THE DIFFERENTIAL EFFECT OF USP7 ON BREAST CANCER CELL LINES

by
Taha Bartu Hayal

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APPROVED BY:

Prof. Dr. Fikrettin Şahin ……
(Thesis Supervisor)

Prof. Dr. Ahmet Arman ……

Prof. Dr. Dilek Telci ……

Prof. Dr. Ertuğrul Kılıç ……

Assist. Prof. Dr. Ayşegül Doğan ……

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ABSTRACT

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Breast cancer has the highest mortality rate among women compared to other various types of cancer. As a multi-stage and complicated cancer type, breast cancer might be initiated by various stimuli. Most of the breast cancer patients have been diagnosed with a familial predisposition due to the poor genetical heritage. Investigation of mutated proteins and affected pathways are bare essential to develop an effective cure for targeted therapy.

In the current study, potential anti-cancer activity of USP7 gene supression has been evaluated by *in vitro* cell proliferation analysis, the examination of alterations in gene and protein expression profile, three dimensional sphere formation capacity and colony forming unit assays. The small molecule inhibitor of USP7 enzyme in addition to small interfering RNA (siRNA) and lentiviral short hairpin vector (shRNA) has been used as targeting strategies against USP7. MCF7 and T47D human breast cancer cell lines, which maintain different P53 gene status, have been used to determine whether the anti-cancer effect of USP7 enzyme is p53 dependent or in-dependent.

Overall, a significant anticancer activity of USP7 inhibition has been detected *in vitro* for the breast cancer cell lines. Many molecular, physiological and morphological changes have been observed upon USP7 inactivation in all of the three different experimental strategy indicating the potential anti-cancer activity of USP7 blockage. USP7 inactivation inhibited the cancer cell proliferation, invasion and metastasis in MCF7 and T47D cell lines. However, further investigation should also be conducted with the purpose of comprehending the exact molecular mechanism which underlies in the total effect of USP7 on tumorigenic progression. This is the first study in literature which offers a critical examination of the differential effects of targeting USP7 enzyme by both at protein and gene levels in MCF7 and T47D cell lines.

ÖZET

SEVİYESİ DEĞİŞEN USP7 PROTEİNİNİN MEME KANSERİ HÜCRE HATLARI ÜZERİNE ETKİSİ

Dünya çapında bir çok ülkede, kadınlar arasında, kanser dolayısı ile olan ölümlerin önemli bir kısmını oluşturan meme kanseri, birçok farklı etmenden dolayı oluşabileceği gibi ailesel yatkınlık ve genetik mirasın da meme kanseri oluşumunda etkisi olduğu bilinen bir gerçektir. Özellikle hastalığa neden olabilecek proteinlerin belirlenmesi ve onlara özel gen terapi yöntemlerinin geliştirilmesi meme kanseri vakalarında tedavinin önündeki engelleri kaldırabilir.

Bu çalışmada; USP7 enziminin aktivetisinin engellenmesinin veya gen seviyesinde USP7 geninin anlatımının susturulmasının, meme kanseri üzerindeki etkileri; hücre canlılık testleri, koloni oluşturabilme kapasiteleri, detaylı moleküler mekanizmaların gen ve protein bazında incelenmesi ve üç boyutlu küresel yapılar oluşturabilme potansiyelleri üzerinden incelenmiştir. P53 geni mutasyonu bakımından farklılık gösteren MCF7 ve T47D hücreleri kullanılarak USP7 hedeflenmesinin antikanser aktivitesinin p53 bağımlı olup olmadığı incelenmiştir.

USP7 enziminin kanser gelişiminde rol oynadığı bütün moleküler mekanizmaların teşhisi çalışmaları devam ediyor olsa da; USP7 blokajının potansiyel antikanser aktivitesini gösteren üç farklı deneysel stratejide, USP7 inaktivasyonu sonucu birçok moleküler, fizyolojik ve morfolojik değişiklik gözlemlenmiştir. USP7 inaktivasyonun, MCF7 ve ,T47D hücre hatlarının proliferasyon, invazyon ve metastaz kapasitelerini düşürmüştür. Bu çalışma USP7 enziminin hem protein, hem de gen düzeyinde hedeflenmesinin MCF7 ve T47D hücreleri üzerindeki etkisinin karşılaştırıldığı ilk çalışmadır.

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AMPK	AMP-Activated protein kinase
Akt	Protein kinase B
ANOVA	Analysis of variance
BAX	Bcl-2-associated X protein
BCL2	B-cell lymphoma 2
BRCA1	Breast cancer type 1 susceptibility protein
BRCA2	Breast cancer type 2 susceptibility protein
CDKs	Cyclin-dependent kinases
CFU	Colony forming unit
CHK1	Checkpoint kinase 1
CSC	Cancer stem cell
DAXX	Death-domain associated protein
DUBs	Deubiquitination enzymes
EBV	Epstein-Barr virus
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ER	Estrogen receptor
FasR	Apoptosis antigen 1 receptor
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FOXO	Forkhead box protein
FOXO4	Forkhead box protein O4
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HAUSP	Herpesvirus-associated ubiquitin-specific protease
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HER2	Human epidermal growth factor receptor 2
HIF1	Hypoxia-inducible factor 1
HPV	Human papillomavirus
HTLV	Human T-lymphotropic virus

IBC	Inflammatory breast cancer
IKK	Inhibitor of kappa kinase
IκB	Inhibitor of kappa
JNK	c-Jun NH2-terminal kinase
KSHV	Kaposi's sarcoma-associated herpes-virus
MCF7	Michigan cancer foundation-7
MCV	Merkel cell polyomavirus
MDM2	Mouse double minute 2 homolog
MOI	Multiplicity of infection
MTS	3-(4,5-di-methyl-thiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4 sulfo-phenyl)-2H-tetrazolium
NF-κB	Nuclear factor kappa B
NLS	Nuclear localization signal
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PSA	Penicillin/Streptomycin/Amphotericin
PTEN	Phosphatase and tensin homolog
PTMs	Post-translational modifications
RASSF1	Ras association domain-containing protein 1
Rb	Retinoblastoma
ROS	Reactive oxygen species
TGF-β	Transforming growth factor beta 1
TLR	Tool like receptor
TNFR	Tumor necrosis factor receptor
TNFα	Tumor necrosis factor alpha
TSP-1	Thrombospondin 1
USP7	Ubiquitin-specific-processing protease 7
VEGF	Vascular endothelial growth factor
ZEB1	Zinc finger E-box binding homeobox 1

1. INTRODUCTION

1.1. CANCER

As a word, cancer is originated from Greek word; *karkinos* which can roughly means crap. Hippocrates (460–370 B.C) is the first person who used this term for the form of carcinoma tumors because of its vascularity which has the appearance of crap's feet [1]. First known evidence for breast cancer was found in mummies in ancient Egypt died in 1500 B.C [1]. As cancer has 3 thousand years of history, it is crucial to know the cancer well to find a cure.

Cancer can be described as an abnormal cell growth with the invasion and metastasis capacity. Uncontrolled cell proliferation and growth are main characteristics of all the cancer types. One of the main reasons for abnormal cell growth is the undifferentiated state of cancer cells [2]. Cancer is a complex disease with various features. Therefore, each cancer type has individual properties in terms of molecular and cellular mechanisms. Common features of all cancers were identified as hallmarks of cancer [3]. Originally, there were 6 main hallmarks of cancer which were stated by well-known publication of Hanahan and Weinberg [4]; cancer cells can express growth factors, cancer cells also immunize themselves against growth inhibitory signals, cancer cells can escape from apoptosis, cancer cells can divide multiple times, cancer cells can grow blood vessels for an energy source (angiogenesis), cancer cells can invade different tissues via blood vessels (metastasis).

After eleven years an updated version was published [5]. This updated article includes four more hallmarks which are; Irregular metabolic pathways, mutation and instable genome, avoiding the immune system, promoting tumor inflammation
ten hallmarks of cancer identifies the all properties of cancer and mortal consequences caused by cancer [5].

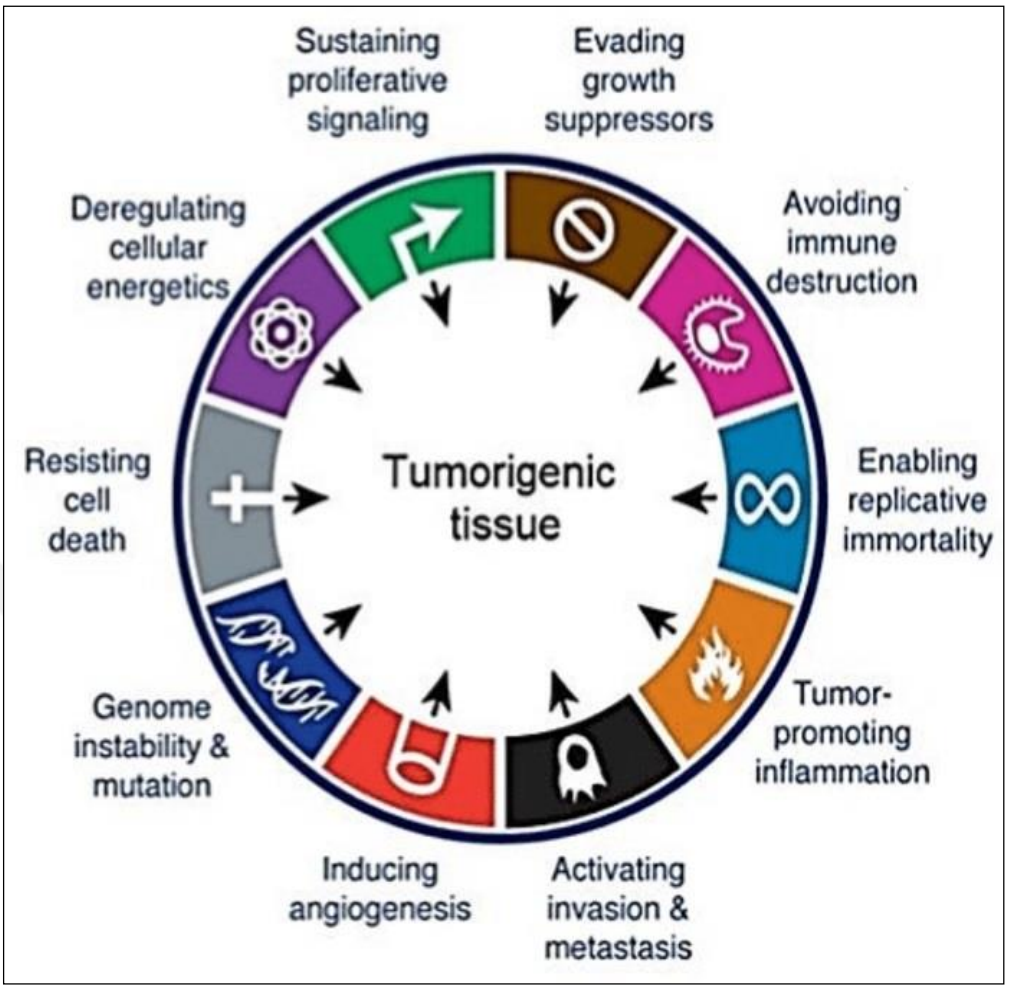


Figure 1.1. Hallmarks of cancer [5].

Proliferation and growth of the cells are controlled by production and secretion of signals that are called as growth promotion signals. Cell homeostasis and division depends on those signals in healthy cells [4, 5]. Change in growth signals which can be seen in multiple ways is the main reason for uncontrolled cell division. Cancer cells do not only obtain required growth signals *via* autocrine signals, but also have the ability to manipulate its environment to provide assorted growth factors from surrounding tissue [4-6]. For instance, tyrosine kinase overexpression increased growth factor receptors on the cancer cell surface which gives the ability to recognize more growth factors at once. Hence, cancer cells become more sensitive to small amounts of growth factors compared to healthy cells. Furthermore, downstream regulators of growth factor receptors are mutated to provide proliferative functions [4, 5]. Additionally, various anti-growth or anti-proliferative signals can be blocked successfully by cancer cell *via* several mechanisms. Besides increased growth stimuli, tumor suppressor genes which are guards of the cell cycle are dysregulated in cancer.

cancer cells can avoid programmed cell death as well [4, 5] and apoptosis pathway genes [7] might be changed in cancer cells. Although alterations in growth and metabolism related genes are fundamental for tumorigenesis, tumor cells are not able to form a tumor tissue that has endless division capacity or life time without changing the telomerase activity. Telomerase is responsible for regulation of life span [8]. Normally, telomerase activity decreases at each cell division because of telomere shortening. Decreased telomerase activity triggers many mechanisms which end up with cell senescence and eventually cell death. Cancer cells overcome the limitation of telomerase which is necessary for protection of neoplastic state and potential of replication [4, 5].

To maintain regular tissue growth and homeostasis, nutrient and oxygen balance is mandatory. Low oxygen levels in cancer cells are very common due to enhanced metabolic activity [9]. Accordingly, cancer cells need more oxygen to for proliferation. In order to overcome this problem, cancer cells provide oxygen with newly developed blood vessels by t angiogenesis. Adult vascularization begins during embryonic development and stay quiescent except extreme situations such as if no disease occurs such as wound healing, atherosclerosis, inflammation or cancer [5, 10]. There are two main factors for turning on the angiogenesis in adults such as expression of growth factors like VEGF (Vascular Endothelial Growth Factor) or FGF (Fibroblast Growth Factor) [4] and inhibition of inhibitor molecules such as (thrombospondin-1-TSP-1) [11, 12]. Healthy cells do not have the ability to migrate to the distant parts of the body. However, cancer cells have the ability to migrate from its origin to different parts of the body which is one of the main reasons for cancer caused deaths. Cancer cells easily migrate by using their invasive characteristic. Invasion procedure involves the migration through blood vessels. Cell to cell and cell to extracellular matrix interactions are also important for migration and cancer cells are usually manipulates its surrounding tissue. De-attachment from tissue is the first step of invasion. Dramatic decrease of E-cadherin and sharp increase of N-cadherin is commonly recognized during invasion. Both metastasis and invasion procedures begin with regional invasion to surrounding tissue by the action of various proteases. Once cancer cell reaches the blood or lymph (lymphoangiogenesis) system, it simply permeates to the new tissue with high oxygen or nutrient level [4, 5, 13]. Since cancer cells can do unlimited cell division, they need more energy compared to healthy cells. For that reason, regular energy metabolism is not enough for cancer cells and needs to be re-adjusted like increased glucose uptake or enhanced the

amount glycolysis to ensure the proliferative and replicative advantage [9]. Typically, immune system can detect and destroy cancer cells inside the body. However, cancer cells have the ability to manipulate immune responses and suppressed immune system cannot detect or hinder the cancer development. Therefore, it is crucial to explain deeply the new hallmark of cancer 'evading immune response' [5].

There are some common properties of cancer; however, the classification of the cancer is based on the differences of origin and localization. To diagnose or treat cancer, its stage and grade is used for classification as origin and histological location. If the cancer is originated from epithelial cells, it is called carcinomas. Mesenchymal originated cancers are called sarcoma. Glandular epithelium derived cancer types are classified as adenocarcinoma [2, 3, 14]. Since cancer is a multistage process that interacts with several intra or extra cellular elements, there are many possible factors which cause cancer. Cellular, metabolic and molecular level variations are main reasons for cancer development. Chemical carcinogens are the most widely studied reason of cancer. Carcinogens are the final form of the carcinogenic chemicals and they can react to several molecules except alkylating agents. Metabolic activation is followed by interaction with macromolecules to initiate carcinogenic action. DNA mutations at oncogenes and tumor suppressor genes are the main reason to show tumorigenic properties [15, 16]. For example, blocking the de-activation procedure of oncogene Ras and blocking the process that activates tumor suppressor gene p53 are commonly observed in cancer patient populations [14]. Besides, epigenetic changes like histone methylation and acetylation, may be the reason of oncogene activation and tumor suppressor genes expression suppression [14]. Carcinogenesis process originates from hypermethylated promoters of tumor suppressor genes many times [17]. This effect might be caused by chemical agents as well as UV radiation. DNA damage triggers the repair mechanism which usually causes mutations that lead to tumorigenesis [14, 18, 19]. Since the cells are oxygen dependent for almost any metabolic activity, reactive oxygen species (ROS) which are correlated with aging, can be formed easily. ROS can interact with different molecular metabolism steps thanks to its remarkable reaction affinity than normal oxygen. This interaction capacity of ROS causes DNA and protein damage as well as membrane distortion leading to carcinogenesis [14, 20].

It has been demonstrated that, viral infection can also cause cancer in humans since viruses have the ability to affect host's genome. In addition, viruses can cause cancer indirectly *via*

changing cellular machinery such as proliferative phase [14]. EBV (Epstein-Barr virus), HPV (Human papilloma viruses), KSHV (Kaposi's sarcoma-associated herpes-virus), HBV (Hepatitis B virus), HCV (Hepatitis C virus), HTLV (Human T-lymphotropic virus) and MCV (Merkel cell polyomavirus) are the seven identified viruses that can cause cancer [21].

In summary, cancer as a complex and multiple staged diseases can be summarized as accumulation of mutations, unsuccessful DNA damage repair mechanisms and gain malignant phenotype. To acquire rapid proliferation phenotype, acquisitions of new properties such as genetic instability or cellular alterations are evolved [14, 22]. Malignant phenotype acquisition is mainly controlled by some biochemical acts on the cells. The process which leads to differentiation of the healthy cell into malignant cell is called transformation. Several paracrine or autocrine growth factors control the malignant transformation process. Paracrine and/or autocrine secretions are responsible for proliferation of the cell along with aggressiveness of tumorigenic tissue. Mitosis (active cell division) is also controlled by growth factors in two different ways: either division or remaining at quiescent state [23]. CDKs (cyclin-dependent protein kinases) and their inhibitors regulate the transition from G1 phase into S phase of the cell cycle in eukaryotic organisms. Besides oncogenes, tumor suppressor genes as retinoblastoma (Rb) and p53 are equally important in terms of cancer development. In many cancers, Rb and/or p53 mutations are detected. Rb gene is a key regulatory protein for cell cycle check point control. Similarly, p53 is also required to cell cycle phase transmissions. Moreover, p53 protein is an essential protein to repair DNA damages. There are several studies that describe p53 protein as the guardian of the genome [24-26]. P53 and/or Rb mutations break down the communication between Rb and p53 which is crucial to decide cell cycle arrest or going through apoptosis after DNA damage [27].

1.1.1. Tumor Suppressor Genes and Oncogenes

Alterations in genetics, physiology and biochemical compounds accumulate to cause cancer as it can be described as a complex and multistep process [28]. When the oncogenic growth has been taken into consideration, the genes can be categorized as two main subgroups. Healthy cell proliferation depends on the perfect harmony between oncogenes and tumor suppressor genes. However, in order to keep cancer cells' dividing potential at maximum,

oncogenic gene activation is required as well as suppression of tumor suppressor genes [29]. The hypothesis of somatic mutation speculates that specific mutations at genes trigger carcinogenesis [30]. In any way (such as insertion, invasion, duplication and so on) of mutations at oncogenic genes, might activate the oncogenic genes while other mutations might inactivate tumor suppressor genes [31]. Cells tend to give a direct or indirect response to growth stimuli from the environment. Usually, growth stimuli initiate a cascade that is formed out of oncogenic proteins. In any case, activated oncogenic protein keeps the cascade activate which is responsible for limitless growth signal with or without growth stimuli [29]. For instance, many of the human tumors have mutated Ras oncogene. Normally, growth stimuli bind the membrane protein to activate Ras protein but if the Ras gene has a mutation that keeps Ras protein active at any moment, without the growth stimulation the cell proliferates constantly [32].

Tumor suppressor genes also play critical role in carcinogenesis since they are responsible for cell growth regulation. Unsurprisingly, the mutations at the tumor suppressor genes which cause functional loss are also provoke tumorigenesis. Two-hit hypothesis speculates that, there should be mutation at both alleles of tumor suppressor genes [33]. Mutations at one allele of a tumor suppressor gene (haploinsufficiency) can be tolerated because remaining allele is functional [34]. Tumor suppressor genes are the gatekeepers and the caretakers of the cell [35]. Protection, prevention and repair of DNA mutations are related to caretakers' responsibility. Moreover, gatekeepers are responsible for induced apoptosis and initiation of the cellular senescence [36]. Best-known gatekeepers are retinoblastoma (RB) and TP53 [37] which can affect other tumor suppressor proteins' pathways. In many cancer type, modified Rb and p53 pathways can be seen [31].

TP53 is the one of the most studied tumor suppressor gene and its protein product, p53 protein, acts as a transcription factor that simply decides if the cell should go to programmed cell death or not [38-40]. Additionally, it also plays crucial role in DNA synthesis, apoptosis and DNA repair mechanism [41]. Unsurprisingly, p53 mutations are very common among many cancer types during almost in every stages of carcinogenesis [42]. Another well-known tumor suppressor, Rb is the main cause of childhood tumor, retinoblastoma [43]. Retinoblastoma has two forms; hereditary or sporadic. In order to develop cancer caused by Rb protein, both of the alleles should be mutated [29]. Notwithstanding, the Rb gene mutations is not only responsible in retinoblastoma, but also play role in many other cancers

as well. Rb protein is key modulator in cell cycle, differentiation and cell survival. Furthermore, Rb controls many pathways that differ in the carcinogenesis of various cell types. Rb protein is crucial for many cancer types because it regulates several pathways including cellular growth [44].

"Stability genes" are also play a critical role in tumorigenesis. This third class of genes are involved in DNA repair mechanisms and consequences of DNA damage [31, 45]. Most likely, cells try to avoid the effects of mutations in tumor suppressor genes by increasing growth inhibitory signals such as transforming growth factor –β (TGF-β) [29]. Even in low concentrations, TGF-β down-regulates cell growth [46]. However, mutated Rb may cause the defected response of TGF-β. Loss of TGF-β response ends up with continuous cell growth [47].

1.1.2. Cancer Initiation, Promotion and Progression

As cancer progression can be described as a multistage process, tumorigenic initiation and progression processes can be categorized in three steps which are initiation, promotion and progression [14, 48]. Agents that have initiating and promoting activity are required for initiation and promotion of cancers [14]. Progression phase can be characterized by various genetic alterations and genomic instability which are translocations on chromosomes and mutations [28]. Genetic changes induce invasive and metastasis capacity as well as aggressive cellular growth characteristics in cancer cells [14]. Unlikely, carcinogenesis promotion is not slow as initiation phase because of the long agent exposure time [14]. Promotion phase is the step where cell proliferation takes place and tumor formation occurs when clonal cells enters the progression phase caused by a damage. Molecular pathways and the exact mechanisms behind the carcinogenesis are complicated and can be categorized under three steps [49]. Direct (several mutations) or indirect (epigenetic changes) changes in DNA trigger the initiation phase [50]. The main difference is the time point when the molecular events take place. Although initiation and progression phases depend on various genetic alterations, promotion phase does not require genetic changes. During the promotion phase, several situations (wound formation, inflammation or changes in environment) called promoting agents, stimulate tumorigenesis [14, 51]. Briefly, promotion phase can be described as cell proliferation step during carcinogenesis. The ultimate step of all phases

includes differentiation, invasion and metastasis [14]. In the last stage, cells that have proliferative capacity adapt to harsh conditions such as low oxygen levels or low pH and gain the ability to invade to different tissues [52]. Limitation of increased cell proliferation and cellular growth is low oxygen levels by abated blood supply [50]. As a result, cells should migrate into other tissues in order to accomplish tumor formation. Mature tumors have the invasion capacity with high mortality rate.

1.1.3. Cancer Metabolism

As metabolic activity determines the fate of a cell in terms of cell cycle regulation, cellular growth, cell death or energy consume, cancer metabolism has been the main target to find a cure. Like all of the cells in the body cancer cells use glucose as an energy source, under regular oxygen levels. It is known that cancer cells need more oxygen than healthy cells since cancer cells' dividing potential is limitless. Moreover cancer cells can inhibit the cell cycle arrest mechanisms or can invade into other tissues [53]. In order to produce more ATP, cancer cells uses glycolysis (oxygen dependent) together with lactic acid fermentation. The process of combining two different energy sources is called Warburg effect [53, 54] Although exact mechanism behind the metabolic changes has been enlightened [9], there are several discovered molecular pathways. For instance, increased glucose uptake is associated with protein kinase B (Akt) protein which is phosphorylated (and activated) by phosphoinositide 3-kinase (PI3K) [53, 55]. Homeostasis and cellular growth are complex metabolic activities that are controlled by various pathways like lipid biosynthesis [56]. Correlation between lipid biosynthesis and cancer has not been described completely. However, it is known that biosynthesis of lipids is an obligation for cellular growth and structural stability. Since lipid production is key regulator for cellular growth, Akt protein plays a major role in cellular growth rather than the glycolytic pathway [57]. Hypoxia inducible factor (HIF1) which is another protein involved in downstream of PI3K pathway, is also associated with glucose metabolism by improving the glucose transportation and conversion [58]. Opposite to healthy cells which increase HIF1 levels under low oxygen conditions, cancer cells can increase HIF1 levels independent of oxygen levels to increase the oxygen metabolism [53]. AMPK (AMP-activated protein kinase) is responsible for blockage of cellular proliferation when energy stress is observed. The blockage of proliferation by HIF1 is correlated with oxidative metabolism [59]. Besides its role in DNA

damage response and apoptosis, p53 can also be defined as glucose metabolism related protein in cancer cells. P53 activation is important for synthesis of several enzymes that are involved in glycolic pathways. Thus, glycolytic phenotype is also related with p53 suppression [53].

1.1.4. Cancer Diagnosis

Diagnosis of cancer is crucial for several patients all around the world. In order to find proper treatment, identification of a tumor at the correct stage and time is required. Although cancer cells differentiate from healthy cells, they differ in many fields such as metabolic activities, genetic codes, phenotypic perspective or biological content. Identification of cancer by epigenetic and genomic changes as well as biomarkers and elector-magnetic systems [14] is important for developing the cure. Markers, based upon DNA mutations, hypermethylation or detection of viral DNA, are called DNA based markers. On the other hand, several enzymatic activity analysis and microarrays, antibody based methods, are also used for cancer identification [60]. Earliest techniques for cancer determination are based on the microscopic observations. Even though, microscopic analysis is not reliable, it is important to estimate the stages of cancer. For diagnosis of numerous cancer types, serum markers are used as they are highly specific. However, more than one serum marker should be used to diagnose cancer [61].

DNA microarrays aim to develop more specific markers for cancer diagnosis purposes. DNA microarrays are used to develop patient based treatment as all cancer patients have different gene expression levels and DNA mutations [62]. Histological analysis and serum markers can be described as traditional diagnosis methods which should be supported with more sensitive advanced molecular approaches [60].

1.1.5. Cancer Epidemiology

As a worldwide disease, cancer incidence varies according to the daily habits (alcohol consume, smoking, diet etc.), country, population and the sex. Numerous factors can affect the epidemiology of cancer. Exposure to tumorigenic agents as environmental factors, genetic factors that affect the aggressiveness of a tumor, genetic inclination and age (as in

prostate cancer [14]) can determine the prevalence of cancer. The epidemiology of cancer changes due to the sexuality as well. While, the lung cancer is the most popular type in men, the breast cancer is mostly seen in women [63]. Moreover, stomach, uterine and cervix cancers are the most observed cancers in developing countries but prostate and colorectal cancers are commonly seen in developed countries. Epidemiology analysis is hard in some cancers that has unknown origin. Though, there are various reasons, it is generally highlighted that lung cancer results from smoking [64]. In order to find the way to prevent from cancer, epidemiologic analysis is a must to determine the risk factors that are correlated with cancer initiation.

1.2. BREAST CANCER

Tumors in breast tissue appear when breast cells start to divide abnormally. These abnormal cells form a tumor which can be observed on an x-ray. If the abnormal cells have invasive or metastatic capacity to further areas of the body, the tumor is called cancerous (malignant). Almost every women and certain amount of men are under risk of breast cancer [65, 66].

Breast cancer has two main origins in the breast. Usually breast cancers start in the ducts which transfer the milk to the nipples. If the cancer starts in the ducts, it is called ductal cancers [67, 68]. Breast cancers can also initiate in glands which produce milk. If the cancer starts in the glands, it is called lobular cancers [68]. Less commonly, there are more types of breast cancer;

Inflammatory breast cancer (IBC): Instead of the single tumor, IBC patient's breast (or breasts) is warmer and looking red. As a consequence of having no actual lump or tumor, IBC will not be observed in mammogram. This makes IBC hard to diagnose at early stages. IBC has more metastatic capacity then ductal or lobular carcinomas [69].

Paget disease of the nipple: Originally starts from breast ducts and expand to the nipple skin and areola. Nipple and areola areas usually bleed because of the cancer and patient will feel burning and itching before bleeding stage [70]. Mastectomy will be required to treat paget disease.

Phyllodes tumor: Origins from connective tissue (stroma) of the breast. This kind of tumor is usually benign but in certain cases it can be malignant. Malignant kind of

phyllodes tumor has metastatic capacity and usually can be treated with chemotherapy [71].

Angiosarcoma: Origins from lymph vessels or blood vessels. Angiosarcoma usually forms as a result of radiotherapy for other tumors at breast. It could be seen 5 to 10 years after radiotherapy [72].

The most terrifying property of breast cancer is its ability to metastasis to lymph system [73] which includes lymph nodes, vessels and lymph fluid that flows through whole body like small veins. Instead of carrying blood, lymph vessels carry immune system cells, waste products and tissue fluid [74].

According the statistics of American Cancer Society in United States for 2018; In upcoming year 268,670 new patients will be added to invasive breast cancer patients' statistics. Moreover, 63,960 new patients will be added to carcinoma in situ (CIS) patients' statistics and approximately 40,920 women will die because of breast cancer [75].

Ubiquitination is the main mediator of post-translational modifications of proteins that control major breast cancer pathways. Although the previous studies mainly focused on phosphorylation events which is another mediator of post-translational modifications, recent studies prove that ubiquitination has equivalent part in various signaling and cell regulatory pathways in breast cancer [76].

1.2.1. Molecular Biology of Breast Cancer

As malignant cancers, breast cancer is seen as a result of multistep process which affects many different cellular pathways. Identification of molecular mechanisms that are responsible for breast cancer development is important in order to develop more sensitive treatments.

1.2.1.1. BRCA1 and BRCA2

Although most of the breast cancer cases are sporadic, 5-10 per cent of breast cancer cases are caused by familial heritage. Numerous genes are involved in breast cancer development however, *BRCA1* (breast cancer 1) and *BRCA2* (breast cancer 2) genes are found to be

responsible for familial breast cancer inclination. *BRCA1* gene is located on 17q21 and *BRCA2* gene is located on 13q12. Both of them play a key role in regulation of cellular proliferation and transcription, DNA damage response and regulation in cell cycle [77, 78]. Mutations that inhibit BRCA1 or BRCA2 proteins' functions provoke tumorigenic development.

1.2.1.2. Epidermal Growth Factor Receptor (EGFR)

The epidermal growth factor receptor family members, EGFR, HER2, HER3 and HER4, have tyrosine kinase activity. EGF receptors have the ability to form heterodimers and initiate the cascade of many other cellular pathways, after the activation *via* external stimuli. The cascade which triggered by epidermal growth factor binding to its receptor, includes many oncoproteins such as Ras, Raf and MAPK [79]. Any oncogenic mutations during this cascade, stimulates uncontrolled dividing. In the half of breast cancer patients, overexpression of epidermal growth factor receptor is noticed [80]. Overexpression of EGFR members is also correlated with poor prognosis [81]. For instance, HER2 mutation is observed among 20-25 per cent of breast cancer patients [80].

1.2.1.3. CerbB-2 (HER2/Neu)

CerrB-2, also known as HER2/Neu, is located on 17q12 and involved in cell proliferation [82] and differentiation [83]. 15-30 per cent of breast cancer patients overexpress HER2 gene that is resulting in around hundred-fold increase in HER2 protein [84]. Abnormalities in HER2 gene transcription is correlated with younger diagnosis and decreased life expectation. Moreover, HER2 overexpression is associated with amount of the axillary nodes, morphological differences and the missing ER (estrogen receptor) and PgR (progesterone receptor). In nearly half of *in situ* ductal carcinomas HER2 overexpression is observed [85]. Although, cancers that have high amount of HER2 is highly sensitive to various chemotherapeutic drugs, they grow a resistance to hormone therapy and increased chance of metastasis to brain is observed [86].

1.2.1.4. c-Myc

c-Myc is, 8q24 located proto-oncogene, observed to be mutated in 1/3 of breast cancer patients [87]. Most of the breast patients overexpress the c-Myc gene. Moreover, increased transcription and translation of MYC gene is estimated around 30-50 per cent of breast cancer patients depending on the grade of a tumor. Estrogen treatment was observed to be effective to decrease c-Myc levels in the cell [88] likewise; anti-estrogen drugs cause overexpression of c-Myc [89]. It is also observed *via* clinical studies that increase in c-Myc protein level is correlated with the transformation of tumor from *in situ* to invasive carcinoma [90], together with increased aggressiveness [91, 92] and less life expectation [91, 93-96].

1.2.1.5. Ras

Ras proteins are classified as members of G protein family that has the ability to bind guanine nucleotide [97]. Ras superfamily members (H, K, N and M-Ras) are GTP dependent proteins and show GTPase activity. Ras proteins are activated by extracellular stimuli [98]. Ras protein is activated *via* the initiation of EGF to its receptor EGFR. Ras protein activation is correlated with cellular proliferation [99]. Various mutation on the Ras gene cause inhibition of de-activating mechanism of Ras [100]. Especially, K-Ras is observed to be mutated, as a gain of function mutation, in 30 per cent of all cancers in humans [101]. Ras overexpression is the most common type of mutation among breast cancers. Overexpression of H-Ras is observed in lymph node metastasis and in advanced histological phenotype of the cancer [102].

1.2.1.6. p53

As a tumor suppressor, P53 gene plays vital roles in DNA damage, DNA repair, cell cycle regulation and apoptosis [103]. Rossner P *et al*. demonstrated that, 15 per cent of breast cancer patients have p53 mutations. Moreover, 39 per cent of breast cancer patients overexpress p53 protein. Additionally, not the p53 overexpression profile, but any missense or other mutations in P53 gene is correlated with the deaths from breast cancer [104]. p53

mutations are correlated not only with breast cancer [105], but also with many cancers such as colon cancer [106], myeloma [107] and lymphoma [108]. p53 mutations are found to be highly correlated with the aggressiveness of a tumor [109, 110].

1.2.1.7. Ataxia telangiectasia mutated (ATM)

Mutated ATM protein like all tumor suppressor genes, both alleles should be mutated for ATM caused cancer. However, the women that have heterozygote mutation of ATM gene, have increased risk for breast cancer [111]. ATM protein is known to be critical protein for p53 activation after DNA damage. As a serine/threonine kinase, ATM activates p53 by phosphorylation of its feedback mechanism protein mdm2. Phosphorylation of mdm2 triggers self-ubiquitination property of mdm2 protein which locates p53 into nucleus as a transcription factor of many other tumor suppression genes [112]. USP7 can regulate this process [113, 114] in almost every step. Since ubiquitination is very important regulatory mechanism in homeostasis and tumorigenesis, it is crucial to learn more about ubiquitination and de-ubiquitination mechanism in tumor progression.

1.3. UBIQUITINATION

Ubiquitination can be described as; marking proteins with ubiquitin signal take proteins into degradation via proteasomal activity. Ubiquitination of proteins controls various cellular mechanisms such as, cell cycle progression [115, 116],apoptosis [117, 118] and regulation of transcription factors [119].

E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzymes and E3 ubiquitin–protein ligases work together to start ubiquitination of proteins [120]. E1 enzyme gathers ubiquitin proteins with the energy from ATP in order to activate ubiquitin. Active ubiquitin interacts with E2 enzyme. E3 ubiquitin-protein ligases work as 'docking proteins'. E3s bind to substrate and specific E2s and close the gap between substrate and E2 ubiquitin-conjugating enzymes which results in ubiquitin transferred from E2s to substrate [121]. Ubiquitinated substrate goes either degradation via proteasomal activity or deubiquitination by deubiquitinating enzymes such as USP7. Deubiquitination restores substrate and it can function in its natural state. Ubiquitination process usually leads to degradation of substrate

following the polyubiquitination process. Whereas, monoubiquition switches off the target substrate (Figure 1.2).

1.3.1. HAUSP as A Deubiquitinating Enzyme

Since ubiquitination is crucial each molecular mechanism for every eukaryotic cell [122, 123], it is clear that de-ubiquitination regulates every pathway in the cell. USP7 which is a member of Ubiquitin Specific Protease (USP) deubiquitination family is a unique example for the relevant regulation [124]. USP7 is found to be interacted with many cellular proteins [125]. USP7- protein interactions are occurred via deubiquitination ability of USP7. Thus USP7 recycles associated proteins and prevents them from proteasomal degradation. Because of the critical role of USP7, it should be regulated anxiously. The regulation of USP7 starts with its C-terminal domain since its activity depends on this domain. Another inhibition strategy of USP7 is blocking its interaction with targeted proteins. P5091 (Figure 1.3) which is an inhibitor of USP7 is one of the most studied chemical inhibitor of USP7. P5091 is shown to inhibit USP7 specifically and even can overcome the drug resistance against bortezomib in multiple myeloma [126]. Regulation of USP7 maintains its importance as knock down USP7 in mice is found to be deadly [127, 128].

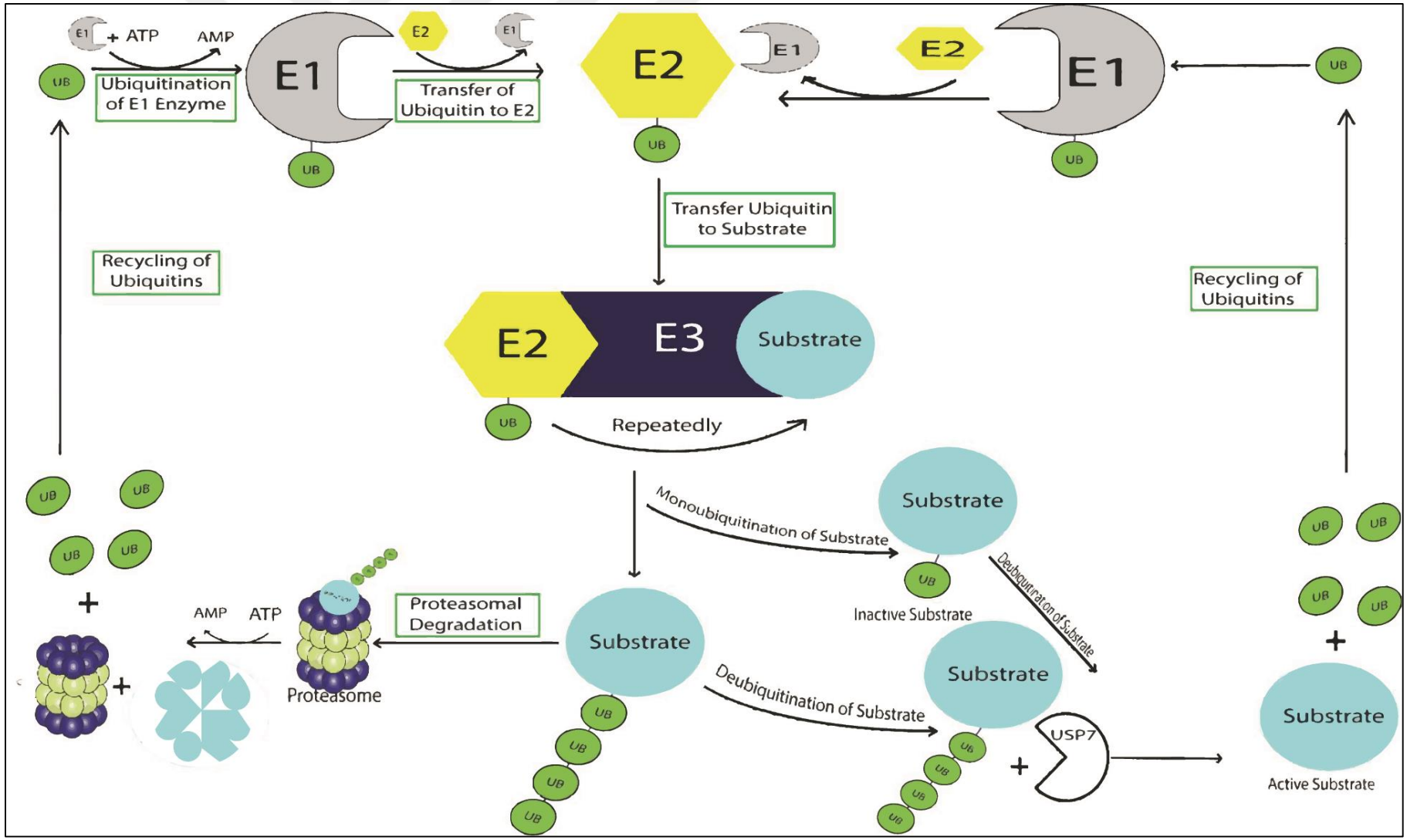


Figure 1.2. Schematical representation of ubiquitination pathway.

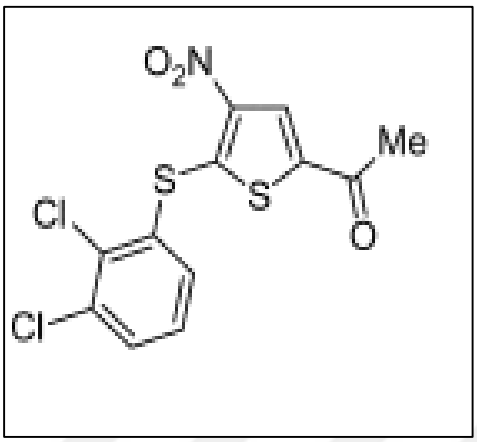


Figure 1.3. P5091 as a structural perspective [129].

USP7 has been shown to interrupt with many different pathways [130] such as, viral interactions, immune response, tumor suppression and DNA damage response.

1.3.2. Viral

It has been shown that, Herpes virus-associated ubiquitin-specific protease (HAUSP) correlates with proteins from *Herpesviridae* virus family's all three subfamilies (*Alphaherpesvirinae, Betaherpesvirinae and Gammaherpesvirinae*) [131, 132]. The interaction between ICP0 (Human Herpes Virus Infected Cell Polypeptide 0) and HAUSP is well established [133]. ICP0 is Herpes Simplex Virus-1's (HSV-1) early protein which is required for efficient lytic infection and reactivation of latent and quiescent viral genomes [134]. USP7 also interacts with Kaposi's sarcoma-associated herpes virus (KSHV) proteins, LANA (latency-associated nuclear antigen) and vIRF4 (viral interferon regulatory factor 4) [135, 136]. LANA protein is one of the most important protein for KSHV. This protein expressed in all KSHV related malignancies [135, 136]. Epstein-Barr virus (EBV) protein EBNA is also target protein for deubiquitinase USP7. EBNA protein competitively binds to USP7 with p53. Because of the competition, EBV infection shuts down process of DNA damage response mechanism by destabilization of p53 protein [137-139]. USP7 interacts with cytomegalovirus's protein UL35 that plays critical role in viral replication and particle formation [140]. Besides Herpesvirus targets, it has been shown that USP7 gets in touch with at least one adenoviral protein called, E1B-55k. E1B-55k forms a complex with E4-orf6. The E1B-55k/E4-orf6 complex in infected cells tacks together with

cellular proteins in order to form ubiquitin ligase, E3 [141]. Inhibition or downregulation of USP7 makes unstable E1B-55k and inhibits viral replication [142, 143].

In summary, three out of seven known oncoviruses (HSV-1, KSHV and EBV) are correlated with USP7 protein so far [144]. Therefore, studies to elucidate the mechanism of USP7 on oncoviruses have immense importance.

1.3.3. Immune Response

Immune response can be described as a reaction of a cell to threats such as virus or bacteria. NF-κB is a master regulator of immune response pathway. NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a transcriptional regulatory protein that plays critical role in inflammation progression and immune response [145, 146]. IκB (inhibitor of kappa) kinase (IKK) complex activates NF-κB in cytoplasm. Active NF-κB is translocates into nucleus. Limitation of NF-κB transcriptional activity include proteasomal degradation control that induced by ubiquitination in the nucleus. This ubiquitin mediated degradation is DNA binding dependent. Colleran *et al*. have shown that USP7 regulates NF-κB by deubiquitination [145]. Therefore, USP7 recycles transcription factor, NF-κB, in order to regulate gene expression. It has been observed that TNFR (TNFα (Tumor necrosis factor alpha) receptor) mediated gene expression is also controlled by USP7. Furthermore, inhibition of USP7 pharmacologically reduces inflammatory gene expression.

Tool like receptor (TLR) is the main receptor for recognizing any antigens in the human body. TLR is also called as “pattern-recognition receptors” [147]. The correlation between USP7 and ICP0, EBNA1 and HSV1 leads to induced nuclear exportation of USP7 after viral infection [148]. ICP0 protein is responsible for inhibition of TLR-mediated NF-κB response in infected cells by TRAF6 and IKKγ deubiquitination. Interaction between ICP0 and USP7 leads ICPO to work in inhibition when nuclear localization signal (nls) is functioning. In the case of HSV infection the role of USP7 to regulate TLR response is displaced by ICP0 to abolish immune response [148]. For all those reasons, correlation between NF-κB and USP7 might be a novel therapeutic target [145].

1.3.4. Tumor Suppression

There are various proteins that can protect cells from tumorigenesis called tumor suppressor proteins or anti oncoproteins [149]. USP7 interacts with some of them to rescue from ubiquitination.

Interestingly, USP7 also regulates tumor suppressor proteins' levels by deubiquitination of oncoproteins, which controls tumor suppressor proteins' amount in the cell. Studies have shown that USP7 correlates with death-domain associated protein (DAXX) in order to protect (Murine Double Minute 2) MDM2 from self-ubiquitination. This protection improves the activity of MDM2 on p53 which leads to p53 degradation [150]. MDM2 protein, also known as E3 ubiquitin-protein ligase MDM2, inhibits transcriptional activation of p53 and as an E3 ubiquitin-protein ligase ubiquitinates itself and p53 to proteasomal degradation [151, 152]. Ras association domain-containing protein 1 (RASSF1) is a tumor suppressor protein that is responsible for self-ubiquitination of Mdm2. RASSF1 disturbs Mdm2-DAXX-USP7 complex which leads to self-ubiquitination [153]. DAXX protein is a regulatory protein of apoptosis which connects death receptor Fas (FasR) with c-Jun NH2-terminal kinase (JNK) [154]. It is widely considered that USP7 rescues p53 from proteasomal degradation due to ubiquitination [114]. Nevertheless, decreased level of USP7 causes upregulation of p53 and p53-dependent G1 arrest. In addition, respective alteration causes in a reduction in Mdm2 level which also means that USP7 has critical role in regulating the stability of Mdm2 [113, 155, 156]. USP can directly interact with DAXX and this interaction is independent from p53/tp53. Furthermore, DAXX-USP7 interaction is also independent from MDM2 [157] (Figure 1.3).

PTEN (Phosphatase and Tensin homologue deleted on chromosome 10) is a vital regulatory protein in oncogenic PI3K/AKT pathway [158, 159]. PI3K/AKT pathway promotes cell proliferation and PTEN ceases this pathway at the earliest step. PTEN is normally functional in the cytoplasm. However, several studies remarked that the more nuclear-exclusion phenotype of PTEN expression, the more aggressive cancer is observed [160-165]. Similarly, mutations at PTEN's ubiquitination sites (such as Lysine 289) damages nuclear export of PTEN that causes increased tumorigenic behavior [160]. Those findings indicate that PTEN localization can be controlled by ubiquitination. Song M.S. *et al.* showed that

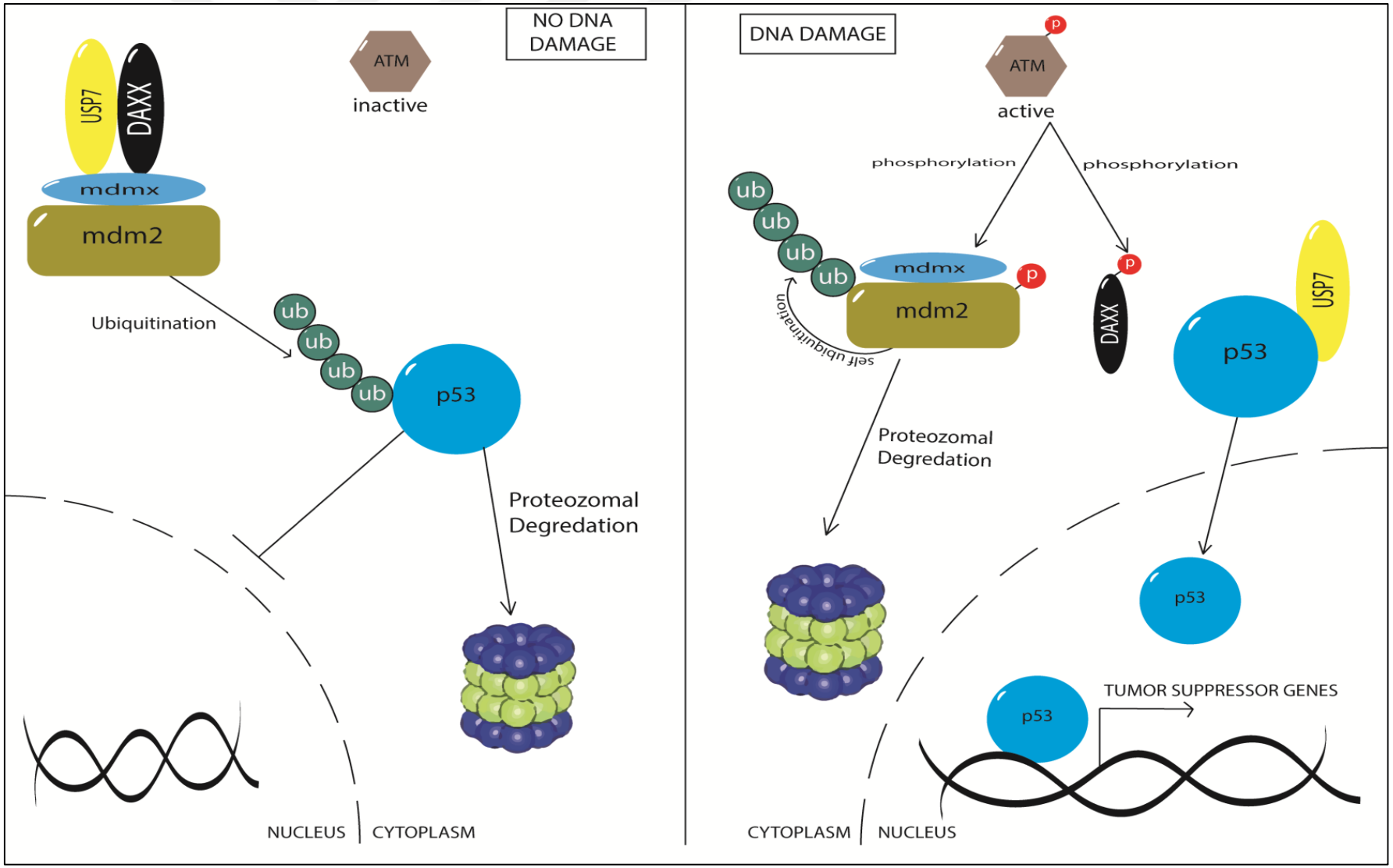


Figure 1.4. Schematical representation of p53/mdm2 and USP correlation pathway.

monoubiquitination of PTEN, mostly stays in the nucleus correlated with great apoptotic potential [166]. Forkhead box O (FOXO) transcription factors are involved in regulation of critical pathways such as apoptosis and regulation of cell cycle [167]. It is known that FOXO regulated by reactive oxygen species (ROS) and it has been shown that increasing cellular oxidative stress causes nuclear translocation of FOXO [168, 169]. FOXO proteins mostly regulated by post-translational modifications (PTMs) such as acetylation, phosphorylation and ubiquitination [170]. Protein Kinase B (PKB) mediated phosphorylation causes discharging of FOXO from nucleus. When oxidative stress in the cell increased, FOXO is induced to be nuclear. This relocalization is controlled by post-transcriptional modifications. Only limitation of this modifications is that PTM enzymes are extraordinarily similar between p53 and FOXO. In addition, both p53 and FOXO4 are deubiquitinated by USP7 protein [114, 171]. Furthermore, in the condition of elevated cellular oxidative stress, FOXO4 is monoubiquitinated by Mdm2 through acting as E3 ligase [172].

1.3.5. DNA Damage Response

In human body every ~1013 cells go through thousands of damages in their DNA every day [173]. Those damages can block expression of the genes responsible for transcription or replication. Repairing damages can reactivate those genes. However, incorrect repairs lead to irreversible mutations.

p53 is one of the major proteins in DNA damage response pathway. It coordinates repair process with DNA replication to determine undergoing apoptosis or not [174, 175]. As discussed before USP7 controls p53 or Mdm2 widely. Moreover, regulation of USP7 is a key to DNA damage signal transmission. Stabilizing Mdm2 and similarly downregulation of p53 in healthy cells, is performed by an isoform of USP7 (USP7S) that is phosphorylated by protein kinase CK2 at 18th serine. In stress condition (for instance ionizing radiation) ATM-dependent protein phosphatase PPM1G dephosphorylates USP7S which causes decrease in Mdm2 level and increase in level of p53. ATM (Ataxia telangiectasia mutated) protein is a serine/threonine protein kinase which is triggered by double-strand DNA damages. This protein kinase phosphorylates considerable amount of proteins and this phosphorylation activates different pathway proteins such as p53 and BRCA1 (breast cancer type 1 susceptibility protein). Those pathways also correlated with DNA damage response [176].

EMT (Epithelial-mesenchymal transition) is related with aggressive behaviors of breast cancer stem cells, such as resistance to chemotherapy and radiotherapy [177, 178]. Zinc finger E-box binding homeobox 1 (ZEB1) is a transcription factor which induces EMT that works as a regulatory mechanism of DNA damage response [179]. In breast cancer cells, ionizing radiation causes the hyper activation of ATM and that increases amount of ZEB1. ZEB1 is phosphorylated by ATM kinase in DNA damage response and phosphorylated ZEB1 directly cooperates with USP7 in order to deubiquitinate CHK1 (Checkpoint Kinase 1). Stabilized CHK1 regulates radioresistance and DNA repair after deubiquitination.

1.4. AIM OF THE STUDY

USP7 has a complicated and multi-staged mechanism of action in terms of cellular regulation. Although there are limited number of studies in the literature, underlying molecular mechanism and affected pathways are not well known. The aim of this study is to establish novel approach to use USP7 as a cancer therapeutic agent. Changing the levels of USP7 in order to find a novel pathway behind the mechanism of deubiquitination in cancer progression will be promising for future gene therapy applications. The purpose of the current study is to discover the effects of USP7 regulation; in p53 mutated, T47D, cell line and p53 wild type, MCF7, cell line as an *in vitro* model system for breast cancer.

2. MATERIALS AND METHOD

2.1. CELL LINES

T47D (HTB-133, human breast cancer cell line) and MCF7 (HTB-22, human breast cancer cell line) cell lines provided from American Type Culture Collection (ATCC, Rockville, MD). T47D cells were incubated in Roswell Park Memorial Institute medium (RPMI, #11875093, Invitrogen, Gibco, UK). On the other hand, MCF7 cell line was incubated in Dulbecco's Modified Eagle's Medium (DMEM, #41966-029, Invitrogen, Gibco, UK). Both of the mediums were supplemented with 10 per cent fetal bovine serum (FBS, #10500-064, Invitrogen, Gibco, UK) and 1 per cent Penicillin/Streptomycin/Amphotericin (PSA, Invitrogen, Gibco, UK). Humidified incubator's conditions were 37 °C and 5 per cent CO_2. For passage and/or seeding, cells were detached from the surface using 0.25 per cent trypsin-EDTA (#25200-056, Invitrogen, Gibco, UK). Also cells remained proper confluence during experimental growths (≈80 per cent).

2.2. SMALL MOLECULE INHIBITOR PREPARATION

DUB incubator, (p5091, #S7132, Selleckchem, TX) was prepared in dimethyl sulfoxide (DMSO, #472301, Sigma-Aldrich, Germany) as described in elsewhere [180]. To obtain 10 mM of stock concentration, 1 mg of p5091 was dissolved in 287.2 µl of DMSO.

2.3. siUSP7 TRANSFECTION

USP7 specific small interfering RNA was purchased from ThermoFisher Scientific (#AM16708, siRNA ID:105065) (Figure 2.1). Transfection protocol was performed with using Lipofectamine® RNAiMAX (#13778150, ThermoFisher, Invitrogen, CA) as literature [181, 182]. Briefly, cells were seeded onto 12 well plates prior to transfection in 80 per cent confluence (5 x 10^4 cells/well). Overnight incubation was followed by preparation of transfection components. siRNa's (Control siRNA (#AM4611, ThermoFisher Scientific, CA) or siUSP7) and transfection reagent groups were prepared separately. In every group,

Opti-MEM (#31985062, ThermoFisher, Gibco, CA) was used in order to reduce possible inhibition of transfection caused by antibiotics and/or serum. 75 µl of Opti-MEM medium was mixed by RNAiMAX and another 75 µl of Opti-MEM was mixed with desired siRNA. After 5 minutes of incubation at room temperature, the groups were mixed together to incubate another 20 minutes in room temperature. Before the application of siRNA-RNAiMAX mixture, cells were washed carefully with PBS to get rid of any remaining serum or antibiotic. Later on, cells were treated with siRNA and Lipofectamine mixture for two days.

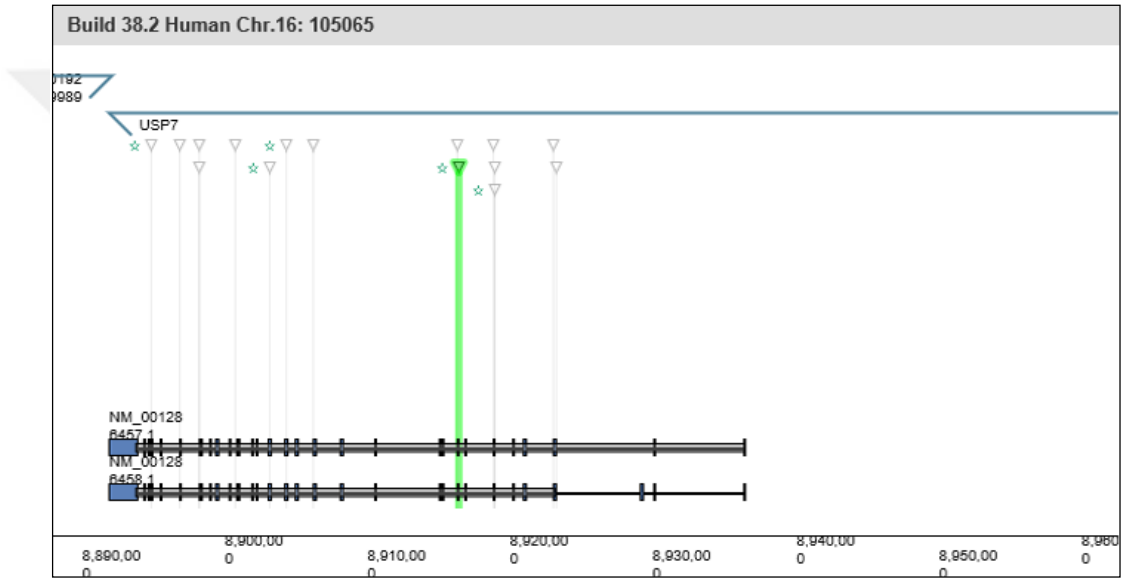


Figure 2.1. Genomic map of #105065 identification numbered siRNA which was validated and specific for USP7. Target sequence shown in green [183].

2.4. GENE THERAPY

2.4.1. Preparation of Competent Cells with $CaCl_2$ Method

Competent cells for transformation of USP7 plasmids were prepared by using $CaCl_2$ method [184]. Briefly, a fresh DH5α colony was cultured at 37°C in 3ml of Luria-Bertani broth (LB, #12780052, ThermoFisher, Invitrogen, CA) overnight with vigorous shaking. After incubation 500µl of inoculant were transferred into 500ml of LB broth in a sterile Erlenmeyer (#CLS431143, Corning Plasticware, Corning, NY) and incubated at 37°C approximately 2 hours until OD600 reaches 0.3-0.4. Then cells were transferred into ice-

cold 50-ml polypropylene tubes (#CLS430290, Corning Plasticware, Corning, NY) and cooled by storing in ice. When cooled, cells were pelleted by centrifugation at 4000 rpm for 15 minutes at 4°C. Supernatant was removed and tubes were inverted to remove the remaining LB broth. After removing LB broth completely, cell pellets were re-suspended in 10ml filter-sterilized ice cold 0.1 M calcium chloride (CaCl2, #C1016, Sigma-Aldrich, Germany). Mixture was stored on ice for 5 minutes (pellets in each 50 ml polypropylene tubes were combined in 10ml $CaCl_2$ in this step). Cells were pelleted by centrifugation at 4000 rpm for 15 minutes at 4°C. Supernatant was removed and tubes were inverted to remove the remaining $CaCl_2$ in tube. After removal of $CaCl_2$ cells were re-suspended in 2ml of 0.1 M $CaCl_2$ and will be stored on ice for 2-3 hours. 200µl of suspension of competent cells were used for transformation or were frozen immediately in liquid nitrogen and stored in -80 °C.

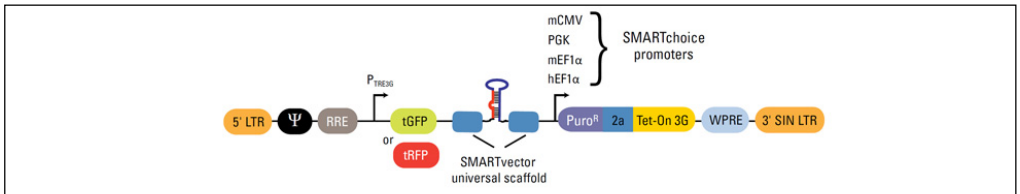
2.4.2. Plasmid Isolation

A colony from transformed bacteria was inoculated in LB containing the corresponding selection antibiotic (250ml) overnight at 37 °C. After incubation, cells were pelleted with centrifuge and the supernatant LB was discarded. Plasmids were isolated by using ZymoPURE™ Plasmid Maxiprep Kit (#D4200, Zymo Research, CA). Concentrations of isolated plasmid were measured by using Nanodrop 2000 UV-Vis Spectrophotometer (#ND-2000, Thermo-Fisher, UK). In order to control the successful isolation of plasmids, approximately 500 ng of plasmid was loaded into 1 per cent agarose (#16500500, Thermo-Fisher, UK) gel and results were compared to the original size of the plasmid integrity of plasmid was understood.

2.4.3. Transformation of USP7 Lentiviral Vector into Competent Cells

USP7 Lentiviral Vector (#V3SH11252, Dharmacon Inc., UK) (Figure 2.1) was transformed to competent *Escherichia Coli* with heat-shock method. Briefly, competent cells were thawed on ice prior to transformation. For each plasmid transformed, to 50µl competent bacteria, 250ng of plasmid DNA was mixed. Competent cell/DNA mixture was placed on ice for 30 minutes. After incubation on ice, mixture was incubated at 42 °C for 55 seconds

then mixture was placed back on ice for 2-3 minutes. 250-500 µl LB broth or SOC broth (without antibiotics) was added to mixture and mixture was let to grow in 37°C shaking incubator for 45 minutes. After incubation cells were spread to agar plates containing kanamycin (50-100 µg/µl) and incubated in a 37°C incubator overnight. Appearance of colonies was count as the sign of successful transformation and the colonies were used for plasmid isolation.



Vector Element	Utility
5' LTR	5' Long Terminal Repeat necessary for lentiviral particle production and integration of the construct into the host cell genome
Ψ	Psi packaging sequence allows viral genome packaging using lentiviral packaging systems
P_{TRE3G}	Inducible promoter with Tetracycline Response Element is activated by the Tet-On® 3G protein in the presence of doxycycline
tGFP or tRFP	TurboGFP or TurboRFP reporter for visual tracking expression upon doxycycline induction
SMARTvector universal scaffold	Optimized proprietary scaffold based on native primary microRNA in which gene-targeting sequence is embedded
$Puro^R$	Puromycin resistance permits antibiotic selection of transduced cells
2a	Self-cleaving peptide enables the expression of both PuroR and Tet-On® 3G transactivator from a single RNA pol II promoter
Tet-On® 3G	Encodes the doxycycline-regulated transactivator protein, which binds to P_{TRE3G} promoter only in the presence of doxycycline
WPRE	Woodchuck Hepatitis Post-transcriptional Regulatory Element enhances transgene expression in target cells
3' SIN LTR	3' Self-inactivating Long Terminal Repeat for generation of replication-incompetent lentiviral particles

Figure 2.2. Elements of the SMARTVector inducible shRNA lentiviral backbone [185].

2.4.4. Lentiviral Packaging and Confirmation of Lentivirus Occurrence

Prior to lentiviral packaging, 293T (#CRL-3216, human embryonic kidney cell line, ATCC, Rockville, MD) cells were thawed and cultured with DMEM cell culture medium. Cells were then seeded on 6-well plates left on incubation (37 °C and 5 per cent CO2) overnight.

Plasmid induction to 293T was performed by using transfection reagent (Xtreme HD transfection reagent, #6366244001, Sigma-Aldirch, Germany) was done by using manufacturer's protocol.

USP7 Lentiviral Vector (6.5 µg/µl) was given to 293T cells together with transfection reagent (25 µl) was introduced to cells. 24 hours after viral induction cell culture medium was refreshed and observation of GFP protein was the criteria for successful transfection. Culture medium was collected for viruses for 48 hours. Medium was filtered by 0.22 µm syringe filters (#CLS431219, Corning Plasticware, Corning, NY) and lentiviral occurrence

in collected medium was examined with Lenti-x Go Sticks (#631280 Takara, Japon), by using manufacturer's protocols. Remaining viruses were stored in -80°C.

2.4.5. Transduction of Packaged Viruses to MCF7 and T47D Cells

After viruses were collected and filtered, MCF7 and T47D cells were cultured into 24-well plates (#CLS3526, Corning Plasticware, Corning, NY) 24 hours before viral infection at a density of $5x10^4$ cells per well and cultured with regular cell culture medium. After overnight incubation, culture medium was refreshed with 0.5 ml new complete media containing 10 µg/ml polybrene (Hexadimethrine bromide, # 107689, Sigma-Aldrich, Germany). After determination of an effective MOI (4×10^4 transducing units/ml), viral particles were added to the medium containing polybrene and cells were cultured with this medium in 37 °C and 5 per cent CO_2 humidified incubator, overnight. After overnight incubation with viruses, cell media were refreshed with complete culture medium and cells were incubated for additional 24 hours. In the following days cells were split and after observation of GFP in cells, puromycin (#P7255, Sigma-Aldrich, Germany) selection was started for 15 days before conducting further experiments. After puromycin selection, cells were pelleted and cryopreservated for further experiments.

2.5. CELL VIABILITY ASSAY

Effect of DUB inhibitor, p5091, on cell viability of T47D and MCF7 cells was measured with 3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium (MTS) assay (#G3582, CellTiter96 AqueousOne Solution; Promega, Southampton, UK). Briefly, cells were seeded on 96 well-paltes (#CLS6509, Corning Plasticware, Corning, NY) at a cell density of $5x10^3$ cells/well and incubated in 37 °C and 5 per cent CO_2 humidified incubator for 24 hours. After surface attachment of the cells were treated with various (2µM, 5µM, 10µM and 20µM) concentrations of p5091. 24, 48 and 72 hours later, p5091 containing medium was removed and replaced with PBS containing 4.5 g/L glucose and 10 per cent MTS as described elsewhere [186]. Cells were incubated at growing conditions to let them metabolize MTS reagent. One and a half hour later, viability

changes were measured with the absorbance rates at 490nm by using an ELISA plate reader (Biotek, Winooski, VT).

2.6. REAL TIME PCR

Specific primers for Ubiquitin Specific Protease 7 (USP7), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Protein kinase B (Akt), Bcl-2-associated X protein (BAX), B-cell lymphoma 2 (BCL2), Caspase-3, Caspase 7, Forkhead box protein O4 (FOXO4), Epidermal growth factor receptor (EGFR), Nuclear Factor Kappa B (NF-κB), Mouse double minute 2 homolog (MDM2) and (table 2.1) were produced with Primer-BLAST software (National Center for Biotechnology, Bethesda, MD, USA) and Macrogen (Seoul, Korea) synthesized the primers. All reagents that were used in real time PCR procedure were summarized in table 2.2 and PCR conditions were given in table 2.3. Total RNAs were isolated with Trizol reagent (#15596026, Invitrogen, CA) according to the manufacturer's instructions. Isolated mRNAs were converted into cDNA's via transcriptor first strand cDNA synthesis kit (#04379012001, Roche, USA) prior to reverse transcription polymerase chain reaction (RT-PCR) with SYBR Green method which was used to detect mRNA levels of the target genes [187]. Mixture of primers, cDNAs, SYBR-mix (#K0221, Fermentas, USA) and PCR grade distilled water (#SH30538.02, Hyclone, Utah, USA) were prepared in a 10µl of final volume. GAPDH was used for housekeeping gene as literature [188]. RT-PCR assay was performed by using CFX96 RT-PCR system (Bio-Rad, Hercules, CA).

Table 2.1. Real time PCR primer sequences

Gene	Side	Sequence
USP7	Forward	5' GGAAGCGGGAGATACAGATGA 3'
	Reverse	5' AAGGACCGACTCACTCAGTCT 3'
MDM2	Forward	5' GGCTCTGTGTGTAATAAGGGAGA 3'
	Reverse	5' GGACTGCCAGGACTAGACTTTG 3'
P53	Forward	5' GCCCAACAACACCAGCTCCT 3'
	Reverse	5' CCTGGGCATCCTTGAGTTCC 3'
BAX	Forward	5' TGCAGAGGATGATTGCCGCCG 3'

	Reverse	5’ ACCCAACCACCCTGGTGTTGG 3’
BCL2	Forward	5’ AACGGAGGCTGGGATGCCTTTGTG 3’
	Reverse	5’ ACCAGGGCCAAACTGAGCAGAGT 3’
Caspase 3	Forward	5’ GAGGCGGTTGTAGAAGAGTTCGTG 3’
	Reverse	5’ TGGGGGAAGAGGCAGGTGCA 3’
Caspase 7	Forward	5’ GGAGAAAGCTCATGGCTGTGT 3’
	Reverse	5’ TCCCCTTGGCTGTGTTTTG 3’
EGFR	Forward	5’ AATGCAACATCCTGGAGGGG 3’
	Reverse	5’ AGGTGATGTTCATGGCCTGG 3’
PTEN	Forward	5’ TGTGGTCTGCCAGCTAAAGG 3’
	Reverse	5’ ACACACAGGTAACGGCTGAG 3’
FOXO4	Forward	5’ GACTGCGAGTCCATCATCCT 3’
	Reverse	5’ GGGCTGAGTCGAAGTTGAAG 3’
Akt	Forward	5’ GAAGCTGCTGGGCAAGGGGCA 3’
	Reverse	5’ GTGGGCCACCTCGTCCTTGG 3’
NF-κB	Forward	5’ GCCACCCGGCTTCAGAATGGC 3’
	Reverse	5’ TATGGGCCATCTGCTGTTGGCAGT 3’
GAPDH	Forward	5’ AAGGTGAAGGTCGGAGTCAAC 3’
	Reverse	5’ GGGGTCATTGATGGCAACAATA 3’

Table 2.2. Reagents of RT-PCR

Reagents	Volume
Maxima™ SYBR Green qPCR Master Mix	5 µl
Primer Forward (10pmol)	0,3 µl
Primer Forward (10pmol)	0,3 µl
Distilled water	2,9 µl
Template (100ng/ml)	1,5 µl

Table 2.3. Conditions for RT-PCR

Cycle	Repeats	Step	Duration	Temperature
Initial Denaturation	1	1	3 minutes	93 °C
Denaturation	36	1	30 seconds	93 °C
Annealing		2	40 seconds	59 °C
Extension		3	45 seconds	72 °C
Final extension	1	1	10 minutes	72 °C
Melt curve	110	1	12 seconds	-0.5 °C/cycle
Hold	1	1	-	4 °C

2.7. WESTERN BLOT

All chemicals used in immunoblotting assays were purchased from Biorad Laboratories (Richmond, CA). Solutions prepared for western blot analysis is given in table 2.4. In order to understand the change in synthesized protein amount, total protein was isolated from cell pellets with RIPA buffer (#sc-24948, SantaCruz, TX) after changing USP7 levels via various methods. To sum up, cell pellet was washed twice with ice cold PBS. While washing step, RIPA buffer cocktail was prepared with the addition of PMSF (Phenylmethylsulfonyl fluoride), protease inhibitor and sodium orthovanadate to RIPA solution (10µl each for every milliliter of RIPA solution). RIPA cocktail was added into washed cell pellets and mixed carefully on ice. By the end of the 10 minutes of incubation this mixture was centrifuged at 14.000 g 4°C for 15 minutes. Supernatant includes proteins which can be stored at -80°C. Protein concentrations from each sample were determined with BCA assay (Pierce BCA Protein Assay, # 23225, ThermoFisher, UK). Change in USP7 amount and total mono- and poly-ubiquitination in total protein were determined with western blot analysis. Proteins were loaded in Mini-PROTEAN Precast Gel (#4561023, BioRad, CA) with the protein ladder (Page Ruler (#26619, ThermoFisher, Life Technologies, UK). After running the gel at 110 V for approximately 90 mins gel was transferred to PVDF membrane (#1620177, BioRad, CA) with wet transferring method. After transfer membrane was blocked with TBST containing 5 per cent Blotting-Grade Blocker (#1706404, BioRad, CA) for 1 hour.

USP7 specific antibody (#4833, Cell Signaling Technology, MA) or mono- and polyubiquitinated conjugates antibody (#BML-PW8810, Enzo Life Science, Germany) was applied in blocking solution overnight (4°C). Primary antibody was washed in TBST for 3 times (room temperature) and membrane was incubated with anti-rabbit secondary antibody (sc-2004, dilution 1:5000, Santa Cruz Biotech Inc., USA) for 2 hours (room temperature). Lastly, after the washing step of secondary antibody, membrane was incubated with Amersham ECL™ Detection Reagents (#GERPN2105, SigmaAldrich, Germany) for 30 seconds. GAPDH was used as an internal control and images were taken by using the luminometer system (Biorad, USA). Band intensities were calculated using Image J software and normalized to the respective GAPDH band intensities. Results were represented as fold change of control.

Table 2.4. Western blotting solutions

TBS-T	20mM Tris-HCl 150mM NaCl 0.1 per cent Tween 20, pH 7.6
Running Buffer	25mM Tris base 190mM Glycine 0.1 per cent SDS, pH 8.3
Transfer Buffer	25mM Tris base 190mM Glycine 20 per cent Methanol
Blocking Buffer	5 per cent Non-fat dry milk prepared in TBS-T

2.8. SCRATCH ASSAY

To identify the changes in metastatic capacity scratch assay protocol was performed as described in literature [189]. Briefly, cells were seeded on twelve-well plate dishes (#CLS3512, Corning Plasticware, Corning, NY) as 10^5 cells/well in appropriate medium for

each cell and let them to attach in humidified 37 °C and 5 per cent CO_2 conditioned incubator. The attachment of the cells was followed by scratching a wound with sterile 200 µl pipette tip. De-attached cells were washed once with 1 x PBS solution followed by fresh medium addition which includes p5091, siUSP7 or shUSP7. Images were taken *via* ZEISS microscopy (Zeiss Primo Vert, Göttingen, Germany), AxioCam ICc 5 camera and ZEN 2 (blue edition) computer application.

2.9. COLONY FORMING UNIT (CFU)

Colony-forming capability, of the cells after changing USP7 levels, was determined via CFU assay [190]. To perform CFU assay, T47D and MCF-7 cell lines, which grow as a monolayer, were harvested and counted via neubauer hemocytometer (#MARI0640011, VWR, England). T47D and MCF7 cell lines were plated in six-well plates (#CLS3506, Corning Plasticware, Corning, NY) as 300 cells/well in RMPI-1640 for T47D and DMEM high glucose for MCF7. Platting was followed by USP7 inhibitory, siUSP7 and lentiviral shUSP7 treatment. After appropriate time, that is described in MTS, siUSP7, shUSP7 and the inhibitory containing medium was changed in every 48 hours into fresh growth medium (10 per cent FBS containing RMPI-1640 and DMEM high glucose) until the colony formations could be observed (10 to 15 days). Lastly, fixation and staining with crystal violet (#C3886, Sigma-Aldrich, Germany) were performed in order to see colonies clearer under the microscope. Briefly, colonies were washed with 1X PBS once that followed by addition of 4 per cent PFA for 30 minutes, room temperature (RT) for fixation. For staining, colonies were washed once with 1X PBS before crystal violet treatment (30 minutes, RT). The pictures were taken with ZEISS microscopy, AxioCam ICc 5 camera and ZEN 2 (blue edition) computer application.

2.10. CELL CYCLE

In order to determine the effect of differential effect of USP7 protein on T47D and MCF7 cells, cell cycle assay was performed accordingly [191], cells were plated (3×10^5 cells/well) in six well tissue culture plates and after 24h incubation, media was refreshed with fresh media containing viruses that contains desired plasmi (shRNA), siRNA or p5091. After

changing the amount of USP7 protein in the cells, fixation of the cells with 4 per cent PFA (#P6148, Sigma-Aldrich, Germany) was performed. After fixation, cells were suspended and incubated in 500 µL staining solution (40 µg/mL RNase A (#EN0531, ThermoFisher, UK), 33 µg/mL Propidium Iodide (PI, #P1304MP, ThermoFisher, UK), 0.2 per cent nonyl phenoxypolyethoxylethanol (NP-40, #FNN0021, ThermoFisher, Invitrogen, UK) in PBS) at 37°C for 30 min. BD FACS Calibur (BD Bioscience, USA) was used for flow cytometry and data were analyzed by using BD-Cell Quest Pro software (USA).

2.11. HANGING DROP ASSAY

To identify various levels of USP7 on sphere formation capability of cells, hanging drop assay was carried out like described elsewhere [192]. Succinctly, after downregulating USP7 levels through shRNA transduction, transfection with USP7 specific siRNA or inhibiting USP7 levels with p5091, cells were counted and diluted to reach 1x 10^3 cells per 40 µl of media containing 500 µg/ml of collagen-I. Cell and collagen fibrils were mixed well and transferred drop-by-drop to 100 mm inside of a Culture Dish's (#430167, Corning Plasticware, Corning, NY) lid. Every 40 µl volume drop had approximately diameter of 1.4 mm. Appropriate amount of PBS was added into culture dish to minimize unwanted evaporation. Lid that has cell drops on, gently but rapidly flipped upside down to leave cells alone with gravitational force for 24 hours. Spheres that were formed by the help of collagen fibrils and gravity were transferred into cell culture compatible 24-well plate for examination under the light microscope. To have further information about tumor dissemination, pre-formed spheres were examined for two days, gradually. Spheres were formed regularly, after spheres were completely formed, USP7 level was downregulated by shUSP and siRNA or USP7's enzyme activity decreased by p5091.

3. RESULTS

3.1. CELL VIABILITY

MTS assay was performed in order to understand the effect of USP7 inhibitor, p5091, on the cell viability for three days. Several doses of p5091 were tested on T47D and MCF7 cell lines for three separated days. Light microscopy pictures were taken at the determined time for the dose of IC_{50}. Results have shown that cell viability decreased by approximately 50 per cent in MCF7 and T47D cell lines after 2 and 3 days, respectively (Figure 3.1).

Effect of siRNA application on cell viability was also examined by microscopic demonstration and calculation of viable cells were done. siUSP7 treatment decreased the cell viability of MCF7 cells from 180 x 10^3 living cells to 40 x 10^3 cells (77.78 per cent decrease) (Figure 3.2). Additionally, the viable T47D cells amount decreased from 245 x 10^3 to 165 x 10^3 which is 32.65 per cent decrease (Figure 3.3).

Furthermore, lentiviral shUSP7 application's effect on cell viability was also determined as it was performed in siRNA application. Successful transduction was shown by fluorescent microscopic evaluation of green fluorescent protein since the vector includes GFP region (Figure 3.4). Silencing of USP7 via lentiviral application decreased live MCF7 cell number by 67 per cent (from 285 x 10^3 to 93 x 10^3) (Figure 3.5) and viable T47D number by 31.88 per cent (from 138 x 10^3 to 94 x 10^3) (Figure 3.6).

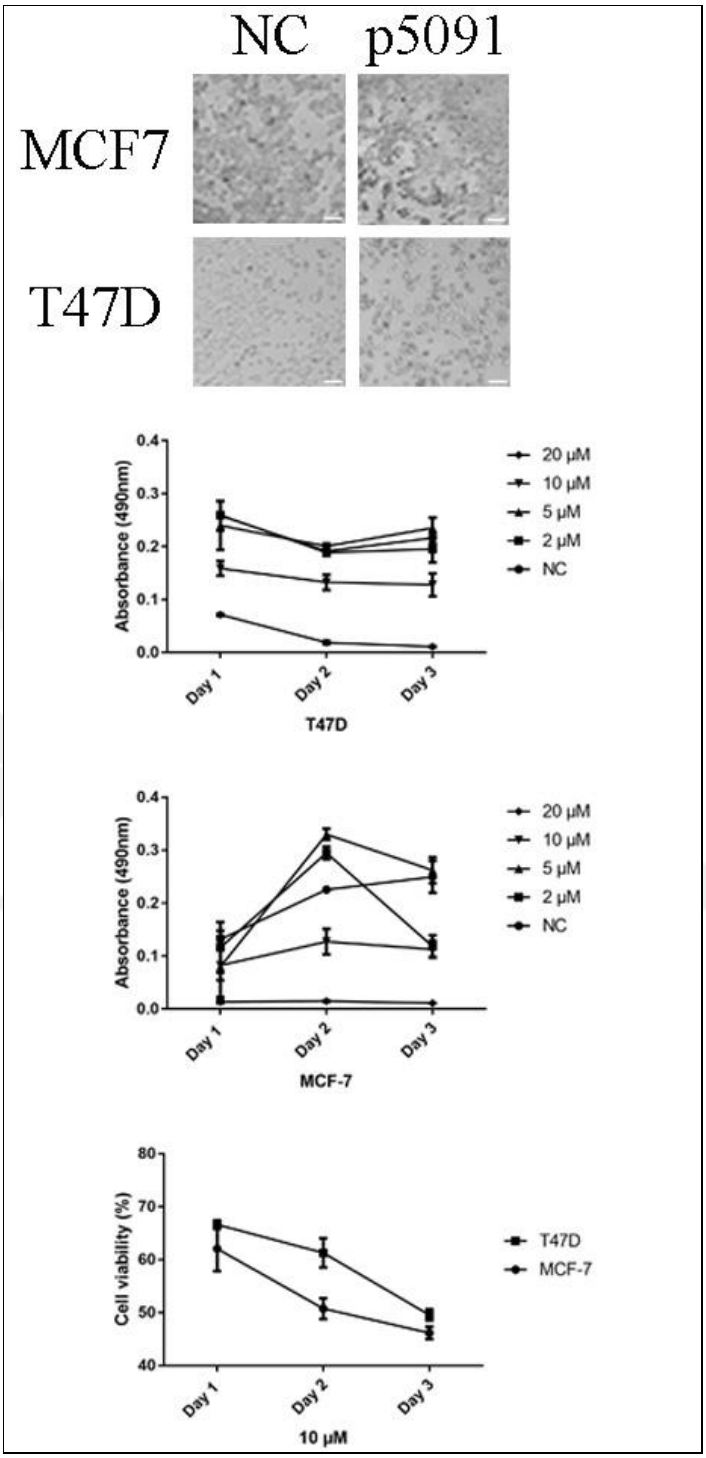


Figure 3.1. Effect of p5091 on MCF7 and T47D cell lines' cell viability. Upper panel shows fifty times magnified light microscopy image prior and after p5091 application. Line graphs demonstrate various p5091 doses' effect on each T47D and MCF7. The ultimate line graph shows selected p5091 dose's effect on both cell lines' viabilities by percentage.

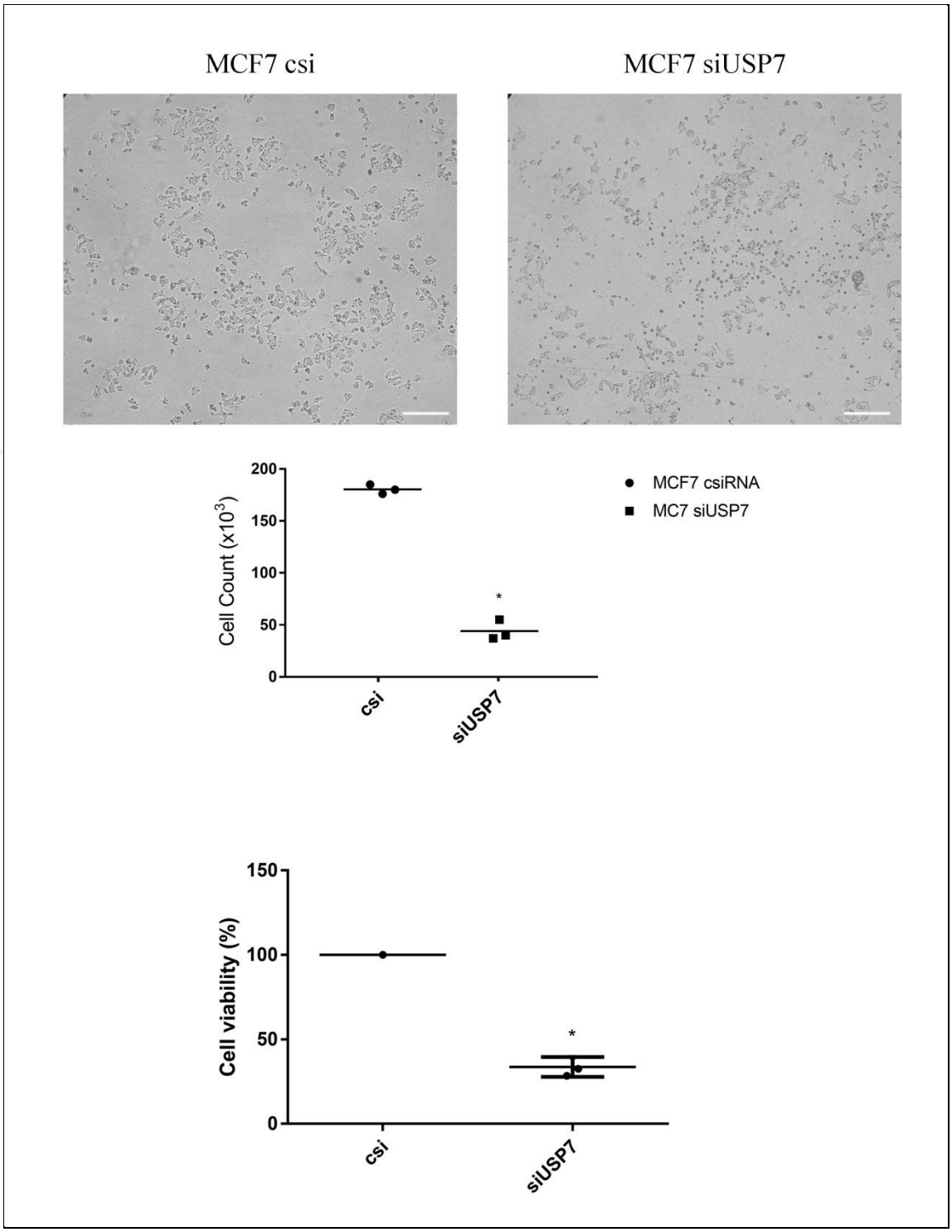


Figure 3.2. Effect of siUSP7 on MCF7 cell viability. Microscopic pictures were taken with fifty times magnification. *P<0.05. Results were analyzed by one-way ANOVA and Tukey's test using GraphPad Prism Version 7.00. Images were taken after 48 hours. csi: control small interfering RNA.

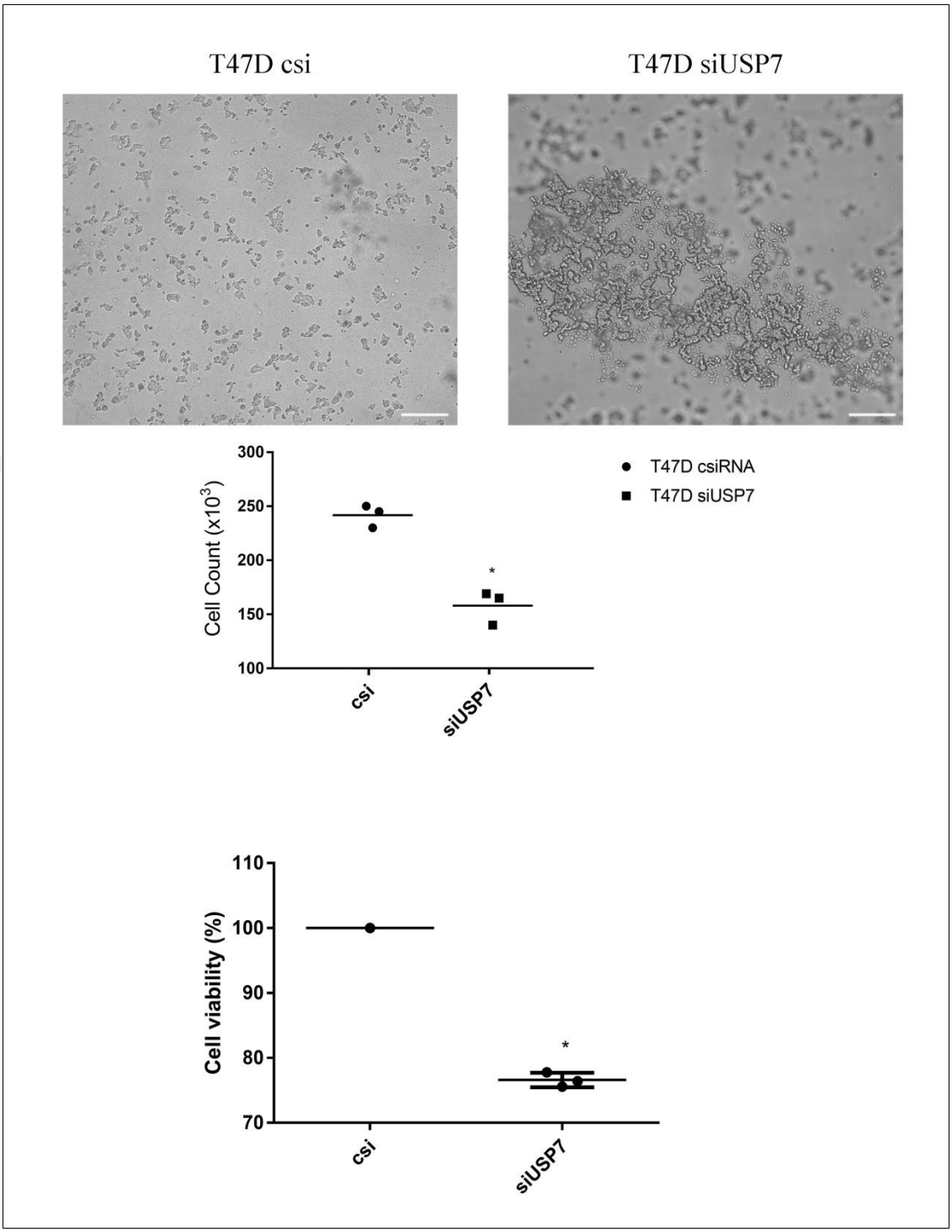


Figure 3.3. Effect of siUSP7 on T47D cell viability. Microscopic pictures were taken with fifty times magnification. *P<0.05. Results were analyzed by one-way ANOVA and Tukey's test using GraphPad Prism Version 7.00. Images were taken after 48 hours. csi: control small interfering RNA.

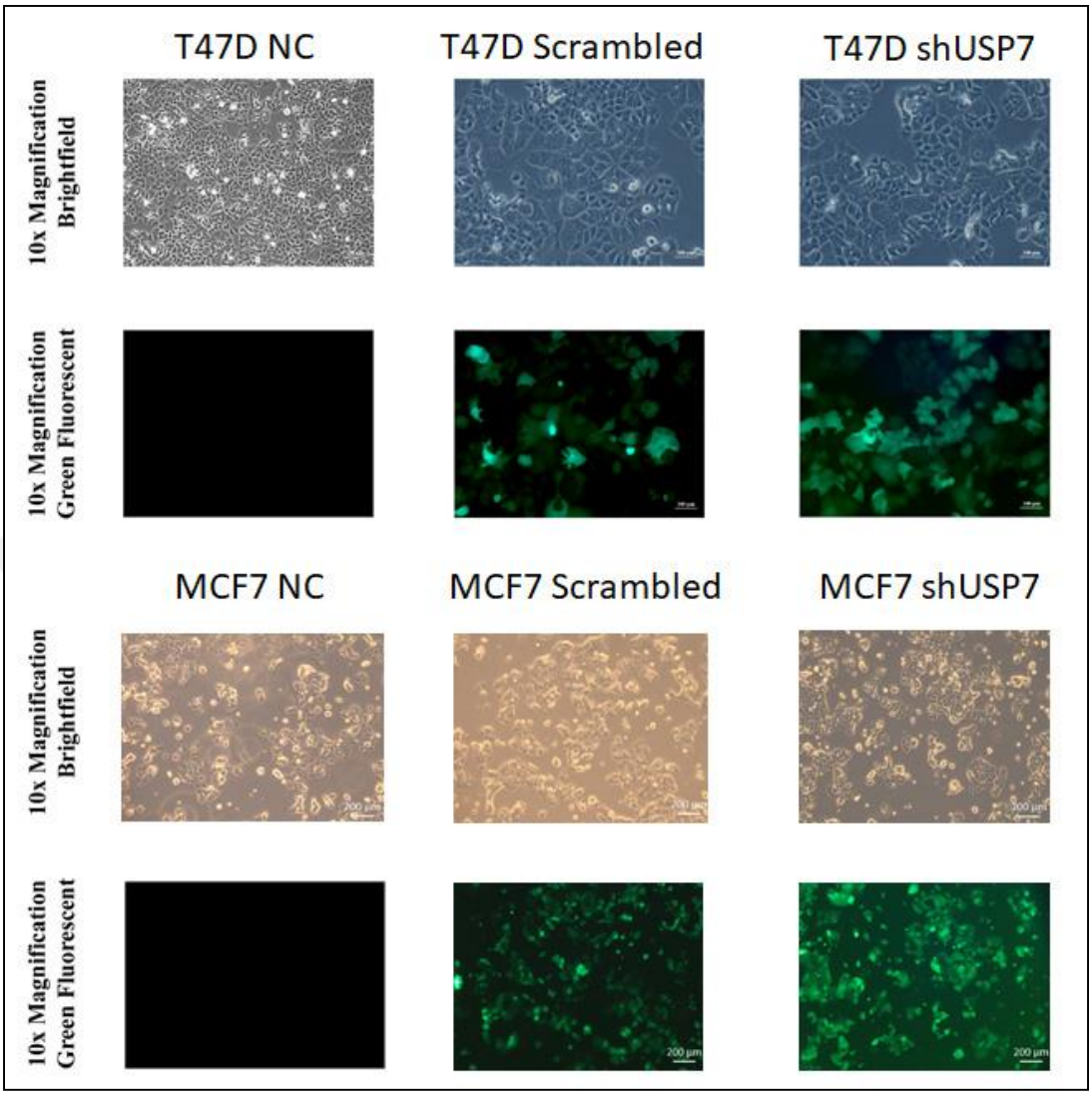


Figure 3.4. Fluorescent and bright filtered images of T47D and MCF7 after lentiviral infection. NC represents negative control group and both scrambled and shUSP7 plasmids have green fluorescent genes.

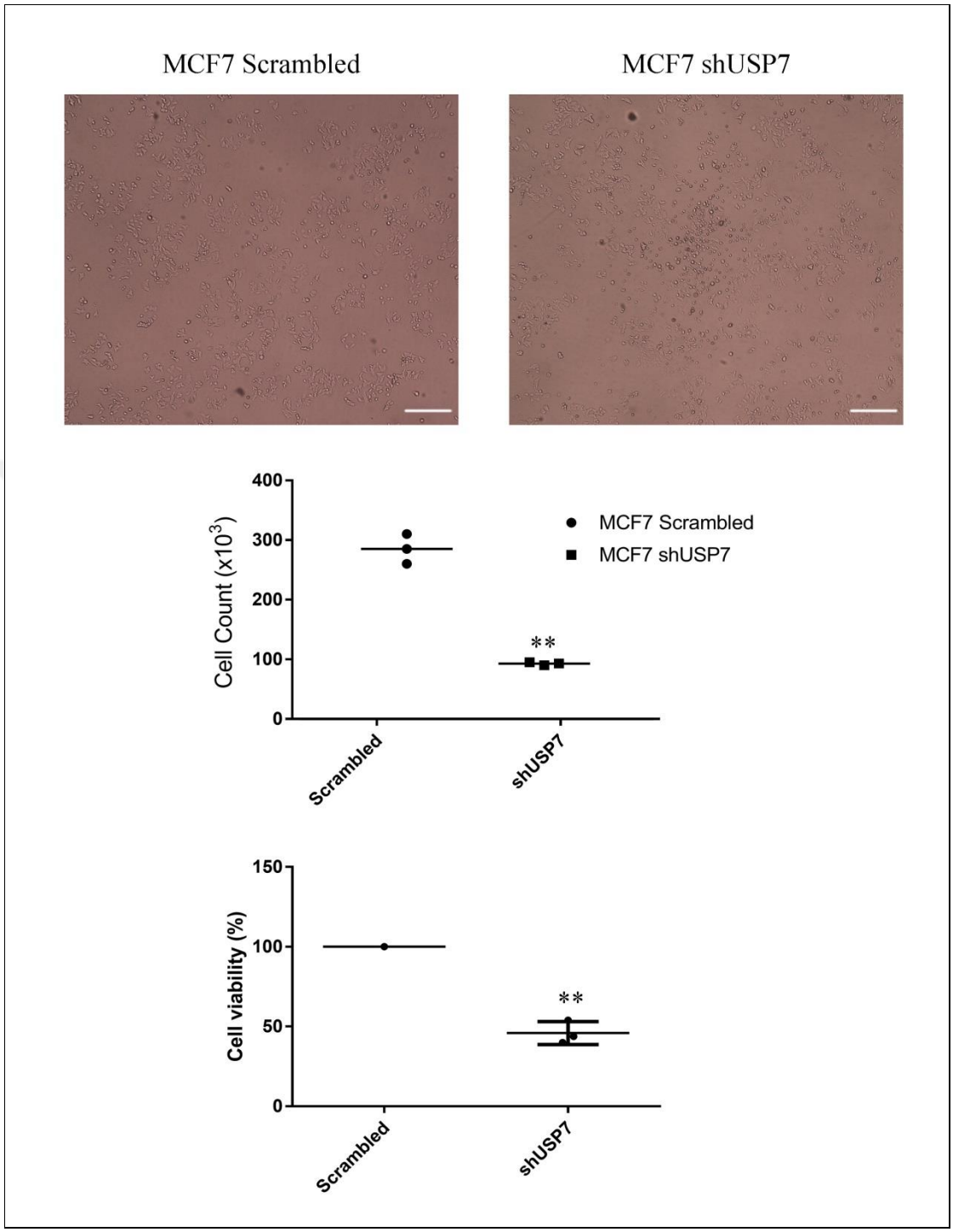


Figure 3.5. Effect of shUSP7 on MCF7 cell viability. Microscopic pictures were taken with fifty times magnification. **P<0.01. Results were analyzed by one-way ANOVA and Tukey's test using GraphPad Prism Version 7.00.

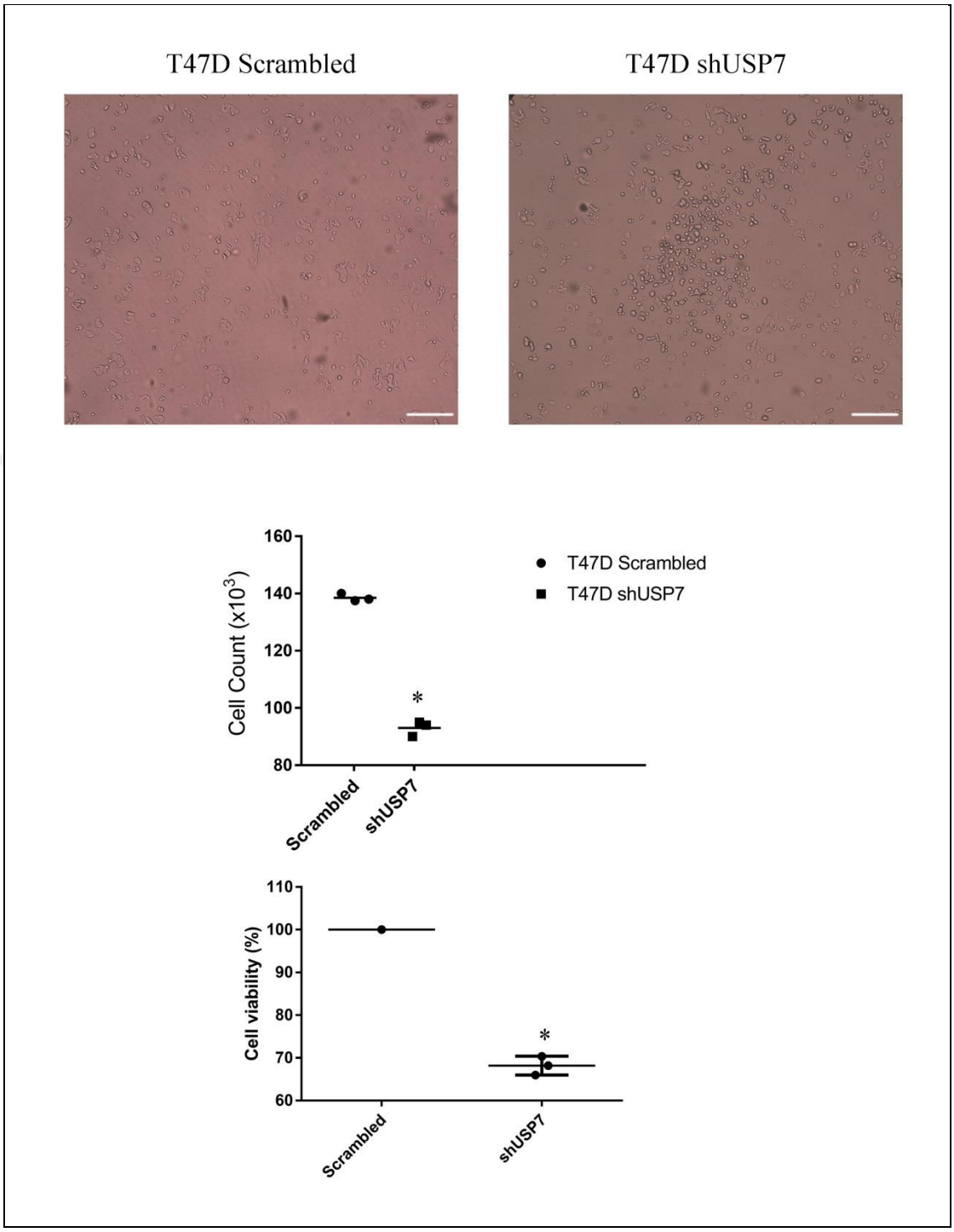


Figure 3.6. Effect of shUSP7 on T47D cell viability. Microscopic pictures were taken with fifty times magnification. *P<0.05. Results were analyzed by one-way ANOVA and Tukey's test using GraphPad Prism Version 7.00.

3.2. RT-PCR ANALYSIS

Level of deubiquitinase enzyme, USP7, was decreased in MCF7 and T47D cells by siUSP7 and shUSP7 and USP7 enzyme activity was abated with its' allosteric small molecule inhibitor, p5091 in order to examine gene expression levels of oncogenic and apoptotic genes. All significant changes in gene expression profiles were statistically analyzed by one-way ANOVA and Tukey's test. Significant changes were calculated as $P<0.05$.

3.2.1. p5091

RT-PCR results have shown clearly that p5091 treatment in MCF7 cell line, have no significant effect on USP7 gene expression levels as well as BAX, BCL2, PTEN and EGFR mRNAs. However, 3 fold increase of TP53 gene expression was observed. Thus, MDM2 expression levels were also increased (≈1.5 fold). Caspase 3 and caspase 7, also known as executioner caspases, gene expression levels were increased by 1.9 fold and 1.8 fold, respectively. 2.3-fold increase in FOXO4 mRNA level was also observed. NF-kB gene expression level was also increased (≈2 fold). However, the gene that is correlated with tumor growth and proliferation, Akt, was abated 0.67 fold (Figure 3.7).

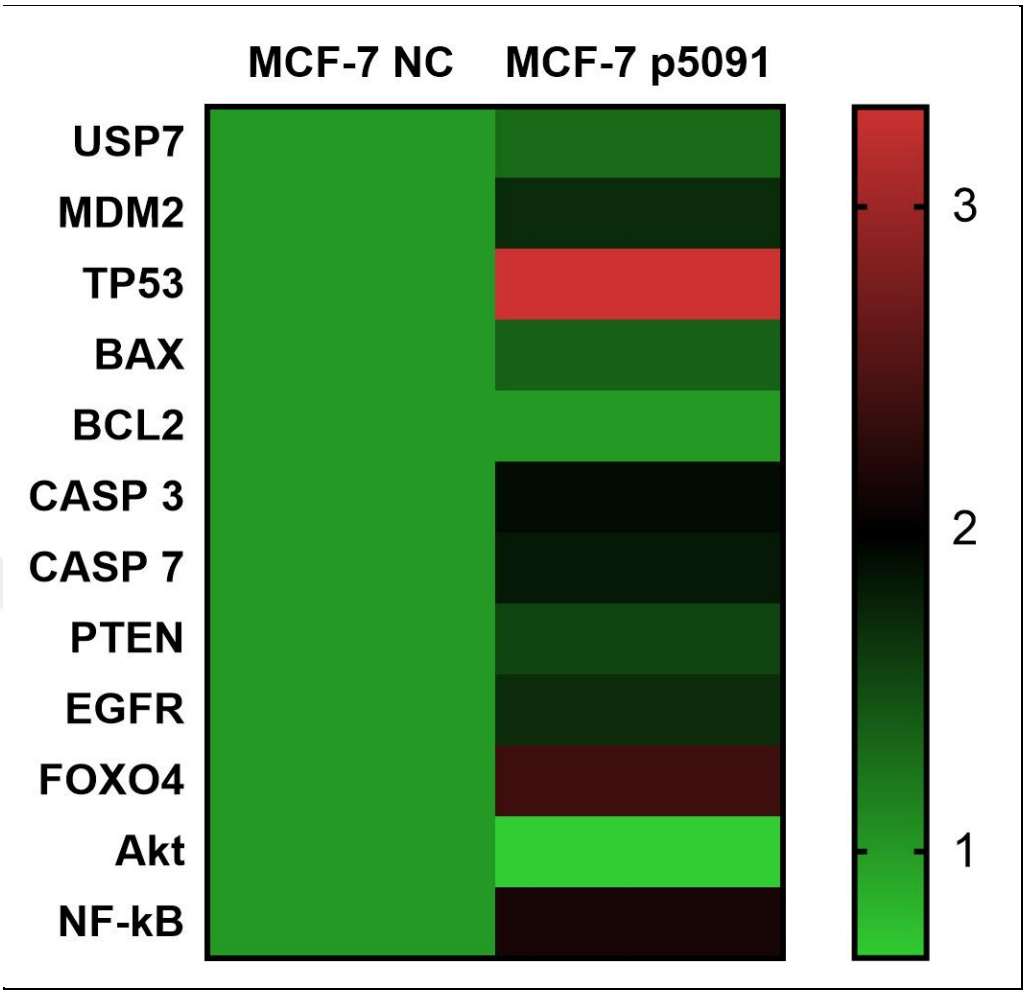


Figure 3.7. Heat map representation of p5091 effect on gene expression profile of MCF7 cells. NC: Negative control.

p5091 had a similar effect on T47D cell's gene expression profile with no significant change in USP7, BCL2 and PTEN and increased FOXO4 (3.9 fold), CASP 3 (≈2.5 fold), CASP 7 (≈2 fold), TP53 (2 fold) and MDM2 (1.8 fold) mRNA levels. Notwithstanding, p5091 caused 5.7 fold increase in BAX. Small molecule inhibitor of USP7 also caused no significant change in Akt and NF-kB (Figure 3.8).

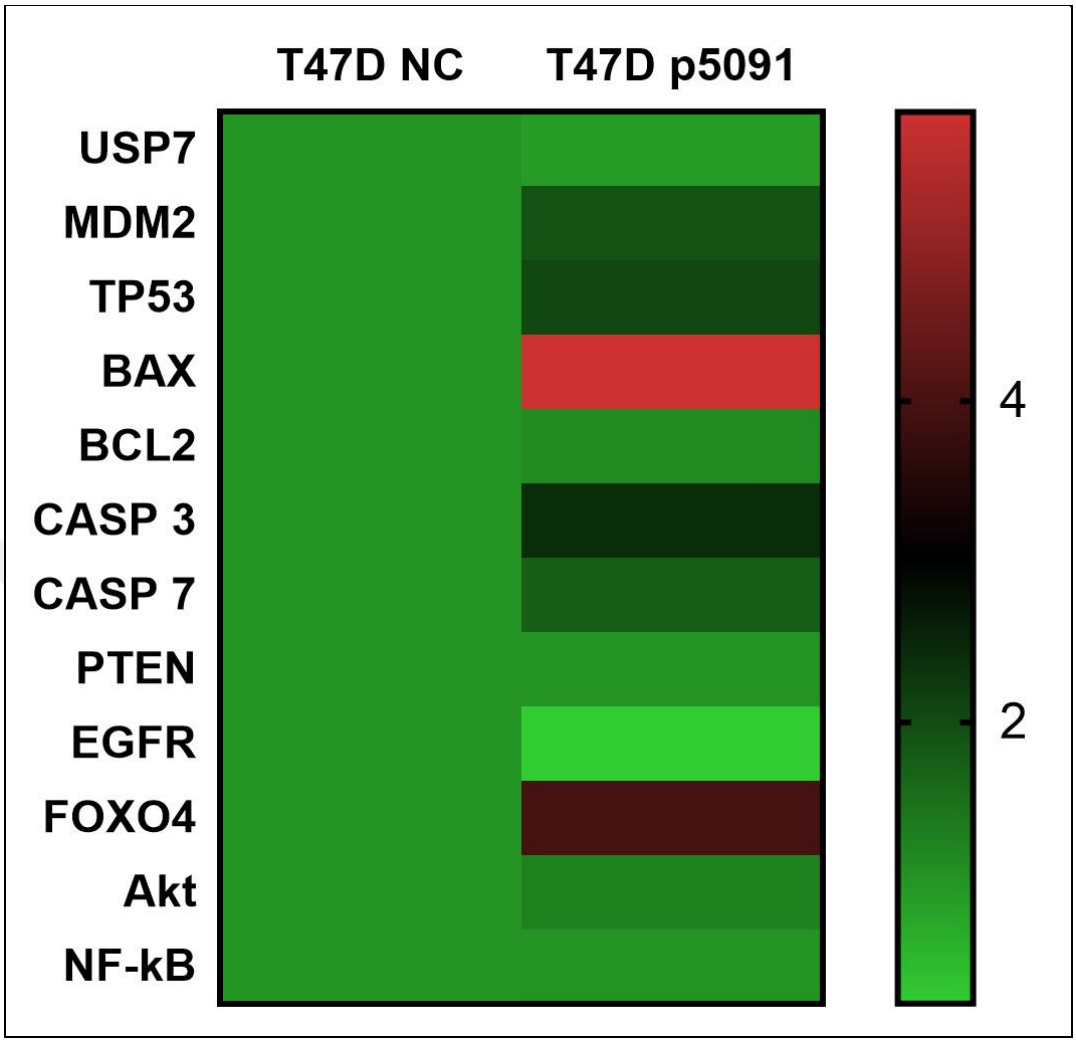


Figure 3.8. Heat map representation of p5091 effect on gene expression profile of T47D cells. NC: negative control.

3.2.2. siUSP7

siUSP7 treatment on MCF7 cell led to a dramatic decrease in USP7 gene expression (0.2 fold). USP7 decrease had no noticeable effect on PTEN, FOXO4, TP53 and MDM2 genes. On the other hand, BAX, CASP 3 and CASP 7 mRNA levels were increased 1.6 fold, 6.8 fold and 4.4 fold respectively. BLC2 mRNA level was observed by to be 0.4 fold lessened after siUSP7 application. NF-kB and EGFR gene expressions were also decreased (≈0.7 fold for both) but Akt gene expression was increased by 1.6 fold (Figure 3.9).

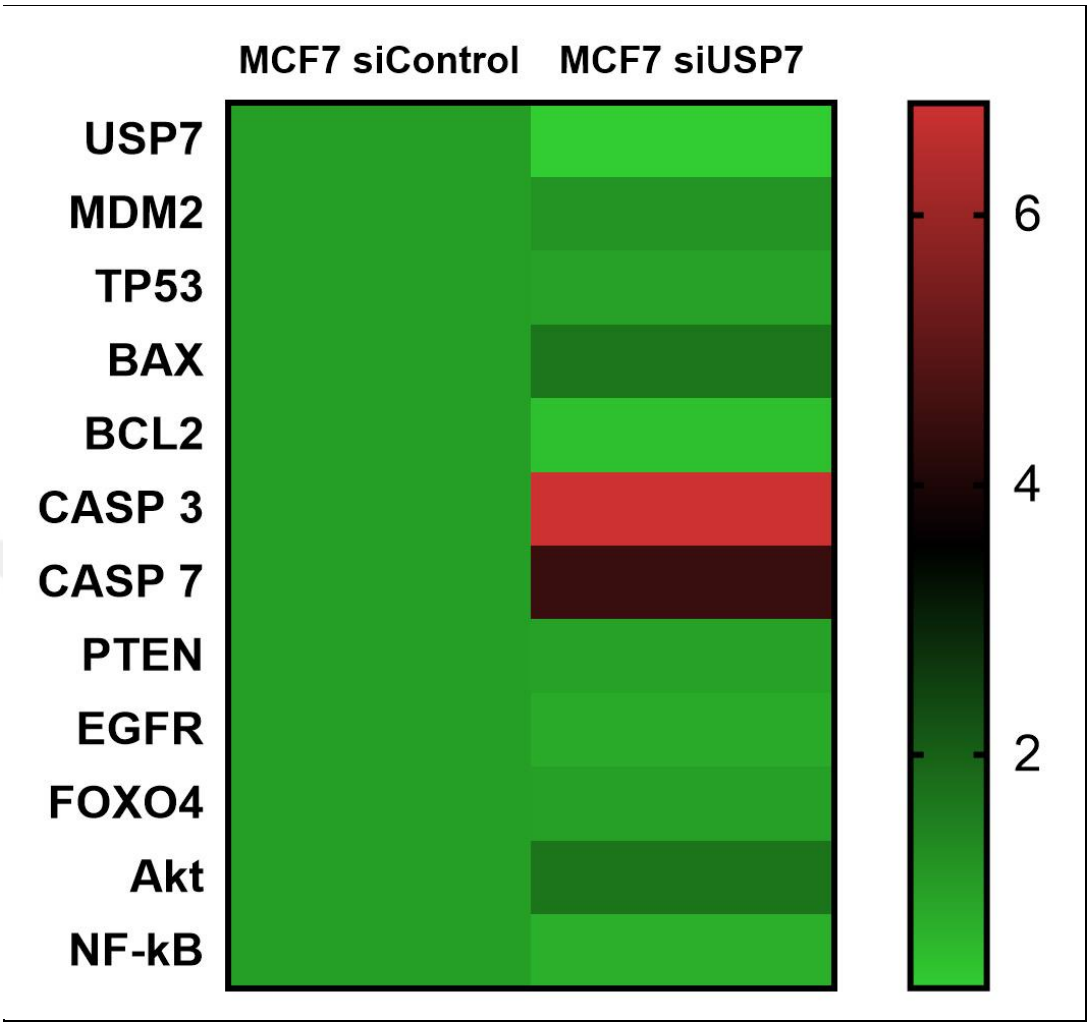


Figure 3.9. Heat map representation of siUSP7 effect on gene expression profile of MCF7 cells. csi: control small interfering RNA.

siUSP7 treatment on T47D cell, had not only caused a decrease in UPS7 (0.1 fold), but also in MDM2 (0.1 fold), BCL2 (0.2 fold), EGFR (0.8 fold), Akt (0.07 fold) and NF-kB (0.47 fold). On the other hand, increase in TP53 (3.4 fold), CASP3 (6.5 fold) and CASP 7 (4.2 fold) mRNA levels were observed. BAX, PTEN and FOXO4 mRNA levels showed no significant difference compared to control siRNA (Figure 3.10).

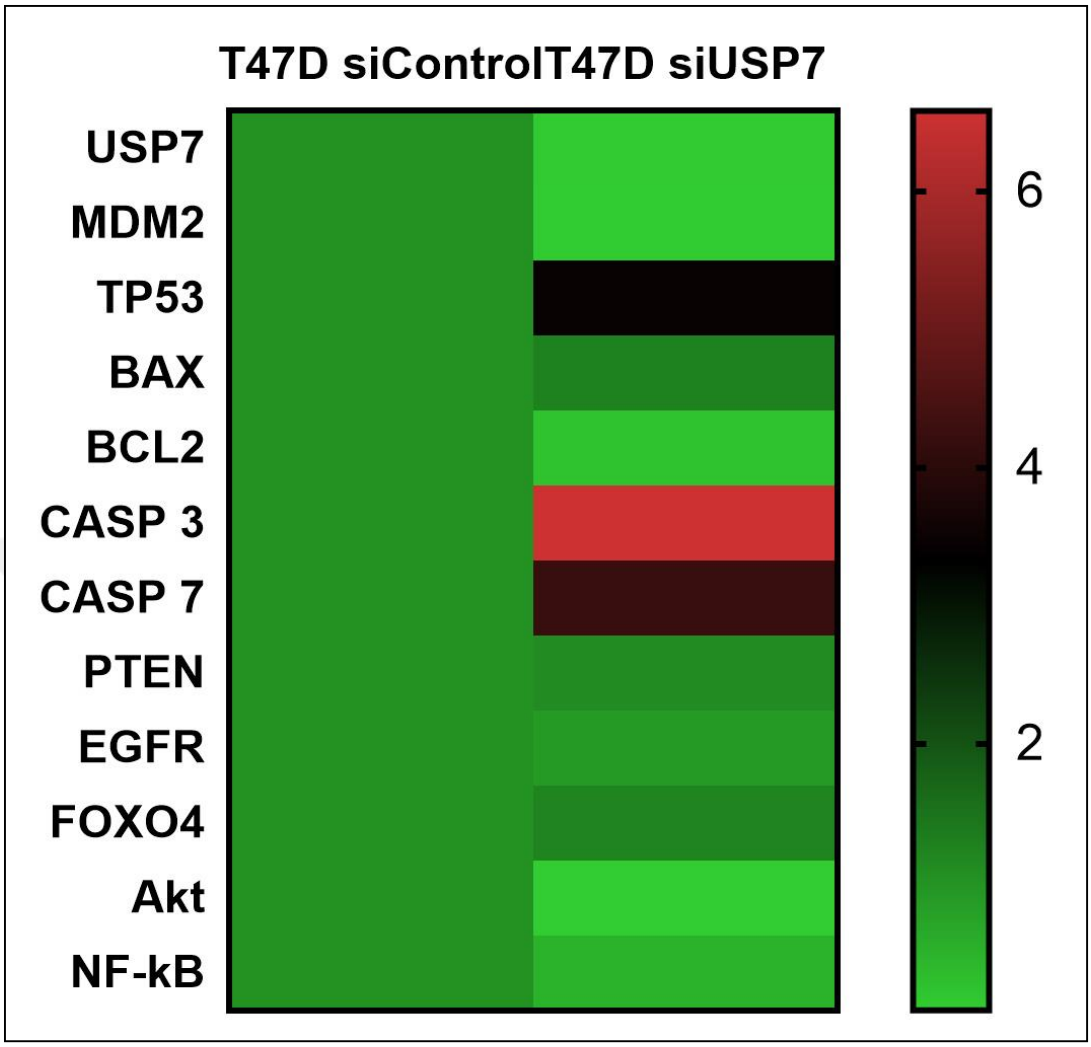


Figure 3.10. Heat map representation of siUSP7 effect on gene expression profile of T47D cells. csi: control small interfering RNA.

3.2.3. shUSP7

Low MDM2 (0.47 fold), BCL2 (0.08 fold), EGFR (0.05 fold), Akt (0.3 fold) and NF-kB (0.1 fold) levels were detected after lentiviral silencing of USP7 (0.4 fold) in MCF7 compared to the scrambled plasmid. However, TP53, Bax, CASP 3, CASP 7, PTEN and FOXO4 levels were found to remain unchanged (Figure 3.11). Similarly, MDM2, TP53, BAX, BCL2, CASP 3, CASP 7, FOXO4 and NF-kB levels were found to be unchanged in USP7 silenced T47D cells. Nevertheless, expression levels for the remaining genes including USP7 (0.5 fold), PTEN (0.8 fold), EGFR (0.45 fold) and Akt (0.3 fold) were found to be decreased(Figure 3.12).

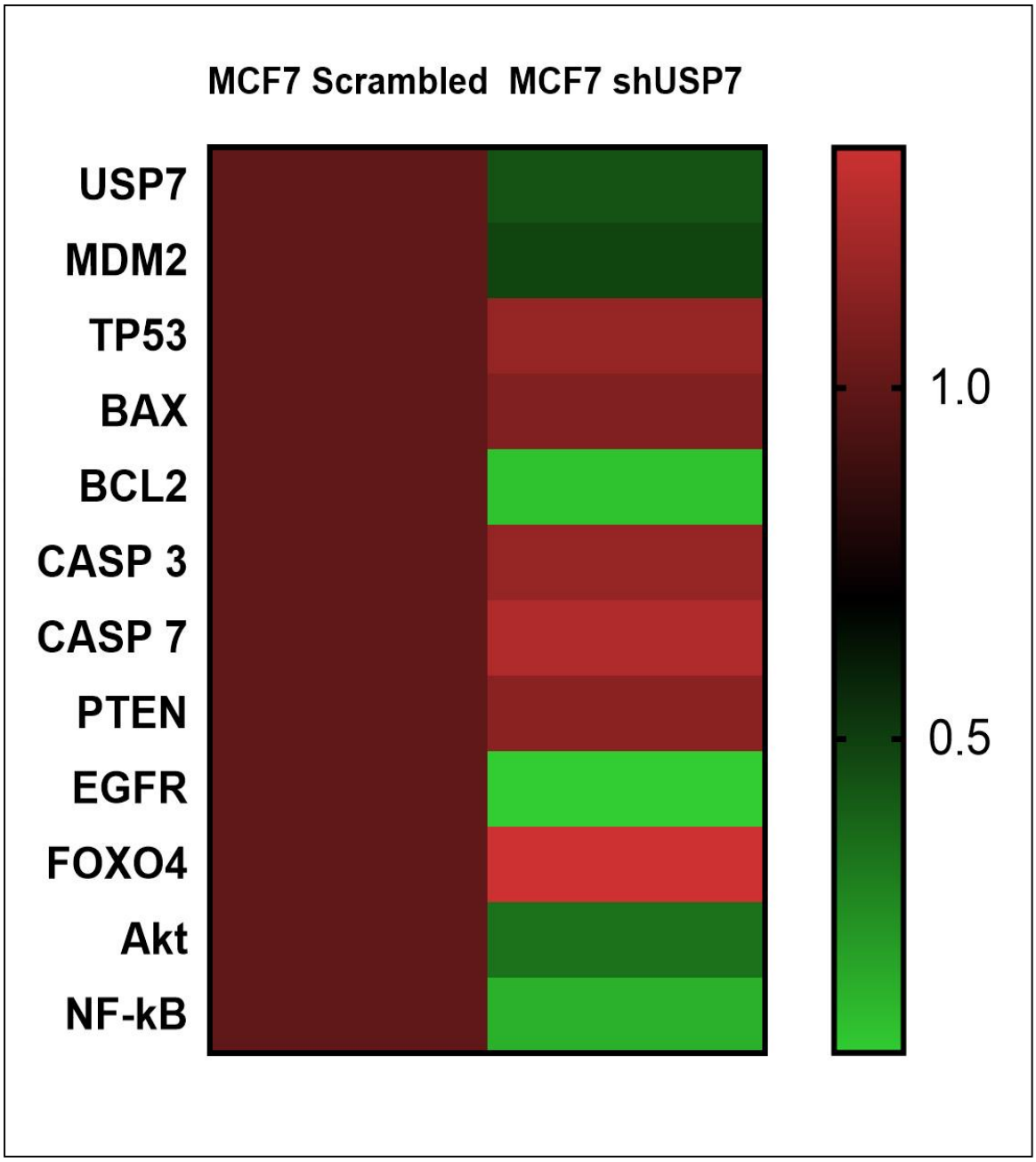


Figure 3.11. Heat map representation of shUSP7 effect on gene expression profile of MCF7 cells.

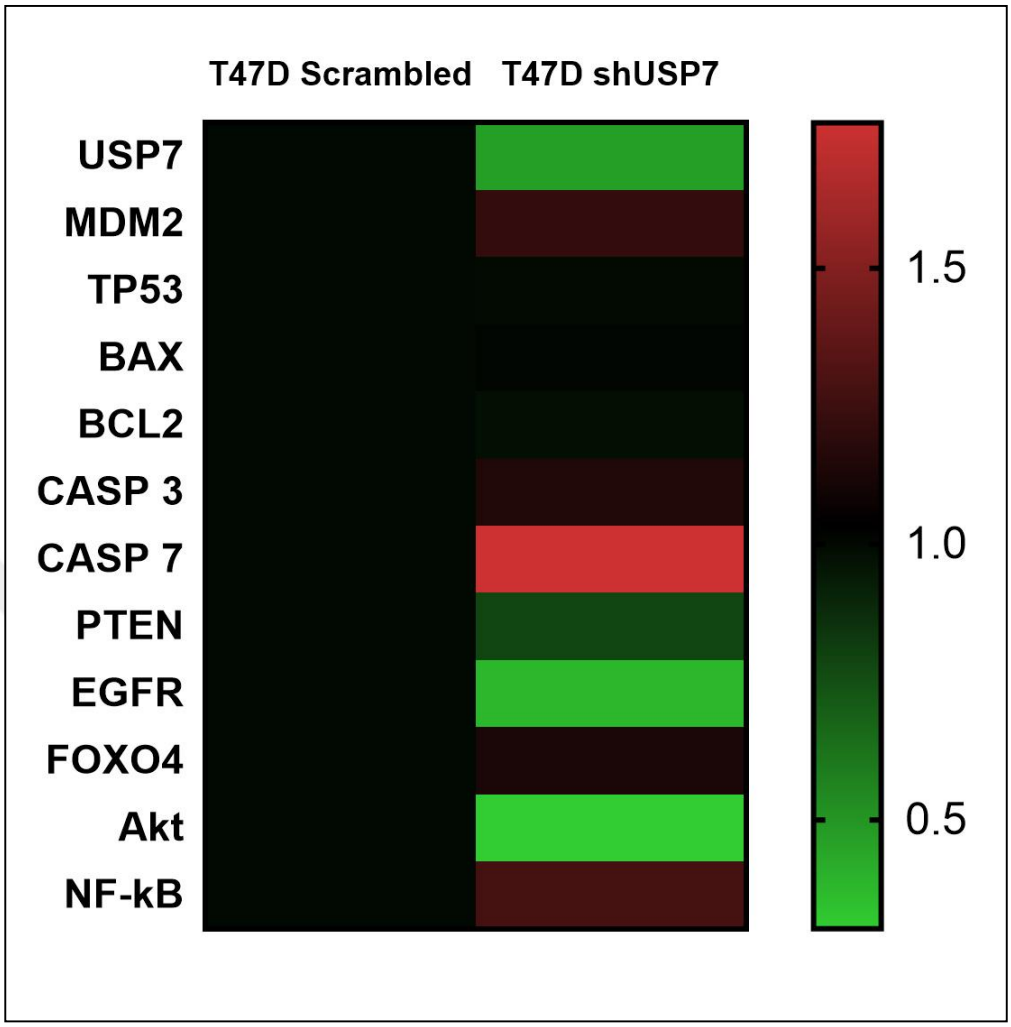


Figure 3.12. Heat map representation of shUSP7 effect on gene expression profile of T47D cells.

3.3. WESTERN BLOT

Possible changes in USP7 protein level after small molecule inhibitor, siUSP7 and shUSP7 treatment was displayed with western blot analysis. Treatment with small molecule inhibitor of USP7 had no effect on USP7's protein amount in both MCF7 and T47D. However, total ubiquitination levels significantly increased (≈4 fold) (Figure 3.13). siUSP7 strategy decreased the USP7 levels by 0.2 fold and 0.4 fold for MCF7 and T47D respectively (Figure 3.14). Likewise, lentiviral application of shUSP7 caused 0.3 fold decreased USP7 in MCF7 and 0.2 fold decreased USP7 protein in T47D cells (Figure 3.15).

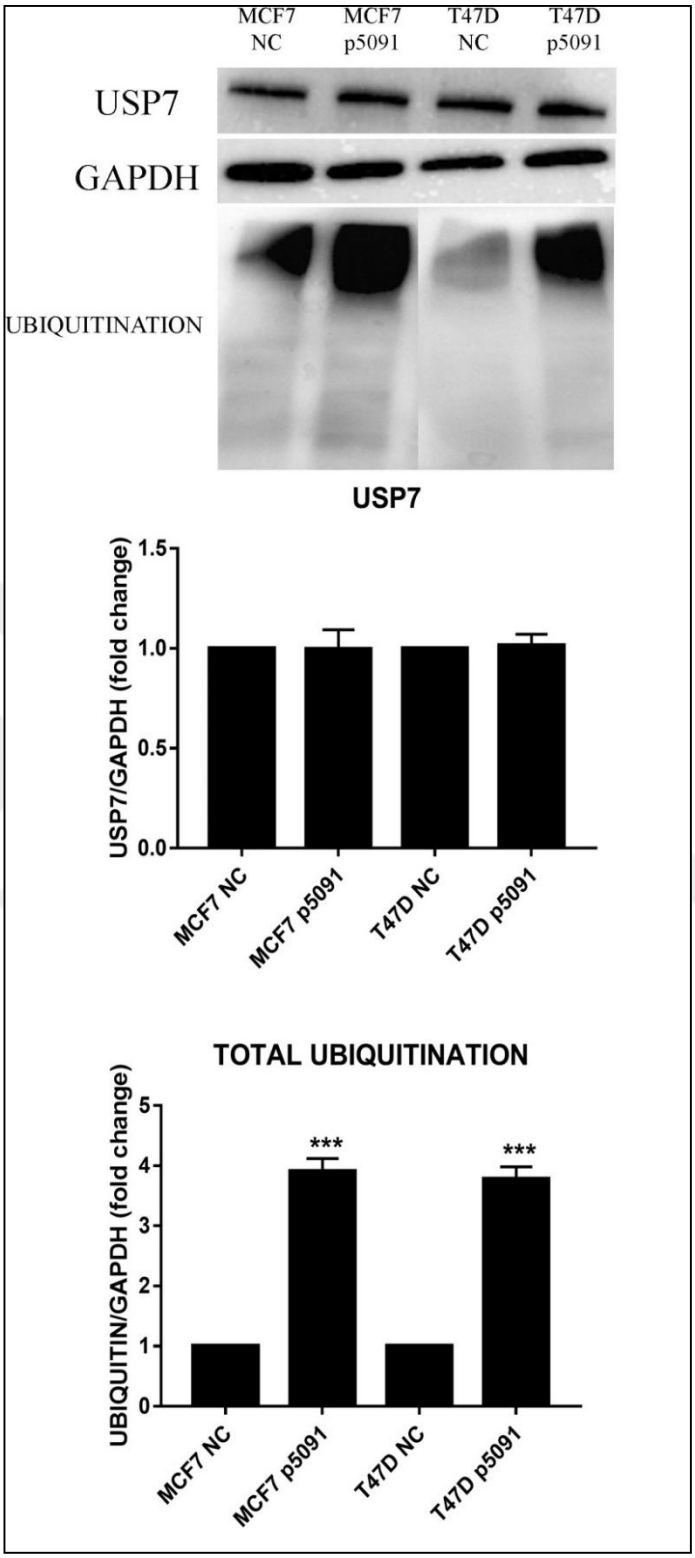


Figure 3.13. Total USP7 and ubiquitinated protein levels of MCF7 and T47D cell lines. GAPDH antibody was used as a loading control. ***P<0.001. Results were analyzed by one-way ANOVA and Tukey's test using GraphPad Prism Version 7.00. NC: negative control.

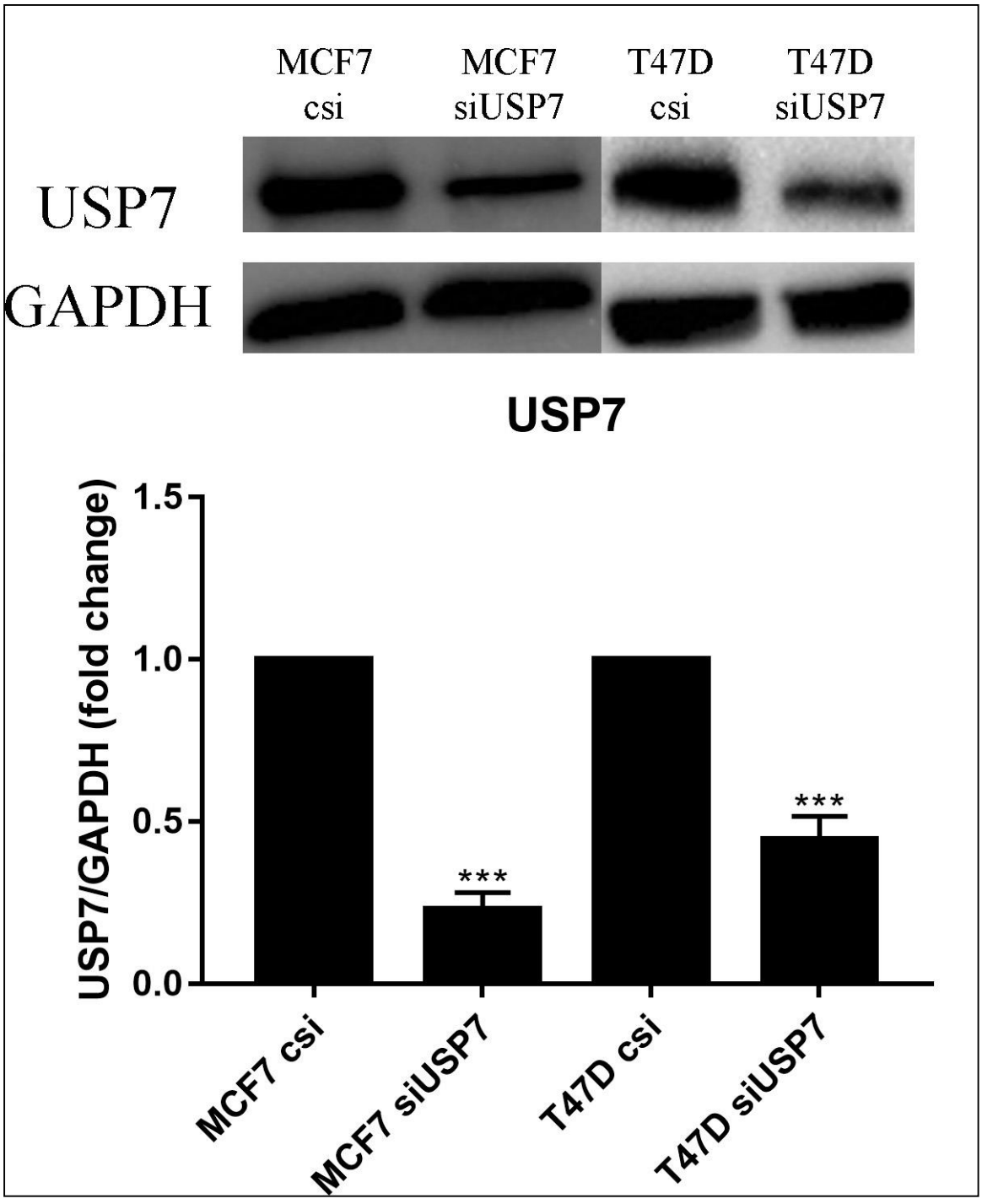


Figure 3.14. Total USP7 protein levels of MCF7 and T47D cell lines after siRNA applicaiton. GAPDH antibody was used as a loading control. ***P<0.001. Results were analyzed by one-way ANOVA and Tukey's test using GraphPad Prism Version 7.00. csi: control small interfering RNA.

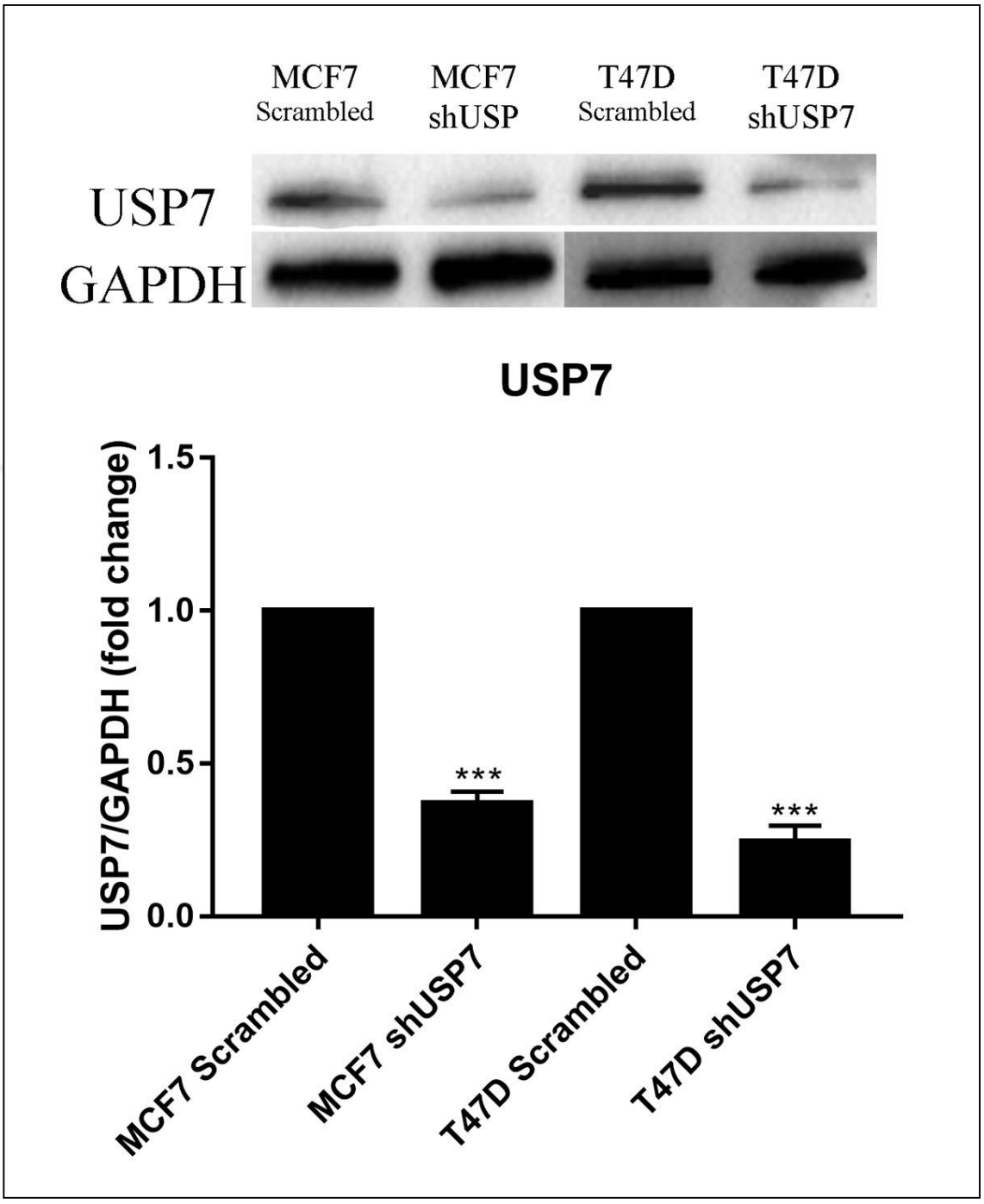


Figure 3.15. Total USP7 protein levels of MCF7 and T47D cell lines after lentiviral application of shUSP7. GAPDH antibody was used as a loading control. ***P<0.001. Results were analyzed by one-way ANOVA and Tukey's test using GraphPad Prism Version 7.00.

3.4. SCRATCH ASSAY

To determine the effect of de-ubiquitinase USP7 on cell migration, scratch (*in vitro* cell migration) assay was performed. Obtained results clearly highlight the deactivation of USP7 protein with its allosteric inhibitor, p5091; gap closure capability of T47D cells has displayed a decrease of 95 per cent (Figure 3.16). Additionally, figure 3.17 clearly shows that P5091 has a similar effect on MCF7 cell line (87.5 per cent decrease was observed). Besides, compared to control siRNA group, siUSP7 application also decreased the wound healing capacity of T47D (Figure 3.18) and MCF7 (Figure 3.19) cell lines by 66 per cent and 64 per cent respectively. As figure 3.20 and 3.21 respectively demonstrates, silencing of USP7 protein by lentiviral shUSP7 plasmid caused a lost in the ability of wound closure, belonging to T47D cell line by 85 per cent and MCF7 by 72 per cent.

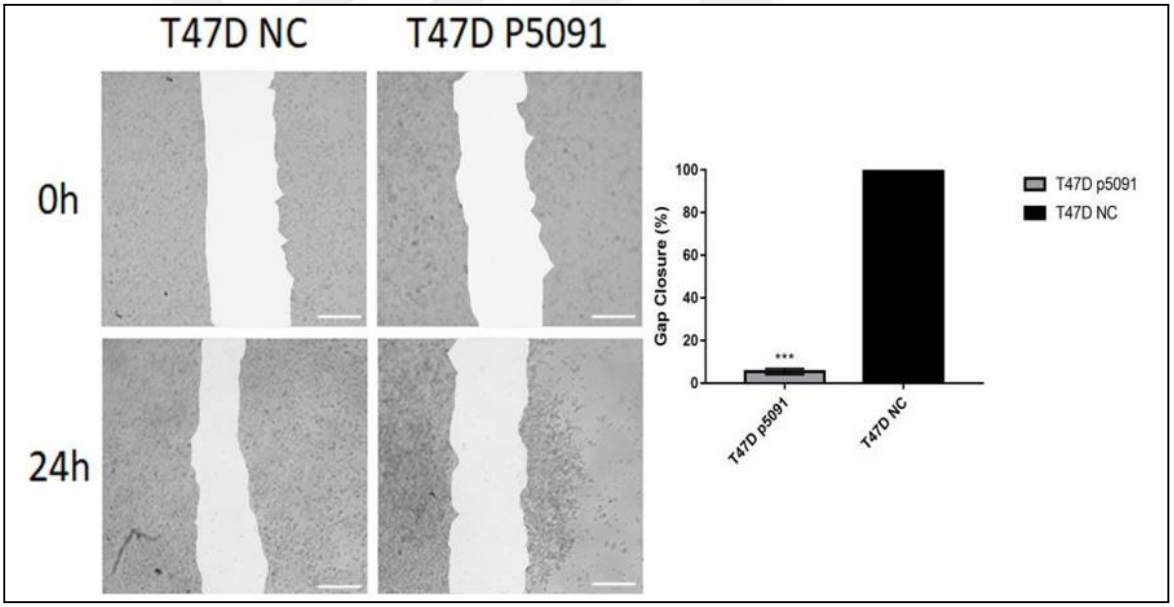


Figure 3.16. Effect of USP7 inhibitor, p5091, on T47D cell line's migration capacity. *In vitro* scratch assay was performed to determine wound healing efficiency**.** ****P*<0.001. Results were analyzed by one-way ANOVA and Tukey's test using GraphPad Prism Version 7.00. NC: negative control.

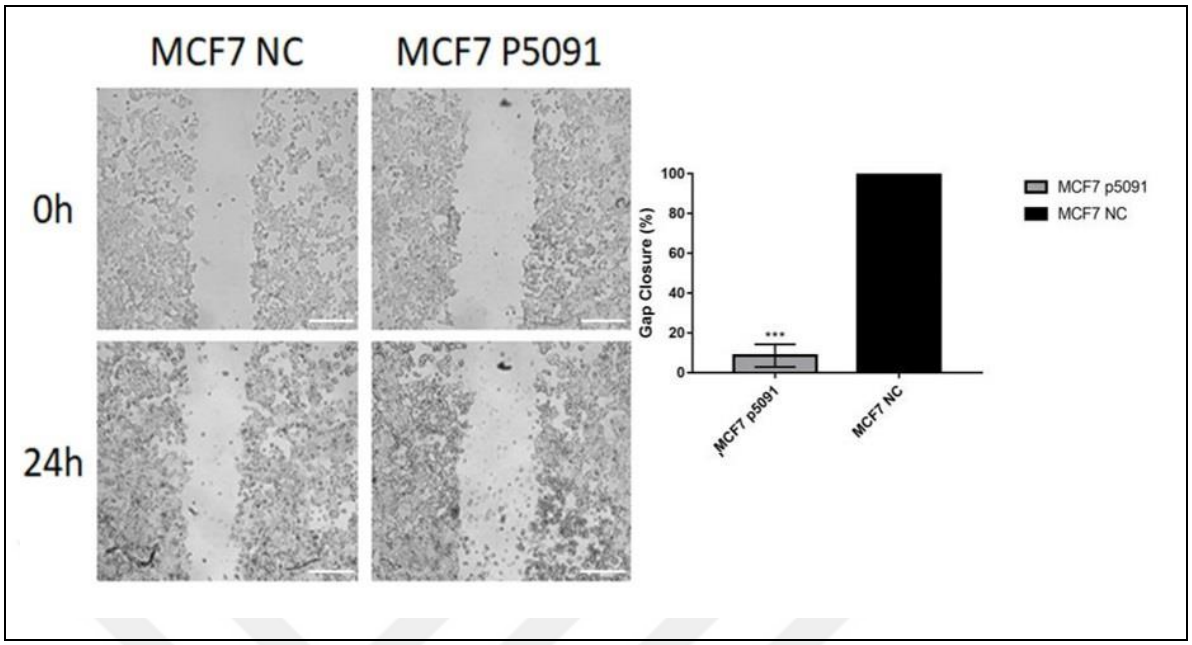


Figure 3.17. Effect of USP7 inhibitor, p5091, on MCF7 cell line's migration capacity. *In vitro* scratch assay was performed to determine wound healing efficiency**.** ****P*<0.001. Results were analyzed by one-way ANOVA and Tukey's test using GraphPad Prism Version 7.00. NC: negative control.

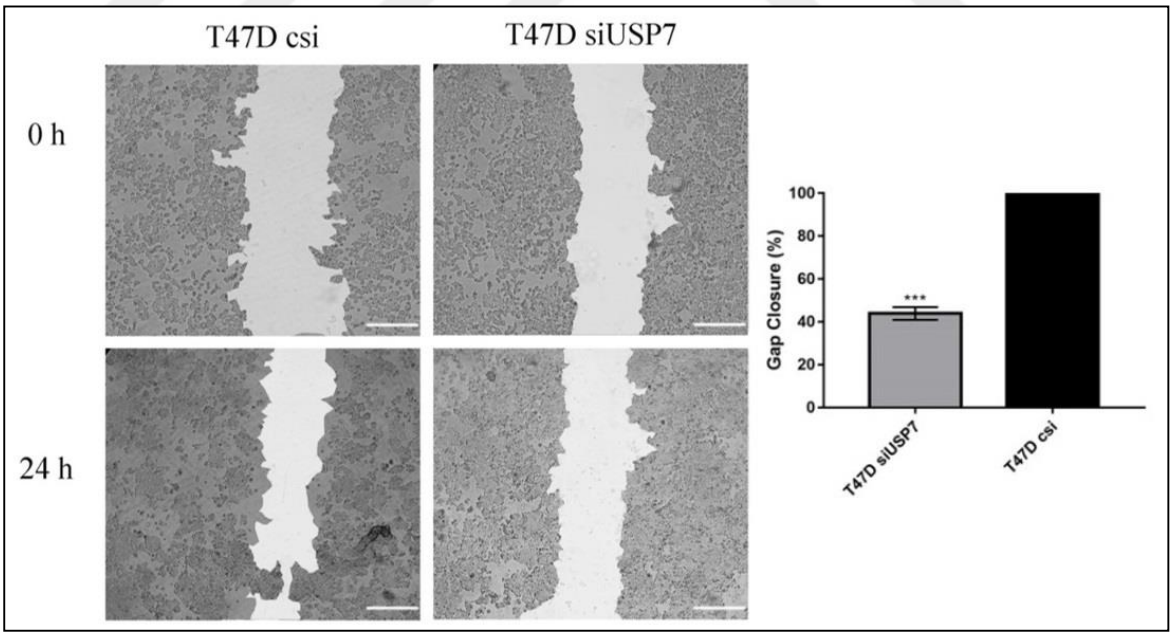


Figure 3.18. Effect of USP7 specific siRNA, siUSP7, on T47D cell line's migration capacity. *In vitro* scratch assay was performed to determine wound healing efficiency. ***P<0.001. Results were analyzed by one-way ANOVA and Tukey's test using GraphPad Prism Version 7.00. csi: control small interfering RNA.

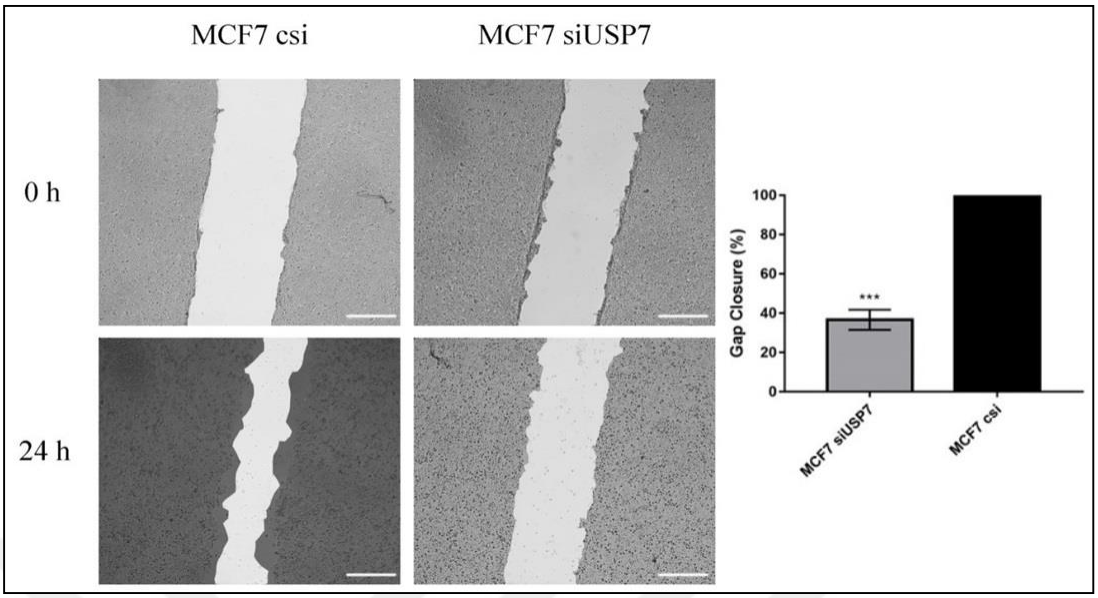


Figure 3.19. Effect of USP7 specific siRNA, siUSP7, on MCF7 cell line's migration capacity. *In vitro* scratch assay was performed to determine wound healing efficiency. ***P<0.001. Results were analyzed by one-way ANOVA and Tukey's test using GraphPad Prism Version 7.00. csi: control small interfering RNA.

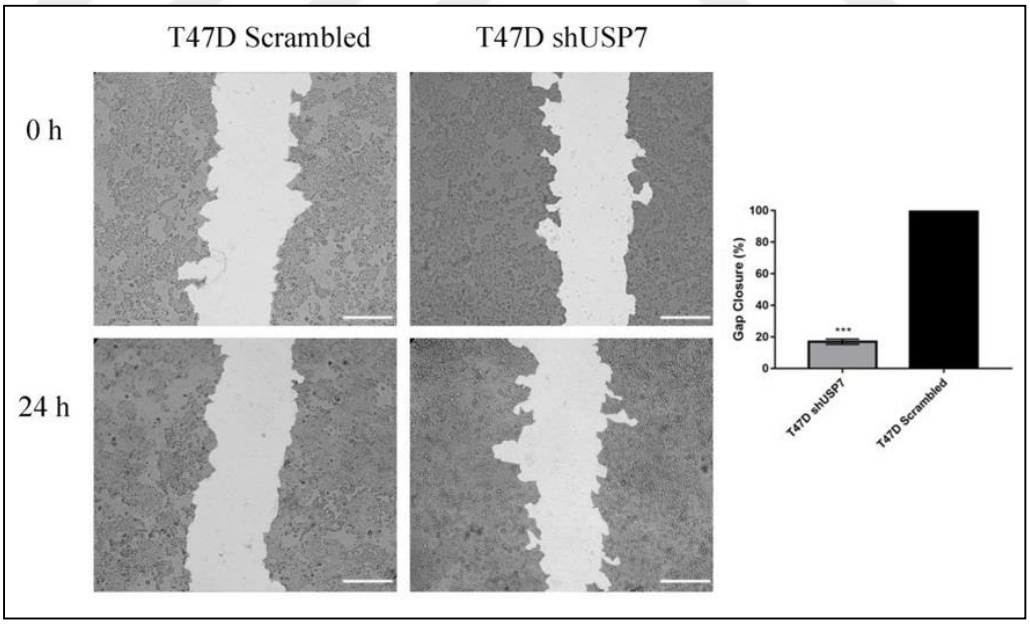


Figure 3.20. Effect of USP7 specific shRNA, shUSP7, on T47D cell line's migration capacity. *In vitro* scratch assay was performed to determine wound healing efficiency. **P<0.001. Results were analyzed by one-way ANOVA and Tukey's test using GraphPad Prism Version 7.00.

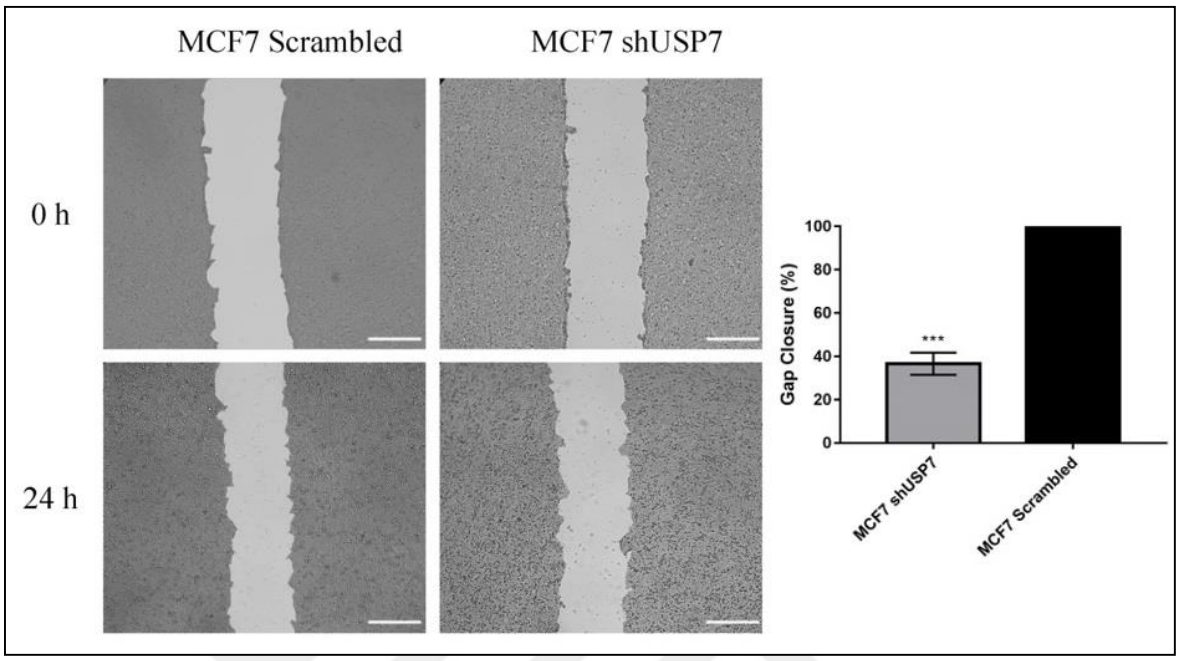


Figure 3.21 Effect of USP7 specific shRNA , shUSP7, on MCF7 cell line's migration capacity. *In vitro* scratch assay was performed to determine wound healing efficiency. ***P<0.001. Results were analyzed by one-way ANOVA and Tukey's test using GraphPad Prism Version 7.00.

3.5. COLONY FORMING UNIT ASSAY (CFU)

CFU assay was performed to identify the shift in colony forming ability of the cancer cells before and after changing the activation statues of USP7 in the cells. The results revealed that when T47D and MCF7 cells have functional USP7 protein, they can form a colony as usual. Nevertheless, de-activation of USP7 by an allosteric inhibitor in T47D cell line, was resulted in the absence of colonies in two experiments out of three. Two colonies were observed in one of the biological replicates. De-activation of USP7 protein not only significantly decreased the number of the colonies but also shrank the volume of colonies by approximately 85 per cent (Figure 3.22). Although MCF7 cells were formed dozens of colonies, they also were detected decreased in diameter (≈45 per cent shrank) and number of colonies that were formed dramatically decreased from 420 to 11 (Figure 3.23). USP7 knock-out was also showed significant difference in CFU amount and diameters of colonies.

Amount of colonies were decreased from 15 to 2 for MCF7 cell. On the other hand, diameter of colonies was decreased from 60 to 12 (Figure 3.24). Similar effect was observed in T47D cell line as well. Diameter of colonies was decreased 50 per cent and number of colonies was decreased from 210 to 13 (Figure 3.25). However, there was no significant change observed after transient downregulation of USP7 in MCF7 (Figure 3.26) and T47D (Figure 3.27).

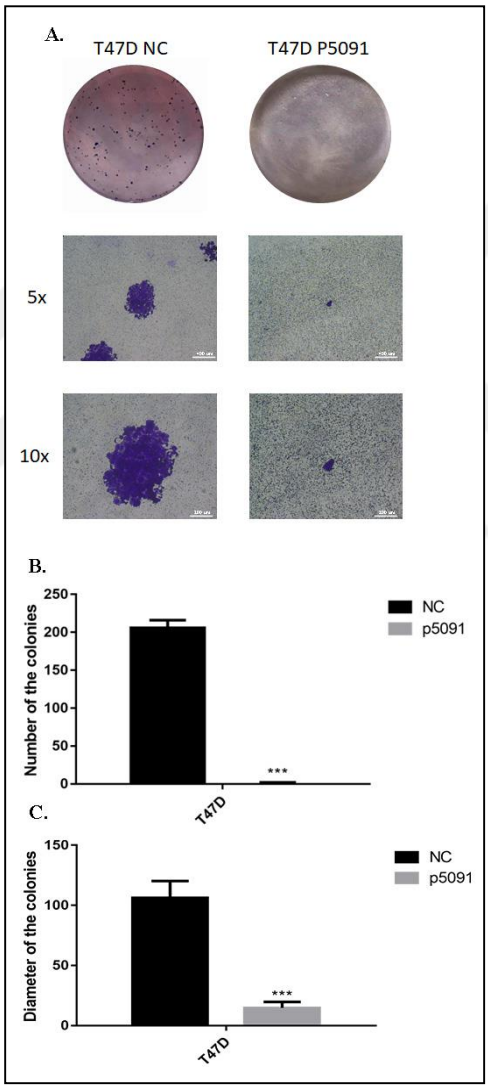


Figure 3.22. CFU assay was carried out to demonstrate the effect of p5091 on T47D cell line in terms of colony forming capability. A. Colony numbers and sizes taken by the magnification of 1x, 5x and 10x. B. Histogram that shows the change in number of the colonies in millimeters. C. Bar graph of diameter change in millimeters***P<0.001. Results were analyzed by one-way ANOVA and Tukey's test using GraphPad Prism Version 7.00.

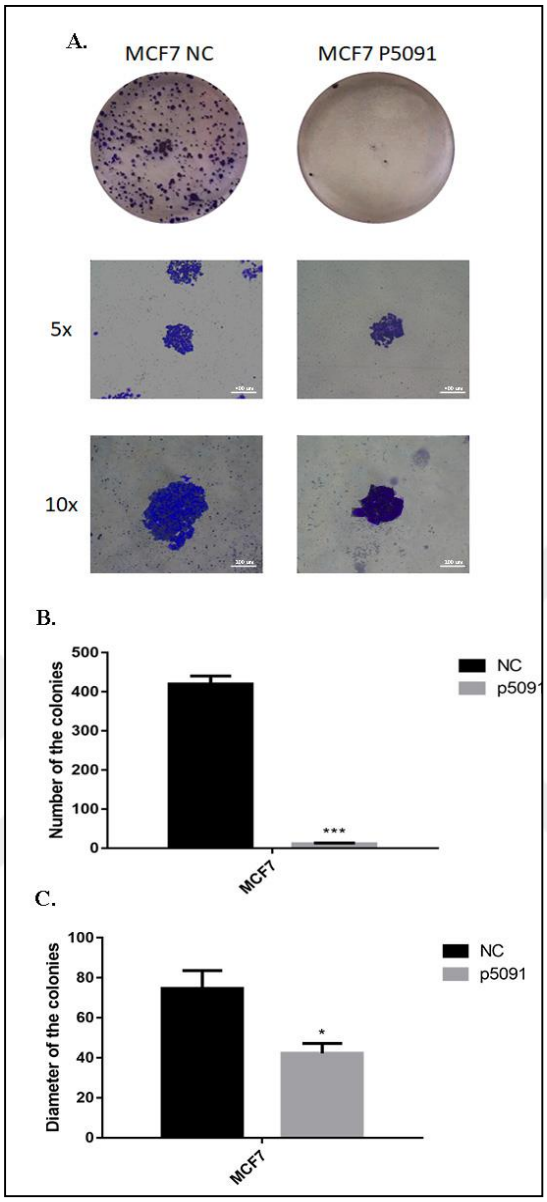


Figure 3.23. CFU assay was carried out to demonstrate the effect of p5091 on MCF7 cell line in terms of colony forming capability. A. Colony numbers and sizes taken by the magnification of 1x, 5x and 10x. B. Histogram that shows the change in number of the colonies in millimeters. C. Bar graph of diameter change in millimeters. ***P<0.001. Results were analyzed by one-way ANOVA and Tukey's test using GraphPad Prism Version 7.00.

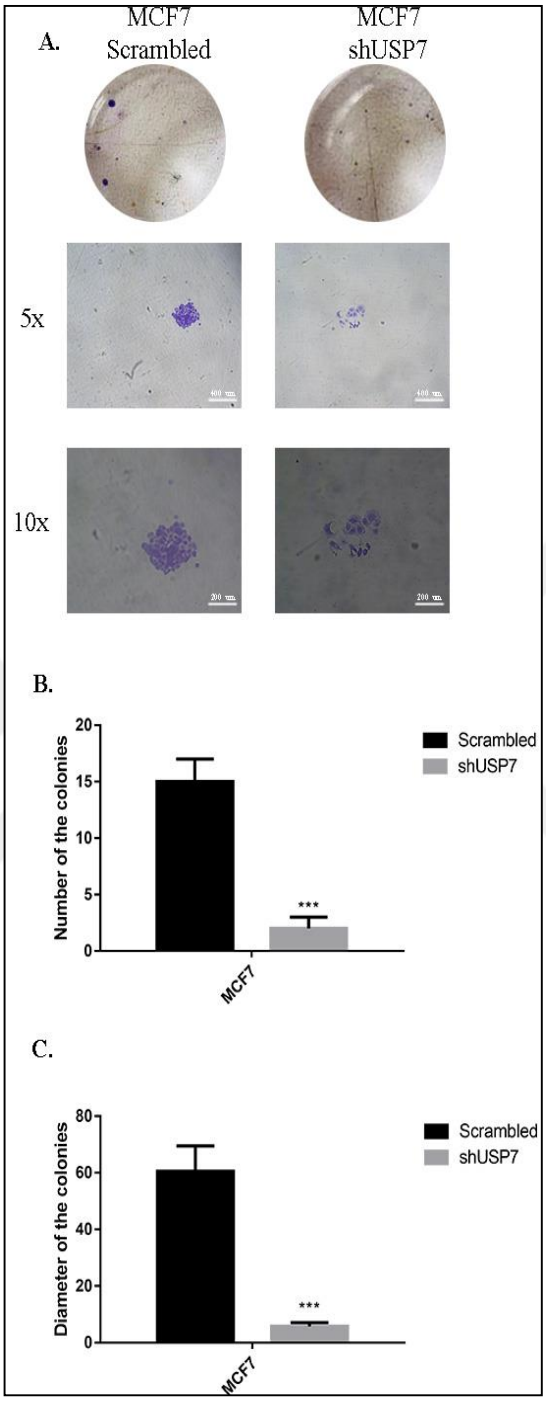


Figure 3.24. CFU assay was carried out to demonstrate the effect of shUSP7 on MCF7 cell line in terms of colony forming capability. A. Colony numbers and sizes taken by the magnification of 1x, 5x and 10x. B. Histogram that shows the change in number of the colonies in millimeters. C. Bar graph of diameter change in millimeters. ***P<0.001. Results were analyzed by one-way ANOVA and Tukey's test using GraphPad Prism Version 7.00.

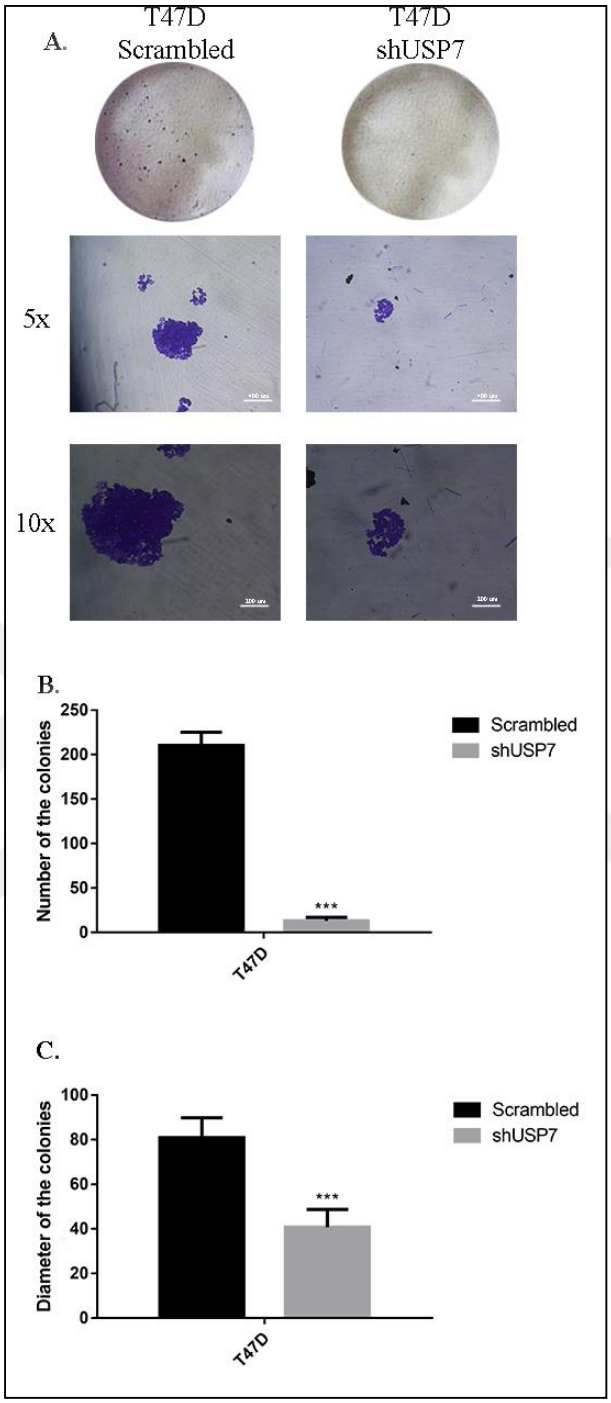


Figure 3.25. CFU assay was carried out to demonstrate the effect of shUSP7 on T47D cell line in terms of colony forming capability. A. Colony numbers and sizes taken by the magnification of 1x, 5x and 10x. B. Histogram that shows the change in number of the colonies in millimeters. C. Bar graph of diameter change in millimeters. ***P<0.001. Results were analyzed by one-way ANOVA and Tukey's test using GraphPad Prism Version 7.00.

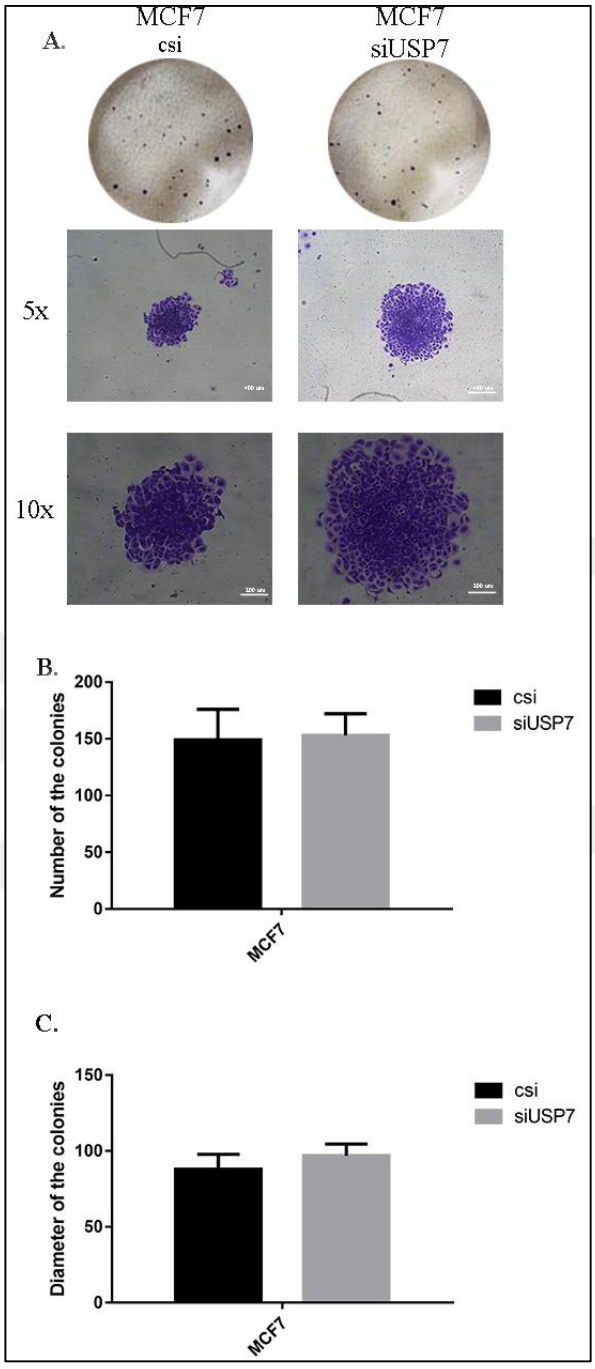


Figure 3.26. CFU assay was carried out to demonstrate the effect of siUSP7 on MCF7 cell line in terms of colony forming capability. A. Colony numbers and sizes taken by the magnification of 1x, 5x and 10x. B. Histogram that shows the change in number of the colonies in millimeters. C. Bar graph of diameter change in millimeters. ***P<0.001. Results were analyzed by one-way ANOVA and Tukey's test using GraphPad Prism Version 7.00. csi: control small interfering RNA.

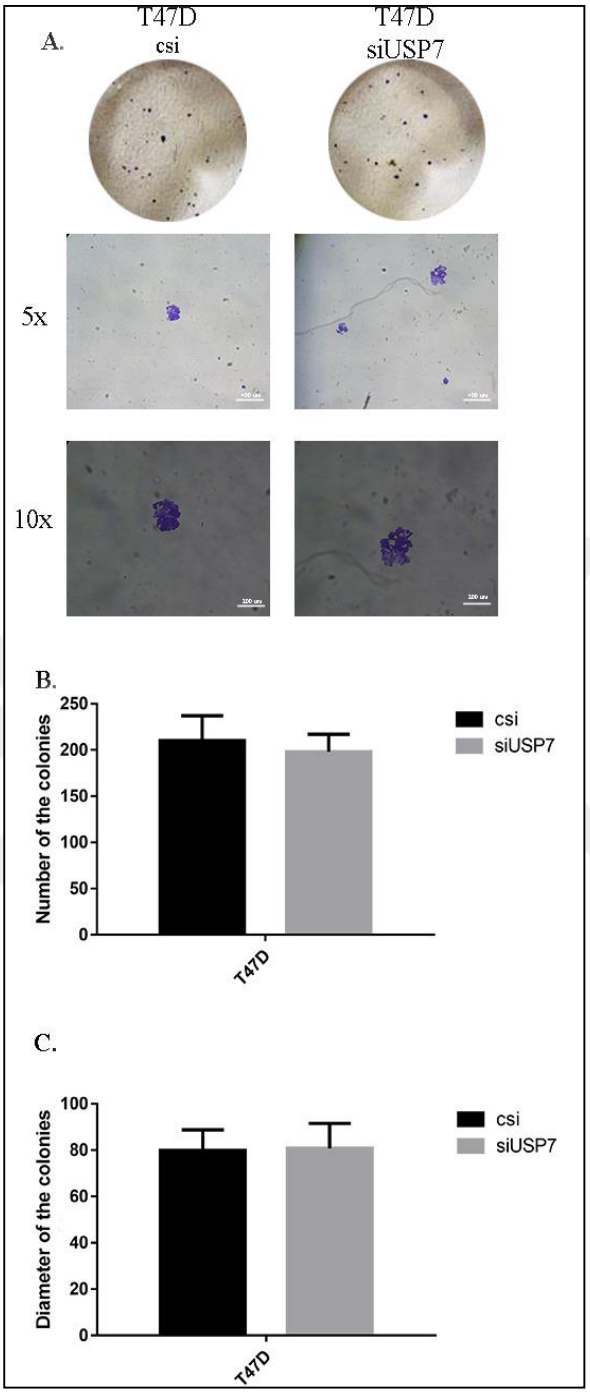


Figure 3.27. CFU assay was carried out to demonstrate the effect of siUSP7 on T47D cell line in terms of colony forming capability. A. Colony numbers and sizes taken by the magnification of 1x, 5x and 10x. B. Histogram that shows the change in number of the colonies in millimeters. C. Bar graph of diameter change in millimeters. ***P<0.001. Results were analyzed by one-way ANOVA and Tukey's test using GraphPad Prism Version 7.00. csi: control small interfering RNA.

3.6. CELL CYCLE

To investigate possible effects of P5091 on cell cycle, DNA content of the cells were stained with propidium iodide (PI) after fixation and permeabilization steps. Changed cell cycle profile was observed after P5091 treatment. Although S phase of T47D cells were barely changed, P5091 treatment was significantly increased the amount of arrested cells at G1 phase 62.95 per cent for control, 77.4 per cent for treated cells). Moreover, G2-M phase of the cells was dramatically decreased at P5091 treated cells (7.89 per cent), compared to control cells (17.62 per cent) (Figure 3.28). Similarly, for MCF7 cells, P5091 treatment caused the accumulation of cells at G1 phase (69.82 per cent for control, 81.36 per cent for P5091 treated cells). It has also cleared that S phase of untreated MCF7 (17.15 per cent) was significantly abated (Figure 3.29).

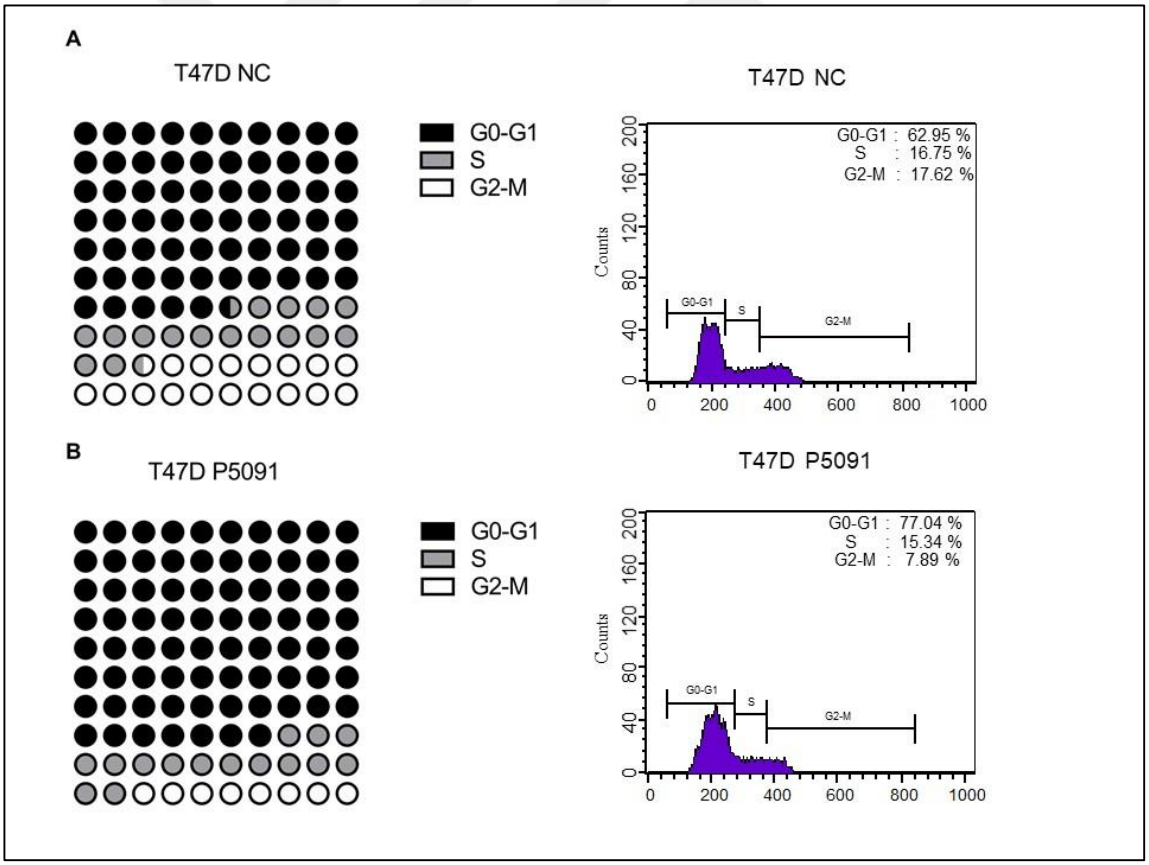


Figure 3.28. Part of a whole graph of the P5091's effect on cell-cycle profile of T47D cells. P5091 led to G1 arrest in T47D cell line. A. Non-treated T47D cell line's cell-cycle profile. B. T47D cells which were treated with 10 μM P5091.

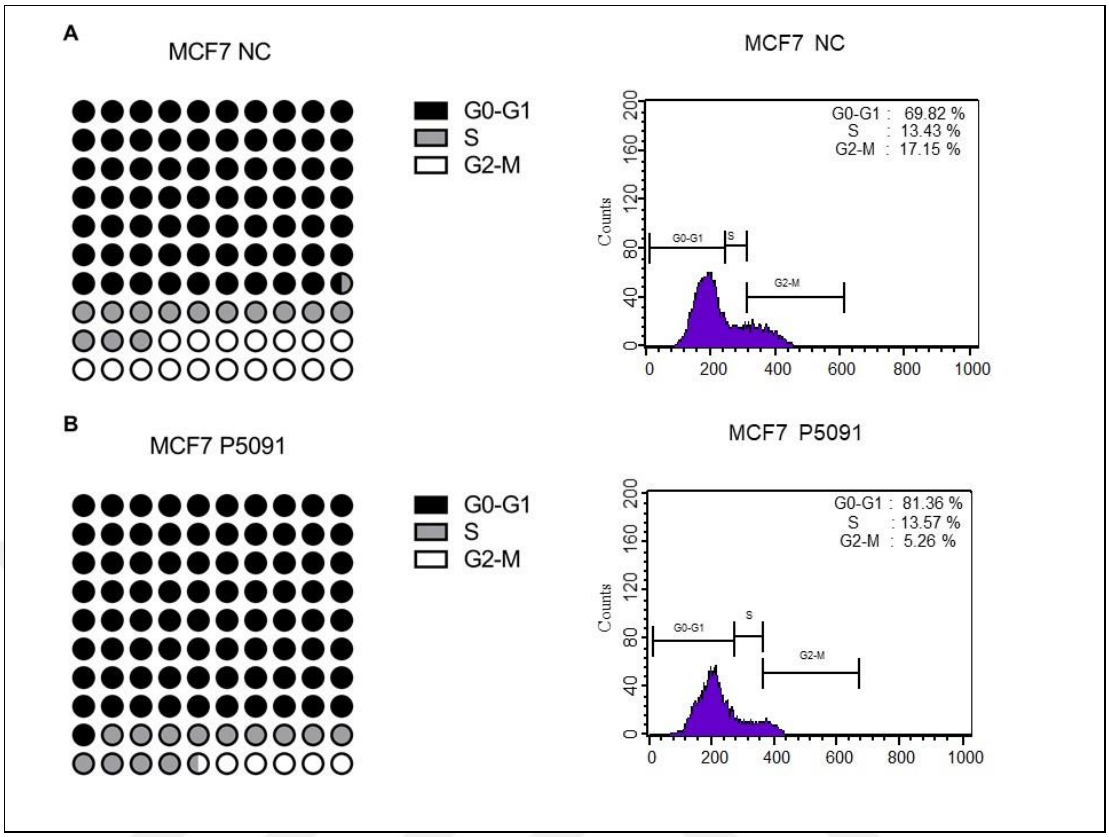


Figure 3.29. Part of a whole graph of the P5091's effect on cell-cycle profile of MCF7 cells. P5091 led to G1 arrest and shortened G2-M phase significantly in MCF7 cell line. A. Non-treated MCF7 cell line's cell-cycle profile. B. MCF7 cells which were treated with 10 μM P5091.

Altered cell cycle profile was also found in siUSP7 treated MCF7 and T47D cell lines. For MCF7 cells, the percentage of G0-G1 was observed 68.63 per cent after control siRNA. 16.33 per cent of the MCF7 cells were at S phase and 15.44 per cent of them were at G2 or M phase. Silencing of USP7 *via* siUSP7 chanced the cell cycle profile into 74.11 per cent cells at G0-G1, 18.14 per cent cells at S and 8.17 per cent cells at G2-M phase. It is obviously seen that in MCF7, siUSP7 application arrested the cells at G0-G1 phase and block them to go through S phase (Figure 3.30). Similar effect was observed in T47D as well. G1 phase were expanded from 76.78 per cent to 82.34 per cent. Extended S phase was found as well (from 10.06 per cent to 13.19 per cent). G2-M phase in T47D was significantly decreased from 13.50 per cent to 4.62 per cent (Figure 3.31).

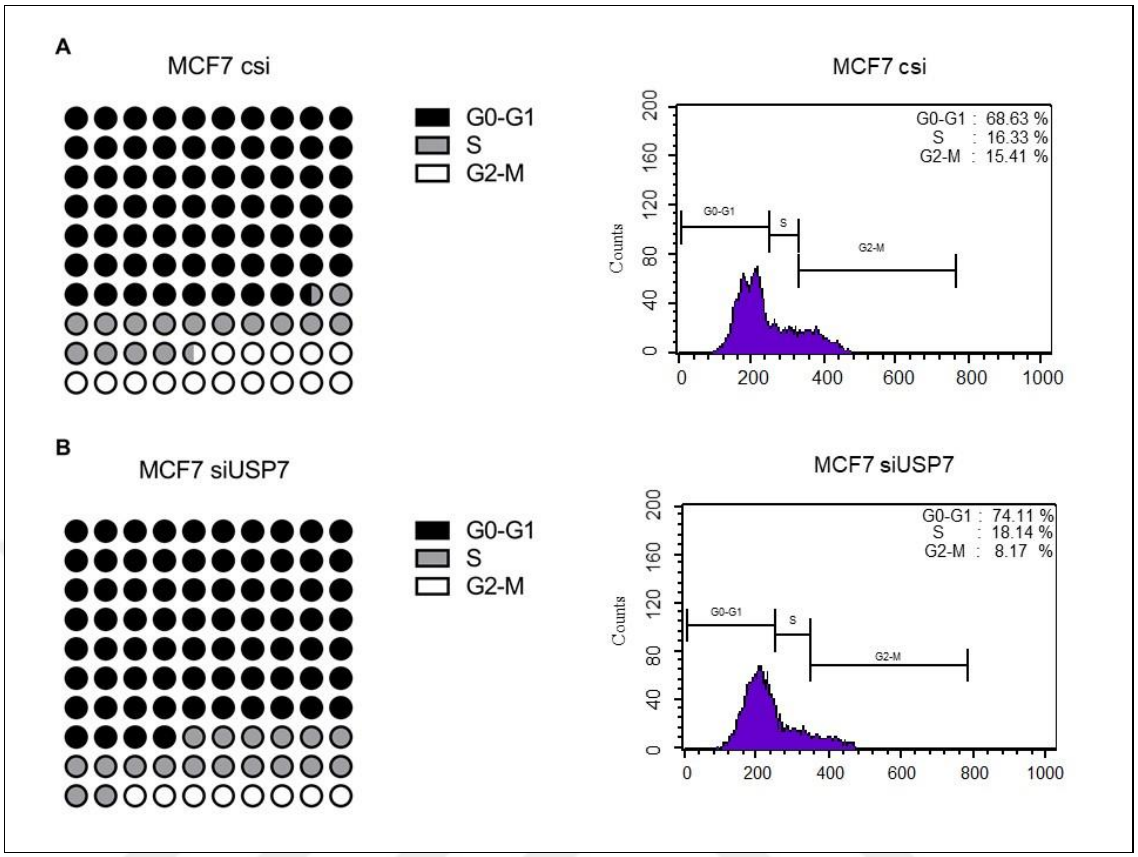


Figure 3.30. Part of a whole graph of the siUSP7's effect on cell-cycle profile of MCF7 cells. siUSP7 led to G0-G1 arrest and diminished G2-M phase significantly in MCF7 cell line. A. Non-treated MCF7 cell line's cell-cycle profile. B. MCF7 cells which were treated with siUSP7. csi: control small interfering RNA.

Lentiviral silencing of USP7 by shUSP7 plasmid had no significant change in MCF7's cell cycle profile compared to scrambled plasmid (Figure 3.32). Nonetheless, in T47D, G0-G1 phase was increased by 3 per cent, S phase found to be decreased by 1 per cent and G2-M phase decreased by approximately 2 per cent (Figure 3.33).

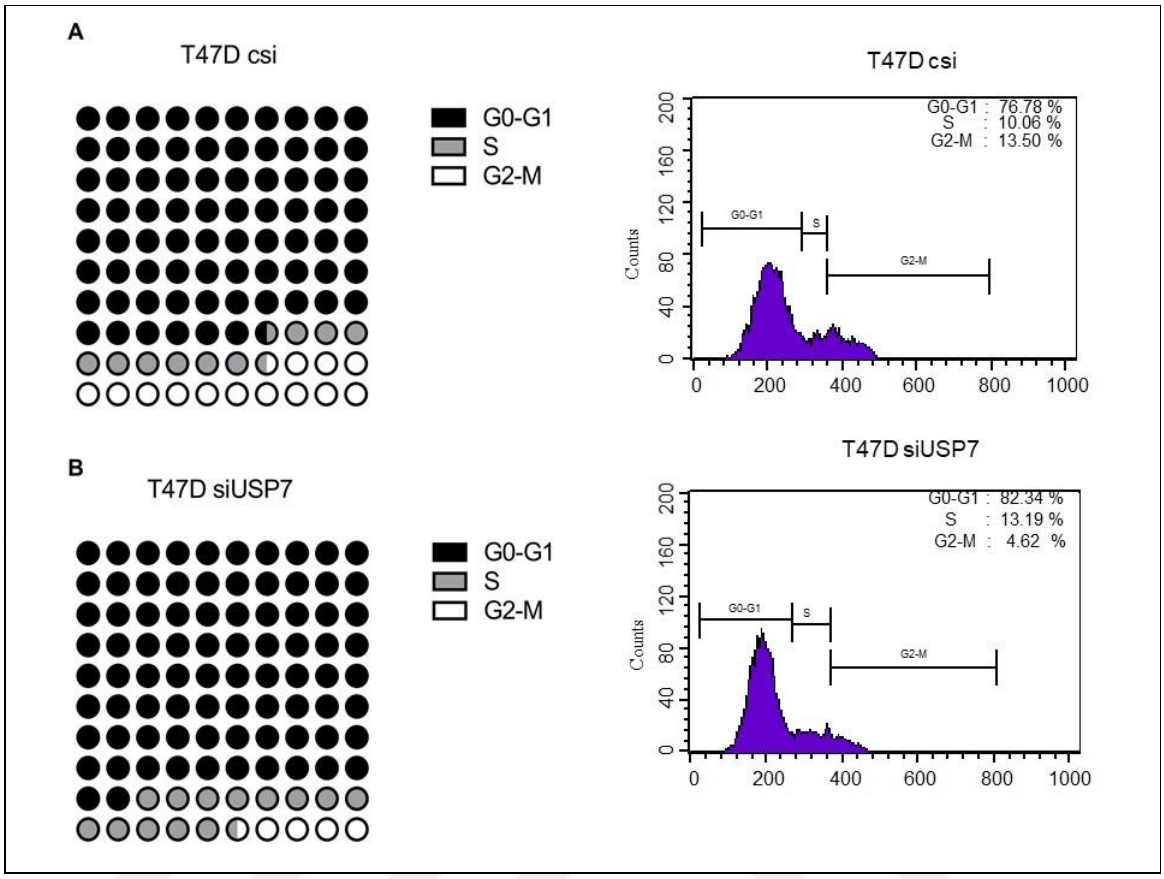


Figure 3.31. Part of a whole graph of the siUSP7's effect on cell-cycle profile of T47D cells. siUSP7 led to G0-G1 arrest and diminished G2-M phase significantly in MCF7 cell line. A. Non-treated MCF7 cell line's cell-cycle profile. B. MCF7 cells which were treated with siUSP7. csi: control small interfering RNA.

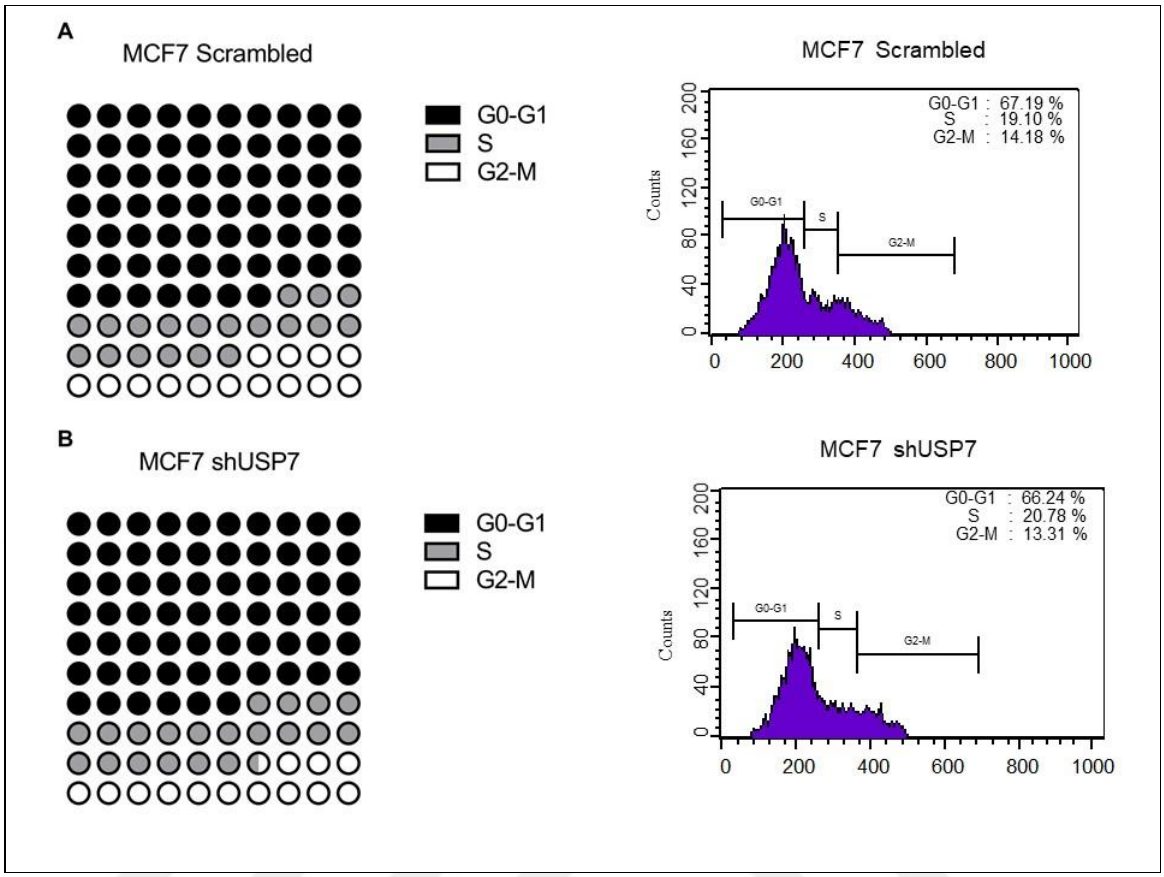


Figure 3.32. Part of a whole graph of shUSP7's effect on cell-cycle profile on MCF7 cell line. A. Non-treated MCF7 cell line's cell-cycle profile. B. MCF7 cells which were treated with siUSP7.

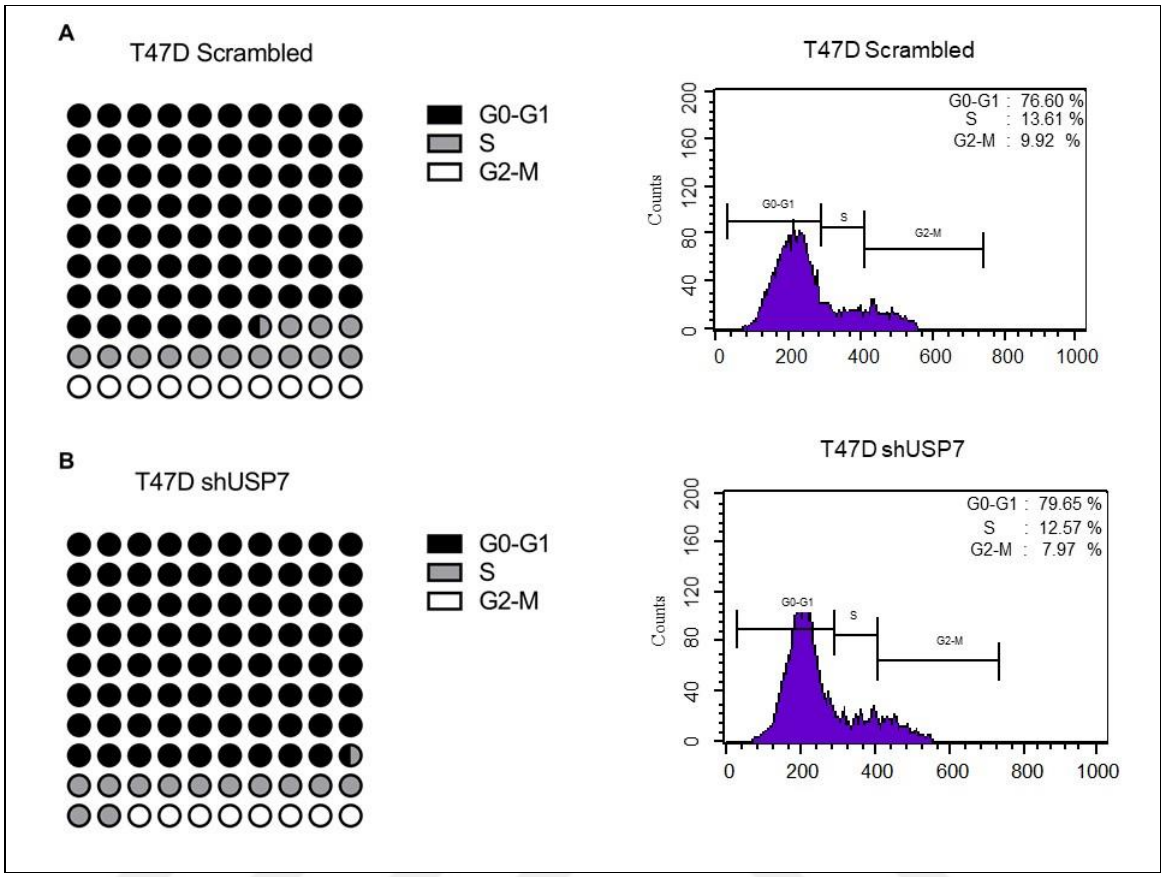


Figure 3.33. Part of a whole graph of shUSP7's effect on cell-cycle profile on T47D cell line. A. Non-treated MCF7 cell line's cell-cycle profile. B. MCF7 cells which were treated with siUSP7.

3.7. SPHERE FORMATION ASSAY

In order to identify the change in durability of the sphere formation, cells were treated with allosteric inhibitor, P5091. The results indicate that inhibition of USP7 activity in both MCF7 and T47D cell lines caused to form wrecked or no spheres at all (Figure 3.34). Under the USP7 inactivating conditions, formed spheres shrank in size by 58.82 per cent for MCF7 and 87.50 per cent for T47D cells (Figure 3.34).

The effect of USP7 on migration was evaluated by the examination of tumor dissemination potential depending on USP7 protein amount in the cell. To identify the changes in cancer migration, pre-formed spheres were transferred into polystyrene 48 well plates. The results were demonstrated that dissemination of the spheres was decreased dramatically for both MCF7 (Figure 3.35) and T47D (Figure 3.36) cells.

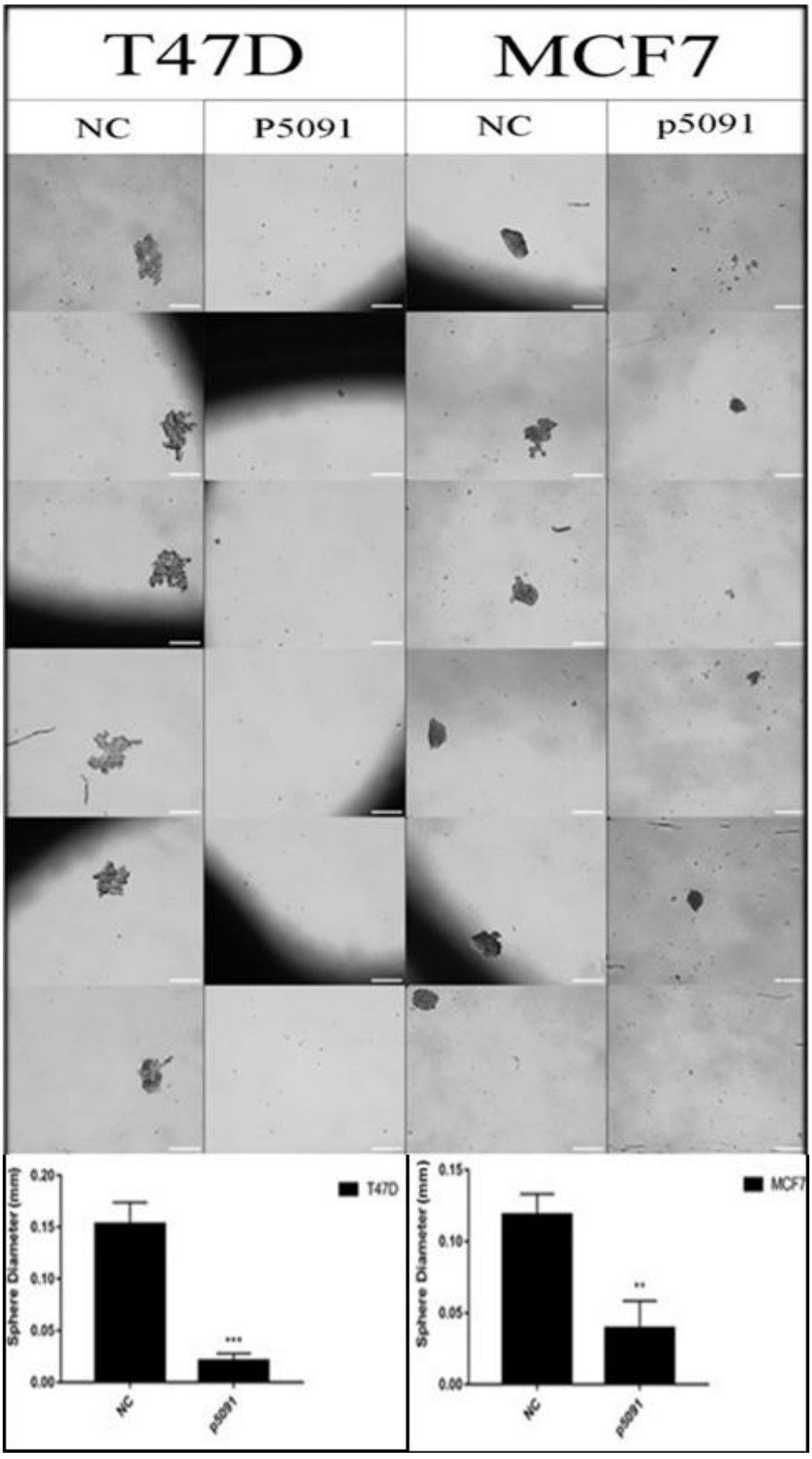


Figure 3.34. Sphere formation capacity of T47D and MCF7 cell lines with presence or absence of USP7 inhibitor, p5091. **P<0.01, ***P<0.001. Results were analyzed by one-way ANOVA and Tukey's test using GraphPad Prism Version 7.00. NC: negative control.

Inhibition of USP7 protein by p5091 harmed the migration capacity of both MCF7 and T47D cells. After 24 hours of treatment, formed spheres' diameters remarkably diminished. Comparing with negative control, MCF7 cells' spheres were 37.5 per cent smaller after 24 hours (Figure 3.37). Similarly, T47D cells formed nearly 45 per cent smaller spheres after 24 hours (Figure 3.38). 48 hours later the difference, between negative control and treatment group, was more obvious: 40 per cent smaller spheres for MCF7 (Figure 3.37) and 60 per cent smaller spheres for T47D (Figure 3.38) were observed. Likewise, correlated results were obtained after transient silencing of USP7 by siUSP7. Although, after 24 hours of treatment, there was no significant change of size in MCF7 cell spheres, 30 per cent decreased diameter of spheres were observed (Figure 3.37). T47D spheres were found to be effected by siUSP7 more. After 24 and 48 hours of treatment, 35 per cent and 46 per cent tinier spheres were observed, respectively (Figure 3.38). Lentiviral silencing of USP7 by shUSP7 plasmid also shrank the size of spheres compared to its control, scrambled plasmid transfection. After 24 hours both MCF7 (Figure 3.37) and T47D (Figure 3.38) formed remarkably smaller spheres (62 per cent smaller in MCF7, 55 per cent smaller in T47D). Eventually, 45 per cent smaller spheres were noticed in MCF7 after 48 hours (Figure 3.37) while T47D had 60 per cent smaller spheres (Figure 3.38). Furthermore, alterations in dissemination of MCF7 and T47D cell lines were determined *via* intensity measurements. Intensity profile of MCF7 (Figure 3.39) demonstrates that USP7 specific inhibitory molecule nearly doubled the intensity of the sphere. 32 per cent and 31 per cent increased intensity of a sphere profile was noticed in the groups that downregulates USP7 *via* siUSP7 and shUSP7, respectively (Figure 3.41). Correspondingly, intensity profile measurements of T47D (Figure 3.40) cleared the fact that siUSP7 application increased the sphere intensity by 44 per cent. Allosteric inhibitor of USP7 was found to be responsible for 32 per cent less disseminated T47D cells. The effect of lentiviral shUSP7 transfection, on T47D cells, was calculated as 15 per cent less capable of migration (Figure 3.41)

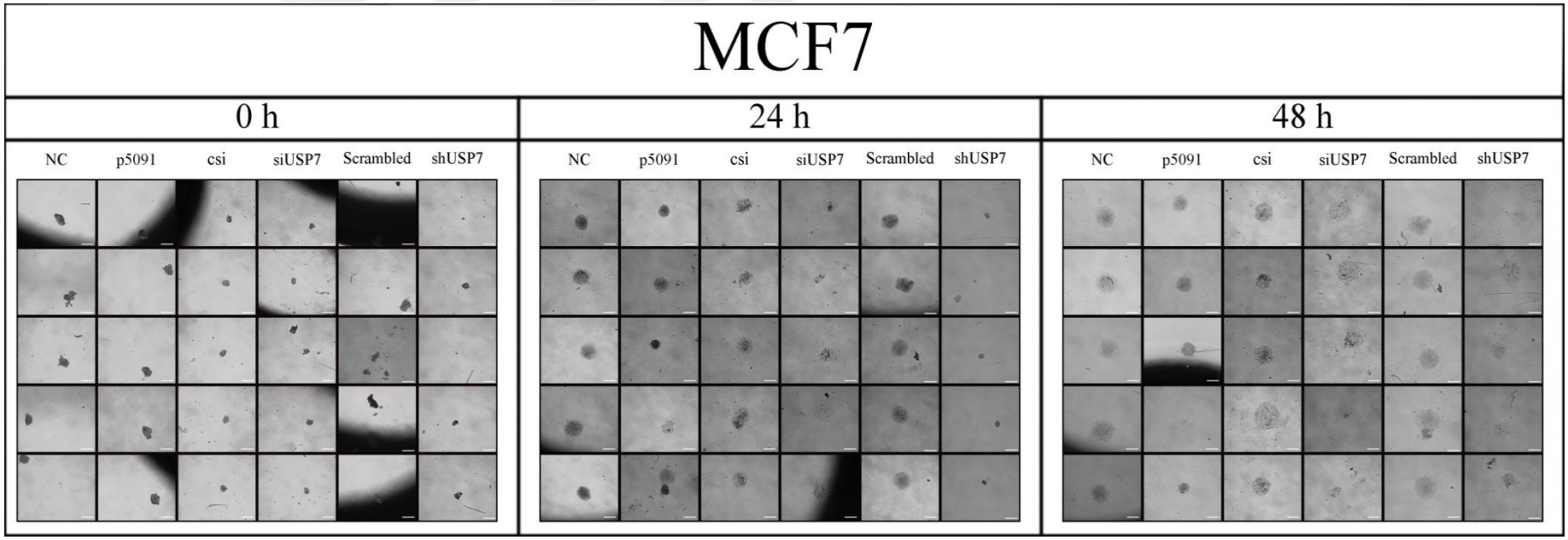


Figure 3.35. Effect of p5091, siUSP7 and shUSP7 on cell migration of MCF7 cell. All replicates were captured by fifty times magnified light microscopy. NC: negative control. csi: control small interfering RNA.

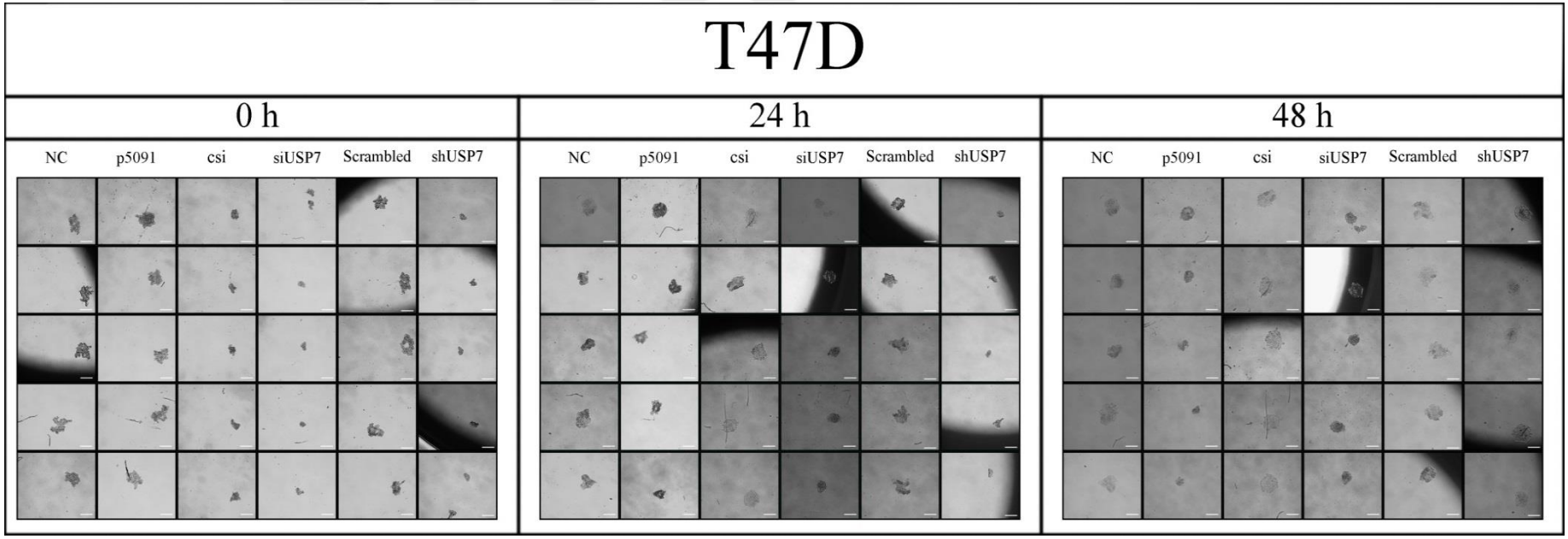


Figure 3.36. Effect of p5091, siUSP7 and shUSP7 on cell migration of T47D cell. All replicates were captured by fifty times magnified light microscopy. NC: negative control. csi: control small interfering RNA.

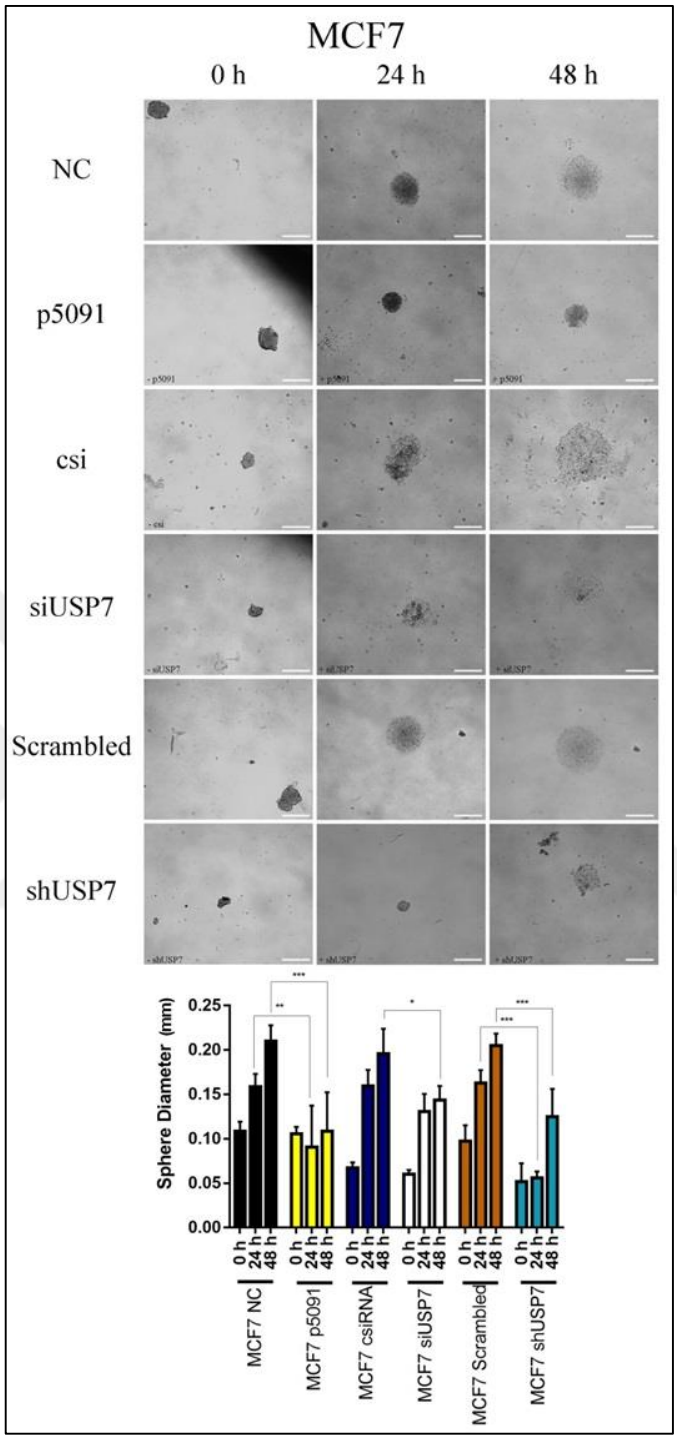


Figure 3.37. Close-up representation of p5091 siUSP7 and shUSP7 effect on cell migration of MCF7 cells. *P<0.5, **P<0.01, ***P<0.001. Results were analyzed by two-way ANOVA using GraphPad Prism Version 7.00. NC: negative control. csi: control small interfering RNA.

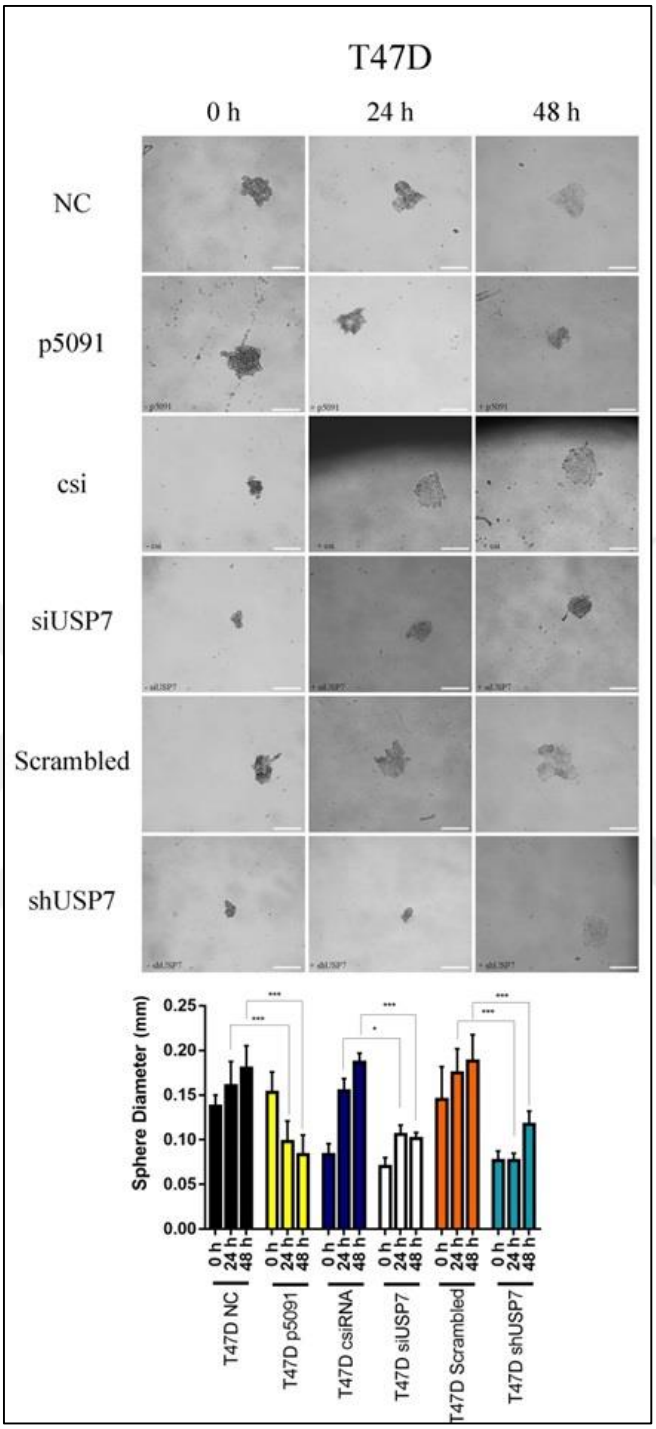


Figure 3.38. Close-up representation of p5091 siUSP7 and shUSP7 effect on cell migration of T47D cells. *P<0.5, **P<0.01, ***P<0.001. Results were analyzed by two-way ANOVA using GraphPad Prism Version 7.00. NC: negative control. csi: control small interfering RNA.

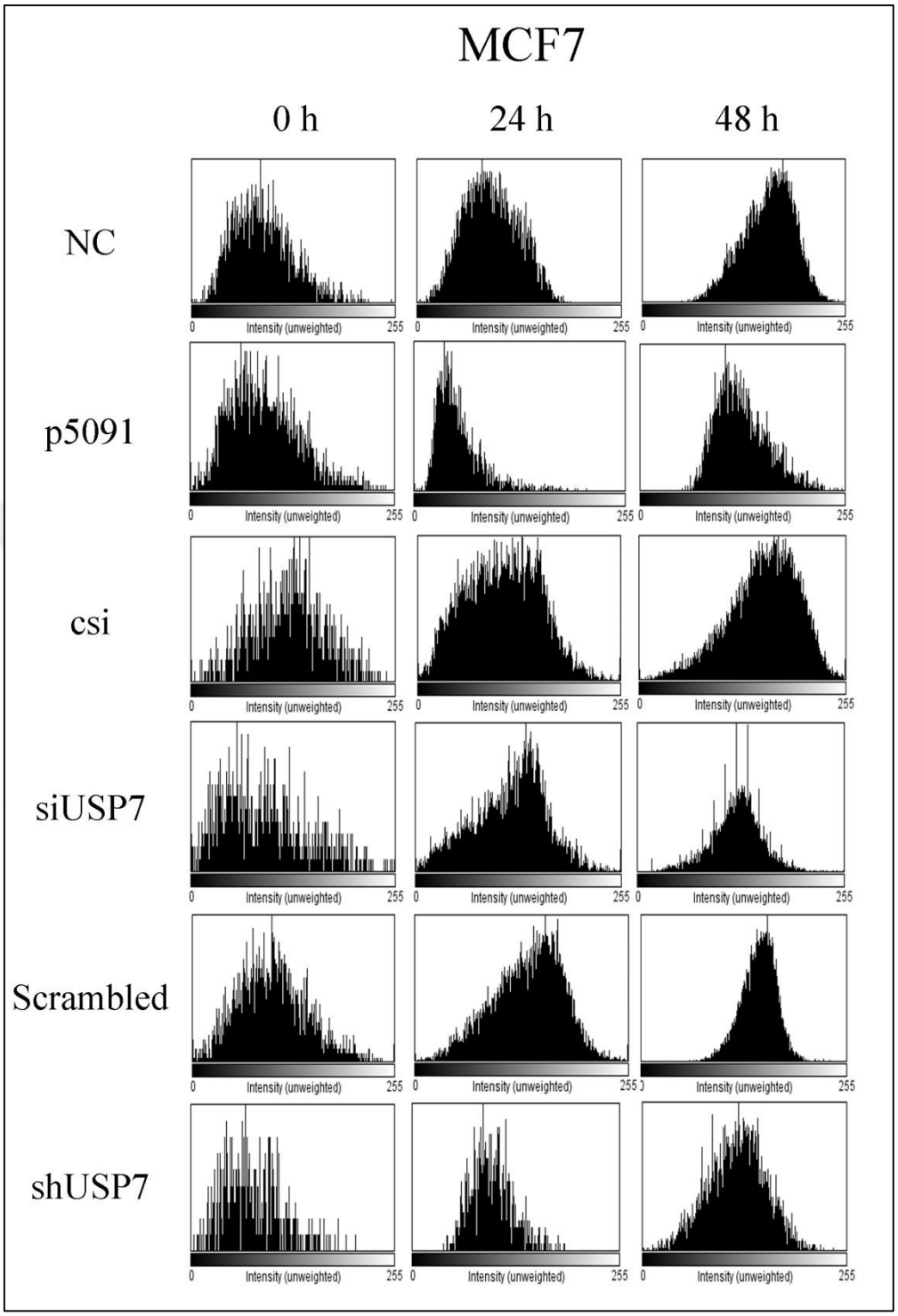


Figure 3.39. Histogramic display of intensity measurements of MCF7 spheres. Images were analyzed using ImageJ software. NC: negative control. csi: control small interfering RNA.

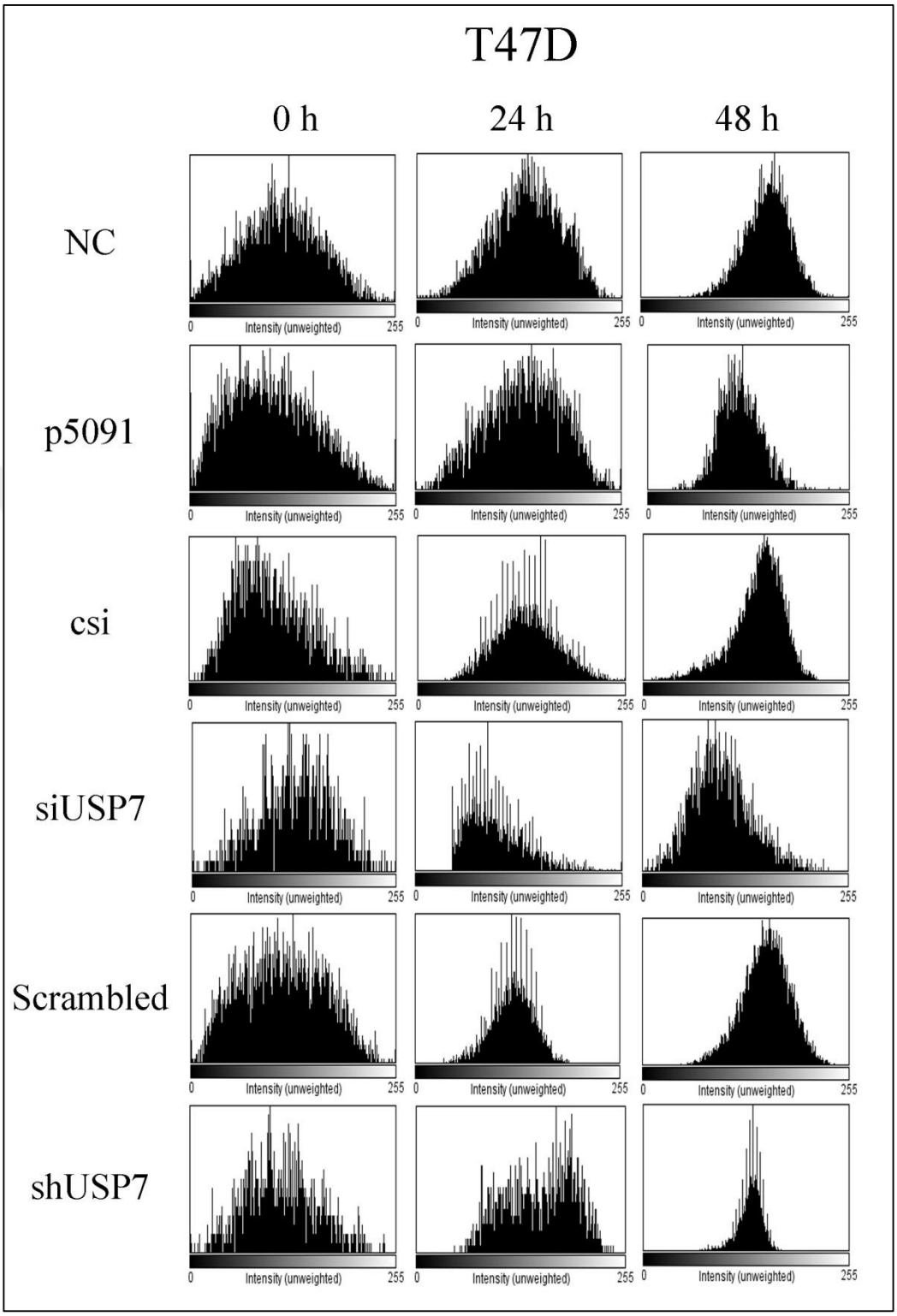


Figure 3.40. Histogramic display of intensity measurements of T47D spheres. Images were analyzed using ImageJ software. NC: negative control. csi: control small interfering RNA.

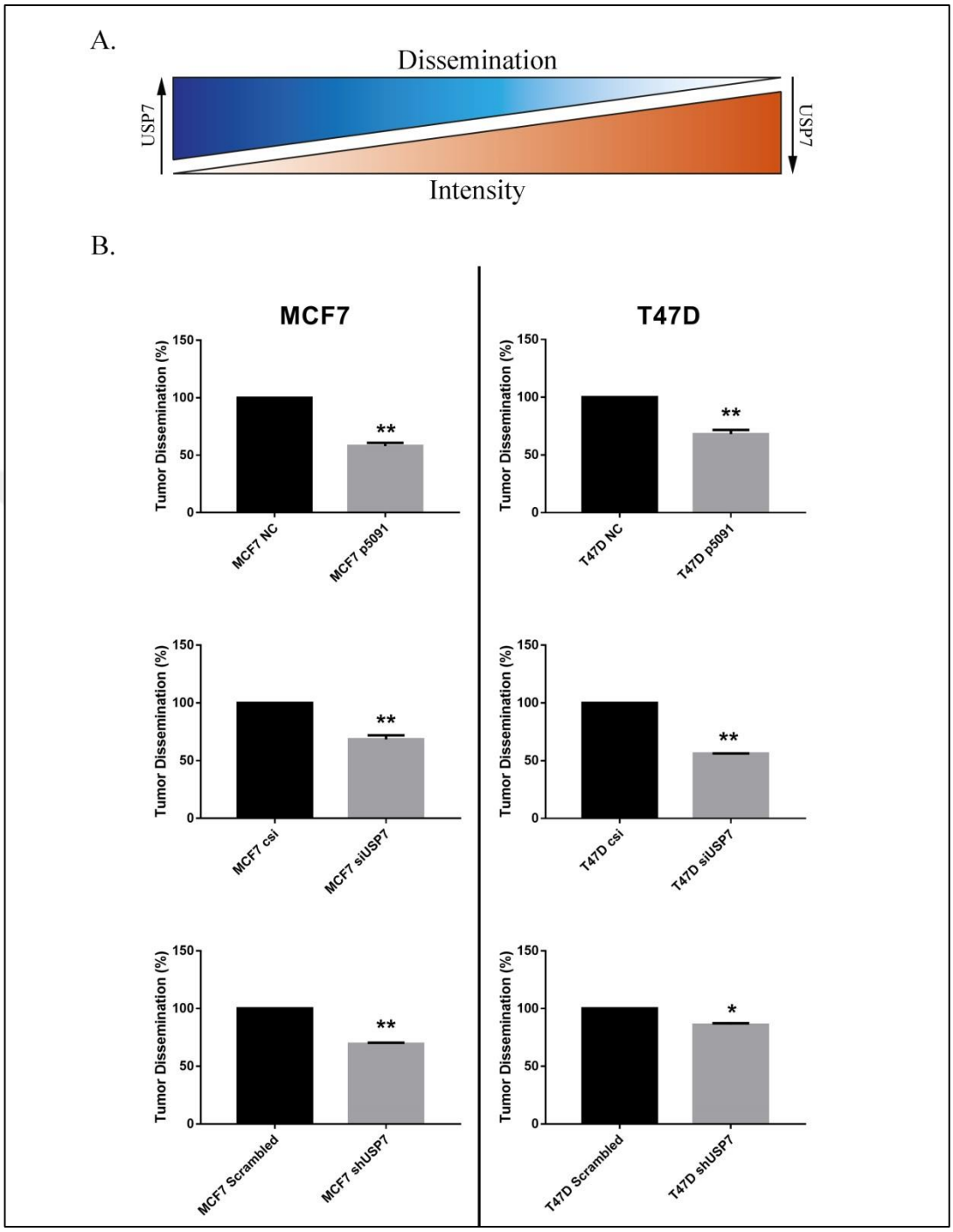


Figure 3.41. Effect of USP7 on tumor dissemination. A. Illustrated chart of the correlation between dissemination, intensity and USP7. B. Bar graphs of tumor dissemination changes with USP7's activity. Results were analyzed by one-way ANOVA and Tukey's test using GraphPad Prism Version 7.00. NC: negative control. csi: control small interfering RNA.

4. DISCUSSION

Cancer is a common disease diagnosed all around the globe. Some progression has been done to understand cancer's development, metabolism, molecular mechanism and progression. There are many questions waiting answers to develop new treatment options. Statistical analyses revealed the fact that cancer is the main reason for death in developing countries [193]. In 104 countries, located in North America, most of the Europe and Asia, northern parts of Africa, breast cancer can be classified as the most popular cancer, and can also be classified as the second most popular cancer in the rest of the world [194]. Unfortunately, mortality rates of breast cancer is high as well with 626,679 estimated deaths annually [194]. Breast cancer originates from either in glands, ducts, fat tissue or connective tissue [195].

Prevention, diagnosis, progression, treatment and therapeutic resistance are main limitations in the way to find a cure for breast cancer. Overcoming all limitations is arduous since breast cancer has heterogeneous phenotype in both clinical and molecular perspective [196, 197]. Alterations in molecular mechanisms can be related to familial heritage. Therefore, family history is important for breast cancer diagnosis. Mutations especially occurred in germline cells caused the spread of mutations into next generations. TP53, BRCA1 and BRCA2 mutations areobserved in the most of the breast cancer patients as well as their subfamilies [198]. Identification of differences in molecular mechanism has been a key instrument for diagnosis of phenotype, genotype and potential subtype classification of breast cancer [199]. There are numerous of studies explaining the importance of molecular mechanisms in clinical breast cancer development [200-202]. For instance, having a HER2 gene expression positive profile could be correlated with poor or good prognosis depending on the alterations in differentiation and proliferation gene expression levels [203]. Therefore, molecular approaches are gaining importance to increase treatment success and life expectation or quality of breast cancer patients.

Proteins has a limited life span with recycle process [204]. Recycling occurred by the process called "ubiquitination". Hershko and Ciechanover, discovered the recycling pathway [205] and awarded with the Nobel Prize for discovering this unique pathway. Aim of the whole ubiquitination pathway is to tag the proteins with ubiquitins [204]. Ubiquitination pathway involves in nearly every molecular mechanism as a regulatory

pathway. Most of the proteins have their feed-back mechanism through ubiquitination. For instance, well known transcriptional factor, p53 is responsible for increased levels of mdm2 protein. MDM2 gene encodes mdm2 protein which is a member of a superfamily called E3 ubiquitin ligases [40].

E3 ubiquitin ligases are responsible for transferring the ubiquitin to the desired protein to be marked. However, ubiquitination process is complicated and energy depended. Ubiquitin should become activated with adenosine triphosphate by E1 ubiquitin activating enzyme. Activated ubiquitin then transferred into E2 ubiquitin-conjugating enzyme family members [206]. Consequently, ubiquitin would be transferred to unwanted protein from E2 ubiquitin-conjugating enzyme with the help of E3 ubiquitin ligase. Recently, several studies have been concentrated on the inhibition of E3 ligases to rescue proteins from ubiquitination [207, 208]. Likewise, in the cell there are some proteins called de-ubiquitination enzymes (DUBs). DUBs are responsible for rescuing proteins from 26S proteasomal degradation [209]. Regulation of ubiquitination process is mainly conducted by DUBs since they do not only de-attach the ubiquitin tags from the protein, but also DUBs can block the ubiquitination sides of proteins [210].

The aim of current study was to identify a new therapeutic target for breast cancer treatment which focused on the molecular mechanisms of ubiquitination and de-ubiquitination. As it is known, ubiquitination mechanism is a crucial mechanism for the determination of the proteins fate. In this dissertation, activity of USP7 were tested on breast cancer cell lines (MCF7 and T47D) using small molecule inhibitor p5091, USP7 specific small interfering RNA and lentiviral transduction of short hairpin sequence of USP7.

To evaluate the effect of small molecule inhibitor on cell proliferation, a critical occurrence during tumorigenic progression, p5091 were tested on cell proliferation. Previous studies have demonstrated that p5091 affects cell proliferation of colon adenocarcinoma, rectal adenocarcinoma, colorectal carcinoma, ovarian cancer, multiple myeloma and prostate cancer cells, negatively [126, 211-213]. The effect of p5091 on breast cancer cell lines, regardless of their p53 mutation situations, was tested. MCF7 have wild type p53 while T47D have mutant p53 protein. IC_{50} value of p5091 was calculated as 10 μM as it was supported by the literature with various cancer types [211, 212]. Moreover, silencing oncogenes with specific siRNAs also perishes cancer cells significantly [214, 215]. Similarly, siUSP7 caused a decrease in cell proliferation of both MCF7 and T47D cell lines,

compared to control small interfering RNA. Obtained results indicates cell proliferation were negatively affected by lentiviral transduction of shUSP7, concordantly to literature [216].

Previous researches demonstrated that allosteric inhibition of a specific protein has some differences [217] from other regulatory mechanisms such as decreasing gene expression levels or blocking translational pathways [218]. However, allosteric inhibition of a protein blocks corresponding pathway with the ability of binding to protein of interest [217]. Therefore, obtained results have shown that allosteric inhibition of USP7 by p5091, affected other protein's gene expression levels rather than USP7 itself. Especially, gene expression of p53 was found to be increased in MCF7 cell line and decreased Akt and BCL2 levels were detected. It is known that decrease in anti-apoptotic proteins while increasing profile of apoptotic proteins were highly correlated with inhibition of a cell proliferation and cell death [219]. Additionally, increased BAX, caspase 3, p53 and decreased EGFR levels were observed for T47D cell line. USP7 gene expression levels did not change significantly. Gene expression analysis of both MCF7 and T47D cell lines suggested increased apoptosis levels in the cells after p5091 treatment. Increase of apoptosis after the inhibition of USP7 is also correlated with the literature [212, 220]. Transient silencing of USP7 was found to be more efficient in apoptosis. Kassler *et al.* showed that inhibition of USP7 by RNA interfering is responsible for some major alterations in different proteins that are participate in almost any cellular event, from cell cycle regulation to DNA replication [221]. Associatively, inhibition of USP7 levels by interfering RNA caused remarkably induced apoptotic protein expression in MCF7 and T47D cells. On the other hand, significantly decreased gene expression levels of oncogenic gene expressions were marked after USP7 knock down. Decreased oncogenic gene expression levels were correlated with growth inhibition [222, 223].

Decreased USP7 levels were also corrected with the protein contents similar to literature [221, 224, 225]. As a de-ubiquitination enzyme, inhibition of USP7, observed to be the main reason for increased total ubiquitination level of MCF7 and T47D proteins, showed parallelism with the literature [126].

To assess whether proliferative features of cancer cells and therefore metastatic potential could be actually decreased by differential amount of USP7, scratch assay was performed. It is known from the literature that the factors that have negative effect on cell proliferation, cells migrate less to the wounded area [226]. Both MCF7 and T47D cell lines were migrated to scratched area significantly less after inhibition of USP7 enzyme activity by p5091.

Unsurprisingly, USP7 knockdown by siUSP7 showed similar effect on MCF7 and T47D cells. Knocking down USP7 by lentiviral transduction also correlates with other experimental groups in terms of gap closure capability.

Though, there are several studies which found an indirect correlation between USP7 and the metastasis procedure [227, 228] our study is the first one to identify the direct dependence between the silencing of USP7 (either knock-down or knock-out) and metastasis capability.

There are many articles which were focused on inhibiting the human tumor colony forming units (CFU) [229-231] since CFU is efficiently used for drug screening [232]. The evoluation of the effect of USP7 on colony forming units is first in the literature. Alterations in CFU determined showed that, permanent decrease of USP7 was found to be highly correlated with poor colony forming capability. Moreover, formed couple of colonies was observed significantly smaller than regularly formed colonies. However, transient inhibition of USP7 had no significant effect on the amount of colonies, as well as diameter of them due to temporary inhibition of USP7 and colony forming unit assay lasting at least ten days, which allowed cells to reproduce USP7 back again. Analyzing the difference between p5091 and negative control, siUSP7 and control siRNA or scrambled vector with shUSP7 vector, gave us the ability to compare each experimental setup individually.

It is fact that, cancer defined as uncontrolled cell division [27]. Cell cycle mechanism is highly important for verification cell cycle steps which are crucial. Therefore, cell cycle is a highly controlled mechanism by cyclins, cyclin dependent kinases and their inhibitors [233]. Numerous of cyclins and cyclin dependent kinases (cdk) are involved in cell cycle regulation mechanism at different stages. For example, correlation between cyclin D and cdk 4 or cdk 6 is the main informant for going through S phase. There are also some inhibitory proteins for cyclin and cdk connections such as p21 [234, 235] . Cell cycle arrest occurred after the inhibition of cyclin-cdk complex and cells could not divide and try to repair mistakes to continue cell cycle without perishing [236]. A certain correlation between USP7 and cell cycle regulation was found. Several studies demonstrated that an increased p21 level was observed after inactivation of USP7 by p5091 [126, 212]. In this study, remarkably higher levels of G0-G1 phase accumulation decreased cell existence in G2-M phase was found after inactivation of USP7 protein regardless to p53 mutation situation. Knockdown of USP7 showed similar effect on both MCF7 and T47D cells. However, no significant change was observed in G0-G1 phase after knockdown of USP7 in MCF7 cells. On the other hand, G2-

M phase found to be shortened. USP7 knockdown in T47D caused a noticeable increase in G0-G1 accumulated cells.

Tumor sphere formation ability is highly correlated with stemness of cancer cells [237, 238]. One of the main limitations in cancer therapy is to find the way to clear cancer stem cells (CSCs) after therapeutic application [239]. Remaining CSCs might cause cancer once again. Most of tumors become immortal and gain the ability to proliferate extensively by the help of cancer stem cells [240]. Hanging drop technique is relatively new approach to examine stemness of a cancer cells [241]. However, examination of sphere forming capacity of breast cancer cells in USP7 protein perspective is first in literature. Here we examined the alterations in stemness of MCF7 and T47D cells comparing with the change in USP7 protein activity. p5091 treatment caused no or remarkably small spheres in both MCF7 and T47D cells. Since spheres are compact, three dimensional structures, under regular *in vitro* conditions, spheres tend to be disseminated into monolayer [242]. Mentioned sphere dissemination is used to determine the change in potential metastatic and invasive ability of a tumor [242]. Changes in dissemination potential of MCF7 and T47D were observed for 48 hours. Obtained results showed decreased metastatic potential after the inactivation of USP7, p53 in-dependently. Moreover, the metastatic capacity of control siRNA treated spheres was found greater than USP7 knockdown group, in both MCF7 and T47D cells. Similar effect was detected in both cells after USP7 knockdown. Moreover, since migrated spheres become less intense, alterations in sphere intensity was measured day-by-day. Results have shown denser spheres were formed after downregulation of USP7. USP7 plays a critical role in tumor dissemination since silencing or inactivating USP7 decreased tumor dissemination significantly.

In summary, an exceptional anticancer activity was demonstrated for USP7 knock-down, and activity inhibition *in vitro* for breast cancer. Anticancer activity of USP7 silencing was found to be p53 independent. Obtained results demonstrated that downregulation of USP7 exerted similar effects for both MCF7 and T47D cell lines at similar levels.

In this study, we revealed some of the molecular and physiological effects of USP7 inactivation in breast cancer. However, further investigations should be conducted in order to understand the exact molecular mechanism underlying the complete effect of USP7 on tumorigenic progression. To explore the effect of USP7 targeted gene therapy, *in vivo* studies should have done by USP7 knock-out mice. Moreover tumor formation activity of USP7

overexpression, knockdown and knockout in breast cancer should be conducted in vivo by using SCID animal models.

5. CONCLUSION

Breast cancer has the highest mortality rate among women. Especially, in developing countries, breast cancer is becoming more dangerous in recent years. Targeted therapy approaches are crucial for identification of a cure since most of the patients have different family stories and various molecular alterations. Limitations and side effects of traditional chemotherapy draw attention for developing personal based drugs. To overcome aforementioned limitations, gene therapy is needed for personalized therapeutic approach.

Overall, as a regulatory mechanism of ubiquitination pathway, ubiquitin specific protease 7 enzyme (USP7) was found to be very important for tumorigenic development. Inhibition of USP7 enzyme activity by p5091 showed remarkable anti-cancer activity in both MCF7 and T47D. Transient or permanent knockdowns decreased tumor proliferation and progression. Current study is the first study in literature explaining the effect of USP7 de-ubiquitination enzyme on breast cancer, regardless to its p53 situation. In this project, we were able to show anti-tumor activity of USP7 silencing in breast cancer as a potential gene therapy approach for the future therapies.

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