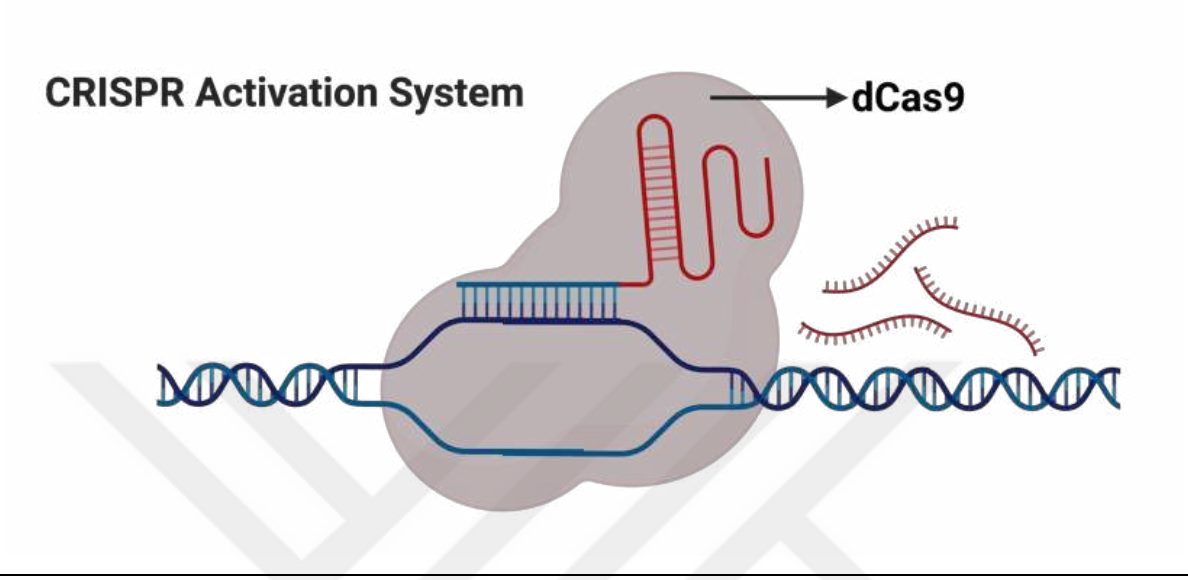

Figure 13. The CRISPR/dCas9 Activation System. *CRISPR activation depends on dCas9, a Cas protein variation lacking endonuclease ability, binding to genes without altering the DNA. (Generated via Biorender.com)*

1.5.2 SAM System

Over the past few years, various CRISPR activation systems have been developed, such as the synergistic activation mediator (SAM) system[209]. SAM system uses dCas9 protein and sgRNA as scaffolds for impressing numerous activators, which has the synergistic function of enhancing the activation of the targeted gene[208]. In the SAM system, the dCas9-VP64 fusion protein is integrated with a modified sgRNA, including two MS2 aptamers. MS2 binding protein is fused with two other activator domains, p65 and HSF1 and this fusion protein (MS2-p65-HSF1) interact with MS2 aptamers[206,208,209,211].

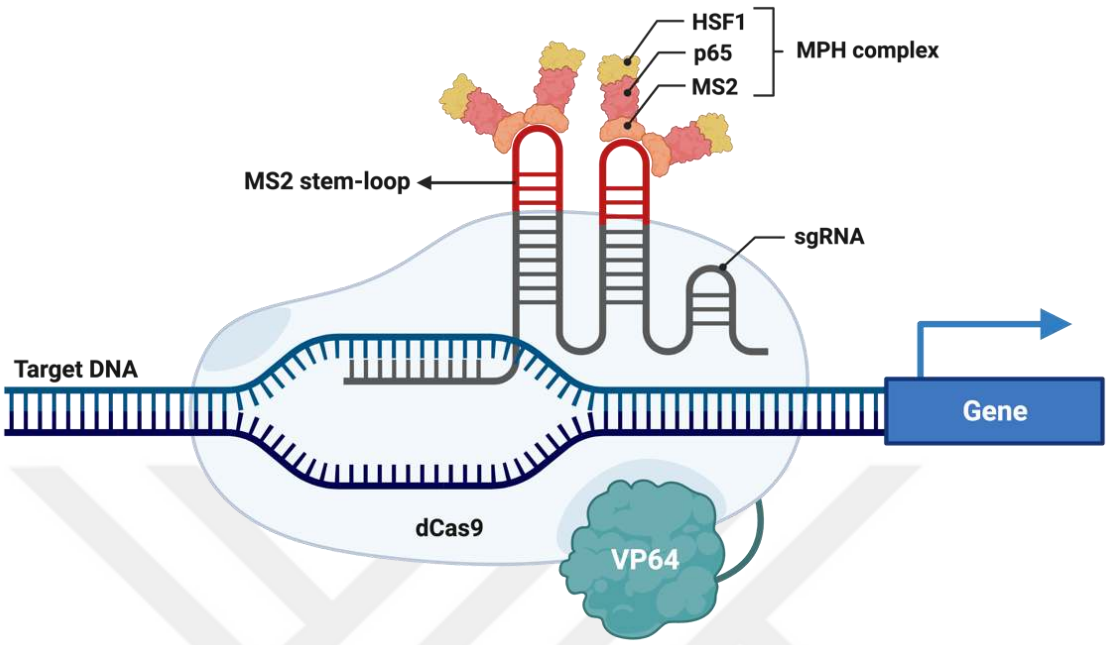

Figure 14. The CRISPR/dCas9 SAM Activation System. *SAM system is the combination of dCas9/VP64 fusion protein that includes aptamers that bind to ms2-p65-hsf1 proteins. SAM uses specific sgRNAs for enhancing transcription. (Generated Biorender.com)*

1.6 CD99

CD99 (also known as MIC or single-chain type-1 glycoprotein) is a 32 kDa transmembrane protein located in the pseudoautosomal region of X and Y chromosomes, and it involves various biological processes as well as apoptosis, migration, differentiation, and protein trafficking[212–214]. Studies have shown that the CD99 gene is highly expressed in cisplatin-resistant cancer cells, and it is also expressed at a lower level in cisplatin-sensitive cells. In addition, the previous studies observed that when the CD99 gene was activated in ovarian cancer cells that were not resistant to cisplatin, they became resistant to cisplatin and these results revealed that the CD99 gene is associated with cisplatin resistance[215,216]. To demonstrate and prove that CD99 is resistant to overexpression cisplatin, they activated the CD99 gene in A2780 and COC1 ovarian cancer cell lines. Then, these cells were treated with cisplatin at different doses for 24 hours along with the controls. They looked at the cell viability of the cells at the end of 24 hours. The results show that cells without activating the CD99 gene showed significant cell viability at IC50 and even above values. In addition, they looked at activation by qPCR to show whether the CD99 gene is activated, and it was observed

that cells resistant to cisplatin treatment were highly expressed compared to the control[215]. In our study, it is aimed to increase the activation of this gene in MSCs to make these cells resistant to cisplatin by genome editing technique. Then, MSCs resistant to cisplatin will be co-cultured with hGRC1 cells, and cisplatin treatment will be applied. The main target is to inquire if MSCs resistant to cisplatin are less affected by cisplatin and provide more protection than non-resistant MSCs.

2

CHAPTER 2: MATERIALS & METHODS

2.1 Chemicals, Reagents, Media and Cell Lines and Instruments

2.1.1 Chemicals, Reagents, Media,

Nuclease-free water. (Eco Tech biotechnology Cat No: DW003L), T4 Ligase Buffer. (New England Biolabs Cat No: M0202S), T4PNK enzyme. (New England Biolabs Cat No: M0201S) PCR devise. (Bio-Rad T100 Thermal Cycler), BsmBI (NEB) Antarctic Phosphatase (NEB), T4 Ligase buffer with ATP (10X) (New England Biolabs Cat No: M0202S), LB (Multicell Cat No: 800-061CG), NucleoSpinR plasmid isolation (Macherey-Nagel) kit (Mini Cat No: 740588.50 Midi Cat No: 740412.10), Fugene (Promega, USA) (Cat No: E2692), M-MLV Reverse Transcriptase (Invitrogen, USA) (Cat No: 28025-013), PBS (Biowest Cat No: L0615-500), DMSO (Sigma Cat No: D2650-100ml), BLAST (Invitrogen Cat No: R210-01), Puromycin (Sigma Cat No: P8833-100mg), Hygromycin (GoldBio Cat No: H-270-1), Puromycin (Cat No: P8833-100mg, Sigma, M-MLV Reverse Transcriptase (Invitrogen Cat No: 28025-013), Qiagen Cyber Green Master, Laemli Buffer 2X (Sigma Aldrich, Cat No: S3401), 2-mercaptoethanol (Sigma Aldrich, Cat No: M3148). DMEM (Cat No: D6429-500ml, Sigma), FBS (Cat No: S181H-500, Biowest), Pen/Strep. (Cat No: L0022-100, Biowest), Trypsin-EDTA (Cat No: 325-542- EL MultiCell), Sarstedt TC Insert (Cat No: 83.3920).

2.1.2 Cell Line and Cell Culture Conditions

hGRC1 (immortalized human granulosa cell line, kind gift from Prof. Dr. Ozgur Oktem) cells were maintained in DMEM (Dulbecco's Modified Eagle Medium) with 10% FBS (Fetal Bovine Serum) and 1% P/S(Penicillin-Streptomycin).

MSC (human umbilical cord-derived mesenchymal stem cell line ATCC PCS-500-010) cells were maintained in DMEM-F12 with 10% FBS and 1% P/S. They were incubated in a 37°C incubator with a humidified 5% carbon dioxide.

2.1.3 Antibodies

Recombinant Anti-CD63 antibody Rabbit monoclonal (ab217345), Recombinant Anti-CD9 antibody Rabbit monoclonal (ab236630), Anti-Calnexin antibody - ER Marker Rabbit polyclonal (ab22595), Fluor 488 goat anti-mouse IgG(H+L) (Invitrogen (Carlsbad) Cat No: A11029), Fluor 488 goat anti-rabbit IgG(H+L) (Invitrogen (Carlsbad) Cat No: ab150077).

2.1.4 Instruments

Micropipette (Eppendorf), 12-well plates (Sarstedt Cat No: 83.3921), Serological pipettes (5 ml, 10 ml, 25 ml, and 50 ml, Greiner Bio-One, Austria), 6well plates (Sarstedt Cat No: 83.3920), 10 cm culture dish (Sarstedt Cat No: 83.3902), Incubator, New Brunswick Galaxy 170S, Centrifugator (Thermo SL-40R), Shaker (Wisd WiseShake SHO2D), LightCycler 480 instrument (Roche, Switzerland) (LightCycler 480 II), chemiluminescence sensing system (Licor), -20°C freezer (Beko, Turkey), and -80°C freezers (Thermo Fisher Scientific Inc., MA, USA), Scanning Electron Microscope (Zeiss Ultra Plus Field Emission Scanning Electron Microscope).

2.2 CRISPR/dCas9

2.2.1 Plasmids

lenti sgRNA (MS2) puro optimized backbone (Plasmid #73797, Addgene), lentiMPH v2 (Plasmid #73797, Addgene), lenti dCAS-VP64_Blast (Plasmid #61425, Addgene).

2.2.2 The Design of the sgRNA

The guide RNA for CRISPR/dCas9-Synergistic Activation Mediator (SAM) systems was designed using published databases. During the designing of the gRNA, we identified the human CD99 promoter sequence from the literature. The EPD (Eukaryotic Promoter Database) tool was used to identify the CD99's promoter region. The sequences for the CD99 gene were selected and imported into the SnapGene program. This program makes it simplify to select the necessary

sequence in the target gene sequence and was used to select the transcription start site (TSS). When designing the gRNAs for the CRISPR/dCas9-SAM activation system, it is fundamental to keep the designed gRNAs between 0 (TSS) and +200 base pairs in length. The SnapGene program was used to look at the distance and chose the closeness of the gRNA sequence discovered for CD99 in the Eukaryotic Promoter Database to the TSS region. The candidate gRNAs were also analyzed using the CHOP-CHOP tool (https://chopchop.cbu.uib.no/) for off-target binding rates. The gRNAs with the lowest self-complementary and off-target binding that was closest to the TSS point were chosen. For the human CD99 gene, one gRNA was created. The designed gRNA for the CD99 sequence is: GCGGGGCGAGGCTGCAGTATCGG.

2.2.3 Annealing

Annealing was performed on the gRNA reached from the manufacturer as top and bottom. The gRNAs were delivered as lyophilized from the manufacturer. The stock solution was prepared at a concentration of 100M in nuclease-free water. An annealing mixture was made using 1 µL top and 1 µL bottom gRNA from the stock solution, 0.5 µL T4PNK 3' phosphor minus enzyme,1 µL T4 DNA Ligase buffer containing 10 mM ATP, and 6.5 µL nuclease-free water. The total reaction mixture was 10 µL. The thermal cycler machine was set to 37 °C for half an hour, 95 °C for five minutes, and progressively cooled to 5 °C and 25 °C during a one-minute period. After that, annealed gRNA was kept at a temperature of -20 °C.

2.2.4 Digestion

To insert the gRNA generated for CRISPR/dCas9-SAM systems, lenti sgRNA (MS2) puro optimized backbone was employed. The restriction enzyme was decided according to manufacturer guidelines and the BsmBI enzyme was used as a restriction enzyme. The restriction mixture was prepared following 5 µg vector backbone, 3 µL 10x 3.1 NEB buffer, 2 µL BsmBI restriction enzyme and a final volume of 30 uL and the remainder was made up with nuclease-free water to a total of 30 µL. The thermal cycler machine was set to 55 °C for 4 hours, 80 °C for 20 minutes and 4 °C for an infinitive hold. After vector backbone cleavage, MN PCR-Clean Up Kit was performed.

2.2.5 Ligation

Ligation is inserting the sgRNA into the previously cut vector from the cut point. At starting of ligation, firstly gRNA was diluted to a concentration of 1:200 in nuclease-free water. The ligation reaction mix was prepared: 50 ng cut vector backbone, 1 µl gRNA, 1 µl T4 Ligase buffer with ATP (10X), and µl T4 DNA Ligase buffer. We generated 50 ng vector, 1 µl gRNA, 5 µl T4 Ligase buffer with ATP (10X), and 1 µl T4 DNA Ligase and the remainder was made up of nuclease-free water to a total of 11 µL. The mixture was incubated at 16 °C for 16 hours. The ligated sample was kept at -20 °C.

2.2.6 Transformation

Bacterial transformation is a technique of plasmid transfer from the environment to bacteria.
By using this technique, ligation products were introduced into competent bacteria. A heat shock transformation technique was performed. 5 µl of ligation product and 50 µl of STBL3 competent bacteria were mixed. The mixture was kept on ice for 15 min then made heat shock at 42 °C for 45 seconds. After heat shock, 150 µl of antibiotic-free LB broth was added and the mixture was incubated at 37 °C at 225 rpm for 1 hour. After incubation, the mixture was centrifugated at 1500 rpm for 5 minutes, and 150 µl of the supernatant was removed. The remaining 50 µl was inoculated on LB agar containing Ampicillin with 1:1000 concentration and was incubated at 37°C for 16 hours.

2.2.7 Colony Formation

After overnight incubation on the LB agar plate, colonies were observed. A single colony was selected and incubated overnight in a shaker at 37 °C in 200 ml LB with Ampicillin (1:1000 concentration).

2.2.8 Plasmid Isolation

The NucleoSpin Xtra Midi Maxi plasmid isolation kit (Macherey Nagel) was used to isolate the plasmids. Success isolated plasmids stored at - 20°C.

2.2.9 Sanger Sequencing Analysis

To verify that whether the gRNAs were inserted into the vectors and replicated properly in the bacteria or not, inserted gRNA plasmid was submitted to the Sanger sequencing. Sequence mixtures were prepared with 500 ng plasmid and 1 µL hU6 primer and the Macrogen company sequence barcodes were stuck to mixture tubes.

2.2.10 Production of Lentiviral Vectors

Before Day 0, 293T cells were seeded at a density of 2.5 x 10^6 cells in a 10 cm TC plate on Day 0. On the evening of Day 1: Transfection mixtures were prepared for a 10cm TC Plate per virus; volumes are written below. For each virus two tubes were labeled. FuGene and DMEM (-/-) had mixed with each other and also DNA mixture was mixed and prepared. After mixing well enough with each mixture, FuGene/DMEM (-/ -) and DNA mixture were mixed thoroughly. The mixture was incubated for 30 minutes at room temperature. After that the mixture was given to 293T cells by dropwise. 293T cells were placed in the CO_2 incubator.

FuGene mixture (10 cm TC plate):

- FuGene: 20 uL
- DMEM -/-: 180 uL

DNA mixture:

- VSVG: 250 ng
- PsPax2: 2250 ng
- Vector: 2500 ng
- DMEM -/- up to 200 uL

On the morning of Day 2: The medium was changed with DMEM with %10 FBS and %1 Pen/Strep. 8 mL complete medium was placed.
On the evening of Day 3: The first collection was made from 293T cells. The medium was gently removed and collected it into the appropriate size tube. Then collected the medium, fresh complete

8 mL medium was replaced and 293T cells were returned to the CO2 incubator. On the evening of Day 4, the collection step was repeated and the medium combined with Day 3 collected medium. After that the collected mediums were filtered with a 45um filter for cleaning the medium of debris and cells. 293T cells and their plates had been bleached and were trashed. After filtering, 1/4 PEG is added to the medium and incubated at +4 degrees for 48 hours. After 48 hours

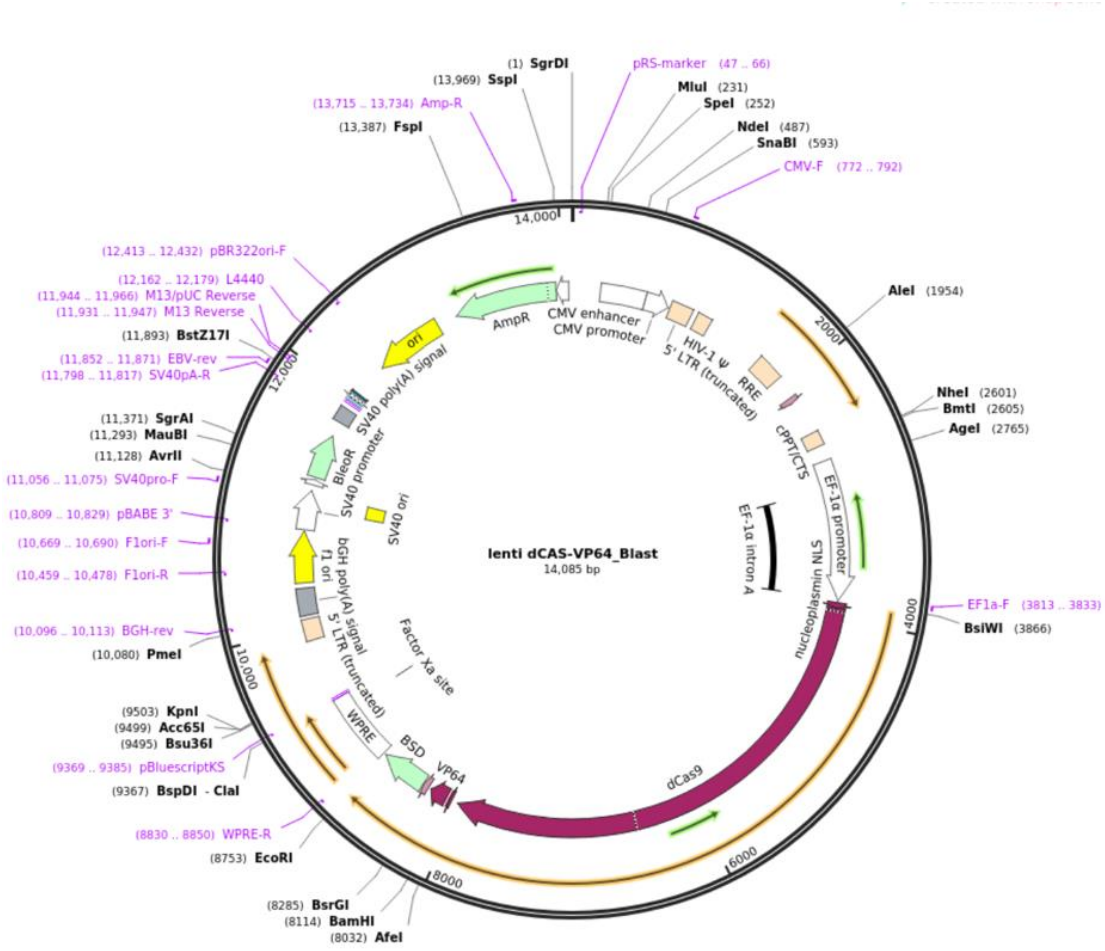

Figure 15. lenti dCas9-VP64 Plasmid. *The plasmid containing the dCas9 and VP64 proteins and the Blasticidin antibiotic resistance gene ready to be transduced into the lentiviral vector.*

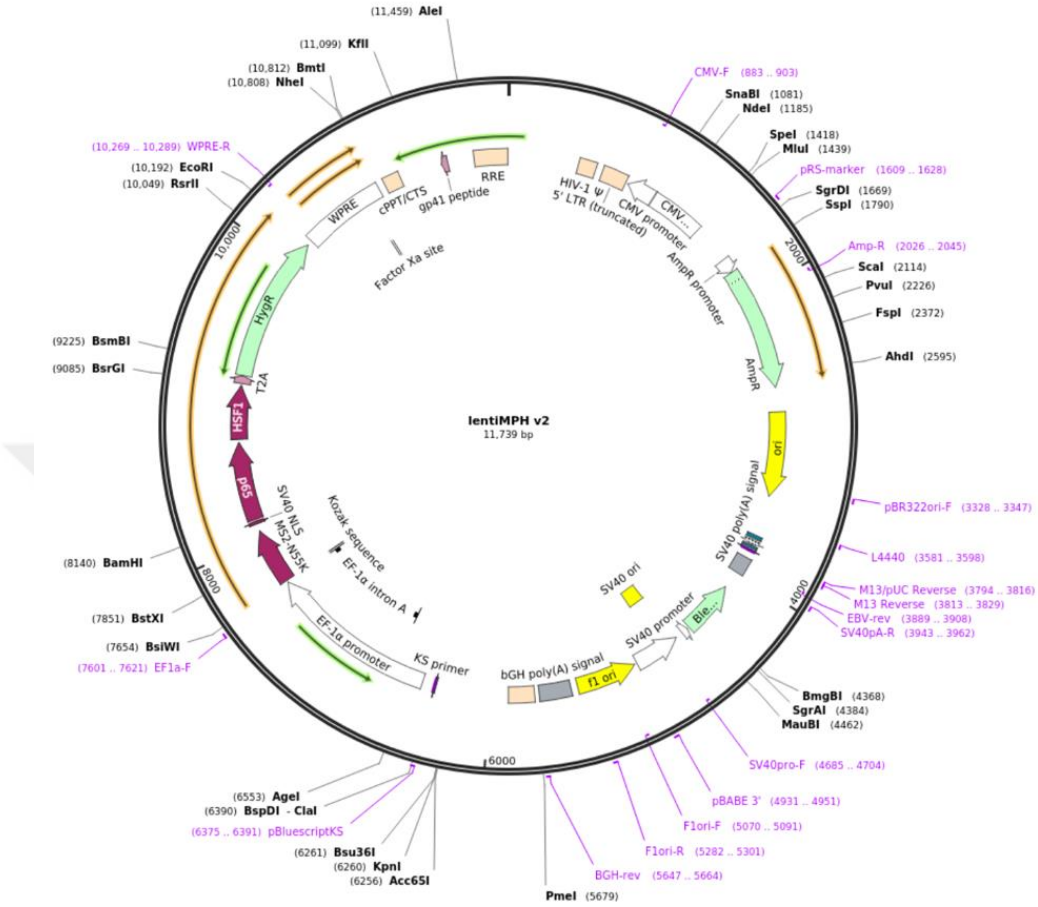

Figure 16. lenti MPH Plasmid. *The plasmid containing the ms2-p65-hsf1 proteins and the Hygromycin antibiotic resistance gene ready to be transduced into the lentiviral vector.*

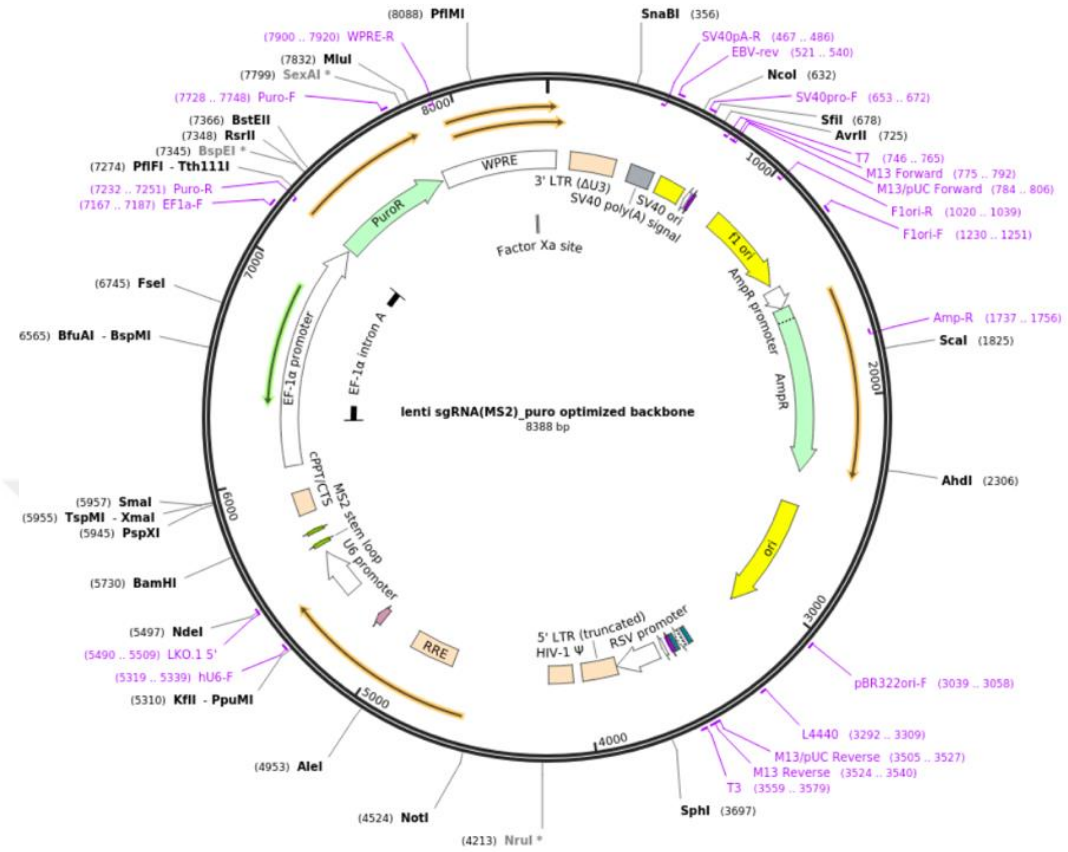

Figure 17. gRNA Vector Plasmid. *The plasmid contains the ms2 stem-loops and the Puromycin antibiotic resistance gene. It has a specific BsmBI restriction enzyme site for inserting sgRNA.*

2.2.11 MOI (Multiplicity of Infection) Calculation

Before transduction into Mesenchymal Stem Cells, the MOI was calculated to compute the number of viruses that could to infect. Lentiviruses that carry the dCas9-VP64, ms2-p65-Hsf1 and gRNA vectors were used. The MOI were determined for each lentivirus and gRNA vectors. 55,000 Mesenchymal Stem Cells have seeded in a separate 12-well culture dish for each lentivirus and gRNA vectors. Virus concentrations were chosen as six different doses: 1 µL, 2 µL, 5 µL, 10 µL, 15 µL, and 20 µL. The medium was changed 16 hours after transduction and selection started the next day after medium change. Each virus has different antibiotics for selection:

Plasmids	Antibiotic	Concentration	Time
dCas9-VP64	Blasticidin	10 µg/mL	6 days
ms2-p65-Hsf1	Hygromycin	100 µg/mL	10 days
gRNAs vector	Puromycin	1 µg/mL	10 days

Table 1. Plasmids, Their Selection Antibiotics, Concentrations Used, and Selection Times. *dCas9-VP64 uses Blasticidin as the selection antibiotic; the selection concentration is 10 µg/mL, lasting 6 days. ms2-p65-Hsf1 uses Hygromycin as the selection antibiotic; the selection concentration is 100 µg/mL, lasting 10 days. gRNA vector uses Puromycin as the selection antibiotic; the selection concentration is 1 µg/mL, lasting 10 days.*

Blasticidin was used at 10 µg/mL concentration and selection was completed within 4-6 days. Hygromycin was used at 100 µg/mL concentration and selection was completed within 8-10 days. Puromycin was used at 1 µg/mL concentration and selection was completed within 8-10 days. After all of the cells died in the virus-negative antibiotic-positive groups (Negative Control), the selection process was finished. The MOI was determined after the cells were removed and counted by using a hemocytometer. After calculation, the mean MOI value was used in future experiments.

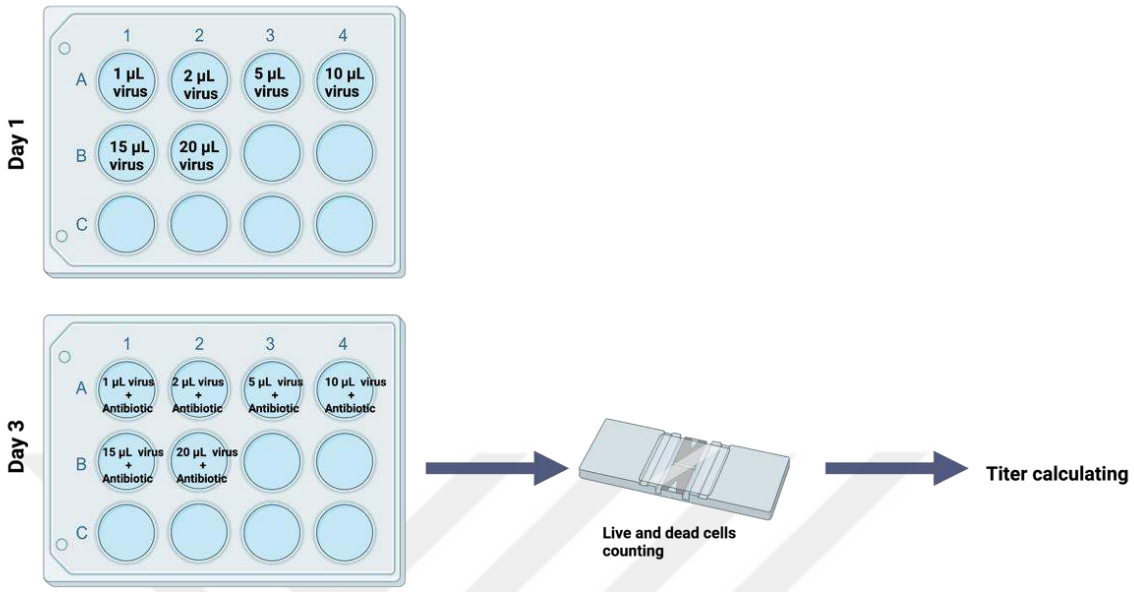

Figure 18. Schematic Diagram of MOI Calculation. *Antibiotic selection starts on the 3rd day for the cells given lentivirus on the 1st day. Three days after selection, cells are treated with Trypsin/EDTA, and viable cells are counted with a hemocytometer, then MOI is calculated. (Generated by Biorender)*

2.3 Cisplatin Resistance in Engineered MSCs

2,000 engineered and normal mesenchymal stem cells were transplanted into sub-transparent black 96-well plates. The next day, cisplatin treatment was applied with serial dilution starting from 100 µg. After 24 hours, the Cell Titer Go (CTG) assay was performed using the manufacturer's instructions. The IC50 value was used for the cisplatin resistance check. Cells that did not die at the IC50 value were accepted as resistance. The resistance ratios of both genes were compared, and the gene most resistant to cisplatin and its sgRNA were selected. Selected gene-activated cells were used in all subsequent experiments.

2.4 Exosome

2.4.1 Exosome Isolation

Mesenchymal stem cells for exosomes were incubated in a serum-free medium. Serum-free medium was collected 48 hours later. Then it was passed through a 0.22 µm mesh filter. After

centrifugation at 10,000 xg at +4 degrees for 30 minutes, the supernatant was taken. Then the supernatant was centrifuged at 100,000 xg at +4 C degrees for 5 hours. After centrifugation, the supernatant was discarded, and the pellet was dissolved with sterile DPBS (Exosomes are in the pellet). The thawed pellet was raised to -80 C degrees for long-term storage.

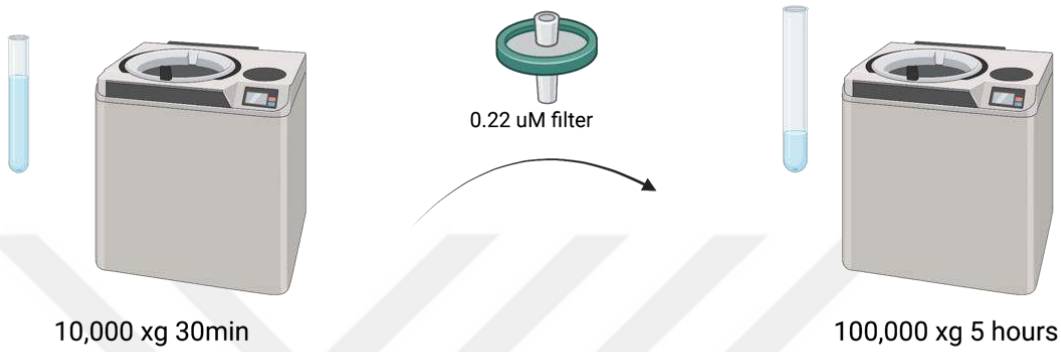

Figure 19. Exosome Isolation. *Exosome isolation is done with an ultracentrifuge device. (Generated by Biorender)*

2.4.2 Exosome SEM Characterization

The isolated exosomes were diluted at 1:1000 with DPBS. 2% PFA was prepared from 16% PFA solution with nanopure water. Then, 1:1000 diluted exosomes were fixed with 2% PFA solution at a ratio of 1:100. 5 µL of fixed exosomes were placed in drop form on a 12mm coverslip and then fixed on the coverslip by airdry method. Then, the coverslip was covered with 5 nm gold, and SEM images were taken under a Zeiss Evo (Germany).

2.4.3 Exosome Western Blot Characterization

After being treated with Laemmli buffer (sodium dodecyl sulfate (SDS) sample buffer with 2-mercaptoethanol), the total exosomes were boiled at 95 °C for 15 minutes to extract the proteins. Before electrophoretically transferred to polyvinylidene fluoride membranes, proteins were split by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE). After that, membranes were blocked with a blocking solution (DPBS with 0.1% Tween 20, containing 5% skim milk) for 1 hour. Afterward, membranes were incubated with primary antibodies (GAPDH, CD63, CD9, and Calnexin) at 4 °C for overnight. Following by 3-time washes (10 minutes each) with DPBS-T, the membranes incubation was done with Horse Radish Peroxidase (HRP)-

conjugated Goat anti-Rabbit IgG (1:1000) for 1 hour at room temperature. In the end, the membranes were incubated with an improved chemiluminescence sensing system.

2.5 Spheroid Generation from MSCs

Approximately 50k cell suspension with a total volume of 20 µL of MSCs was prepared and placed on a 10cm plate cover. Then the cap closed quickly without any dispersion of the drops. It was incubated for 3-4 days for spheroid formation. Approximately 8 mL of medium was placed on the plate.

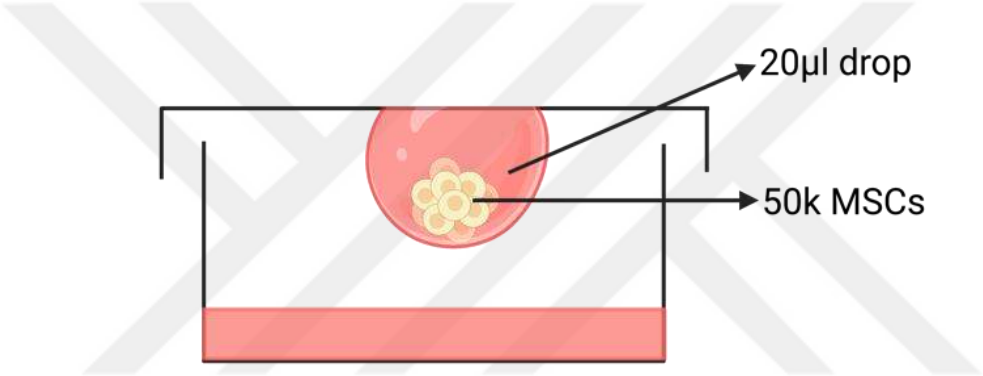

Figure 20. Hanging Drop Technique. *The hanging drop technique is used to create a spheroid, 20 µL of cell suspension contains 50k MSCs.*

2.6 Cisplatin Working Dosage Decision

5.000 hGRC1 cells were inoculated into a sub-transparent black 96 well plate. Then, to find the cisplatin value to be used in co-culture, cisplatin was given as a serial dilution from 100 µg to 0.19 µg. The Cell Titer Go (CTG) assay was performed according to the manufacturer's instructions. The IC50 value was used for cisplatin dose selection.

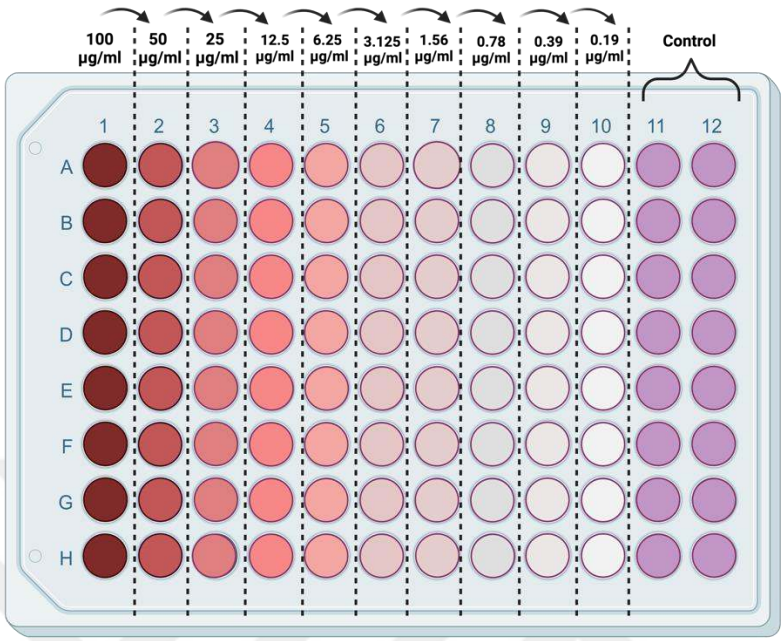

Figure 21. Cisplatin CTG Assay Plate Template. *To determine the cisplatin dose, serial dilution was applied to hGRC1 cells in 96 wells. Serial dilutions were applied from 100 µg/ml to 0.19 µg/ml.*

2.7 Co-Cultures

In *in vitro* studies, the transwell system was used in all groups in co-cultures. When establishing co-culture with transwell, the culture system was always set up with mesenchymal stem cells at the bottom and hGRC1 cells at the insert. The same complete medium (DMEM High Glucose + 10% FBS + 1% Pen/Strep) was used in co-cultures. hGRC1 cells in all groups were co-cultured as follows: 1) Normal MSC, 2) Resistant MSCs, 3) Exosome of normal MSC, 4) Exosome of resistant MSC, 5) Normal MSC+ its exosome, 6) Resistant MSC+ its exosome, 7) Normal MSC spheroid, 8) Resistant MSC spheroid. A control group that was not given cisplatin was formed in the same culture groups. In the cisplatin groups, cisplatin (6.25ug/ml) was given to the common complete medium (DMEM High Glucose + 10% FBS + 1% Pen/Strep). After cisplatin treatment, hGRC1 cells in co-culture groups were collected after 24, 48, 72 and 96 hours.

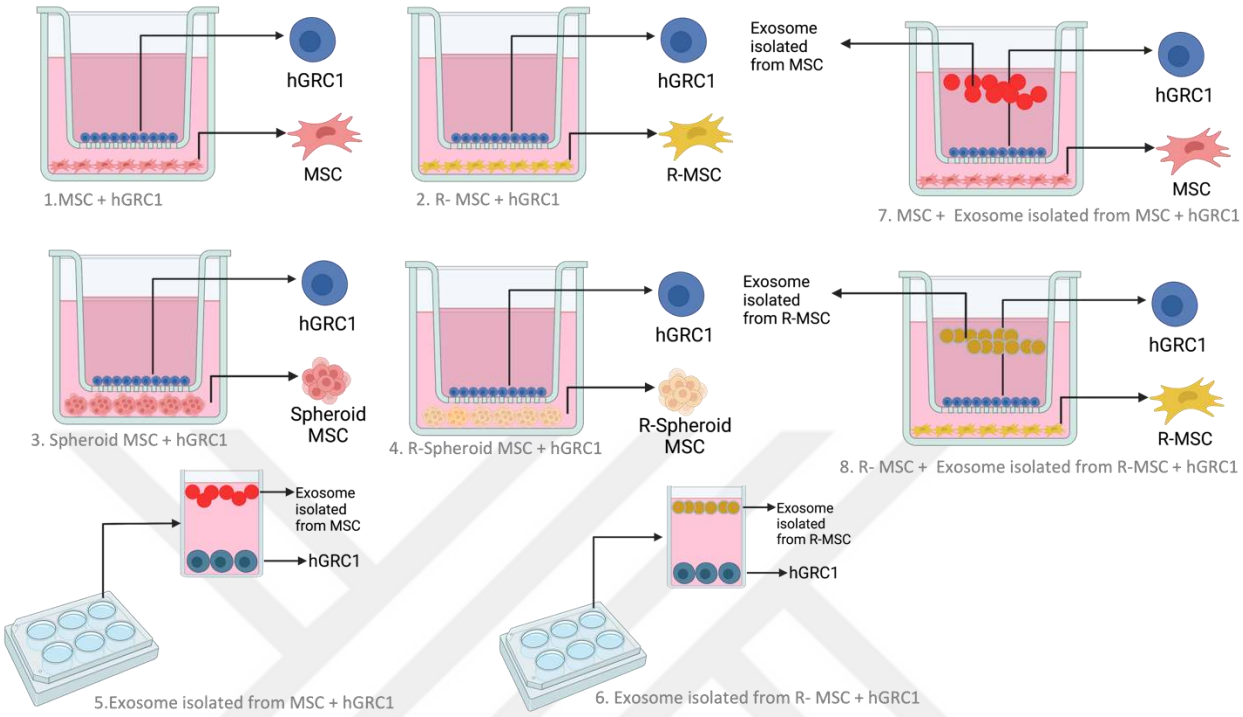

Figure 22. Co-culture Experimental Groups. *1. Mesenchymal stem cell and hGRC1, 2. Resistance mesenchymal stem cell and hGRC1, 3. Spheroid mesenchymal stem cell, 4. Resistance spheroid mesenchymal stem cell, 5. Exosome and hGRC1, isolated from mesenchymal stem cells 6. Isolated from Resistance Mesenchymal stem cells Exosome and hGRC1, 7. Mesenchymal stem cell, Exosome isolated from it, and hGRC1, 8. Resistance Mesenchymal stem cell, Exosome isolated from it, and hGRC1.*

2.8 Cell Viability Assay

After co-culture experiments, hGRC1 cells were removed from the transwell, and a viability assay was done with a hemocytometer before extracting RNA. Only live cells were counted, and Trypan blue dye was used for this assay.

2.9 qPCR

RNA isolation of hGRC1 cells was done with a Macharey-Nagel Nucleospin RNA kit following the manufacturer's instructions. RNA quantification was measured with a spectrophotometric read at 260/280 nm. For making 250 ng cDNA, M-MLV Reverse Transcriptase was used following the

manufacturer's instructions. qPCR experiments were done by Roche LightCycler 480 equipment with triplicate replicate. For reaction, Qiagen Cyber Green Master Mix was used. Roche LightCycler 480 equipment was set up to 95°C for 5 minutes, 45 cycles of 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Cq data were normalized with housekeeping gene (GAPDH) expression. Relative gene expressions were evaluated like fold changes to a reference sample using the delta delta Cq technique.

Genes	Forward Primers	Reverse Primers
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
CD99	GCGAGTGACGACTTCAACCTGG	CGTCCTCCAGGTCGAAGCCTC
IL-10	GTTCTTTGGGGAGCCAACAG	GCTCCCTGGTTTCTCTTCCT
IL-1Ra	GCTGGGGATTAGATGCTCCT	CTGTGGTAGGCTTGGAGTGA
Bax	ATGTTTTCTGACGGCAACTTC	AGTCCAATGTCCAGCCCAT
Bcl-2	TGTTTGTGTGCTTCTGAGCC	CTTATTGGATGTGCTTTGCATTC

Table 2. qPCR Primer List. *Glyceraldehyde 3-Phosphate Dehydrogenase, CD99, Interleukin-10, Interleukin-1 Receptor Antagonist, B-cell lymphoma 2 -associated X protein, B-cell lymphoma 2 were evaluated, respectively.*

3

CHAPTER 3: RESULTS

3.1 CRISPR/dCas9 Design and Sequences

Sanger sequencing was performed on the plasmid in which the gRNAs were cloned. The sequencing results are as shown in the figure, and we showed that the sequence of our gRNA was within the plasmid sequence and was cloned correctly (Figure 23). After this result, CRISPR activation experiments were continued.

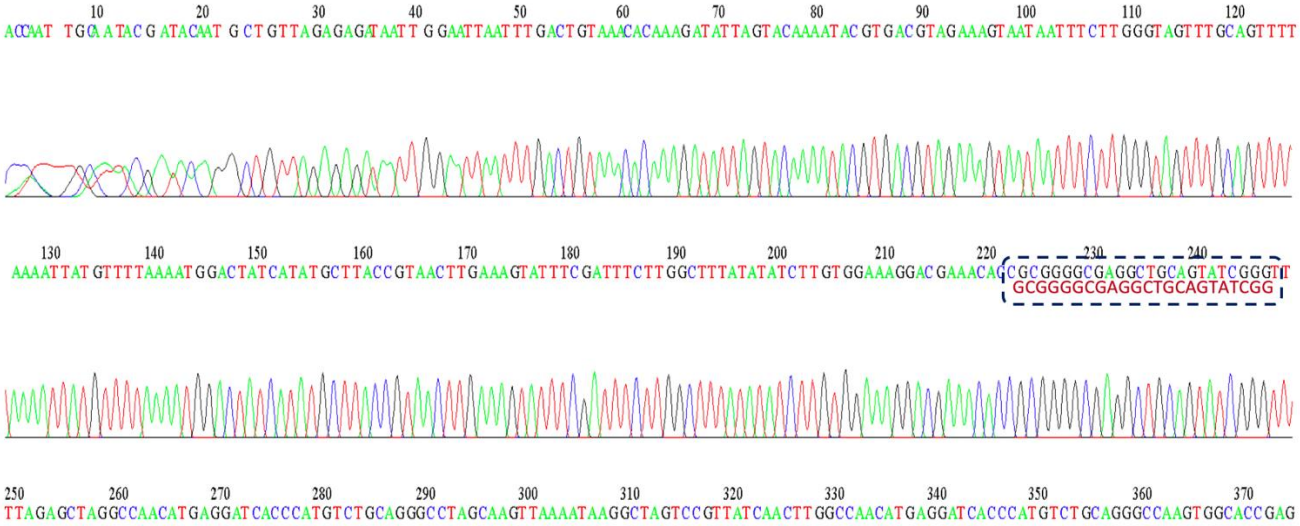

Figure 23. Sanger Sequence Analysis*. CD99's sgRNA was successfully inserted into the gRNA vector plasmid.*

3.2 MOI

Each virus was transduced to a 12-well plate containing 55,000 MSC cells. Virus concentrations were chosen as six different doses: 1 µL, 2 µL, 5 µL, 10 µL, 15 µL, and 20 µL. After approximately 16 hours of transduction, the medium was collected and replaced with a fresh medium. The collected medium was discarded with 10% bleach. Antibiotics selection started the day after the medium change. Blasticidin was used on dCas9-VP64 lentivirus at 10 µg/ml concentrations, and selection was completed on day 10. Hygromycin was used on ms2-p65-Hsf1 lentivirus at 100

µg/mL concentration, and selection was completed on day 10. Puromycin was used on gRNA vector lentivirus at 1 µg/mL concentration, and selection was completed on day 10. Selections were considered complete when all the cells in the negative control, which did not receive the virus but were given antibiotics, were dead (Figure 24).

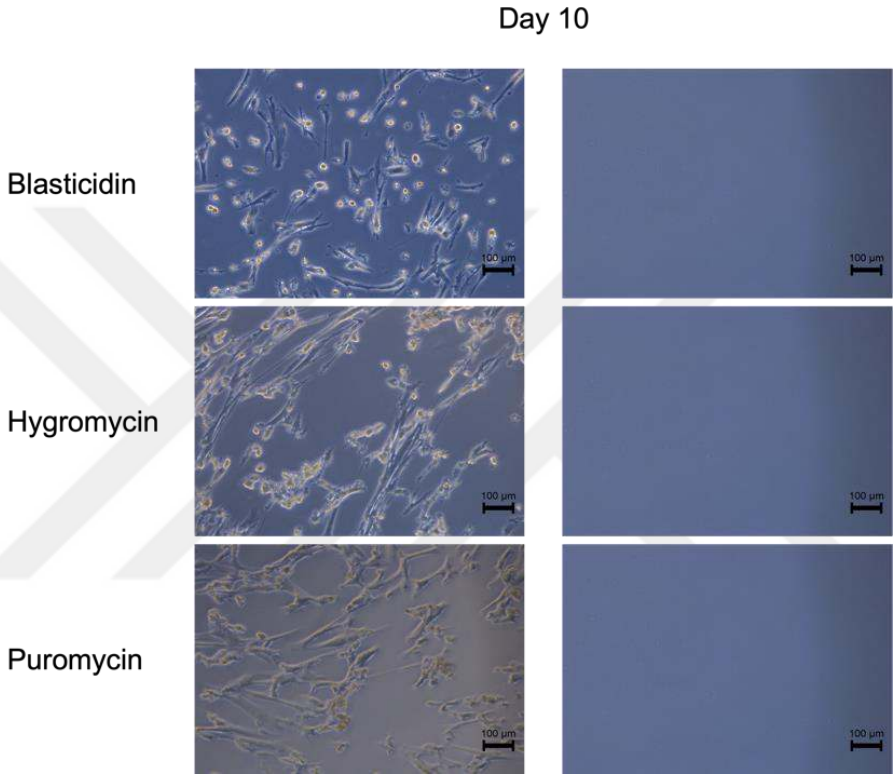

Figure 24. Selection of Blasticidin, Hygromycin, and Puromycin Treatments. *10 µg/ml for Blasticidin, 100 µg/ml for Hygromycin, and 1 µg/ml for Puromycin were the optimum selection concentration.*

3.3 Cisplatin Resistance

After the CRISPR/dCas9 activation system was established, a Cisplatin CTG assay was performed to see if there was resistance against cisplatin in mesenchymal stem cells. In the assay, it was decided whether it was resistant to cisplatin or not by looking at the IC50 value. It was observed that the cells given the activation system did not die and survived at the IC50 value of the control group which is 50 µg/ml, and they were shown to be resistant according to this result (Figure 25).

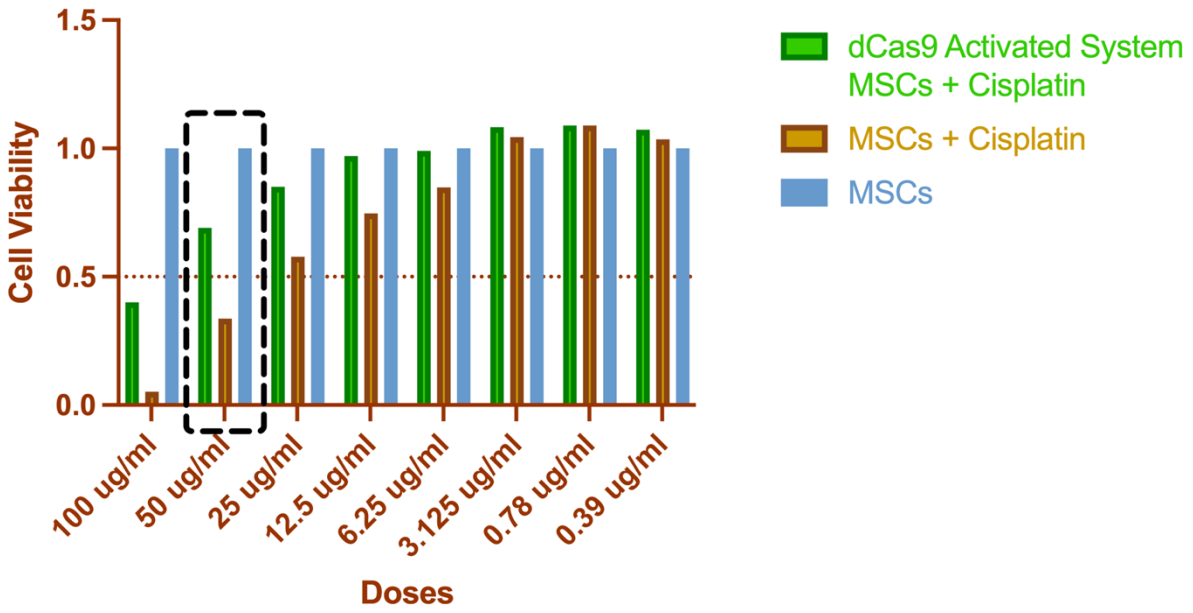

Figure 25. Resistance Check Cisplatin Treatment. *Cisplatin treatment was applied to mesenchymal stem cells by serial dilution. Engineered MSCs were counted resistant by showing more than 50% cell life at the control group's IC50 value (50 µg/ml).*

3.4 Exosome Characterization

3.4.1 Exosome Characterization by Western Blotting

A Western blotting was performed for the characterization of exosomes. Exosomes are vesicles that are CD63 and CD9 positive and Calnexin negative. Western blotting results has showed that while CD63 and CD9 are positive in the exosomes we isolated, and calnexin was negative. It has been shown that the exosomes we isolated here are characterized as exosomes (Figure 26).

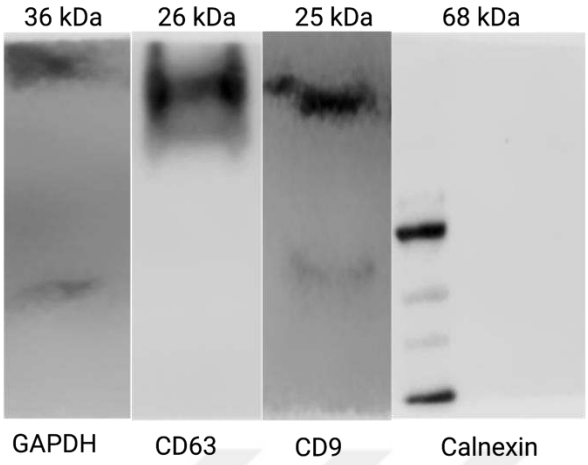

Figure 26. Exosome Characterization by Western Blot*. Western blot showed the presence of CD63 and CD9 protein and the absence of Calnexin protein in exosomes.*

3.4.2 Exosome Characterization by SEM

In addition to Western blotting characterization, scanning electron microscope image characterization was also performed for exosomes. SEM images of exosomes were taken. The SEM image shows that the isolated exosomes are round as in the literature, and their sizes are between 100-200 nm on average (Figure 27).

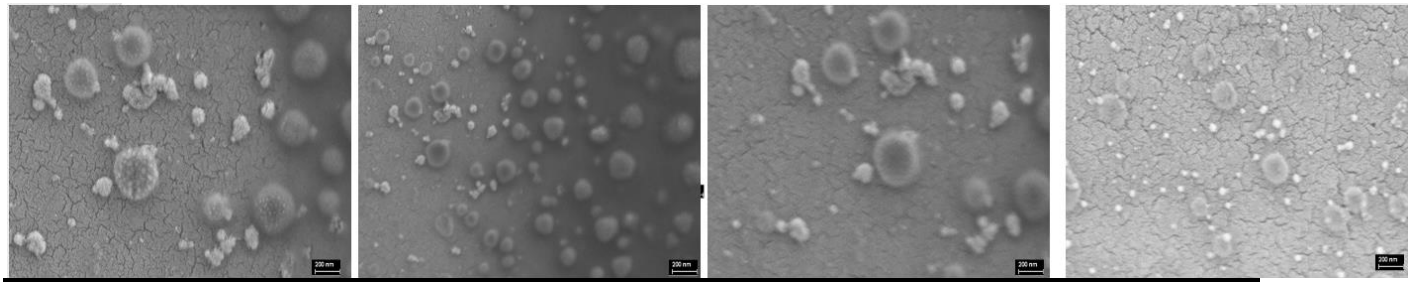

Figure 27. Exosome Characterization by SEM. *The SEM image shows that the isolated exosomes are round or cup-shaped, and their size is between 20-200 nm.*

3.5 Spheroid Generation

At the end of 4 days, MSC suspensions, which were left in drop form, were checked with the hanging drop technique, and it was observed that they took the shape of a spheroid. When

examined under a phase contrast microscope, MSCs spheroids were found to be similar in size and shape, compared to those depicted in the literature (Figure 28).

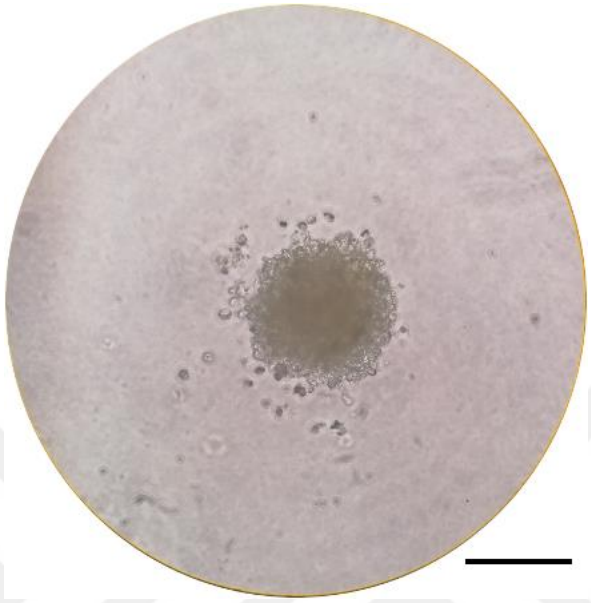

Figure 28. Spheroid Shape of MSCs. *The phase contrast microscope image shows that at the end of the 4th day, MSCs formed a spheroid shape by the hanging drop method. Size of spheroid 143µm. Scale bar 100 µm.*

3.6 Cisplatin Working Dosage Decision

The cisplatin dose was determined for the co-culture of MSCs and hGRC1 cells to be grown together. Cisplatin CTG assay was performed on hGRC1 cells to determine this dose. A working dose of 6.25 µg/ml cisplatin concentration, which is the IC50 value of hGRC1 cells, was chosen (Figure 29).

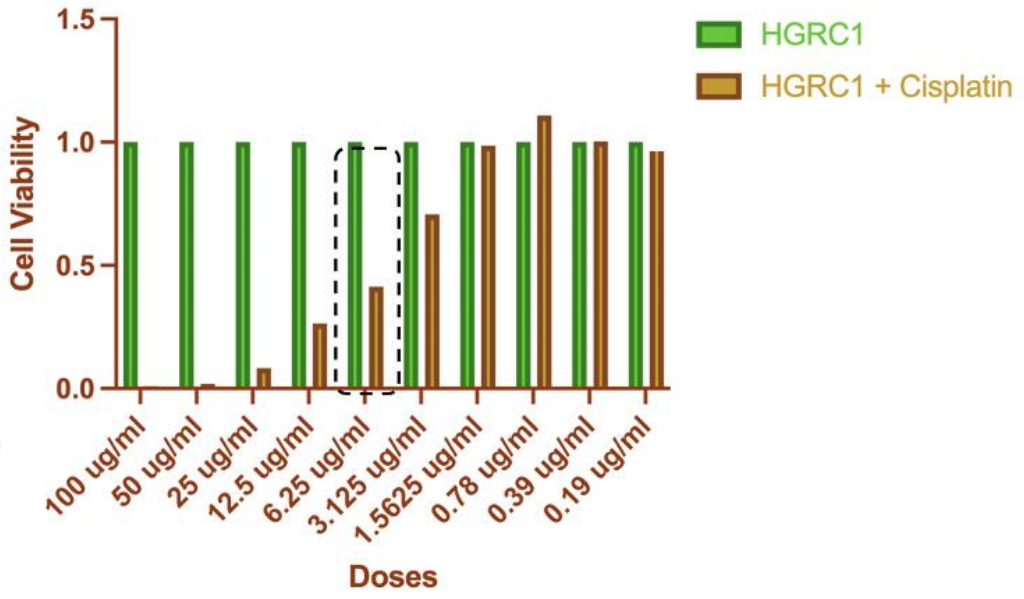

Figure 29. Cisplatin Dosage Decision. *The IC50 value (6.25 µg/ml) was determined as the working dose in the cisplatin serial dilution treatment in hGRC1 cells.*

3.7 Cell Viability Assay

Cisplatin was given to the experimental groups in different set-ups for 24, 48, 72, and 96 hours. Then, after each hour group, hGRC1 cells to be treated with 0.05% Trypsin/EDTA were counted as living cells with trypan blue (Figures 30-33).

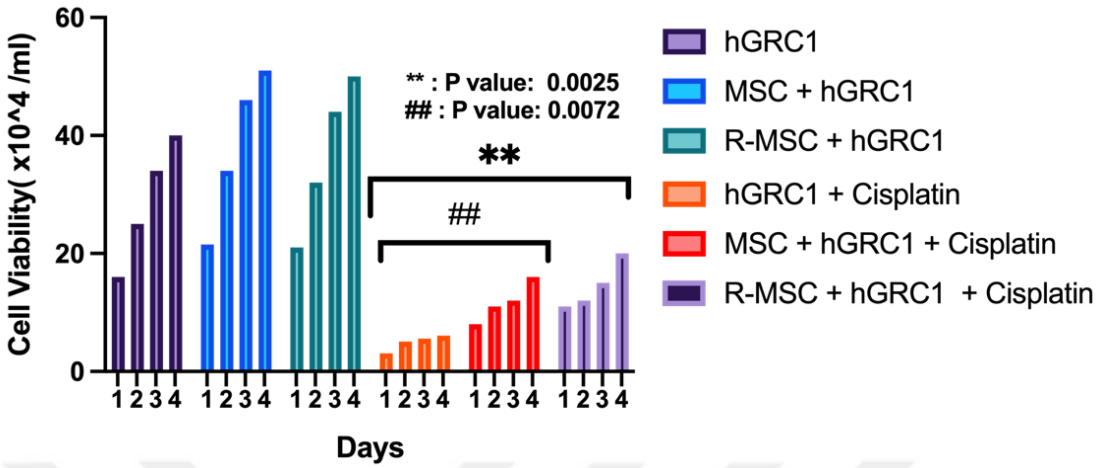

Figure 30. Cisplatin Treatment - MSC and R-MSC Groups. *MSC and R-MSC groups were exposed to cisplatin, and hGRC1 cells were harvested at 24, 48, 72, and 96 hours. Both groups provided significantly more protection than the control group, with more viable cells. It was also observed that the R-MSC group provided more protection than the MSC group. (**: p value : 0.0025 and ## : p value : 0.0072)*

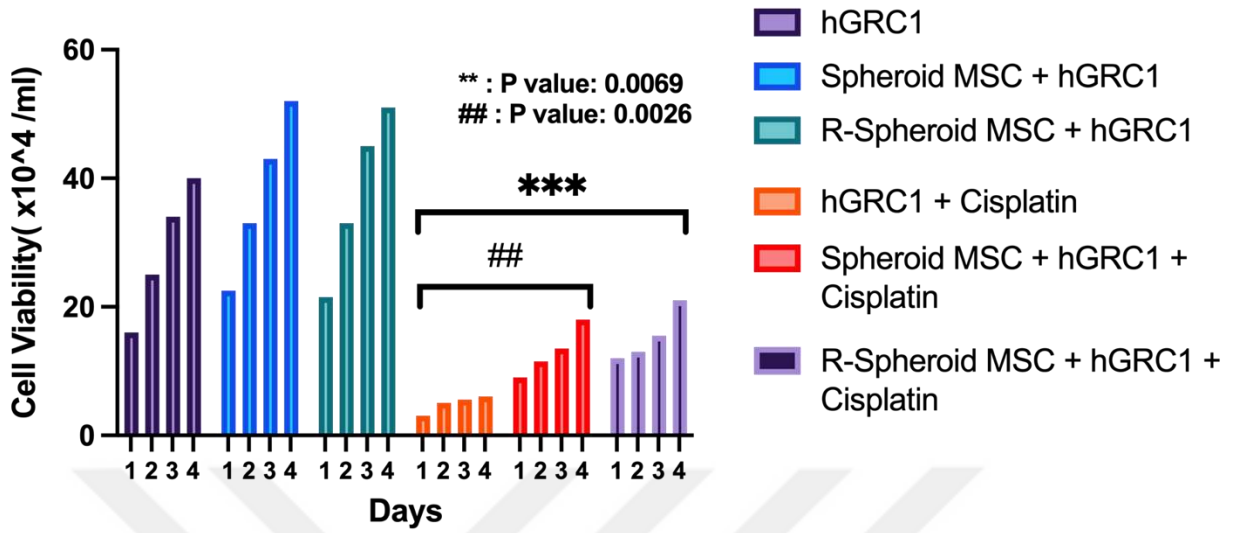

Figure 31. Cisplatin Treatment - Sp-MSC and R-Sp-MSC Groups. *Sp-MSC and R-Sp-MSC groups were exposed to cisplatin, and hGRC1 cells were harvested at 24, 48, 72, and 96 hours. Both groups provided significantly more protection than the control group, with more viable cells. It was also observed that the R-Sp-MSC group provided more protection than the Sp-MSC group.(** : p value : 0.0069 and ## : p value : 0.0026)*

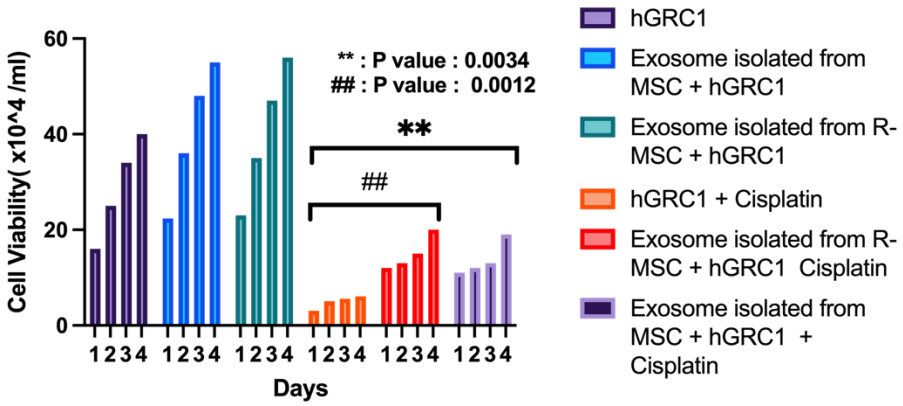

Figure 32. Cisplatin Treatment Ex and R-EX Groups. *Ex and R-Ex groups were exposed to cisplatin, and hGRC1 cells were harvested at 24, 48, 72, and 96 hours. Both groups provided significantly more protection than the control group, with more viable cells. (** : p value : 0.0034 and ## : p value : 0.0012)*

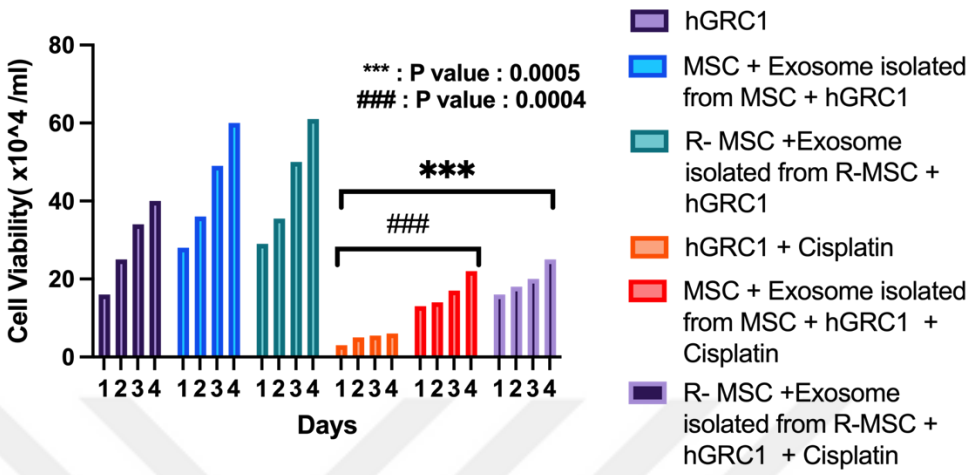

Figure 33. Cisplatin Treatment – MSC + Ex and R-MSC + R-EX Groups. *MSC + Ex and R-MSC + Ex groups were exposed to cisplatin, and hGRC1 cells were harvested at 24, 48, 72, and 96 hours. Both groups provided significantly more protection than the control group, with more viable cells. It was also observed that the and R-MSC + Ex group provided more protection than the MSC + Ex group. In addition to these, these experimental groups are the group that provides the most protection compared to the control, and they have more viable cells that have provided more protection than the other groups. The R-MSC + R-Ex group was the group that provided the most protection among all experimental groups. (** : p value : 0.0005 and ## : p value : 0.0004)*

Cell viability assay results show that all our experimental groups protected hGRC1 cells against cisplatin compared to control. The experimental groups had more viable cells per day than the control (Figure 35). Resistant cells were also found to provide more protection than non-resisting cells (Figure 35). Especially at the 96th hour, resistant cells provided significant protection compared to both non-resistant and control. Among all the experimental groups, the group that provides the most protection every day is the group in which resistant mesenchymal stem cells and their exosomes are given together. This group protected significantly against both the mesenchymal stem cell group and the control each day. At the 96th hour, the resistant mesenchymal stem cells and their exosomes group provided the most protection and significantly differed significantly from the others (Figure 35).

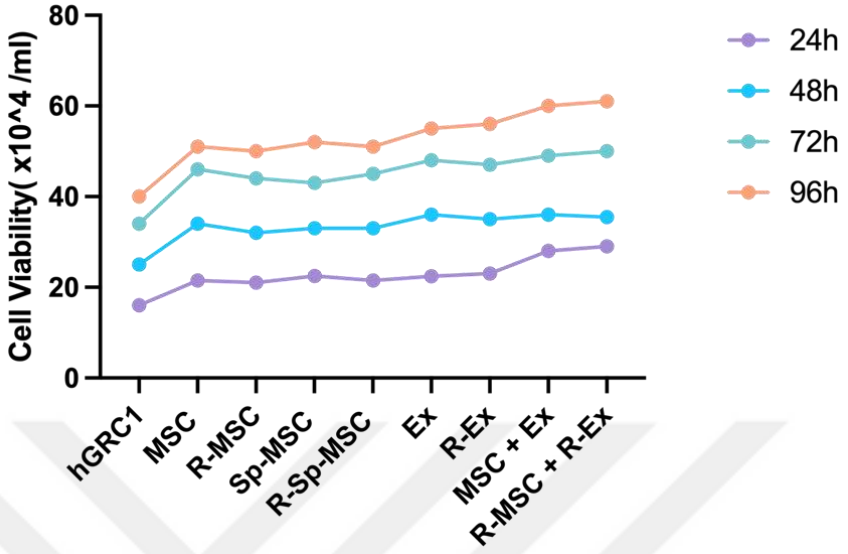

Figure 34. Groups Not Given Cisplatin. *Neither group had a toxic effect on hGRC1 cells on their own, and they continued to proliferate without death as in control.*

The experimental groups were co-cultured without Cisplatin, and it was examined whether MSCs or exosomes isolated from them had any toxic effects on hGRC1. The results show that neither MSCs nor exosomes isolated from them have a toxic effect. Interestingly, some co-cultures even had a higher cell count than the normal control (Figure 34).

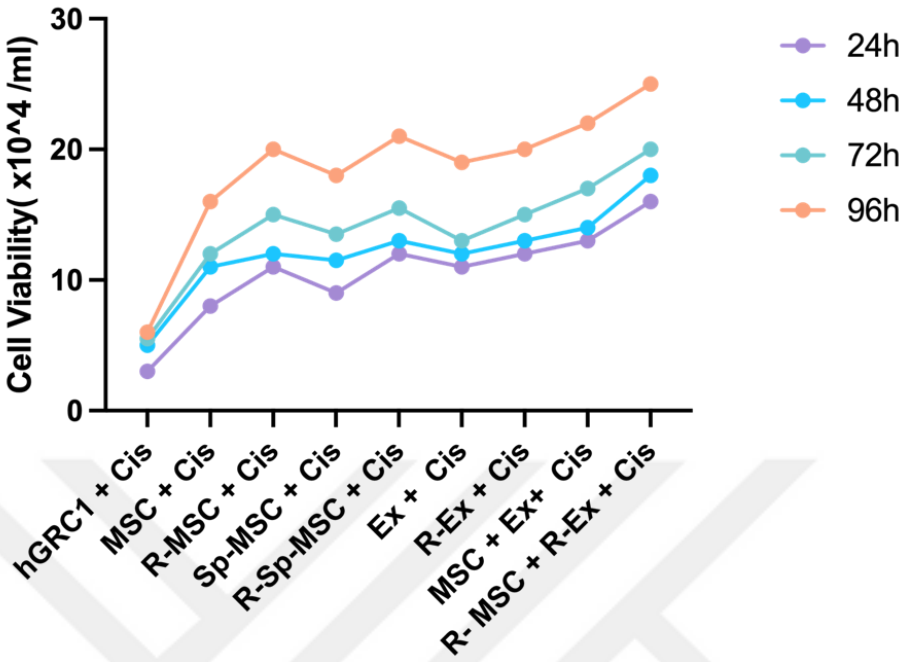

Figure 35. Analysis of Experimental Groups. *The cell count of the experimental groups is shown according to 24, 48, 72, and 96 hours and the difference between the other groups according to the hours. In addition, the most significant difference between the control group among all-time groups and the most protection was at the 96th hour.*

When the experimental groups were compared with each other, the R-MSC + R-Ex group provided significantly more protection than the other groups at all hours (24, 48, 72, and 96). In addition, when the groups are looked at within themselves, Resistant groups provide more protection than non-resistant ones, which shows that an MSC treatment with resistant cells is more effective than a non-resistant MSC treatment (Figure 35).

No significant difference exists between the exosome groups isolated from resistant cells and those isolated from normal cells (Figure 35). However, the Exosome groups provided a high level of protection overall. Each hour group gave more protection than the group co-cultured with MSC alone.

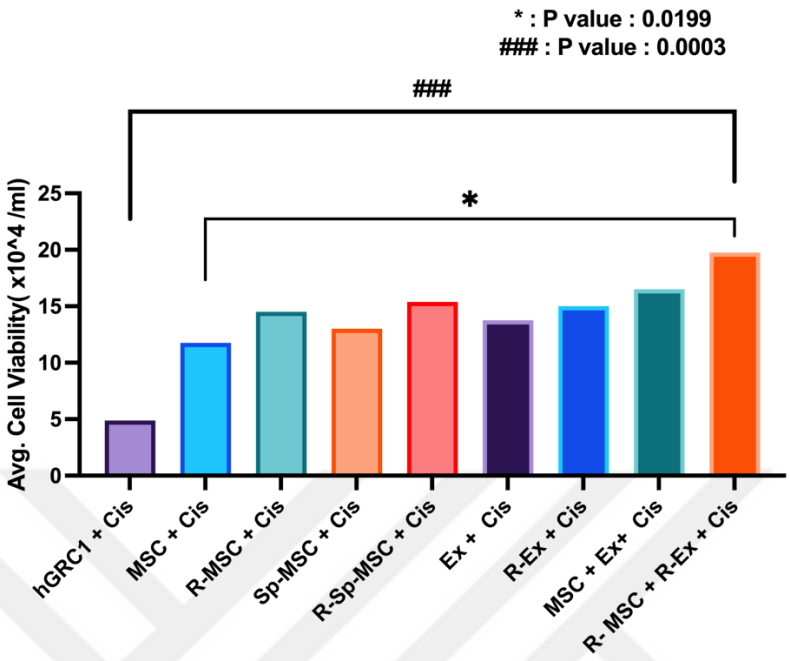

Figure 36. Average Viability Comparison. *The average cell viability results show that all groups had more viable cells than the control group, providing protection. While the resistant experimental groups provided more protection than the non-resistant ones, the R-MSC + R-Ex group provided the most protection among all groups. Significant somewhat protected over both the control and MSC groups.*

All these cell viability results show that using MSC cells simultaneously with cisplatin creates a protective effect. Engineered cisplatin-resistant mesenchymal stem cells, which we created to improve the treatment at the same time with cisplatin, are even less affected by cisplatin, providing greater protection. Mesenchymal stem cells were also used in the treatment in 3D (spheroid) shapes and their exosomes were isolated, as well. In this way, it was aimed to find the most effective treatment. Separate data of 24, 48, 72, and 96 hours show that resistance mesenchymal stem cells and their exosomes provide a significant level of protection when we compare them with the control and MSC experimental groups when used at the same time. R-MSC + R-Ex group was the most effective protection group at every hour. At the 96th hour, it provided significant protection compared to the other groups and the control and had the highest number of viable cells. In addition, when all these groups were cultured without cisplatin, it was shown that there was no toxic effect on hGRC1 cells (Figure 36).

3.8 Gene Expression Analysis

qPCR was used to examine how mesenchymal cells and their exosomes affect the inflammation and apoptosis gene expression of hGRC1 cells after cisplatin treatment.

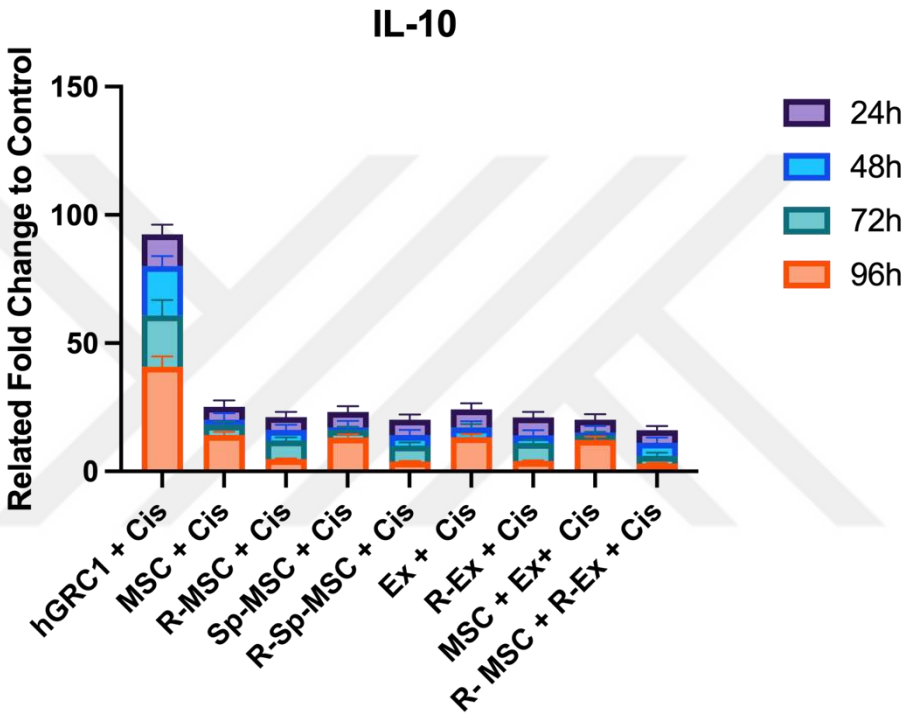

Figure 37. IL-10 Gene Expression in the Experimental Groups. *While the IL-10 value is significantly higher in control, it is less in the experimental groups. There is a significant decrease in IL-10 value, especially in the experimental groups at 96 hours. The R-MSC + R-Ex group is the group with the lowest IL-10 expression.*

While IL-10 was significantly highly expressed in the control group, especially at 24 hours, it was significantly less expressed in the experimental groups. In comparison, there was a decrease in IL-10 level in each group at similar rates at 48 and 72 hours; IL-10 expression decreased differently between groups at 96. hours. Especially resistant MSCs groups showed less IL-10 expression compared with non-resistance MSCs groups in parallel with the cell viability results. The group with the lowest IL-10 level is the R-MSC + R-Ex group. This is a significantly parallel result with the cell viability assay (Figure 37).

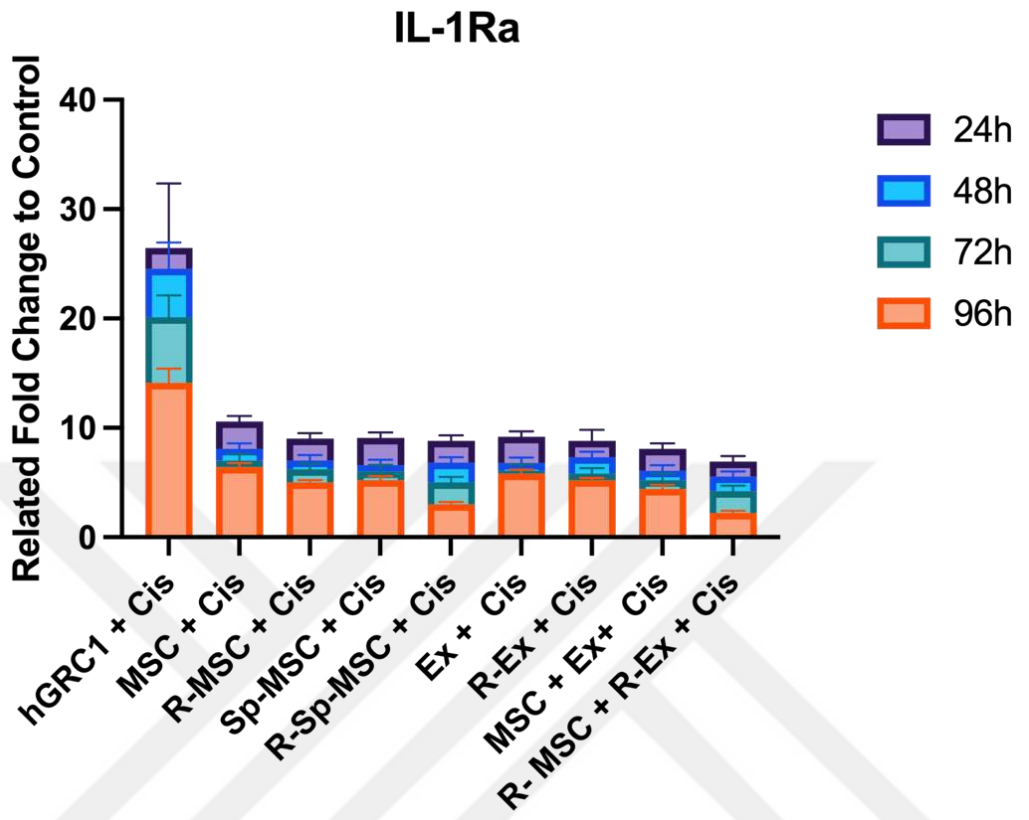

Figure 38. IL-1Ra Gene Expression in the Experimental Groups. *While the IL-1Ra value is significantly higher in the control, it is less in the experimental groups. Only a significant decrease in R-Sp-MSC and R-MSC+R-Ex groups at 96 hours. The other experimental groups show a significant decrease from 24 to 96 hours. The R-MSC + R-Ex group is the group with the lowest IL-1Ra expression.*

IL-1Ra is also a marker of inflammation like IL-10. Likewise, as in IL-10, IL-1Ra expression was highest at 24 hours and in the control group. However, as in IL-10, there are only two groups that decreased significantly at the 96th hour, these are the R-Sp-MSC and R-MSC + R-Ex groups. Other groups showed a steady decrease in expression up from 24h to 96 h. In IL-1Ra, there is no significant difference between resistant and non-resistant in MSC and Exosome groups, but there is a significant change in spheroid and MSC + Ex groups, especially at 96 hours. Again, the R-MSC + R-Ex group showed the lowest expression in IL-1Ra (Figure 38).

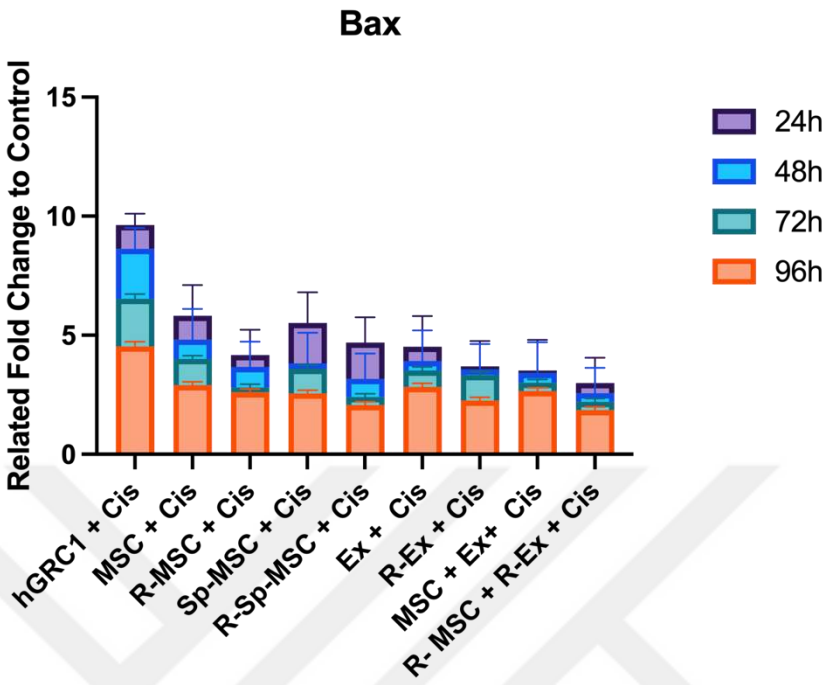

Figure 39. Bax Gene Expression in the Experimental Groups. *All experimental groups had lower Bax expression than the control groups; the lowest expression was in the R-MSC + R-Ex group.*

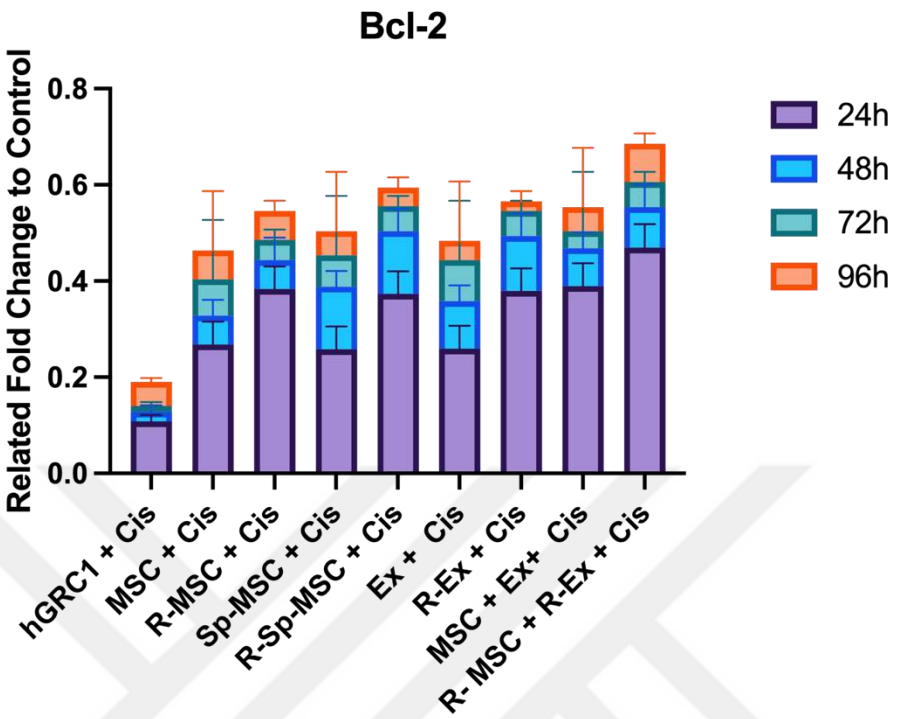

Figure 40. Bcl-2 Gene Expression in the Experimental Groups. *All experimental groups had higher Bcl-2 expression than the control groups; the highest expression was in the R-MSC + R-Ex group.*

Bax / Bcl-2 ratio is related to apoptosis markers and, Bax and Bcl-2 work as inhibitors of each other. Cells with high Bax expression enter apoptosis faster. Bcl-2 works as an apoptosis inhibitor; cells with high Bcl-2 expression are also away from apoptosis.

As a result of the experiments, the control group at 24 hours had the highest Bax and the lowest Bcl-2, which means the group with the most apoptosis. The lowest amount of apoptosis is at the 96th hour. Bax and Bcl-2 expression result graphs show that resistant groups have less apoptosis signals than non-resistant groups; less Bax expression is seen, while more Bcl-2 expression is seen. As in Cell viability, IL-10 and IL-1Ra expressions, the group with the lowest Bax and highest Bcl-2 values among the experimental groups, with the least apoptosis, is the R-MSC + R-Ex group. At the 96th hour, it had the least Bax and the highest Bcl-2 values significantly compared to the other groups (Figure 39, 40).

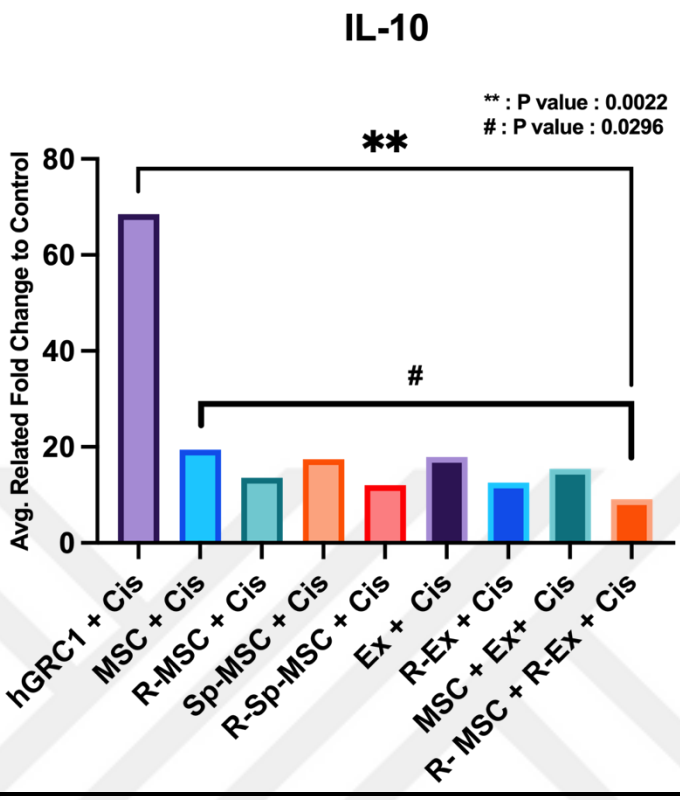

Figure 41. Comparison of Average IL-10 Expression. *When the expression of the experimental groups in IL-10 was compared on average, the R-MSC + R-Ex group had significantly less expression than both the control and MSC groups. (** : p value : 0.0022 and # : p value : 0.0296)*

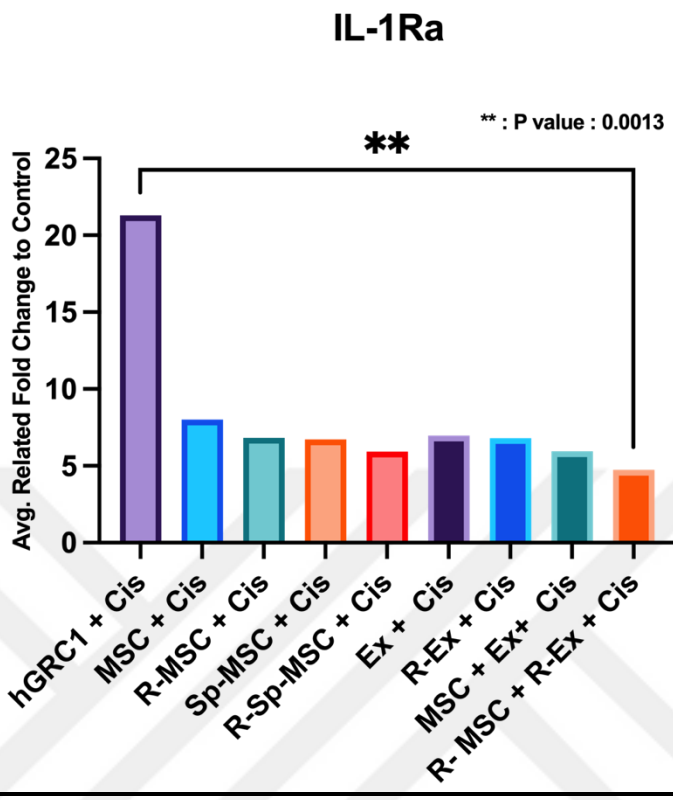

Figure 42. Comparison of Average IL-1Ra Expression . *When the expression of the experimental groups in IL-1Ra was compared on average, the R-MSC + R-Ex group had significantly less expression than the control group. There was no significant difference between the experimental groups for IL-1Ra. (** : p value : 0.0013)*

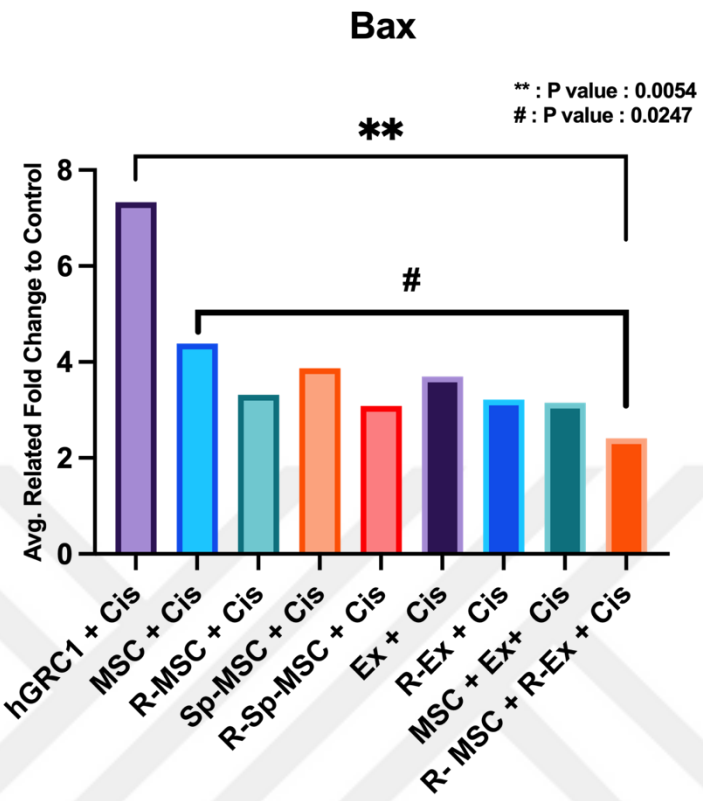

Figure 43. Average Bax expression comparison. *When the expression of the experimental groups in Bax was compared on average, the R-MSC + R-Ex group had significantly less expression than both the control and MSC groups. (** : p value : 0.0054 and # : p value : 0.0247)*

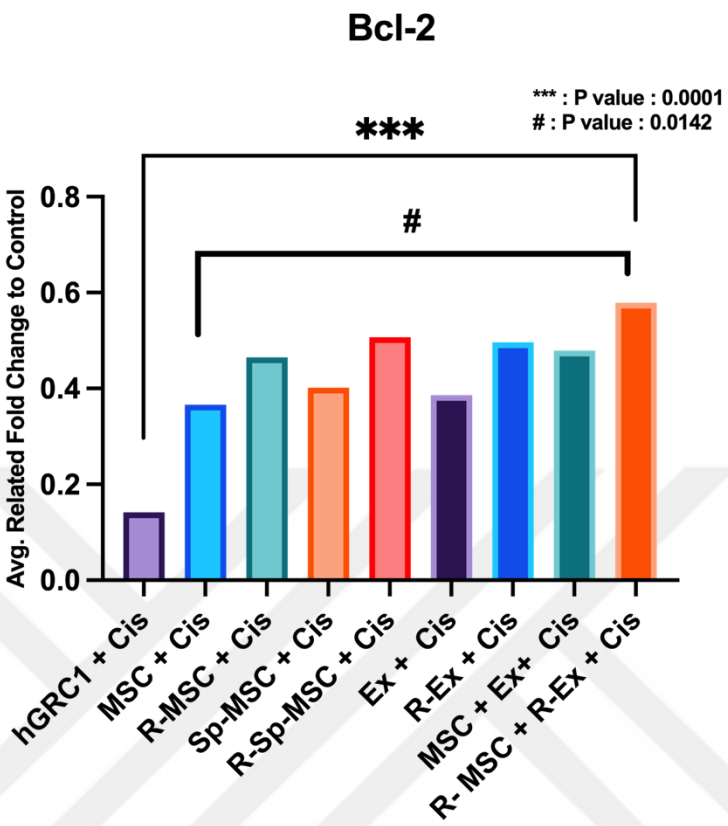

Figure 44. Average Bcl-2 expression comparison*. When the expression of the experimental groups in Bcl-2 was compared on average, the R-MSC + R-Ex group had significantly higher expression than both the control and MSC groups.(***: p value : 0.0001 and # : p value : 0.142)*

The results of the inflammation and apoptosis markers expressions show that each experimental group has less inflammation and apoptosis expressions than the control group. When the groups are evaluated within themselves, resistant groups have less inflammation and apoptosis expression than non-resistant groups. It has been shown that using resistant mesenchymal stem cells in cisplatin treatment is more advantageous and effective, as it protects hGRC1 cells more and further reduces the expression of inflammation and apoptosis. In addition, statistical analyses show that the R-MSC + R-Ex group had significantly less inflammation and apoptosis expression than the control and MSC groups. These results agree with the cell viability results. According to all these results, the R-MSC + R-Ex group is the most effective experimental group in treatments. It has both the highest number of viable cells and the lowest inflation and apoptosis values (Figures 41-44).

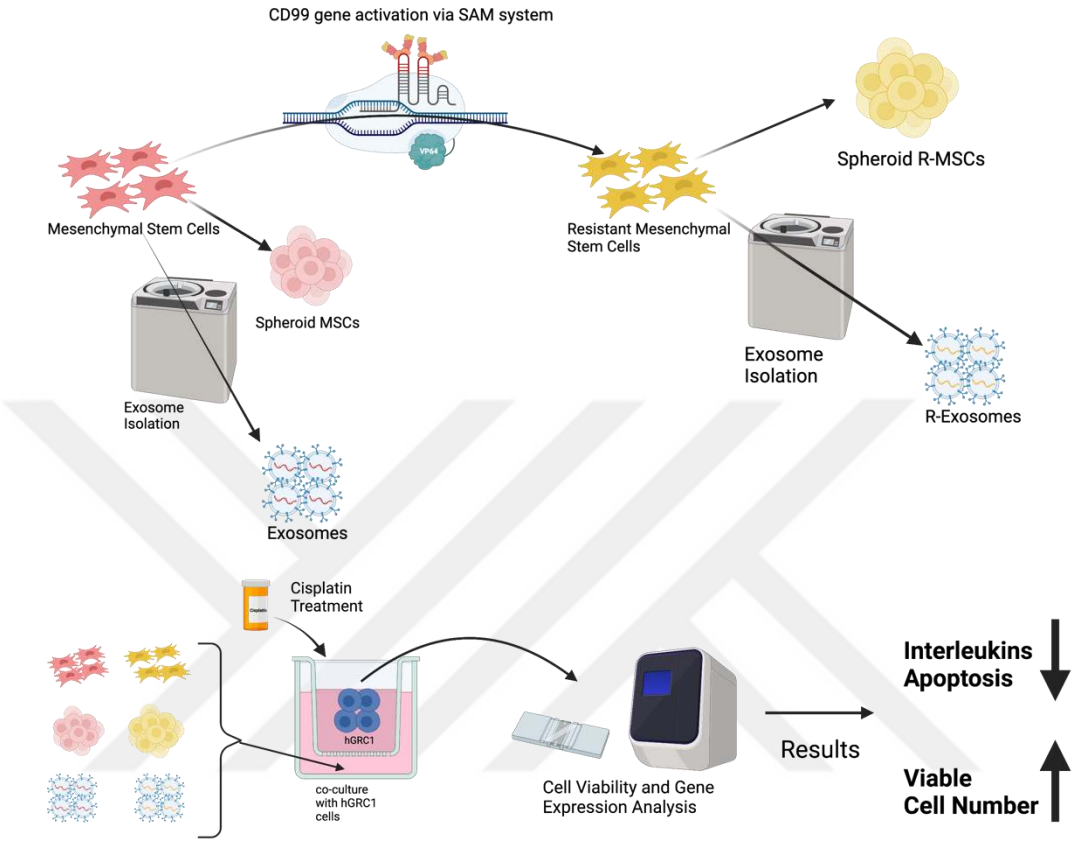

Figure 45. Summary of Experiments. *Overview of experiments and results.*

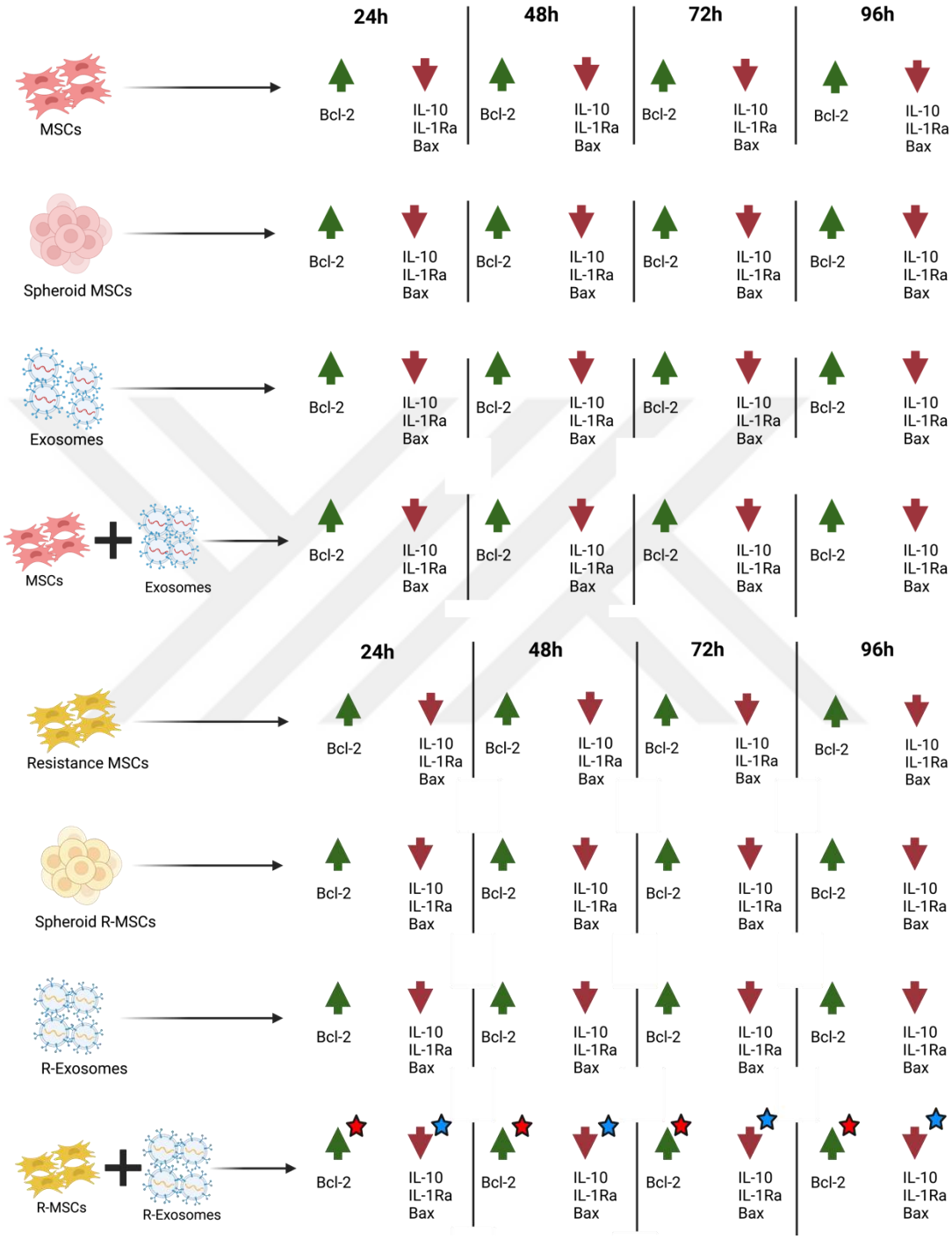

Figure 46. Summary of Gene Expression Results. *The red star represents the highest value increaser of all groups. The blue star represents the most depreciating of all groups.*

4

CHAPTER 4: DISCUSSION

Cancer is one of the diseases that cause millions of new cases and deaths each year[3]. There are many different types and causes of cancer, and various treatment methods depend on them. The most common treatment methods are surgery, chemotherapy, radiotherapy, hormone therapy, and immunotherapy[7].

The prevalence of cancer is higher in women than in men[18]. Millions of women are newly diagnosed with cancer each year, and thousands of women die of cancer. In parallel with the increasing number of cases, the number of women receiving cancer treatment is also increasing. Breast and gynecological cancers are common, especially in women. Gynecological cancers are cancers of the reproductive organs[19].

Chemotherapy is a cancer treatment method with a drug, and chemotherapeutic drugs target rapidly proliferating cancer cells in the body and kill them[22]. The targets and mechanisms for chemotherapeutic medicines vary from drug to drug.

Cisplatin is a platinum-based chemotherapy drug used in almost all types of cancer, including breast and gynecological cancers. In short, when cisplatin enters the cell, it becomes positively charged and binds to nucleophilic molecules such as DNA, mRNA, or protein, preventing their proliferation and blocking their functions[51].

Chemotherapeutic drugs, including cisplatin, affect and kill not only cancer cells but also other rapidly proliferating cells in the body. These include follicular cells in the ovaries. Therefore, chemotherapy-induced ovarian failure due to chemotherapy is seen in women. Chemotherapy-induced ovarian failure can cause a condition of decreased ovarian reserve in women and is one of the main causes of infertility in cancer patients [136,217]. Chemotherapy-induced ovarian failure is a widespread condition after cancer treatments, and infertility due to cancer treatments is one of the most common causes of infertility in women[136]. Given that cisplatin is a commonly used agent in most type of cancer, especially the gyecological ones, we decided to create an alternative treatment against the detrimental effects of cisplatin on cancer cells. While trying to do this, we have thought of using stem cells since they are also very beneficial, as summarized below.

Stem cells are the primary cells that form the tissues and organs naturally found in our body that can reproduce and differentiate into other cells. Stem cells can be divided into five different groups according to their potential to differentiate: totipotent, pluripotent, multipotent, oligopotent, and unipotent[218].

Multipotent stem cells are adult stem cells. Mesenchymal stem cells also belong to this adult stem cell family. Mesenchymal stem cells have different sources in the mammalian body: Bone Marrow, Dental Pulp, Adipose Tissue, Umbilical Cord, and Placenta. MSCs can also differentiate into different types of cells. MSCs can differentiate into various cells such as Adipocyte, Chondrocyte, Neuron, Myocyte, and Osteoblast[150,151,219].

Mesenchymal stem cells have recently become one of the most widely used in stem cell therapies. The most important reasons are that MSCs are naturally found in the body, isolation is easy, cost-effective, ethical problems are low, and these cells can secrete many anti-apoptotic, anti-inflammatory, growth factors, cytokines, and angiogenic molecules. These make the use of these cells in stem cell therapies an advantage[11,151,166]. Based on these reasons, Mesenchymal stem cells, which secrete high levels of anti-apoptotic and anti-inflammatory secretions, were used in this study to reduce cisplatin-induced apoptosis and inflammation in granulosa cells and to protect granulosa cells.

In treatments, not only mesenchymal stem cells but also exosomes released from mesenchymal stem cells have been used[15]. Exosomes are extracellular vesicles and play a critical role in cell-cell communication. They contain many RNA and different proteins, especially mRNA, miRNA, and anti-apoptotic signals[169,175]. In the light of this knowledge easily available exosomes can be satisfactory options for cellular treatments along with MSCs. The size is also very advantageous, and it enables the exosomes to reach the target area. As exosomes are also known to produce decreased inflammatory reactions, they seem to be an efficient player in most types of treatments in the future, including infertility, as well.

Mesenchymal stem cells are also used in the reproductive field. In infertility treatments they have been tried for as adjunct options for treatment of cancer or different diseases[152,220,221]. However, all of the studies so far have been conducted for cases where therapy is given after the patients take

chemotherapy; MSCs for prophylactic purposes have not been used. In our study, we used MSCs in cancer treatments to protect the ovaries against chemotherapeutic drugs during the treatment.

CRISPR genome editing is one of the most studied topics in the technology science committee. By using CRISPR technology, DSB, insertion, deletion, or base editing can be done in the desired region of the DNA[190,210]. dCas9, also called dead Cas9, is a protein with other features of the Cas9 protein, except for its nuclease activity[211]. The researchers created the dCas9-VP64 fusion protein by attaching activators to these proteins. This fusion protein binds to the gene targeted by the sgRNA and increases the transcription of that gene[210]. Gene edition is a very powerful tool and use of dCAS9 does not silence the gene and its effects are not dramatic but more efficient in terms of clinical use. One important advantage that we have obtained due to dCAS9 use was that, we have produced stable MSC lines that are all resistant for cisplatin, which will be important for any other related experiments, such as the ones that use various types of chemotherapeutic agents.

When they examined cancer cells resistant to chemotherapeutic drugs, they observed that some genes were overexpressed depending on the cancer type and the drug used. In a study of ovarian cancer cells, researchers found that when they activated the CD99 gene, these cells became resistant to the drug cisplatin[213,216]. Unlike most cancer cell lines, MSCs do not easily get infected by the virus. In this sense, having them infected with CD99 for the first can be considered as a significant success. Given that CD99 expression has been found to be related to development of cisplatin resistance in the cell, such an approach can also be valid for other types of stem cells.
Based on these studies, we aimed to use mesenchymal stem cells during cancer treatment to protect the ovaries against chemotherapy drugs, minimize the damage caused by these treatments, and prevent these patients from becoming infertile. For this, we first activated the CD99 gene in mesenchymal stem cells and made these cells resistant to cisplatin. We then set up different experimental groups to find these cells' most effective protection. In our experimental groups, we used mesenchymal stem cells and exosomes that we isolated in these cells and 3-dimensional spheroid structures that we created with mesenchymal stem cells. We co-cultured these experimental groups in vitro with hGRC1 cells using transwell. We exposed cells to cisplatin for up to 96 hours. For analysis, we examined the cell viability, inflammatory, and apoptosis markers of hGRC1 cells.

Our experimental results showed us that when we co-cultured mesenchymal stem cells and hGRC1 cells at the same time, there were more living cells and lower inflammatory and apoptosis compared to the control group. In our study, we also found that resistant cells provided more effective protection than non-resistant ones. The group with the most protective effect was resistant mesenchymal stem cells, and the exosomes isolated from these MSCs. This group showed the most viable hGRC1 cells and the lowest level of inflammatory and apoptosis. To our knowledge, this is the first study that uses dcas9 edited MSCs and their exosomes for protecting human granulosa cells from the detrimental effects of a strong chemotherapeutic agent, such as cisplatin.

Our study was carried out only *in vitro*; in the light of these results, we found in further studies, *in vivo* experiments can be started. In addition, mesenchymal stem cells can be made resistant to different chemotherapy drugs by using the CRISPR/dCas9 system. It can also be checked whether protection is provided for the damage caused by these drugs. In addition, our study may evolve from the female reproductive system to the male one, and a protection method can be developed for chemotherapy-induced infertility in men, as well.

5

CHAPTER 5: CONCLUSION

Our current study has showed that the mesenchymal stem cells can become cisplatin-resistant by activating the CD99 gene via the CRISPR/dCas9 system. Resistant mesenchymal stem cells can be used simultaneously with cisplatin to protect granulosa cells against the toxic effect of cisplatin. Not only mesenchymal stem cells but also extracellular vesicular exosomes obtained from these cells can provide protection in granulosa cells when given the same as cisplatin. In addition, when resistant mesenchymal stem cells and their exosomes are used simultaneously, much more effective protection against cisplatin can be used. For the first time, mesenchymal stem cells were made resistant to cisplatin using the CRISPR/dCas9 system. Also, for the first time, protection against chemotherapy was provided by culturing mesenchymal stem cells in granulosa cells at the same time as chemotherapy, not after chemotherapy. For the future perspective use of resistant MSCs and their exosomes *in vivo* with animal cancer models can be applied to evaluate the protective effectivity of the genome edited cells.

VITA

Mehmet Yunus Çomar was born in 1997, Fethiye, Muğla, Turkey. He received his bachelor's degree in Molecular Biology and Genetics from Üsküdar University. After graduating from university, he continued his graduate education in the Reproductive Biology Master's Program at Koç University Graduate School of Health Sciences. His research has focused chiefly on reproductive biology, stem cells, and CRISPR Systems.

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