INVESTIGATING THE EFFECT OF HUMAN SACM1L GENE IN THE p53 WILD TYPE BREAST EPITHELIAL MCF10A AND BREAST CANCER MCF7 CELLS

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ABSTRACT

INVESTIGATING THE EFFECT OF HUMAN SACM1L GENE IN THE p53 WILD TYPE BREAST EPITHELIAL MCF10A AND BREAST CANCER MCF7 CELLS

p53, tumor suppressor protein, plays role in the regulation of many cellular processes. Thus, p53 activity is controlled by a series of mechanisms, one of which is a redox reaction. However, redox regulation of p53 is not well defined in the literature. As a candidate of antioxidant, Sac1 gene mutation resulted in decreased levels of human p53 protein in transformed yeast, but the human homolog of Sac1 (SACM1L) has not been studied yet.

SACM1L is known to function as a phosphoinositide phosphatase, hydrolyzes PI4P in the Golgi and ER. Previous studies demonstrated SACM1L depletion in HeLa cells led to decreased viability and arrest at the G2/M phase. However, no data were found on the association between the SACM1L and either directly p53 or p53 mediated cellular processes. We aimed to investigate the role of SACM1L in p53 controlled cellular processes like cell cycle and apoptosis in p53 wild type (wt) breast epithelial cells MCF10A and breast cancer cells MCF7 in the presence or absence of SACM1L gene.

We demonstrated that SACM1L knockout MCF7 cells were arrested in the G1 phase, and number of proliferating cells was reduced, whereas overexpression of SACM1L did not change the proliferation, and cell cycle. Further, the rate of apoptosis was increased in SACM1L overexpressing and knockout MCF10A and MCF7 cells, supported by the findings of transcriptional analysis for p53 target genes.

In conclusion, the greatest effect of SACM1L was observed in the apoptosis, but the underlying mechanisms are still unclear and must be further studied.

ÖZET

p53 VAHŞİ TİP MEME EPİTELYAL MCF10A VE MEME KANSERİ MCF7 HÜCRELERİNDE İNSAN SACM1L GENİNİN ETKİSİNİN ARAŞTIRILMASI

Tümor baskılayıcı protein olan p53 bir çok hücresel olayda görev alır. Bu yüzden birbirinden farklı mekanizmalar tarafından control edilir. Bu mekanizmalardan biri de redoks reaksiyonudur. p53'ün redoks reaksiyonları ile regülasyonunu sağlayan farklı içsel ve dışsal faktörler olmasına ragmen, litaratürde yer almayan başka faktörlerde olabilir. Bir antioksidan adayı olarak SAC1 geni bu faktörlerden biri olabilir. Daha önceki çalışmalarda, p53 transformasyonu yapılmış maya hücrelerinde SAC1 genini mutasyona uğrattıklarında, p53 aktivitesinde azalma olduğu gözlemlenmiştir. Fakat, SAC1'in insan homoloğu olan SACM1L geni daha önce bu amaç doğrultusunda çalışılmamıştır.

SACM1L (SAC1 benzeri Fosfatidilinositid Fosfataz) geni, Golgi ve endoplazmik retikulumda lokalize olan ve öncelikli olarak fosfatidilinositol 4-fosfat hidrolize eden bir fosfoinositid fosfataz olarak işlev gören integral membran proteinini kodlamaktadır. Daha önceki çalışmalarda, SACM1L'in HeLa hücrelerinde silinmesi sonucunda canlılığın azaldığı ve hücre döngüsünün G2/M fazında durduğu gözlemlenmiştir. Fakat, SACM1L direkt olarak p53 ile ve/veya p53'ün görev aldığı hücre yolakları arasındaki ilişki ile ilgili çalışmalar bulunmamaktadir. Bu bağlamda, SACM1L geninin varlığında veya yokluğunda p53 vahşi tip (wt) meme epitel hücreleri MCF10A ve meme kanseri hücreleri MCF7'de hücre döngüsü ve apoptoz gibi p53 kontrollü hücresel süreçlerde SACM1L'nin rolünün araştırılması hedeflenmiştir.

Sonuç olarak MCF7 hücrelerinde SACM1L nakavt edildiğinde hücre döngüsü G1 fazında duraklarken, bölünen hücre sayısında da azalma gözlemlenmiştir. Bunun yanı sıra, SACM1L'nin aşırı ekspresyonu herhangi bir değişime sebep olmamıştır. Apoptoz deneyleri sonucunda, SACM1L'nin aşırı ekspresyonu ve silinmesi durumlarında iki hücre hattı içinde hücre ölümleri önemli ölçüde artış göstermiştir. Bu sonuçlar aynı zamanda p53 hedef genleri için transkripsiyonel analiz bulguları ile de desteklenmiştir.

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CHAPTER 1

INTRODUCTION

1.1. TP53 Gene and p53 protein

The p53 protein, one of the most known tumor suppressor proteins, was firstly discovered in 1979 as a tumor antigen associated with the T antigen of the SV40 virus in tumor cells. Because p53 accumulation in tumor cells leads to growth arrest in knockout p53 tumors, so p53 is initially defined as an oncogene. The idea of "oncogene p53" has changed since wild type (wt) p53 is responsible for inhibiting cell growth and mutations that cause the inactivation of p53 in the tumors. Thus, p53 status is altered from oncogene to the tumor suppressor gene [1]. The name of p53 came from its molecular weight in the SDS-page, although p53 has known 12 isoforms that have various sizes [2, 3]. The gene name of p53 is given as tumor protein 53 (TP53) [1]. The human TP53 gene is localized on the short arm of chromosome 17 (17p13.1) as some other essential tumor suppressor proteins such as BRCA1 and HIC-1, especially in breast cancer [4 5].

TP53 gene expression is low in normal cells, regulated primarily by MDM2 protein. When cells get under stress conditions, the TP53 gene is activated, increasing the p53 protein level. Those stress conditions are DNA damage, decreased O_2 level (hypoxia), oncogenic stress, ribosomal stress, etc. The activated TP53 gene expresses p53 protein that plays a role in determining cell fate by regulating a wide range of genes with the inclusion of autophagy, apoptosis, senescence, growth arrest, DNA repair, and metabolism (Figure 1.1) [5]. p53, as a transcription factor, can bind to target DNA to activate or repress the gene expression. Besides interaction with DNA, p53 can also interact with other proteins to regulate their functions [6]. In addition to more than 100 well-known genes regulated by p53, over 4000 human genes were estimated to have p53 binding sites by bioinformatics tools. Even in the absence of binding sites, some gene

expressions can be suppressed by p53 [7]. Consequently, p53 studies are pivotal and promising, especially for cancer research.

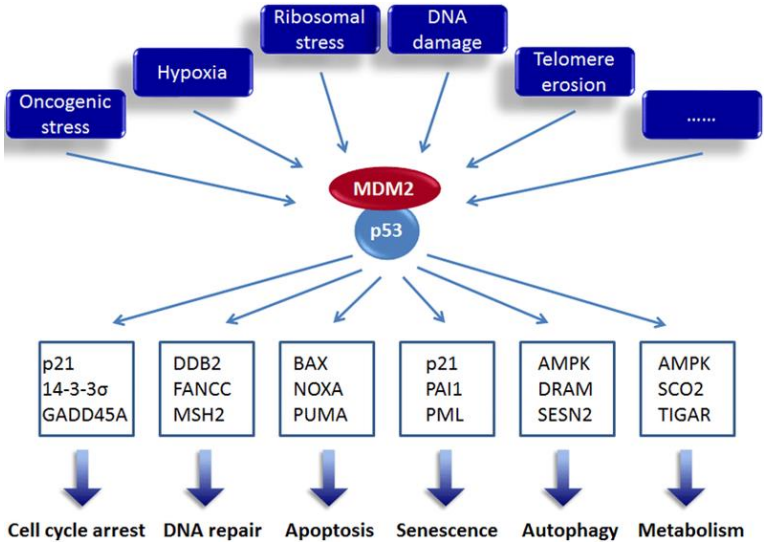


Figure 1. 1: Activation of p53 and p53 regulated pathways [5].

1.1.1. Basic Structure of p53

The p53 protein contains five domains which are transactivation domain (TAD) and proline-rich domain at the N-terminal, DNA binding domain (DBD) is located at the central core, and tetramerization (TD) and regulatory domain at the C- terminal (Figure 1.2). Each domain plays a role in at least one of the p53 functions. The transcription activation domain is responsible for the induction of target gene expression and gene selection. The proline-rich domain comes after the transactivation domain, which aids TAD to activate gene expression and gets involved in cell cycle arrest. DNA binding domain, as its name indicates that contributes to stable DNA interaction by forming tetramer [8]. DBD of p53 does not only interact with DNA but is also committed to proteins in the cytoplasm [9]. The significance of DBD is that most cancer-related mutations occur in this area. In addition to mutation, cysteine residues on the DBD affect the binding capacity to DNA by forming disulfide bridges between them under oxidative stress [8]. The following tetramerization domain has a role in forming tetramer structure and nuclear

localization [9]. If a mutation occurs in the TD domain, p53 cannot properly bind to DNA. Therefore, it is crucial as DBD for functional p53 protein to determine the cell fate [6]. Lastly, the regulatory domain has negative and positive controls on p53 activity [10]. It is the domain where most of the post-translational modifications occur at the C-terminal. The regulatory domain has also been involved in the DNA binding of p53, selection, and expression of target genes [6].

1.1.2. Redox Regulation of p53

The p53 is regulated by various mechanisms. The most familiar one is Mdm2, which is a negative regulator of p53. As an E3 ubiquitin ligase, Mdm2 has the capability to ubiquitinate p53 and result in degradation via proteasome complex. In normal conditions, p53 degradation via Mdm2 occurs continuously. The interaction between p53 and Mdm2 is inhibited by stress factors via various mechanisms. One of the most known is Mdm2 phosphorylation by specific kinases [11]. Inhibition of p53-Mdm2 interaction results in p53 stabilization. Like phosphorylation, there are several post-translational modifications (PTM) such as acetylation, methylation, and so forth. PTMs also regulate the p53 activity and, as a result, cellular fate. Unlike Mdm2, PTMs are substantial modifications in response to stress conditions and occur at the specific site of the p53 domain with certain enzymes. More than one enzyme causes mutual modifications at the single residue of the p53 site, which can generate competition between modifications [6]. Although many studies have reported the regulation of p53 by Mmd2 and PTMs, redox regulation involved in p53 activity is a less known mechanism.

The presence of cysteine residues in the redox signaling, especially if they are reactive cysteines, is the main cause of redox sensitivity for proteins [8]. Thereby, cysteine residues of p53 in the DNA binding domain are the reason why it is affected by the redox changes. Interestingly, there are 10 cysteine residues in the human p53, and all cysteine residues are localized in the DBD (Figure 1.2). Therefore, the binding capacity of p53 to the DNA is affected by the redox state of those cysteine residues. Three of the 10 cysteines in the DBD are responsible for the coordination of zinc ions. The activity of p53 is

influenced significantly by the binding of Zn^{+2} ions. When zinc is removed or cysteines that play role in the zinc-binding are mutated, transcriptional activity and DNA binding capacity of p53 is destroyed. For p53 to maintain its normal functions, cysteines bound with zinc ions must remain reduced. However, due to alteration of cysteine residues and/or loss of zinc ions under oxidative stress, p53 loses its stability and DNA binding function [12].

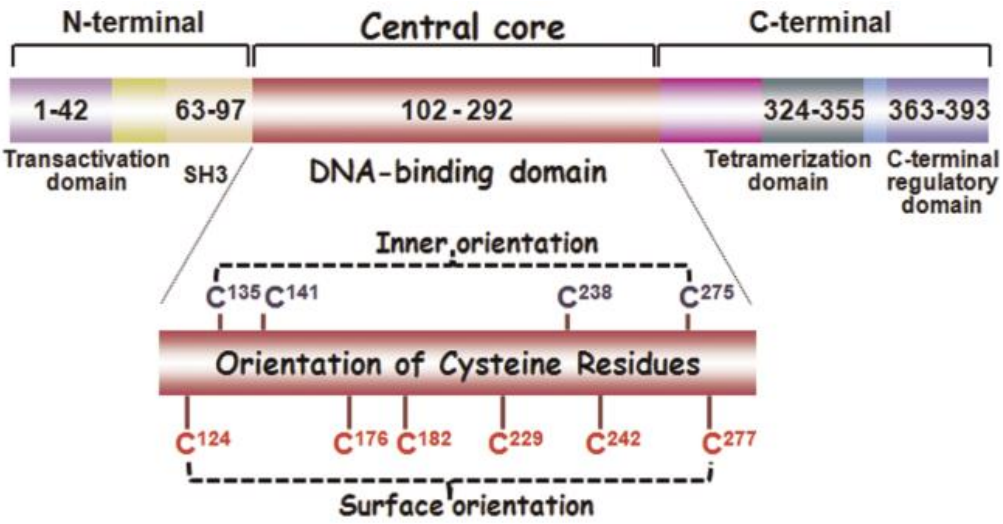


Figure 1.2: Structural domains of p53 and localization of cysteine residues at the DNA binding domain. [9]

The redox status of p53 can be modified by diverse molecules that are endogenously expressed and/or externally provided. The most investigated molecules are thioredoxin/thioredoxin reductase (Trx/TrxR), glutathione (GSH), The Apurinic/apyrimidinic endonuclease 1/reduction-oxidation factor 1 (APE1/Ref-1), and so on. Each molecule has a discrete way to alter the p53 in the redox regulation. For example, Ref-1 is a particular protein that has a dual function. Its C-terminal domain plays a role in the DNA base excision repair (BER) mechanism, whereas the N-terminal domain of Ref-1 participates in regulating redox-sensitive proteins like p53 [9]. [13] suggested that Ref1 controls the p53 activity under mild redox stress conditions to stop p53 mediated apoptosis. However, Ref-1 is oxidized under the increased oxidative stress, p53 can no longer interact with Ref-1, and stabilized p53 activates the apoptotic pathway.

Although the mechanisms of Ref-1 and other proteins reported in many studies have been investigated, there may be other proteins with dual functions in redox regulation. For this purpose, a previous study investigated the different redox-active antioxidant genes that can affect the p53 activity in yeast [14]. The researchers investigated the effects of mutations of genes, which carry redox motifs and have potential redox functions, on the transcriptional activity of p53. It was determined that the deletions of the MAP1, SAC1, and HNT3 genes significantly reduced the p53 reporter gene level in yeast (Table 1.1) [15].

Table 1.1: The list of genes decreased p53 activity in antioxidant gene mutants of yeast [15].

ORF	Gene Name	Human Homologs	Relative p53 activity (%)
WT	-	-	100,0
YKL212W	**SAC1**	**SACM1L**	50,6
YOR258W	**HNT3**	**APTX**	26,2
YLR244C	**MAP1**	**METAP1**	16,5

This study focuses on SACM1L, the human homolog of the yeast SAC1 gene, to investigate its effects on p53-related cellular processes in mammalian cells.

1.2. SACM1L Gene and SACM1L protein

SACM1L (SAC1 Like Phosphatidylinositide Phosphatase) gene is localized on the short arm of chromosome 3 (3p21.3) and encodes transmembrane phosphoinositide phosphatase in the Golgi and endoplasmic reticulum (ER). As a phosphoinositide phosphatase, SACM1L particularly dephosphorylates phosphatidylinositol 4-phosphate (PI4P) generated by PI4K (PI4 Kinases), but it also plays a role in hydrolyzation of phosphatidylinositol 3-phosphate (PI3P) and phosphatidylinosi3,5-bisphosphate

(PI(3,5)P2) with low efficiency [16]. SAC1 was discovered in yeast as a suppressor of actin mutation [17]. Since then, studies have shown that SAC1 plays a role in various cellular processes such as inositol phospholipids metabolism, membrane trafficking, and organization of mitotic spindle and cytoskeleton [18, 19].

1.3. SACM1L Structure

The structure of SAC1 was firstly investigated in yeast, and its "J" like topology was demonstrated due to having two C-terminal transmembrane domains (TMD). The expression "J" like topology was used to identify that both N- and C-terminal ends of the SAC1 protein are towards the cytosol. Mammalian SAC1 (SACM1L) also has the same topology. Although the SAC1 protein has a unique structure, the proteins containing the SAC domain are different in structure as they do not contain TMDs [20]. The C-terminus of SAC1 contains a coatomer protein complex I (COP1) binding sequence (KEKIDD) for localization of SAC1 at the ER, and N-terminus includes leucine zipper (LZ) motif that is specific to SACM1L for transportation to the Golgi (Figure 1.3). The LZ motif provides the oligomerization with the coatomer protein complex II (COP-II) [20, 21].

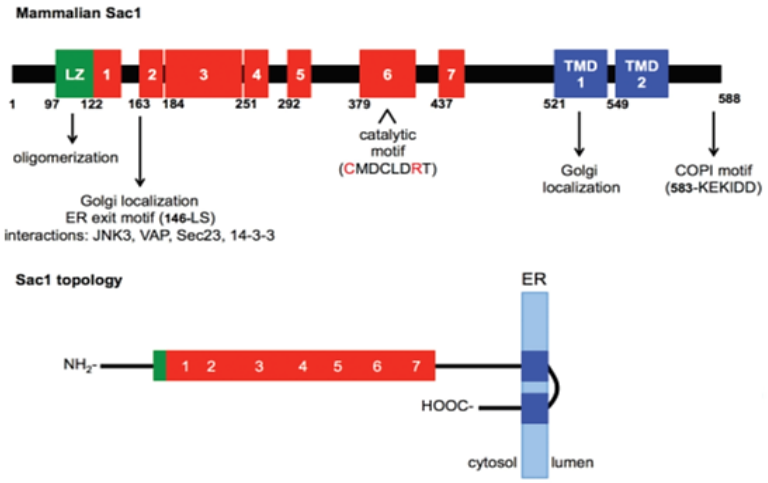


Figure 1.3: Protein structure of mammalian SAC1 (SACM1L) and its ER-localized topology. The SAC phosphatase domain is shown in red, with the number from 1 to 7 representing the conserved motifs.

1.4. SACM1L Function

Knockdown of the SACM1L gene in the various mammalian cell lines (HeLa, MDA-MB-231, MCF7, Hs578T, etc.) has resulted in many different aspects; disorganized Golgi membrane and mitotic spindle malformation decreased cell viability, and consequently arrest at the G2/M phase. There has been research about the mouse SACM1L gene in vivo, but in the embryonic stage, the lethal effect devoid of SACM1L precluded further studies [20].

It was shown that SACM1L silencing significantly reduced the viability of HeLa cells. The data suggested that Golgi disorganization may indirectly activate the apoptotic pathways; however, no data was provided on apoptotic activation [16, 18].

There is evidence that SACM1L expression and PI4P level at the Golgi play a crucial role in regulating tumor progression and metastasis. Previous research established that Golgi localized PI4P controls breast cancer cell lines' migration and invasion capability. Due to the knockdown of the SACM1L gene in MCF7 and SK-BR-3 cells, PI4P level was elevated in the Golgi, inducing focal adhesion with increased expression of CD44 and phosphorylation ezrin/radixin. Ezrin is involved in the invasion and migration of cancer cells by binding to CD44 when activated by phosphorylation. MCF7 and SK-BR-3 cells were known to have few CD44 positive CD24 negative subpopulations. Therefore, It has been suggested that the level of PI4P regulated by SACM1L in the Golgi may influence cancer cell invasion and migration by controlling CD44 expression[22]. Another study related to these findings showed that downregulation of CD44 in A549 and HepG2 cells resulted in induction of apoptosis and inhibition of proliferation [23]. SACM1L may have a role in apoptosis through the regulation of PI4P level in Golgi.

Silencing SACM1L in noninvasive MCF7 cells resulted in increased migration/invasion and decreased cell-cell adhesion depending on the level of PI4P at the Golgi. In addition to PI4P level, E-cadherin and β-catenin localization at the cell adhesion site was altered due to SACM1L depletion. Moreover, silencing the SACM1L gene with siRNA in MCF7 cells, resulting in increased expression of SNAI2, a marker of Epithelial-

to-Mesenchymal Transition (EMT). Therefore, they suggested that SACM1L might have control of the EMT process [24].

1.5. Aim of the Study

This study was aimed to determine the possible relation between the SACM1L gene expression and p53 mediated cellular processes such as cell cycle, proliferation, and apoptosis in the p53 wild type normal breast epithelial cell line MCF10A and breast cancer cell line MCF7. Although extensive research has been carried out on the SACM1L gene, there is no study in which the SACML1 gene is associated with the p53 protein in human or other mammalian systems. On that account, it was aimed to investigate p53 signaling pathways and p53 regulated cellular processes in the presence or absence of SACM1L gene products.

CHAPTER 2

MATERIALS AND METHODS

2.1. Plasmid Constructs

All plasmids were used to produce viral particles by transfection of HEK 293T cell lines. SACM1L cDNA containing pLX304 plasmid (pLX304_SACM1L) was used for overexpression and pLX304_LacZ was the control plasmid (Figure 2.1). LentiCRISPRv2 plasmid shown in Figure 2.2 was used to knock out the target gene. For this, Non-template LentiCRISPRv2 (NTL) was used as a control, and specifically designed gRNAs (Table 2.1) cloned into LentiCRISPRv2 plasmid was used to knockout the SACM1L gene.

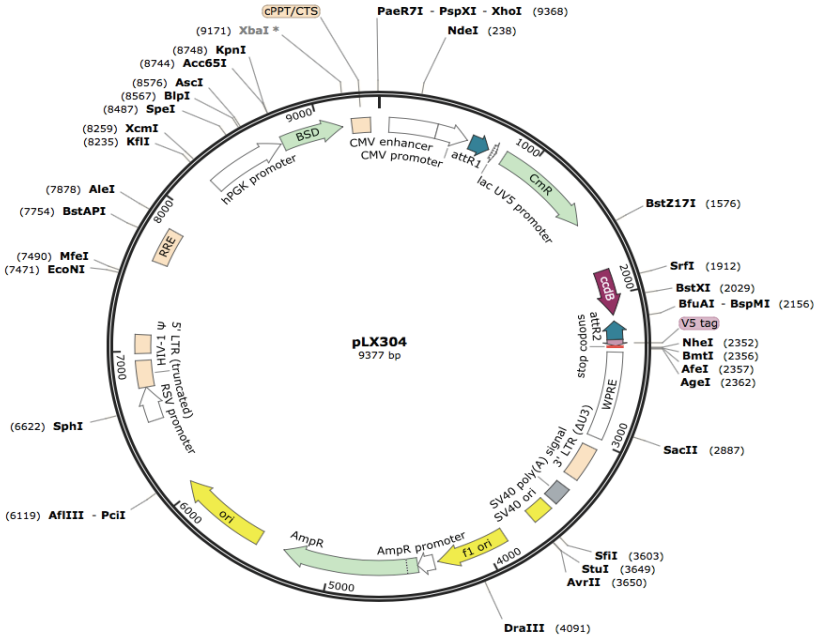


Figure 2. 1: The map of the pLX304 vector.

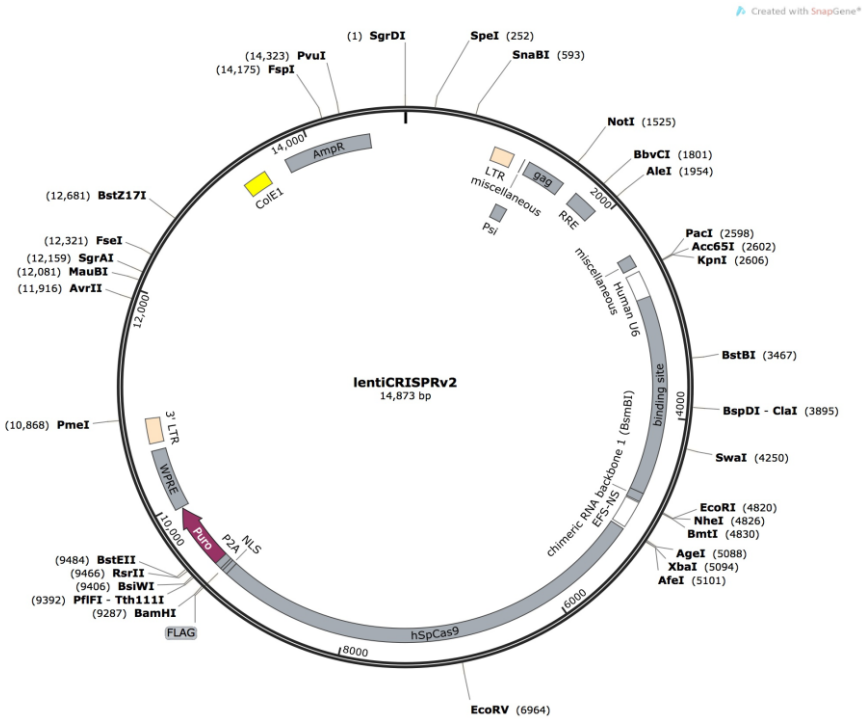


Figure 2. 2: The map of Lenticrispr_v2 vector.

Table 2. 1: List of gRNAs specifically designed for SACM1L gene.

gRNAs	5'	Sequences	3'
gSACM1L-F1	-	CACCGACTGGGCACAATCCATCTGG	-
gSACM1L-R1	-	AAACCCAGATGGATTGTGCCCAGTC	-
gSACM1L-F2	-	CACCGCATTGACCGTGTGTCCACAG	-
gSACM1L-R2	-	AAACCTGTGGACACACGGTCAATGC	-
gSACM1L-F3	-	CACCGATTGGATTCTCATCTCGAGG	-
gSACM1L-R3	-	AAACCCTCGAGATGAGAATCCAATC	-
gSACM1L-F4	-	CACCGGTCTTGTGACAGCTGAAGG	-
gSACM1L-R4	-	AAACCCTTCAGCTGTCACAAGACC	-

2.2. Cell Lines and Maintenance

In this study, MCF10A and MCF7 cell lines were provided by ATCC (American Type Culture Collection) and HEK 293T cell lines were obtained from Prof. Dr. Cathrin Brisken Laboratory (EPFL, ISREC). All cell lines were cultured at 37 °C with 5% CO2.

Maintenance of MCF10A cells were provided by high glucose DMEM-F12 (Gibco, Catalog # 31330038). Supplemented materials were 5% Horse Serum (Gibco, catalog # 16050-122), 0.5μg/mg Hydrocortisone (Sigma, catalog # H0888-1G), 100 ng/ml Cholera toxin (Sigma, catalog # C8052-1MG), 20ng/mg EGF (Sigma, catalog # E9644-.5MG), 10μg/mg Insulin (Sigma, catalog # I1882-100MG), and 1% Penicillin/Streptomycin (Gibco, catalog # 15070063).

Other cell lines, which were MCF7, HEK 293T, were maintained with Dulbecco's Modified Eagle Medium (DMEM) in high glucose (Gibco, Catalog # 41966029). Supplemented materials were 10% Fetal Bovine Serum (FBS) (Gibco, catalog # 10270106) and 1% Penicillin/Streptomycin (Gibco, catalog # 15070063).

2.3. Virus Production

HEK 293T cells were used to produce virus particles. 3.0×10^6 cells were counted and seeded into 10 cm cell culture plates. After 24 hours (h) culturing, 2 μg of designed lentiviral plasmid vector (described in 2.1 plasmid constructs), 0.7 μg of pMD2-VSVG as an envelope plasmid vector, and 1.3 μg of pCMV-dr8.74 as a packaging plasmid vector were used to combine with 12 μl of FuGene HD (Promega, catalog # E2311) in 500 μl of serum-free medium. The mixture was incubated for 30 minutes at room temperature (RT). HEK 293T cells were transfected with the mixture. Medium change was performed 24 hours after transfection. Hereafter, medium including virus particles was collected at 24h and 48h and stored at 80 °C.

2.4. Infection

2.5 x 10^5 MCF10A cells and 3.0 x 10^5 MCF7 were seeded into a 6-well plate. After cells were cultured 24 hours, virus particles were mixed with 8 μg/ml polybrene (Sigma Aldrich, catalog # 107689). Then the mixture was given to the cells and centrifuged at 2500 rpm for 2 hours at 32 °C to increase infection efficiency. After centrifugation, the medium containing virus particles were changed with the culture medium. Cells were incubated at 37 °C with 5% CO_2 for 48 hours. Thereafter, cells infected by pLX304_SACM1L and pLX304_LacZ were harvested and seeded into 6 cm plates with 4μg/ml blasticidin (Santa Cruz, catalog # sc-204655A) containing medium. On the other hand, cells infected by viruses containing LentiCRISPRv2 were harvested and seeded into 6 cm plates with 2μg/ml puromycin. Once every 3 days, if non-infected cells have not died, cells were treated with antibiotics. When non-infected cells were completely dead, cells were taken to a normal culture medium as being stable cells.

2.5. mRNA Quantification

MCF10A and MCF7 cells were counted and seeded into plates. Cells were flash-frozen after 3 and 4 days respectively and kept in -80. The next day, RNA isolation was performed by using a Monarch® Total RNA Miniprep Kit (New England BioLabs, catalog # T2010S) with the protocol provided by the manufacturer. Nanodrop was used to determine the concentration of isolated RNA samples. cDNA was synthesized from a 1 μg RNA sample with random primers by using the RevertAid first-strand cDNA synthesis kit (Thermo Scientific, catalog # K1622). FastStart Essential DNA Green Master (Roche, catalog # 06402712001) was mixed with cDNA of samples and target primers. Each sample was distributed on 96 well plates as three replicas. Expression of RNA level was measured by Roche-Light Cycler 96 Real-Time PCR Detection System. As a housekeeping gene, TAT-Box Binding Protein (TBP) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were preferred to normalize the cycle threshold (Ct)

value of target genes. A comparative method was chosen for the calculation of relative mRNA expression levels. All primers were shown in Table 2.2.

Table 2. 2: List of primers and sequences.

Primer Names	5'	Sequences	3'
PUMA-F	-	GACGACCTCAACGCACAGTA	-
PUMA-R	-	CTGGGTAAGGGCAGGAGTC	-
BAD-F	-	CGGAGGATGAGTGACGAGT	-
BAD-R	-	CCACCAGGACTGGAAGACTC	-
P21-F	-		-
P21-R	-		-
KILLER-F	-	GATGGTCAAGGTCGGTGATT	-
KILLER-R	-	CCCCACTGTGCTTTGTACCT	-
14-3-3-F	-	ACCCAATTCGTCTTGGTCTG	-
14-3-3-R	-	GCTTCATCAAATGCCGTTTT	-
GADD45-F	-	GGAGGAAGTGCTCAGCAAAG	-
GADD45-R	-	ATCTCTGTCGTCGTCCTCGT	-
P53-F	-	AAGACCCAGGTCCAGATGAAGC	-
P53-R	-	GAAGGGACAGAAGATGACAGGG	-
TBP-F	-	TAGAAGGCCTTGTGCTCACC	-
TBP-R	-	TCTGCTCTGACTTTAGCACCTG	-
SACM1L-F		GCTGAAGCTGCATATCACACC	-
SACM1L-R	-	CCTGCCACCAGATGGATTGT	-

2.6. Western Blotting for Protein Analysis

2.6.1. Protein isolation.

To isolate total protein, RIPA lysis buffer was used containing 1mM Na_3VO_4, 50mM NaF, 1mM DTT, protease inhibitor. The mixture of RIPA lysis buffer was placed on flash-freeze cells and the cells were removed from the plate by scrapper. After that, cell lysate was gathered into eppendorf tubes. 26G syringe was used to homogenize cell lysate and then, 20 min. incubation was started on ice for Eppendorf tubes. The centrifuge

was set to 14000 rpm 20 min at 4 °C. After centrifugation of eppendorfs, supernatants were collected into new eppendorf tubes and stored at -80 °C.

2.6.2. Bradford Assay

Bradford assay was used to define the concentration of isolated proteins. Firstly, 5 different concentrations of protein standard were prepared from 20 mg/ml BSA (NEB, catalog # B9000S) with serial dilution with water. 800 µl of water, 10 µl of each standard, and total proteins of interest and 200 µl of 5X Bradford solution were mixed in 1 cm cuvette. Spectrophotometry was set to 595 nm to measure the absorbance of the standard at first. Then, the absorbance of proteins was measured and determined the concentrations by spectrophotometer.

2.6.3. Western Blot

The volumes required for 30-60 µg were calculated using the isolated protein concentrations. The required amount of protein lysate was mixed with 5X loading dye and incubated at 95 C for 5 minutes. A pre-made SDS gel consisting of 5% stacking gel and 10% solvent gel was used to run the sample proteins. After the proteins and color, pre-stained protein standard (NEB, catalog # P7719S) were loaded onto the SDS-gel, an electric current was applied at 18-25 mA until the dye was removed from the gel. Separated proteins in the gel were transferred to the PDVF membrane via wet transfer. The blocking membrane was performed by using 5% of milk powder in 1X Tris-buffered saline-Tween-20 (TBS-T) on the shaker at RT for 2 hours. Once the blocking was complete, the membrane was incubated overnight at 4 °C in primary antibody prepared in 5% powdered milk in TBS-T. The next day, the membrane was washed with 1X TBS-T three times for 5 minutes and then incubated in a secondary antibody which was anti-rabbit IgG, HRP (CST, catalog # 7074S) prepared in TBS-T for 2 hours at RT. The membrane was washed with 1X TBS-T three times for 5 minutes. Visualization of target proteins was performed by using substrate Clarity Western ECL (Bio-Rad, catalog # 170-

5061) via an imaging system (Vilber Fusion SL). Expression of β-actin was determined for the equal loading control and expression analysis. Quantification analyses were performed by using the ImageJ program. The list of antibodies is shown following; polyclonal rabbit anti-SACM1L ab (FN, catalog # FNab07576) with 1:1000 dilution, polyclonal rabbit anti-β-actin (Abcam, catalog # ab75186) with 1:2000 dilution.

2.7. MTT Assay

6.0×10^3 of MCF10A cells overexpressing SACM1L and 7.0×10^3 of MCF7 cells overexpressing SACM1L were seeded into a 48-well plate. Measurements were taken on days 2, 4, 6, and 8. To do this, 10% of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) solution is prepared in a complete medium and the medium was replaced with MTT containing medium. Incubation has lasted for 4 hours at 37 °C with 5% CO_2. Medium with MTT was removed and formazan particles were dissolved with DMSO. When formazan particles were dissolved in DMSO, the solution was replaced into a flat-bottom 96-well plate and measured in spectrophotometry (Thermo Multiskan Spectrum) at 570 and 650 nm.

2.8. PI (Propidium Iodide) Staining

1.5×10^5 and 2.0×10^5 cell numbers were used for the analysis of cell cycles by PI staining. For overexpressed MCF10A cell line, MCF10A-SACM1L infected cells, and MCF10A-LacZ infected cells as control were counted and seeded. For overexpressed and knockout MCF7 cell line, MCF7-SACM1L, and MCF7-LacZ, control of overexpression and MCF7-NTL, control of knockout cells, 1.5×10^5 cells were counted and cultured in a 6-well plate for 72 hours. Afterward, cells were lifted off by trypsinization and collected in a falcon. Centrifugation was applied at 1.2 rpm for 10 minutes. The supernatant was removed, and the cell pellet was first resuspended with 1 ml of cold 1X phosphate-

buffered saline (PBS) and then added 4 ml of cold 100% EtOH. Cells were stored at -20 °C for 24 hours. Thereafter, Cells were started with centrifugation at 1500 rpm +4 °C for 10 minutes. Shortly after, a centrifuge was used at 2000 rpm +4 °C for 1 minute. Supernatants were discarded and resuspended with 1 ml of 1X PBS gently. Cells were passed from falcons to eppendorf tubes. Eppendorfs were placed into a centrifuge and centrifugated at 1500 rpm +4 °C for 10 minutes. The supernatant was removed again. Cell pellets were suspended with 200 µl of 20 µg/ml RNase A in 0.1% Triton X in PBS. Cells were incubated at 37 °C for 30 minutes. After that, 20 µl of the PI solution was added and incubated for 15 minutes in dark. Flow cytometry (BD FACS Canto) was used to analyze cell cycles.

2.9. BrdU Assay

2.5 x 10^5 stable MCF10A cells and 3.0 x 10^5 stable MCF7 cells were seeded on coverslips in 6-well plates. After 48 hours, the medium was changed with serum-free medium for one condition with its control. 24 hours later, 20 µM of BrdU (Abcam, catalog # ab221240) was added into the medium except for negative BrdU conditions. Cells with BrdU were incubated at 37 °C with 5% CO_2 for 4 hours. Then, cells were washed by PBS solution twice for 5 minutes. 750 µl of 4% PFA was placed on coverslips for 45 minutes at 25 °C (RT). Next, PBS washing was done twice for 5 minutes. 750 µl of 1.5M HCl was added for 30 minutes incubation. Afterward, cells on coverslips were washed by PBS solution twice for 5 minutes. For blocking, 5% Donor Horse Serum (DHS) in 0.1% Triton X in PBS was prepared freshly and incubated cells for 1 hour. Then, 1:1000 diluted anti-BrdU ab () was prepared in 2% DHS in 1% Triton X in PBS. Cells were incubated in prepared antibodies at 25 °C overnight. Thereafter, cells were washed by PBS solution 3 times for 5 minutes. 1:1000 dilution for DAPI and 1:600 dilution for Alexa555 (Cell signaling, catalog # 4409S) was prepared in 2% DHS in 1% Triton X in PBS. Incubation was done for 45 minutes at RT. After washing the cells 3 times with PBS, coverslips were mounted on microscope slides and kept at 4 °C in dark. Fluoresce microscopy (Olympus U-LH100HG) was used to acquire the images.

2.10. Annexin V-FITC and PI Staining

2.5 x 10^5 stable MCF10A cells were cultured in a 6-well plate. After cells were cultured 48 hours, 2.5 μM doxorubicin (DOX) was soluble in DMSO for cells overexpressing SACM1L and DOX dissolved in methanol for knockout SACM1L cells were mixed with culture medium, and cells were treated with 2.5 μM DOX containing medium. The same amount of DMSO and methanol were mixed with culture medium and added onto cells for control. After 16 hours of treatment for cells overexpressing SACM1L and 48 hours of treatment for knockout SACM1L cells, cells were harvested by trypsinization. 3.0 x 10^5 stable MCF7 cells were cultured in a 6-well plate. After 48 hours of culture, 20 μM of cisplatin (TRC, catalog # C499500) soluble in 0.9% NaCl was mixed with culture medium, and cells were treated with this medium for 16 hours. The same amount of 0.9% NaCl solution was mixed with culture medium and added onto cells for control. After treatment, cells were harvested by trypsinization. Collected cells were suspended with a culture medium and centrifuged at 800 rpm for 5 minutes. The supernatant was discarded. To wash cells, cold PBS was added and centrifuged again. After the supernatant was removed, the pellet was resuspended in a 1X-annexin binding buffer. Cells were counted and prepared 1.0 x 10^6 cells/ml in tubes. Four eppendorf tubes were prepared for each condition (unstained, PI only, FITC only and, FITC and PI together). 50 μl of cells in 1X annexin binding buffer were distributed in eppendorfs. 2.5 μl of FITC Annexin V dye and 1 μl of PI dye were added into labeled eppendorfs. Cells were incubated at room temperature for 15 minutes in dark. After incubation, 200 μl of 1X annexin binding buffer was added and mixed by pipetting gently. Then, cells were kept on ice and immediately taken to flow cytometry (BD FACS Canto) to analyze apoptosis.

CHAPTER 3

RESULTS

3.1 Overexpression of SACM1L Gene was confirmed in p53 Wild Type MCF10A and MCF7 Cells.

The normal breast cell lines and breast cancer cell lines MCF10A and MCF7, respectively, were selected for their wild type p53 status. SACM1L gene was overexpressed to figure out the effect on p53 wild-type MCF10A and MCF7 cells by using the lentivirus system. Stable cells were selected by antibiotic treatment, and LacZ was used for infection control. After that, the mRNA level was confirmed by quantitative real-time PCR (qRT-PCR), and protein expression of SACM1L was verified by Western Blot. SACM1L mRNA expression was elevated 8.79 times in MCF10A cells whereas, the increment of mRNA level in MCF7 cells was 1.6 times (Figure 3.1). The protein expression of SACM1L was efficaciously increased, as shown in Figure 3.2.

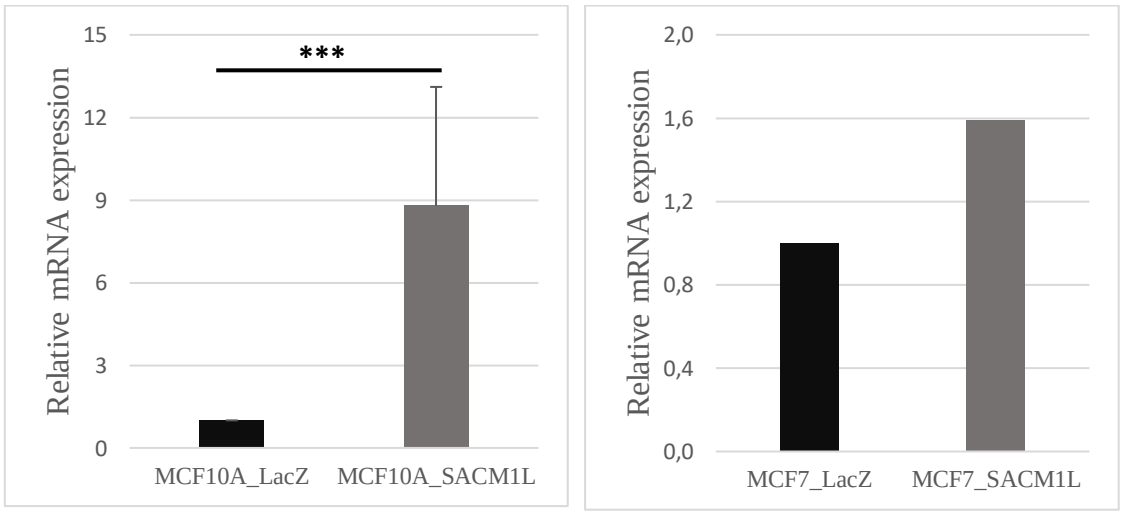


Figure 3.1: Overexpressed SACM1L mRNA levels in stable MCF10A and MCF7 cells were verified by quantitative real-time PCR. The level of SACM1L mRNA

was relative to mRNA expression of control cells (LacZ) and was in proportion to the expression of a housekeeping gene, TATA-binding protein (TBP) Two-tailed student's t-test was used for statistical analysis. (n=5 ***p<0.005; n=1)

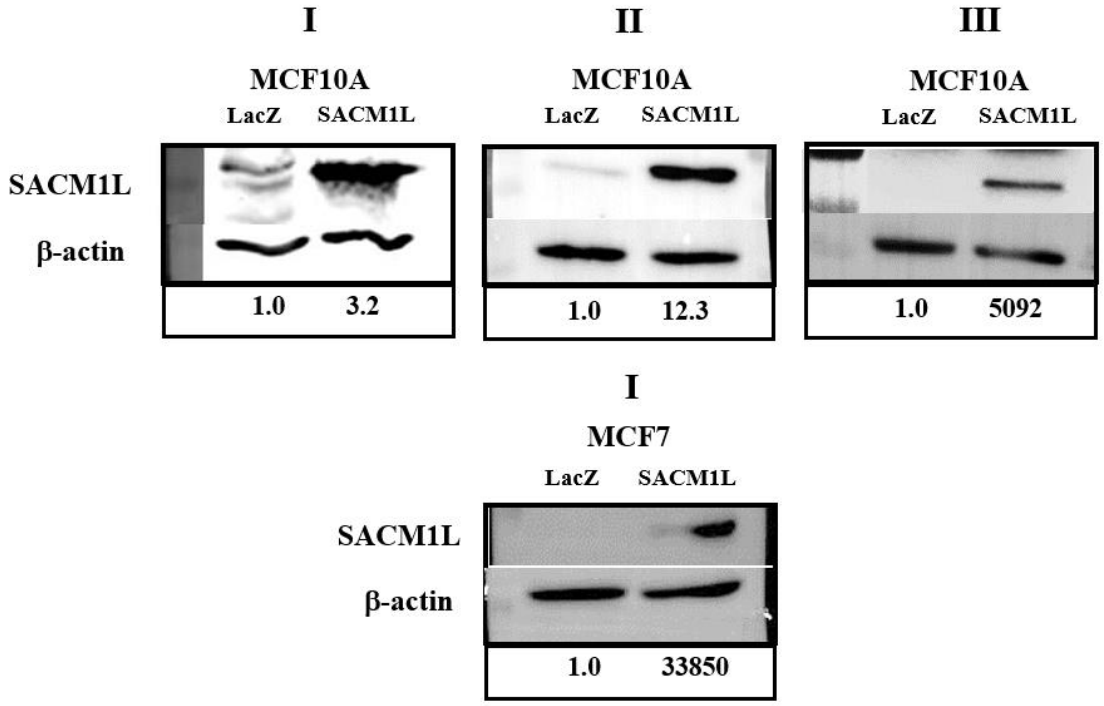


Figure 3.2: Overexpressed SACM1L protein level in stable MCF10A and MCF7 cells were verified by Western Blot. The protein level of SACM1L was relative to control cells (LacZ) and β-actin was used for equal loading control. Quantification of protein level was done with the normalized expression of SACM1L protein to β-actin.

3.2 Proliferation effect of SACM1L gene overexpression was investigated in MCF10A and MCF7 cell lines.

To understand the effect of SACM1L overexpression on proliferation, both p53 wild-type normal breast cells MCF10A and breast cancer cells MCF7 were investigated. Proliferation was determined by MTT assay and BrdU assay. MTT is converted into

formazan crystals by the mitochondrial activity of living cells. The yellow-colored MTT is changed to purple formazan by the enzyme mitochondrial dehydrogenase and quantified by the colorimetric spectrophotometry. It indicates that increased mitochondrial activity is proportional to the number of viable cells [25].

SACM1L overexpression did not change the growth rate of MCF10A and MCF7 cell lines to control for each day (Figures 3.3 and 3.4). All measured data were normalized to day 2. Both MCF10A and MCF7 overexpressed SACM1L cells were normalized to LacZ control of MCF10A and MCF7, respectively.

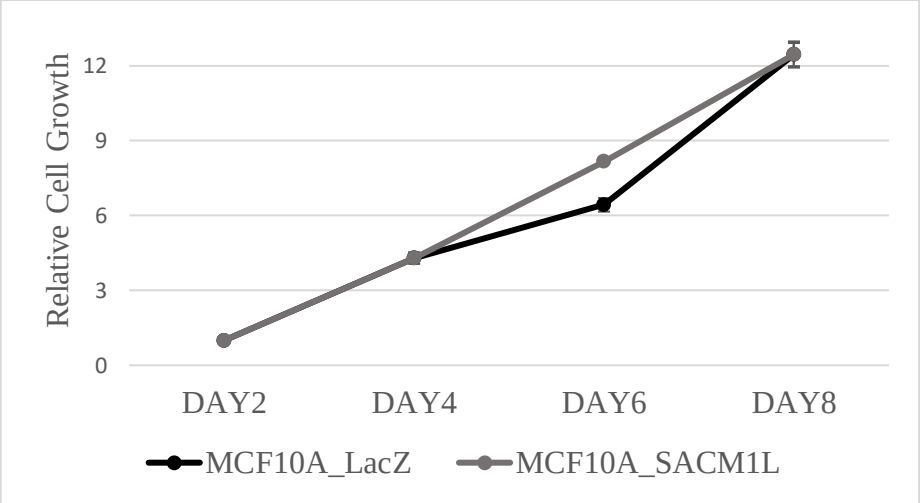


Figure 3.3: Relative cell growth of SACM1L overexpressed MCF10A cells and its LacZ control was analyzed by MTT assay. Measurements were taken on all days normalized to day 2. Statistical analysis was performed with a two-tailed unpaired student t-test. (n=3, ns; not significant for all)

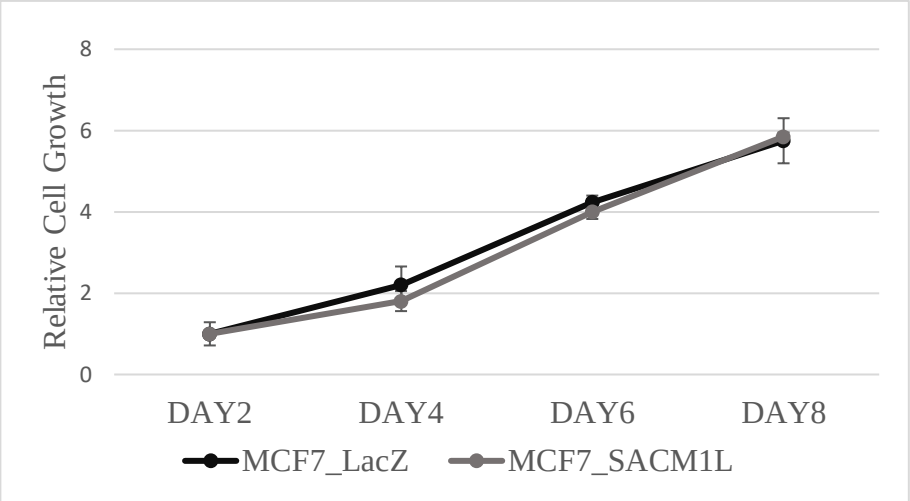


Figure 3.4: Relative cell growth of SACM1L overexpressed MCF7 cells and its LacZ control was analyzed by MTT assay. Measurements were taken on all days normalized to day 2. Statistical analysis was performed with a two-tailed unpaired student t-test. (n=2, n.s: not significant for all days)

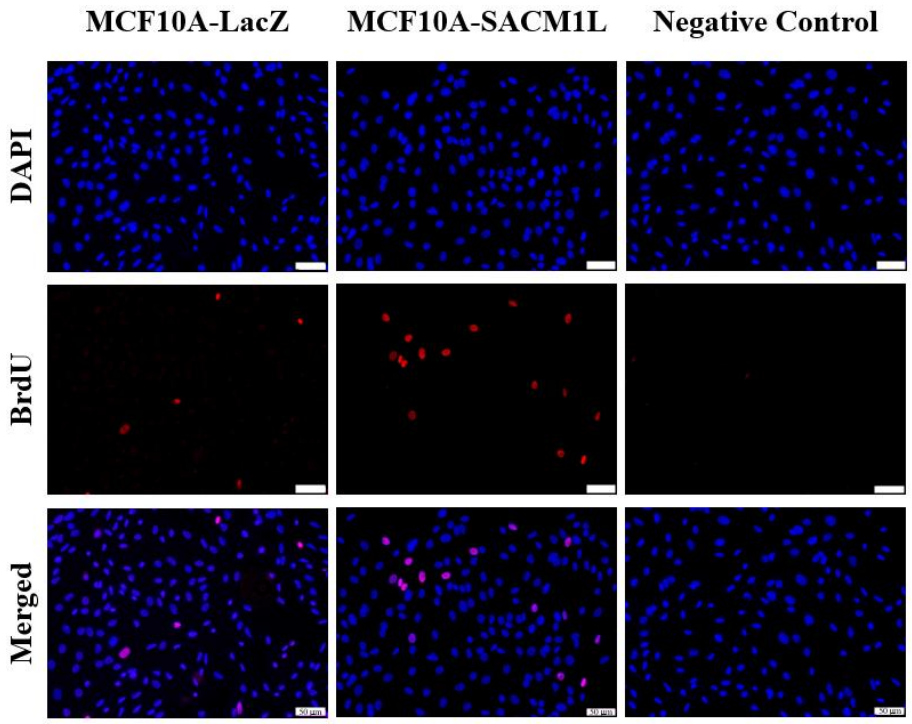


Figure 3.5: Control cells (MCF10A-LacZ) and SACM1L overexpressed MCF10A cells (MCF10A-SACM1L) in the presence of serum were stained to visualize the nucleus DAPI in blue and BrdU in red under fluorescence microscopy. Anti-BrdU antibody specificity was checked by negative control (scale bar: 50 μm).

On the other hand, the BrdU assay also assists in determining the proliferation as the thymidine analog incorporates DNA during the S phase [26]. Representative fluorescent microscope images of MCF10A cells overexpressing SACM1L and control cells LacZ are shown in Figure 3.5. Nuclei were stained blue with DAPI, and BrdU incorporation was indicated in red by the Alexa-555 conjugated anti-BrdU antibody. Negative control was performed with the anti-BrdU antibody alone without BrdU treatment to demonstrate the specificity of the antibody. In the presence of serum, SACM1L overexpressing MCF10A cells, the percentage of BrdU positive cells was obtained, similar to the LacZ control (Figure 3.6).

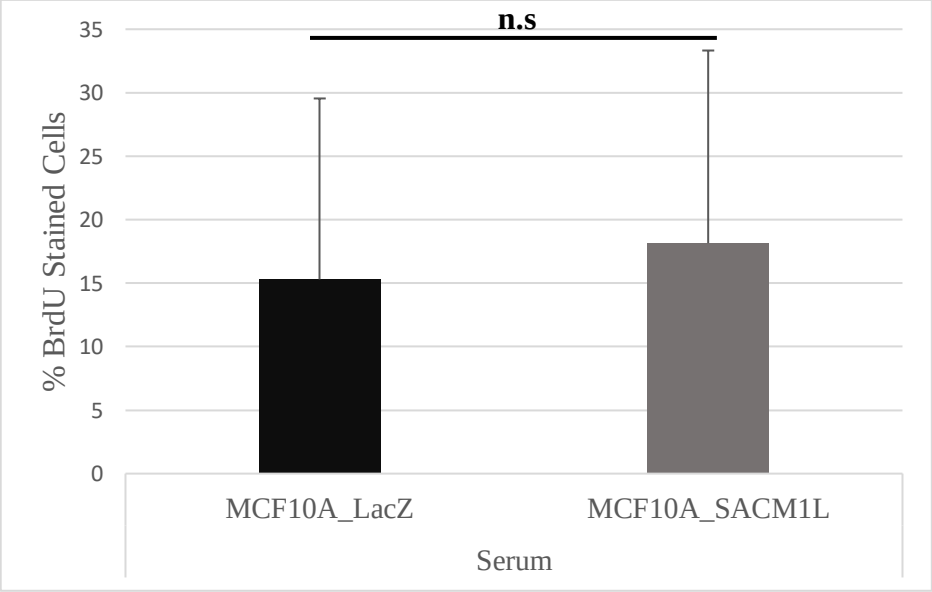


Figure 3.6:BrdU stained cell percentage in control cells (MCF10A_LACZ) and SACM1L overexpressing cells (MCF10A_SACM1L). Two-tailed student's t-test was used for statistical analysis (n=2, n.s: not significant)

Next, representative fluorescence images of MCF7 cells overexpressing SACM1L (MCF7-SACM1L) and control cells (MCF7-LacZ) in the presence and absence of serum conditions are demonstrated in Figure 3.7. DAPI was used to stain nuclei (blue), and BrdU incorporation was indicated in red by the Alexa-555 conjugated anti-BrdU antibody. Negative control was performed with the anti-BrdU antibody alone without BrdU treatment to demonstrate the specificity of the antibody. The proliferation of stable SACM1L overexpressed MCF7 cells had an increasing trend with serum and serum-free

conditions compared to its control cells. However, there was no statistically significant difference between MCF7 cells overexpressing SACM1L and its LacZ control. When control LacZ cells were compared for the presence and absence of serum conditions, a decreasing trend was observed, but the decrease was insignificant. The same was observed in MCF7 cells overexpressing SACM1L (Figure 3.8).

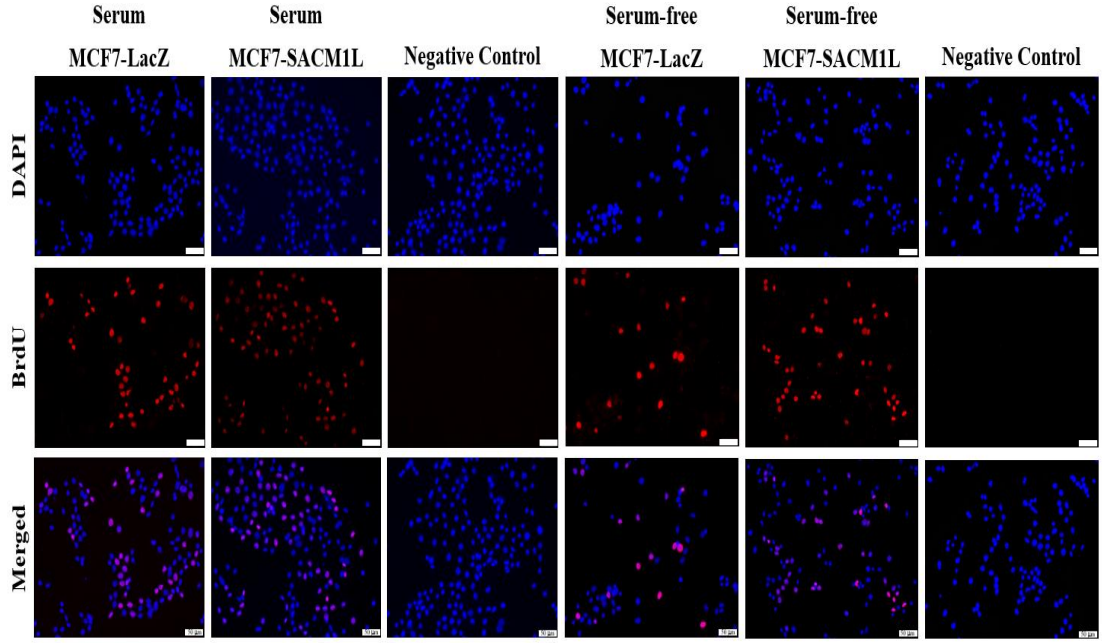


Figure 3.7: Control cells (MCF7-LacZ) and SACM1L overexpressed MCF7 cells (MCF7-SACM1L) in the presence and absence of serum were stained to visualize the nucleus DAPI in blue and BrdU in red under fluorescence microscopy. Anti-BrdU antibody specificity was checked by negative control for both serum and serum free conditions (scale bar: 50 μm).

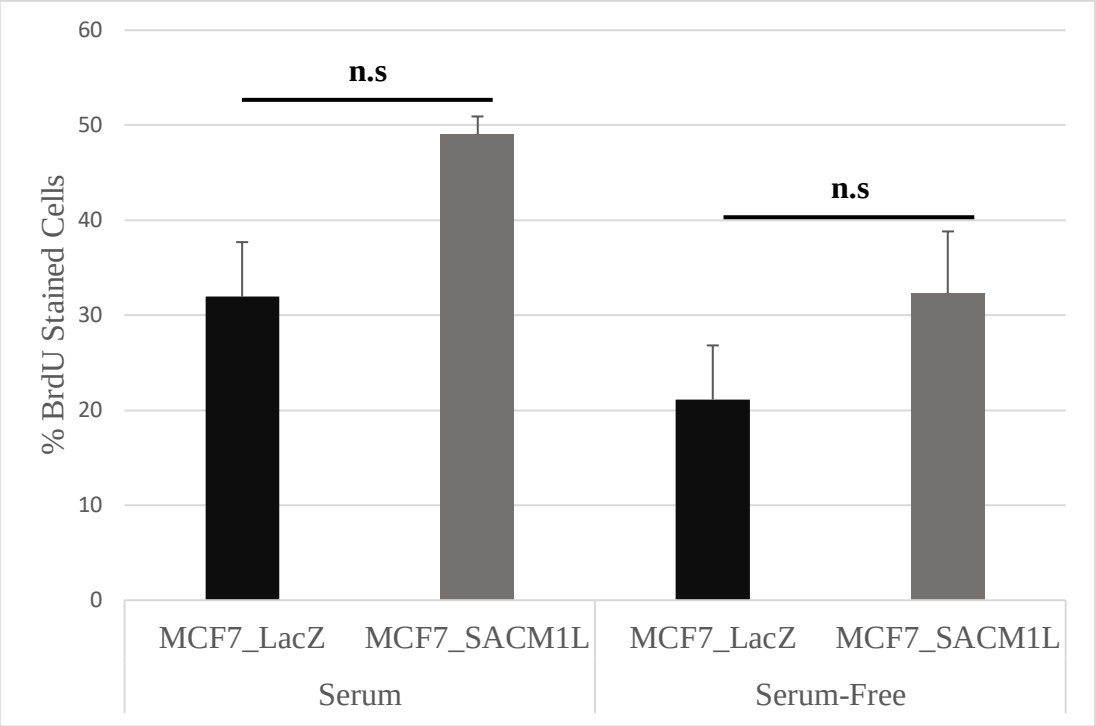


Figure 3. 8: BrdU stained cell percentage in control cells (MCF7_LACZ) and SACM1L overexpressing cells (MCF7_SACM1L). Two-tailed student's t-test was used for statistical analysis. (n=2, n.s: not significant)

3.3 Cell cycle was evaluated in SACM1L overexpressed MCF10A and MCF7 cells.

The effect of SACM1L gene overexpression on proliferation was reviewed by propidium iodide (PI) staining in MCF10A and MCF7 cells. Percentages of cell cycle phases of cells stained with PI were determined by flow cytometry. Cells were categorized according to phases as G1, S, and G2/M. Percentages of all phases were compared to their LacZ controls for MCF10A and MCF7 cells that overexpress SACM1L as shown in Figure 3.9 and Figure 3.10. According to the PI results, there were no significant differences in each cell cycle phase of MCF10A and MCF7 cells overexpressing SACM1L compared to their control cells. However, the G2/M phase of MCF10A cells overexpressing SACM1L increased by approximately 2% but was not statistically significant.

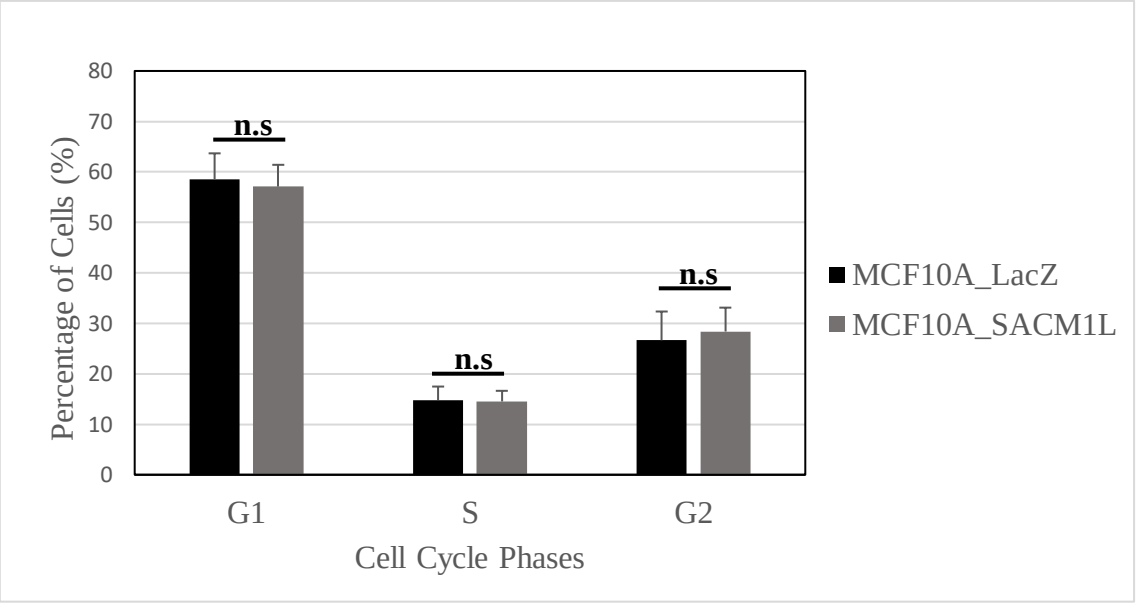


Figure 3. 9: The cell cycle distribution (percentage) of SACM1L overexpressed MCF10A cells at G1, S, G2 phases were confirmed by PI staining analyzed by flow cytometry Two-tailed student's t-test was used for statistical analysis. (n=3, n.s: not significant)

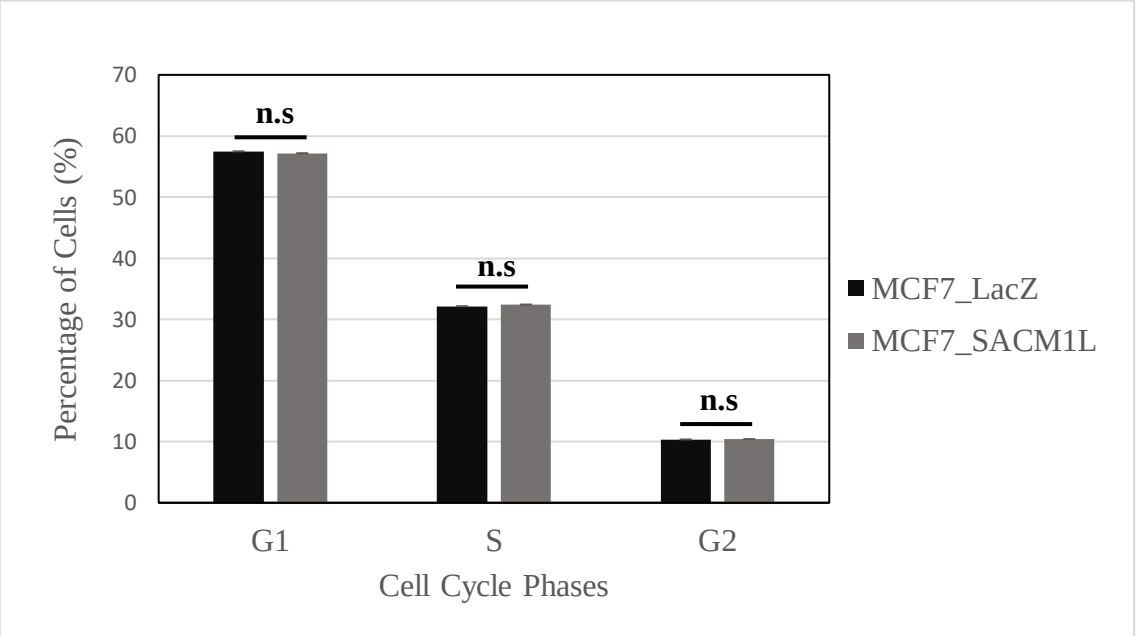


Figure 3.10:The cell cycle distribution (percentage) of MCF7 cells overexpressing SACM1L at G1, S, G2 phases were confirmed by PI staining analyzed by flow cytometry. Two-tailed student's t-test was used for statistical analysis. (n=2, n.s: not significant)

3.4 Overexpression of SACM1L gene induces Apoptosis in MCF10A and MCF7 cells

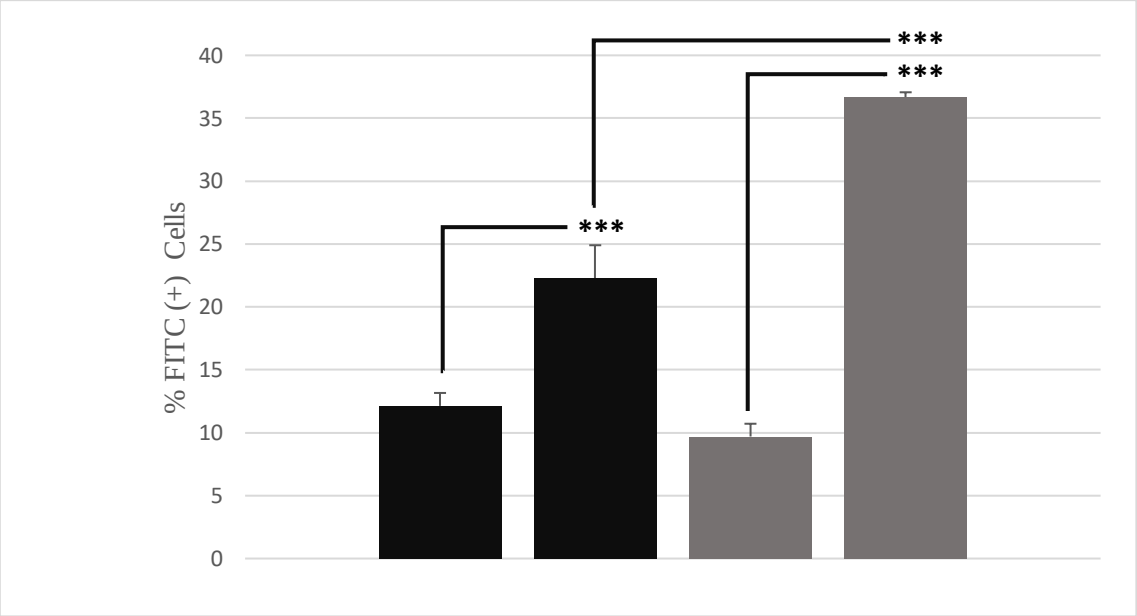
The effect of SACM1L gene overexpression on apoptosis was investigated in the normal breast cell lines MCF10A and breast cancer cell lines MCF7. After 48 hours of culturing the MCF10A overexpressing SACM1L cells and its control cells, LacZ was treated with 2.5 μM of Doxorubicin (DOX) to trigger apoptosis. To control DOX efficiency, its solvent DMSO was also applied to the cells separately. After 16 h DOX incubation, the cells were harvested for apoptosis assay staining with Annexin V conjugated FITC and red fluorescent propidium iodide (PI).

The apoptosis rate was 12% in DMSO-treated control cells, and DMSO-treated SACM1L overexpressing cells had a 9.7% of apoptosis rate. DOX-induced apoptosis was significantly increased in the control cells by 22.3% compared to its DMSO control. Overexpression of the SACM1L gene led to increased apoptosis significantly relative to its DMSO treatment. The apoptotic cell percentage of the cells overexpressing SACM1L was 36.7%. The apoptotic cell percentage was slightly decreased in the DMSO-treated cells overexpressing SACM1L relative to control cells treated with DMSO, but this decrease was not significant. Moreover, DOX induction in the SACM1L overexpressed cells caused a significantly increased apoptosis than DOX-induced apoptosis in the control cells. (Figure 3.11).

For the SACM1L overexpression in MCF7 cells, p53-dependent apoptosis was induced by cisplatin. After the stable cells were cultured for 48 hours, 20 μM of cisplatin was exposed to the cells for 16 hours. The solvent of cisplatin (0.9% of NaCl in water) was also applied to cells to ensure cisplatin efficiency. Apoptotic cells were detected by Annexin V conjugated FITC and PI staining.

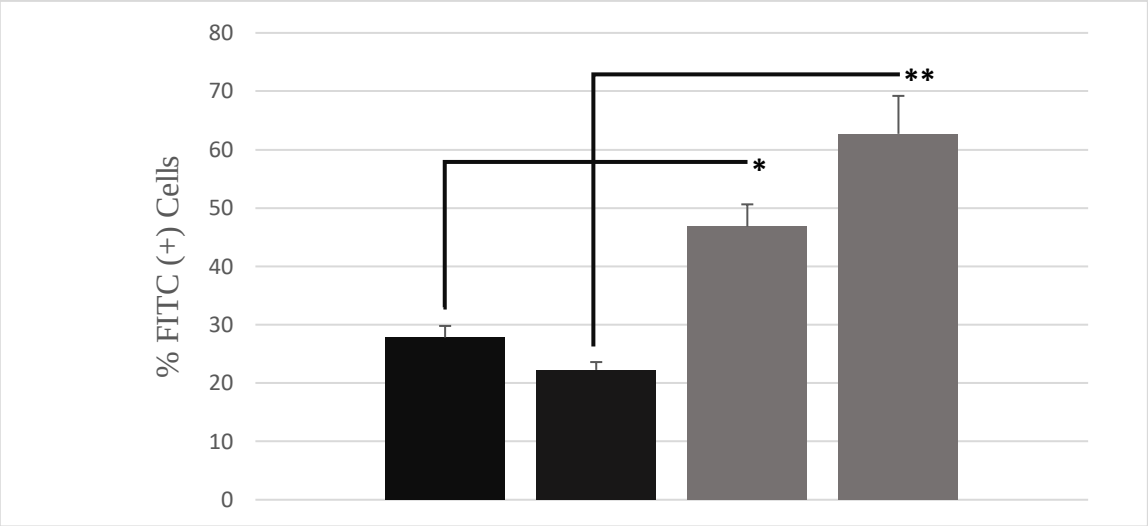
Apoptotic cell percentage of control cells exposed to NaCl was obtained as 27.8% whereas the apoptosis rate of cisplatin-induced control cells was found as 22.3%. This result indicates that cisplatin did not induce apoptosis in the control MCF7-LacZ cells. The rate of apoptotic MCF7 cells overexpressing SACM1L treated with NaCl was detected as 46.8%. SACM1L overexpressed cells were shown to have a significant

increase in the percentage of apoptotic cells compared to control cells treated with NaCl. The rate of apoptotic cells was 62.7% in the cisplatin induction of MCF7 cells overexpressing SACM1L. The increased apoptotic cell percentage in the cells overexpressing SACM1L was significant when comparing cisplatin induced SACM1L and its control LacZ (Figure 3.12).



DOX treatment	-	+	-	+
SACM1L overexpression	-	-	+	+

Figure 3.11:Apoptotic cell percentage in the control cells (MCF10A-LacZ) and SACM1L overexpressing cells (MCF10A-SACM1L). Two-tailed student's t-test was used for statistical analysis. (n=2, ***p<0.005)



Cisplatin treatment	-	+	-	+
SACM1L overexpression	-	-	+	+

Figure 3.12:Apoptotic cell percentage in the control cells (MCF7_Lacz) and SACM1L overexpressing cells (MCF7_SACM1L) Two-tailed student's t-test was used for statistical analysis. (n=1, *p<0.05, **p<0.01)

3.5. Transcriptional profiles of target genes in the p53 signaling pathway were analyzed in the SACM1L overexpressed MCF10A and MCF7 cells

Following apoptosis assay results, quantitative real-time PCR (qRT-PCR) was performed to determine whether SACM1L overexpression in MCF10A and MCF7 cells have transcriptional changes in their p53 target genes. Seven target genes were studied, including p21, Gadd45, 14-3-3, PUMA, Killer, and Bad, as well as p53. mRNA levels of p21, Gadd45, and 14-3-3 genes were inspected to assess p53-mediated cell cycle arrest. PUMA, Killer, and Bad genes regulated by p53, which induces apoptosis, were studied at the transcriptional level. In addition to p53-regulated genes, p53 itself also has been examined at the mRNA level.

In Figure 3.13 and Figure 3.14, the mRNA level of p53 was not changed in the SACM1L overexpressing MCF10A and MCF7 cells compared to their LacZ controls. There was no change in the 14-3-3 mRNA level in MCF10A and MCF7 cells overexpressing SACM1L. However, the mRNA levels of Gadd45 and p21 had an increasing trend in MCF10A cells overexpressing SACM1L but a decreasing trend in the SACM1L overexpressing MCF7 cells. In the MCF10A cells overexpressing SACM1L, the mRNA expressions of PUMA, Killer, and BAD were slightly higher than the control cells. Although the mRNA level of PUMA in MCF7 cells overexpressing SACM1L had a similar trend as in MCF10A cells, the mRNA expressions of Killer and Bad had a decreasing trend in MCF7 cells in contrast to MCF10A.

The increasing trend in the mRNA level of PUMA, Killer, and Bad in MCF10A cells overexpressing SACM1L may be associated with increased apoptosis outcome. Increased mRNA expression of Gadd45 in MCF10A cells overexpressing SACM1L may have resulted in increased cell number in the G2 phase of the cell cycle. Similarly, the result of increased apoptosis may be associated with a trend towards increased PUMA mRNA level in MCF7 cells overexpressing SACM1L. Additionally, reducing Gadd45 and p21 mRNA expression in MCF7 cells overexpressing SACM1L can avoid cell cycle arrest and cause cells to remain at a normal proliferation rate like control cells.

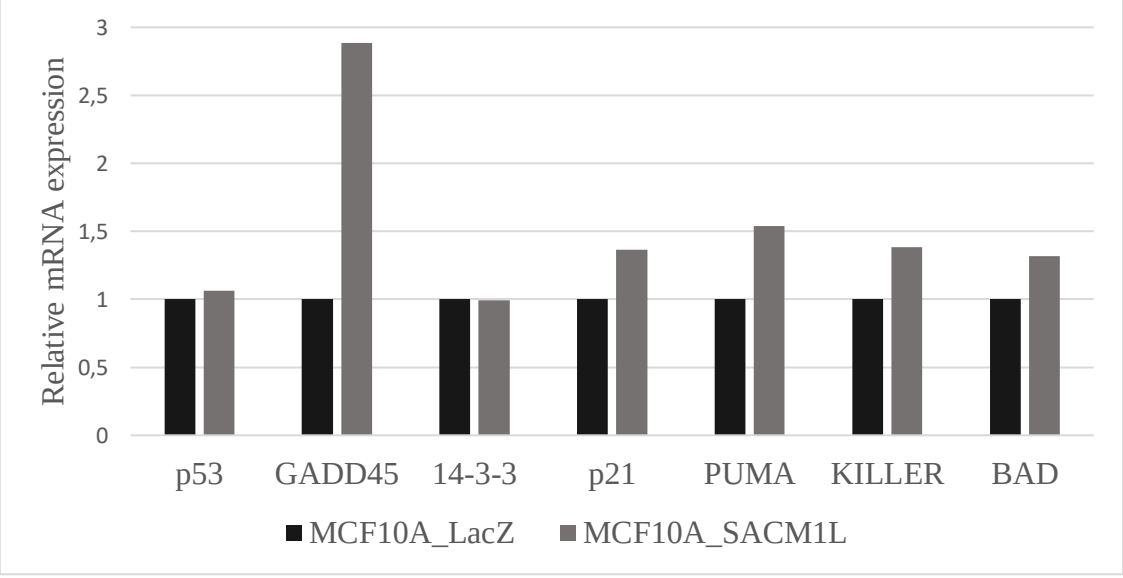


Figure 3.13:mRNA analysis of p53 target genes in MCF10A cells overexpressing SACM1L was verified by quantitative real-time PCR. mRNA levels of target

genes were relative to mRNA expressions of the control cells (LacZ) and were in proportion to the expression of a housekeeping gene, TATA-binding protein (TBP) (n=1)

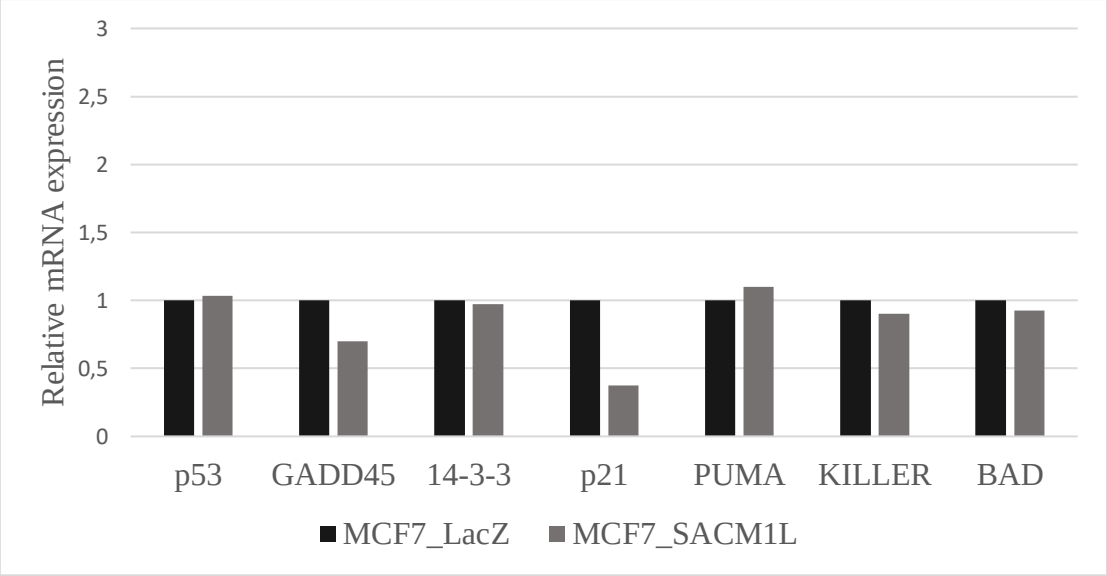


Figure 3.14:mRNA analysis of p53 target genes in SACM1L overexpressed MCF7 cells was verified by quantitative real-time PCR. mRNA levels of target genes were relative to mRNA expressions of the control cells (LacZ) and were in proportion to the expression of a housekeeping gene, TATA-binding protein (TBP) (n=1)

3.6. Knockout of SACM1L Gene was generated in the p53 Wild Type MCF10A and MCF7 Cells.

Overexpression of the SACM1L gene in the p53 wild-type MCF10A and MCF7 cells were investigated in terms of proliferation, cell cycle, and apoptosis. No significant effects on proliferation and cell cycle were observed in these cells, while the number of apoptotic cells was significantly increased. Therefore, to understand the effects of the SACM1L gene on the p53-activated cellular processes in MCF10A and MCF 7 cells, the

SACM1L gene was knockout by the CRISPR-cas9 system. gRNAs that were explicitly designed for the SACM1L gene were cloned into the Lenticrispr_v2 vector. The cells were infected by the lentiviral system, and stable cells were selected by puromycin antibiotic treatment. Non-template Lenticrispr_v2 (NTL) vector was used for infection control. The mRNA and protein expression of the SACM1L gene was confirmed by the quantitative real-time PCR (qRT-PCR) and Western Blot, respectively. Figure 3.15 represents 0.77-fold and 0.29-fold reduced mRNA expression of the SACM1L gene in order of MCF10A and MCF7 cells. The protein expression was significantly diminished in MCF10A and MCF7 cells (Figure 3.16).

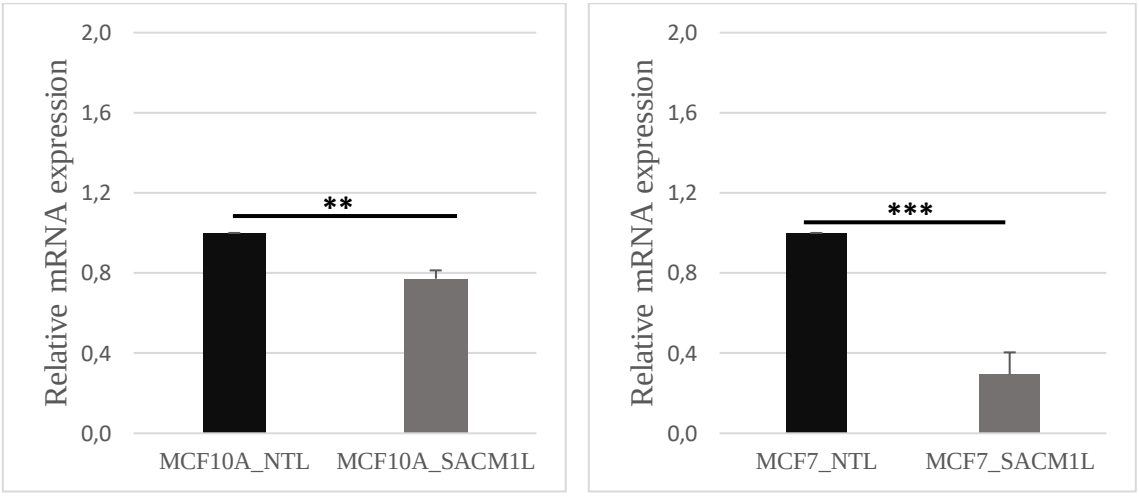


Figure 3.15:Knockout SACM1L mRNA levels in stable MCF10A and MCF7 cells were verified by quantitative real-time PCR. The level of SACM1L mRNA was relative to mRNA expression of control cells (NTLs) and was in proportion to the expression of a housekeeping gene, TATA-binding protein (TBP) Two-tailed student's t-test was used for statistical analysis. (n=2, **p<0.01; n=3, ***p<0.005)

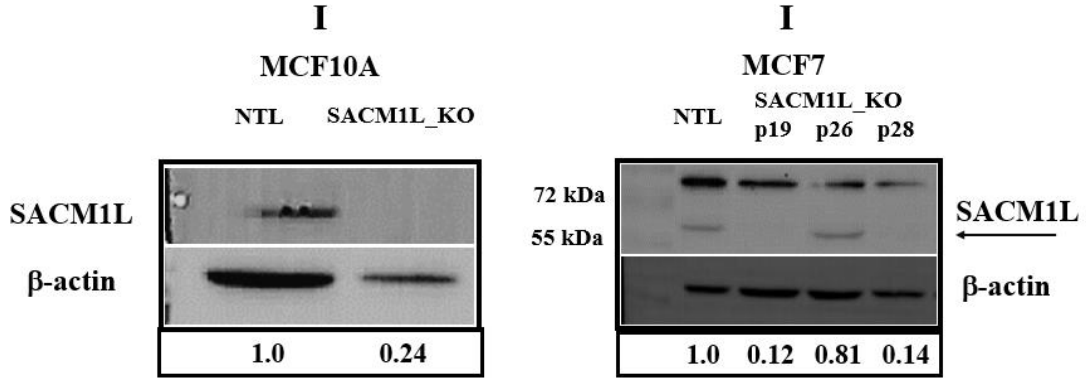


Figure 3.16:Knockout SACM1L protein level in stable MCF10A and MCF7 cells were verified by Western Blot. The protein level of SACM1L was relative to control cells (NTL) and β-actin was used for equal loading control. Quantification of protein level was done with the normalized expression of SACM1L protein to β-actin.

3.7 The effect of knockout SACM1L Gene on cell cycle was assessed on MCF7 Cells.

The cell proliferation capacities of the knockout SACM1L gene in MCF7 cells were evaluated by PI staining. Percentages of stained cells in the G1, S, and G2 phases were determined by flow cytometry. Previous research has established that the knockout SACM1L gene causes reduced proliferation by G2/M arrest in the growth of HeLa cells [18 20].

In the SACM1L knockout MCF7 cells, the percentage of cells at the G1 phase was significantly increased compared to NTL control cells. The increment of the G1 phase was 4.2% which indicates the knockout SACM1L cells were stuck in G0 or G1 phase. On the contrary, the percentage of cells in the S phase was significantly decreased from 30.1% to 27.7%. G2 phase percentage of SACM1L knockout cells was also significantly reduced relative to control NTL cells from 9.9% to 8.2% (Figure 3.17).

This result indicates that the knockout of the SACM1L gene has a proliferation-reducing effect on MCF7 cells.

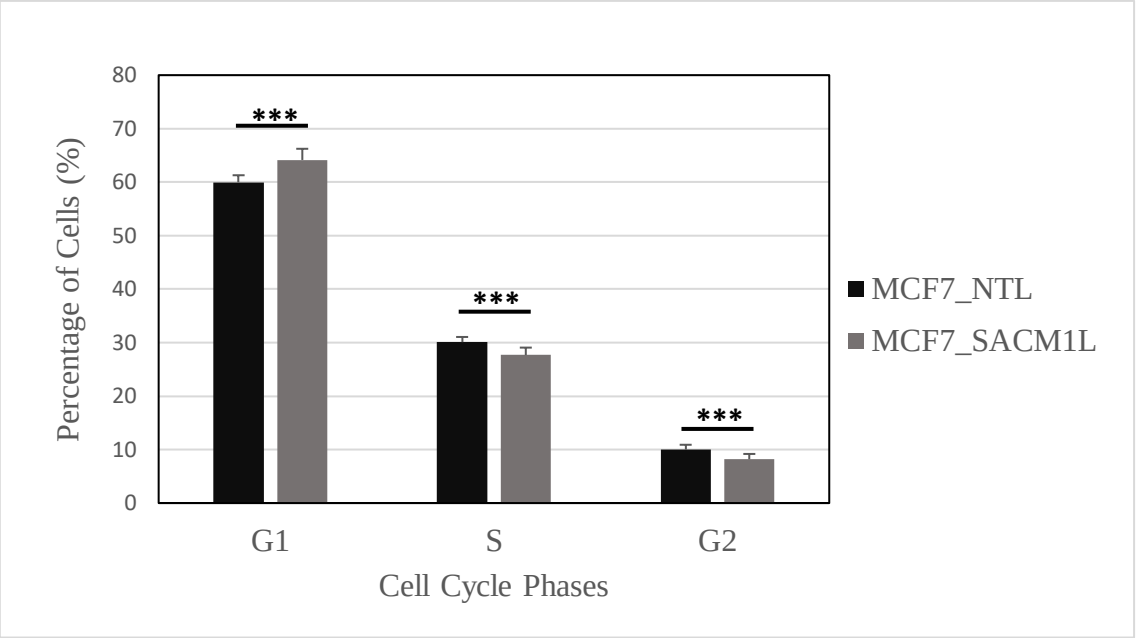


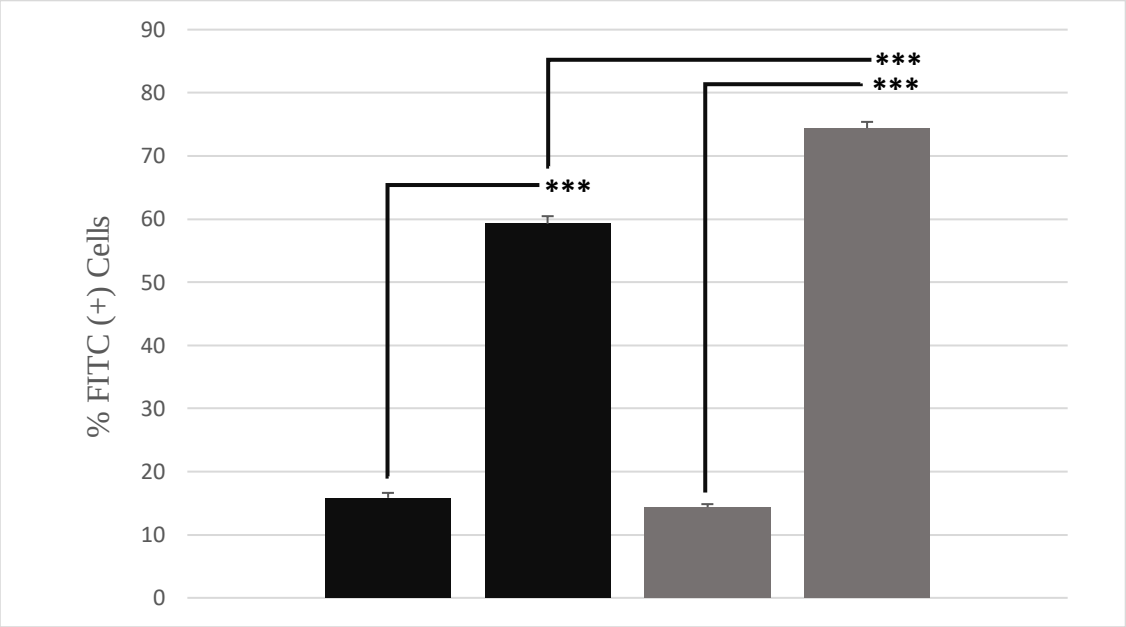
Figure 3.17:Cell cycle distribution (percentage) of knockout MCF7 cells at G1, S, and G2 phases. Two-tailed student's t-test was used for statistical analysis. (n=3, ***p<0.005)

3.8. The effect of knockout of SACM1L Gene on apoptosis was confirmed in MCF10A and MCF7 cells.

The SACM1L overexpression was induced p53 dependent apoptosis in MCF10A and MCF7 cells. Therefore, the effect of the knockout SACM1L gene in MCF10A and MCF7 cells on apoptosis was also examined by Annexin V conjugated FITC and propidium iodide (PI) staining measuring by flow cytometry. After 48 hours of culturing the SACM1L knockout MCF10A cells and its LacZ control cells were incubated with 2.5 μM of Doxorubicin (DOX) dissolved in methanol for 48 hours to induce the p53 dependent apoptotic pathways.

The apoptotic cell percentage of methanol treated control cells (NTL) was observed as 15.8% and the apoptotic cell percentage of DOX treated control cells was 59.2%. The percentage of apoptotic cells increased significantly, indicating that apoptosis

was successfully induced. In SACM1L knockout MCF10A cells exposed to methanol, 14.3% of apoptotic cells were obtained. The apoptosis rate of the DOX-treated knockout cells was significantly increased by 74.4% compared to the methanol treatment. When comparing methanol-treated control and SACM1L knockout cells, the percentage of apoptotic cells was slightly decreased in knockout cells, but this reduction was not significant. Nevertheless, there was a significant increase in DOX-induced knockout cells relative to DOX-treated control cells (Figure 3.18).

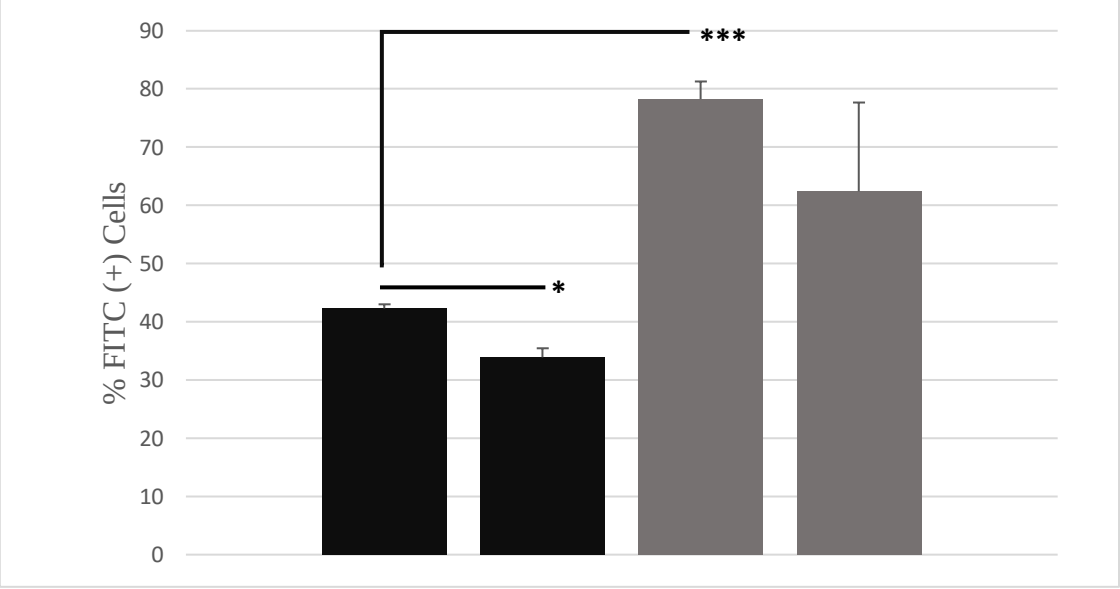


DOX treatment	-	+	-	+
SACM1L-KO	-	-	+	+

Figure 3.18:Apoptotic cell percentage in the control cells **(**MCF10A-NTL) and SACM1L knockout cells (MCF10A-SACM1L) Two-tailed student's t-test was used for statistical analysis. (n=1, ***p<0.005)

For the SACM1L knockout MCF7 cells, p53-dependent apoptosis was induced by cisplatin and the apoptosis assay applied for MCF7 cells overexpressing SACM1L was used similarly for these cells.

The apoptotic cell percentage of control cells (NTL) treated with NaCl was determined as 42.3% and the apoptosis rate of control cells that were exposed to cisplatin was significantly decreased to 33.9%. This dramatic reduction indicates that control MCF7-NTL cells were not effectively induced by cisplatin. For NaCl treated SACM1L knockout MCF7 cells, apoptotic cell percentage was found as 78.25%. When comparing NaCl-treated control and SACM1L knockout cells, apoptotic cells percentage was significantly increased in the knockout cells. Notwithstanding, the apoptotic cell percentage was observed as 62.4% in the knockout cells induced by cisplatin (Figure 3.19). As seen in the control cells, the number of apoptotic cells was decreased in SACM1L knockout cells treated with cisplatin compared to its NaCl treatment. Although cisplatin treatment was not successful, the apoptosis rate was observed to be increased in SACM1L knockout cells treated with cisplatin compared to control cells treated with cisplatin. However, this increase was not significant.



Cisplatin treatment	-	+	-	+
SACM1L-KO	-	-	+	+

Figure 3.19:Apoptotic cell percentage in the control cells **(**MCF7-NTL) and SACM1L knockout cells (MCF7-SACM1L) Two-tailed student's t-test was used for statistical analysis. (n=1, *p<0.05, ***p<0.005)

3.9. Transcriptional profiles of target genes in the p53 signaling pathway were investigated in the SACM1L knockout MCF10A and MCF7 cells

After investigating the proliferation, cell cycle, and apoptosis in SACM1L knockout MCF10A and MCF7 cells, some of the p53-regulated genes were evaluated at the transcriptional level by quantitative real-time PCR (qRT-PCR). The seven previously mentioned target genes were also examined for SACM1L knockout MCF10A and MCF7 cells.

Figure 3.20 and Figure 3.21 show the changes in the mRNA level of p53 target genes of SACM1L knockout normal breast cells MCF10A and breast cancer cells MCF7, respectively. Primarily, the mRNA level of p53 was significantly increased in SACM1L knockout MCF10A cells, whereas it was slightly decreased in knockout MCF7 cells. Genes responsible for p53 mediated cell cycle arrest, Gadd45, and 14-3-3 mRNA levels, were lightly decreased in SACM1L knockout MCF10A and MCF7 cells. However, the mRNA level of p21, which is also involved in cell cycle arrest, was significantly increased in SACM1L knockout MCF10A cells, contrary decreased in knockout MCF7 cells. The transcriptional levels of PUMA, Killer, and Bad, which induce apoptosis, were also evaluated. Although mRNA expression of PUMA was increased in both SACM1L knockout MCF10A and MCF7 cells, the increase was significant in knockout MCF10A cells but not in knockout MCF7 cells. In contrast to PUMA, Killer mRNA expression decreased in both knockout MCF10A and MCF7 cells, but the reduction was significant in knockout MCF10A cells, unlike knockout MCF7 cells. Lastly, the mRNA level of BAD had a decreasing trend in both knockout MCF10A and MCF7 cells. A significant decrease in the mRNA level of Bad was observed in SACM1L knockout MCF7 cells, whereas this decrease in knockout MCF10A cells was not significant.

The increasing trend in the mRNA level of PUMA, in SACM1L knockout MCF10A cells, may be linked to increased apoptotic cells percentage. Similarly, the result of increased apoptosis may be connected to increased PUMA mRNA level in SACM1L knockout MCF7 cells.

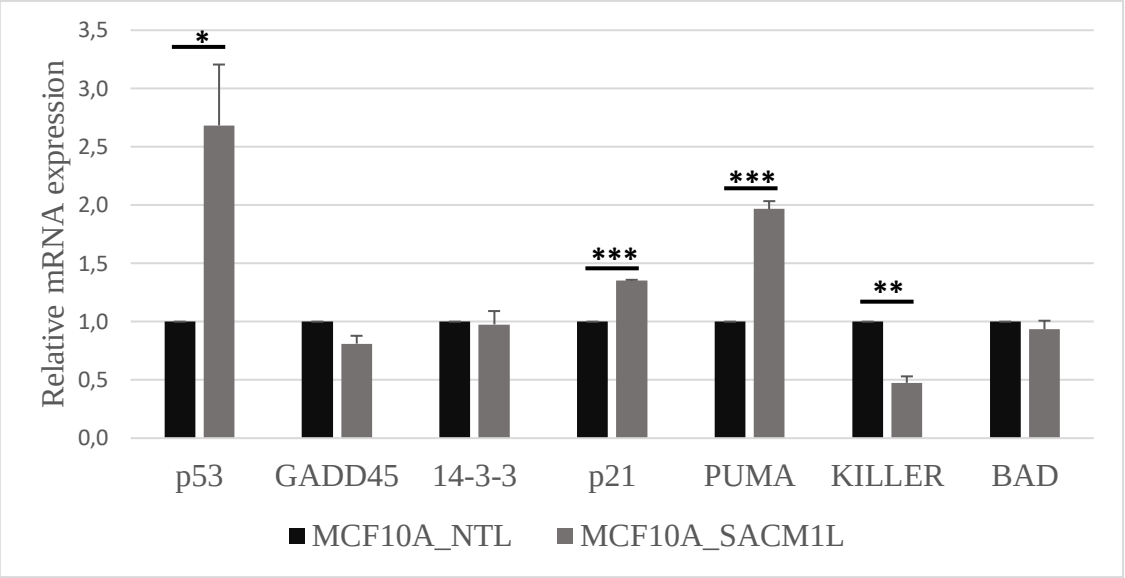


Figure 3.20:mRNA analysis of p53 target genes in SACM1L knockout MCF10A cells was verified by quantitative real-time PCR. mRNA levels of target genes were relative to mRNA expressions of the control cells (NTL) and were in proportion to the expression of a housekeeping gene, (GAPDH) Two-tailed student's t-test was used for statistical analysis. (n=2, *p<0.05, **p<0.01, ***p<0.005)

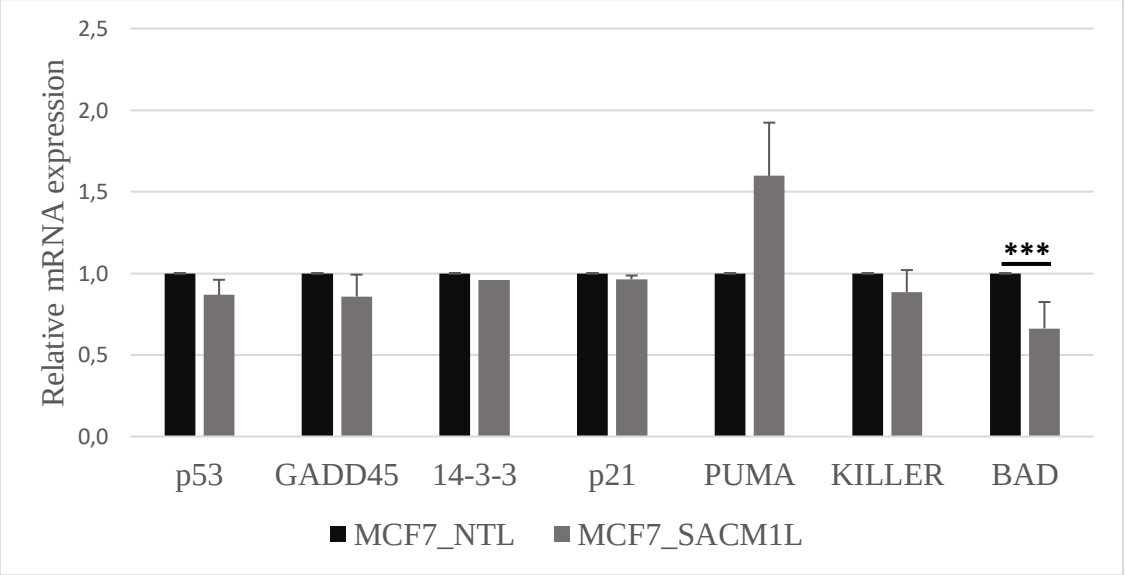


Figure 3.21:mRNA analysis of p53 target genes in SACM1L knockout MCF7 cells was verified by quantitative real-time PCR. mRNA levels of target genes were relative to mRNA expressions of the control cells (NTL) and were in proportion to the expression of a housekeeping gene, TATA-binding protein (TBP) Two-tailed student's t-test was used for statistical analysis. (n=2, ***p<0.005)

CHAPTER 4

DISCUSSION AND CONCLUSION

Since p53 has been extensively studied in cancer research, it continues to be researched because of its tumor suppressor function as a guardian of the genome. p53 is activated by various stress factors and determines cell fate by regulating many genes with post-translational modifications. p53 is mainly involved in regulating the genes that play roles in DNA repair, cell cycle arrest, apoptosis, autophagy, etc. Therefore, TP53 is mutated in many types of cancer [5]. Besides the p53 gene mutation in most cancer cells, in some types of cancer, p53 somehow remains unmutated [27]. While the reason behind some cancer cells expressing wild type p53 is still unknown, this allows exploration of the relationship between wild type p53 and other genes that may aid p53 activity and/or p53 mediated cellular outcomes. Because of high mutation incidence, p53 is tightly regulated by various mechanisms. Although redox regulation is one of the control mechanisms of p53, the details are not well known. A previous study revealed different redox-active antioxidant genes that can affect the p53 activity in yeast[14]. Then, another study introduced the effects of mutations of genes, which carry redox motifs and have potential redox functions, on the transcriptional activity of p53. As a result of these studies, deletions of the MAP1, SAC1, and HNT3 genes showed significantly decreasing p53 activity in yeast[15]. However, there were no data on p53 associations in mammalian homologs of these genes. This study aimed to investigate p53 signaling pathways and p53-regulated cellular processes in p53 wild type normal epithelial breast cell lines MCF10A and breast cancer cell lines MCF7 in the presence or absence of SACM1L gene products.

SACM1L (SAC1 Like Phosphatidylinositide Phosphatase) gene encodes phosphatidylinositol phosphatase, which primarily hydrolyzes PI4P. It is a transmembrane protein that is localized either ER or Golgi. Depending on where SACM1L is localized, the level of PI4P in the ER and Golgi influences cell response

under stress conditions such as starvation. SACM1L also affects other cellular processes, including lipid metabolism, membrane trafficking, and organization of mitotic spindle and cytoskeleton [20]. The absence of SACM1L gene expression in the mammalian cell lines was demonstrated to cause decreasing cell viability, G2/M phase arrest in cellular growth, increasing migration/invasion, and decreasing cell-cell adhesion [16, 22, 24]. However, no data were found on the association between the SACM1L gene and p53 either directly or indirectly.

First, conditions for the presence and absence of the SACM1L gene in the p53 wild type normal breast cell line MCF10A and breast cancer cell lines MCF7 were generated. mRNA and protein expression of the SACM1L gene was checked to ensure that both conditions were successfully established. The overexpressed and knocked out SACM1L gene was significantly produced in MCF10A and MCF7 cells.

SACM1L silencing significantly reduced the viability of HeLa cells and consequently cells trapped in the G2/M phase in the cell cycle [18]. However, no studies were provided on the effect of overexpression of the SACM1L gene on proliferation in mammalian cells. According to the MTT and BrdU assay findings, MCF10A cells overexpressing SACM1L did not show any significant difference in growth rate and proliferation, respectively. Although the PI staining assay showed no significant difference in cell cycle phases, a 2% increase in the G2/M phase of MCF10A cells overexpressing SACM1L was observed compared to control LacZ cells. This increase may be associated with increased mRNA expression of Gadd45 in MCF10A cells overexpressing SACM1L. Because previous studies have indicated that Gadd45 is involved in cell cycle arrest in the G2/M phase in p53 wild-type HCT116 cell lines, but not in p53 mutated HeLa cells [28]. However, the experiment of transcriptional profiles of p53 target genes was performed only once, so it must be repeated to see if it has a significant impact. In addition to the mRNA levels of p53 target genes, their protein expression should also be investigated.

MTT and PI staining assay for MCF7 cells overexpressing SACM1L showed no significant difference in viability and cell cycle relative to control LacZ cells. However, the proliferation of MCF7 cells overexpressing SACM1L has indicated an increasing trend in serum presence and deprivation conditions. This increasing trend was not significant, nonetheless. A decreasing trend was observed when the Gadd45 and p21

mRNA expressions of MCF7 cells overexpressing SACM1L were examined. We have previously mentioned that Gadd45 causes G2/M arrest. Besides this, p21 plays role in the p53 regulated G1 arrest by inhibiting Cdk2 [29]. Decreasing expression of Gadd45 and p21 may cause failure to arrest in the cell cycle of MCF7 cells overexpressing SACM1L. Again, the experiment of transcriptional profiles of p53 target genes was performed only once, so it must be repeated and protein expressions of them also should be examined.

On the other hand, cell cycle analysis of SACM1L knockout MCF7 cells showed a significant increase at the G1 phase, which resulted in G1 arrest. The previous study demonstrated that SACM1L silencing in HeLa cells resulted in an arrest at the G2/M phase. It should be noted that HeLa cells have mutated p53, while wild type p53 expression is present in MCF7 cells. In addition, G2/M arrest resulting from silencing of SACM1L in HeLa cells has been linked to Golgi membrane disorganization [18]. Therefore, further experiments are required to understand whether Golgi membrane dysregulation is present in SACM1L knockout MCF7. Alongside the increased G1, the percentage of cells in the S phase decreased significantly. These results are likely related to decreased viability in the absence of SACM1L expression. Further experiments may be required to investigate the effect of knockout SACM1L on viability such as MTT, XTT, or Alamar blue assays.

To assess apoptosis in MCF10A cells overexpressing SACM1L, 2.5 μm of Doxorubicin (DOX) dissolved in DMSO was administered for 16 h. Cells were treated with only DMSO to control DOX efficacy. Then, an apoptosis assay was performed. The reason for choosing DOX was to induce p53-mediated apoptosis by generating free radicals such as ROS by redox reaction [30]. DOX induced apoptotic cell percentage was significantly increased in MCF10A cells overexpressing SACM1L and the control LacZ cells compared to their DMSO conditions. This means that DOX has successfully induced apoptosis. However, SACM1L overexpression in MCF10A cells alone was not sufficient to induce apoptosis. This is because MCF10A cells overexpressing SACM1L could not increase apoptosis compared to LacZ control cells in the DMSO conditions. What is surprising is that SACM1L overexpression in MCF10A cells caused a significant increase in the percentage of apoptotic cells under the DOX induction compared to LacZ control cells. According to these data, we can infer that SACM1L overexpression induces p53 dependent apoptosis. Therefore, future studies on how SACM1L overexpression induces

apoptosis via p53 are required. It can be started by determining the expression of apoptotic proteins such as caspase 3, 8, and 9 to understand which apoptotic pathway, intrinsic or extrinsic, is triggered. In the light of apoptosis results, transcriptional profiles of p53 target genes PUMA, Bad, and Killer were evaluated in MCF10A cells overexpressing SACM1L. According to the literature, PUMA and Bad are involved in the secretion of cytochrome c from mitochondria into the cytoplasm by the stress induced p53 activity. This initiates caspase 9 and caspase 3 activity, which results in intrinsic apoptosis. Killer, on the other hand, activates extrinsic apoptosis via activating caspase 8 and caspase 3 [29, 31]. The transcriptional expression levels of all these genes were increased in MCF10A cells overexpressing SACM1L. It can be concluded that SACM1L overexpression activates intrinsic and extrinsic apoptotic pathways in MCF10A cells. These findings support the apoptosis results but are not sufficient. In addition to examining the mRNA expression levels of p53 target genes only once, the protein expression, phosphorylation status, and localization of the genes should also be investigated.

Apoptosis assay of SACM1L knockout MCF10A cells and the control NTL cells was conducted with DOX dissolved in methanol. Therefore, cells were treated with only methanol to control DOX efficiency. DOX induced apoptotic cell percentage was significantly elevated in SACM1L knockout MCF10A cells and the control NTL cells compared to their methanol conditions. On the DOX induction, knockout SACM1L in MCF10A cells resulted in a significant increase in the percentage of apoptotic cells compared to the control NTL cells. On the other hand, when we addressed p53 target genes, mRNA expression of PUMA increased significantly while expression of Killer dramatically decreased. It can be assumed that only the intrinsic apoptotic pathway was activated in SACM1L knockout MCF10A cells. Interestingly, p53 mRNA expression was also significantly increased, unlike SACM1L overexpressing MCF10A cells. When we considered all results, the SACM1L gene could play a role in the p53 mediated extrinsic apoptosis pathway.

To examine the effect of overexpression and knockout of SACM1L on apoptosis in the breast cancer cell line MCF7, 20 μm of cisplatin dissolved in 0.9% NaCl was used. Cisplatin is a drug commonly used to induce apoptosis through alkylation to generate DNA damage that triggers p53 activity [32]. 0.9% NaCl was administered to check cisplatin efficacy. Apoptosis analysis of SACM1L overexpression in MCF7 revealed that cisplatin

induction did not work effectively. Because the rate of apoptosis of cisplatin-induced control LacZ cells was lower than that of NaCl treated cells. One explanation for this situation is that the cells could be resistant to the dose we administer. Cancer cells may become resistant to some drugs, such as cisplatin, depending on the dose [32]. Surprisingly, in the cisplatin treated MCF7 cells overexpressing SACM1L showed a significant increase in the percentage of apoptotic cells compared to LacZ control cells. Moreover, it has been shown that NaCl-treated MCF7 cells overexpressing SACM1L have a significant increase in the percentage of apoptotic cells compared to NaCl-treated control cells. It can thus be suggested that SACM1L overexpression may trigger apoptosis in MCF7 cells without cisplatin induction. Different drugs such as DOX, or paclitaxel can be tried to assess whether SACM1L overexpression in MCF7 cells activates apoptosis with or without induction. In addition, paclitaxel may assist in the assessment of p53-dependent or independent increase in apoptosis[33]. According to the result of apoptosis associated p53 target genes, there was an increasing trend of PUMA in mRNA expression of MCF7 cells overexpressing SACM1L, whereas Killer and Bad had a decreasing trend in mRNA expression. This data comes from only one set of experiments, so it needs to be replicated to explain.

Apoptosis assay results of SACM1L knockout MCF7 cells and control NTL cells showed the same trouble with MCF7 overexpressing SACM1L, which was related to cisplatin induction. Unlike SACM1L overexpression in MCF7, SACM1L knockout MCF7 cells treated with NaCl had also decreased apoptotic cells compared to NaCl treated cells. An implication of this is the possibility that the presence of SACM1L may break the resistance to cisplatin for MCF7 cells. Nevertheless, as observed in MCF7 overexpressing SACM1L, NaCl-treated SACM1L knockout MCF7 cells had a significant increase in the percentage of apoptotic cells compared to NaCl-treated control NTL cells. In accordance with the result of apoptosis associated p53 target genes, PUMA had an increasing trend, whereas BAD mRNA expression was significantly decreased in SACM1L knockout MCF7 cells and p53 expression had a decreasing trend. This combination of findings allows interpretation of p53-independent apoptosis increases in MCF7 cells in the presence and absence of SACM1L.

In this study, p53 related cellular processes were investigated in the presence and absence of SACM1L in MCF10A and MCF7 cells, including proliferation, cell cycle, and

apoptosis. In the light of all results, apoptosis was drastically affected by SACM1L, so underlying mechanisms should be further investigated. Future studies should be conducted on the association of SACM1L gene to apoptosis and p53 target gene expressions in p53 wild-type and mutant cell lines. Thus, a direct connection can be evaluated.

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