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THE ROLES OF LONG NONCODING RNA DANCR IN CELLULAR BIOLOGICAL PATHWAYS

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MASTER'S THESIS

DEPARTMENT OF MOLECULAR AND TRANSLATIONAL BIOMEDICINE

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DECLARATION

I hereby declare that; this thesis has been written by me based on the data obtained in line with the scientific rules and ethical principles of responsible conduct of research. All information, data, comments, analyses have been collected and processed through scientific, academic writing style, and literature used have been duly shown by giving reference to the original sources in accordance with the publication ethics. I also announce and emphasize that I have not violated any rules secured by patent and copyrights whilst the conduct and writing of this research.

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ACKNOWLEDGMENT

First and foremost I am extremely grateful to my supervisor, Assist. Prof Dr. Emre Deniz, for his invaluable advice, continuous support, and patience during my thesis. He have encouraged me with his immense knowledge and plentiful experience in all the time of my academic research and daily life. He always showed respect my and patience to my all mistakes. I have to thank also to Asst Prof. Dr. Zeynep TOKCAER- KESKİN and Asst Prof. Dr. Nazlı KESKİN-TOKLU for their encouragement, guidance and supports.

I would like to thanks Başak KAVAKLIOĞLU, İrfan Baki Kılıç, Gizem GENÇAY and Gülin BARAN for their friendship and endless support. I would like to show my thankfulness to Başak and Gülin for their motivation, emotional support, and they became like my sister. I have to thank also to Baki for his patience, kindness and technical support especially in my first year.

Son olarak benim bugünlere gelmemi sağlayan, verdiğim bütün kararların arkasında durup bana destek olan, güvenen, sevgisini her zaman hissettiğim annem Derya Nalbant, babam Erkan Nalbant ve bu çalışmalarım sırasında vefat eden biricik anneannem Zeynep Bilal'e derinden teşekkür ederim. Sizleri çok seviyorum.

I thank to the Scientific and Technological Research Council of Turkey (TÜBİTAK) for supporting me and this Master's thesis. This project was funded by the TÜBİTAK 1003 project entitled "Screening and Modeling of Resistance Mechanisms Against Cytotoxic Drugs Using CRISPR / Cas9 Technology in Cancer Treatments" (Grant Number: 216S404).

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LIST OF SYMBOLS AND ABBREVIATIONS

ASO	Anti-sense oligos
ATP	Adenosine 5'-Triphosphate
crasiRNA	Centromere repeat-associated small interacting RNA
cDMEM	Complete Mammalian Cell Culture Medium
DANCR	Differentiation antagonizing non-protein coding RNA
DANCR KO	DANCR knock-out
DANCR OE	DANCR overexpressed
DEGs	Differentially expressed genes
ddH₂O	Double Distilled Water
DDR	DNA Damage Response
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
DOX	Doxorubicin
DSBs	Double-strand breaks
EDTA	Ethylene Diamine Tetraacetic Acid
EMT	Epithelial-mesenchymal transition
EtBr	Etidium Bromide
EtOH	Ethanol
ES	Enrichment score
EV	Extracellular vesicle

eRNAs	Enhancer lncRNAs
FDA	Food and Drug Administration
FDR	False discovery rate
FM	Freezing Medium
gDNA	Genomic DNA
GO	Gene ontology
GSEA	Gene Set Enrichment Analysis
HCC	Hepatocellular carcinoma
HCl	Hydrochloric Acid
HCT116	Human Colorectal Cancer
IARC	International Agency for Research on Cancer
KO	Knock-Out
LB	Luria Bertani
lncRNAs	Long non-coding RNAs
MgCl₂	Magnesium Chloride
mCAT	Mouse cationic amino acid transporter
miRNAs	MicroRNAs
MMP	Matrix metalloproteinases
MREs	MicroRNA response elements
mRNA	Messenger RNAs
mncRNAs	Medium non-coding RNAs
MSc	Mesenchymal stem cells
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-Diphenyltetrazolium Bromide; Thiazolyl Blue
NaCl	Sodium Chloride

NaOH	Sodium Hydroxide
ncRNA	Non-coding RNA
NES	Normalized enrichment score
NF-YA	Nuclear transcription factor
NMD	Nonsense-mediated decay
Nt	Nucleotide
ORFs	Open reading frames
PBS	Phosphate-buffered saline
PcG	Polycomb-group
PCR	Polymerase Chain Reaction
PC	Prostate cancer
Pei-MAX	Polyethylenimine HCl MAX
Pen-Strep	Penicillin-Streptomycin
PRC	Polycomb Repressive Complexes
PIPES	1,4-Piperazinediethanesulfonic acid
piRNAs	Piwi-interacting RNAs
poly(A) tail	Polyadenylated Region
RISC	RNA-induced silencing complex
RNA	Ribonucleic Acid
RNase A	Ribonuclease A
RNA-Seq	RNA Sequencing
ROS	Reactive oxygen species
RT-PCR	Reverse Transcriptase PCR
RT-qPCR	Reverse Transcriptase-Quantitative PCR

SDS	Sodium Dodecyl Sulfate
sgRNA	Single guide RNA
siRNAs	Small interfering RNAs
snRNA	Small nuclear RNAs
sncRNAs	Short non-coding RNAs
snoRNA	Small nucleolar RNAs
TAE	Tris-Acetate EDTA
telsRNAs	Telomere-specific small RNA
tiRNA	Transcription initiation RNAs
UTR	Untranslated region
WHO	World Health Organization
XCI	X chromosome inactivation

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SUMMARY

The Roles of Long Noncoding RNA DANCER in Cellular Biological Pathways

It has been shown that 2% of the human genome comprised of genes encoding protein. Although 80% of this genome is transcribed, these transcripts are not translated into protein. Long non-coding RNAs (lncRNA), which are longer than 200 nucleotides, are more specifically expressed for tissue or cell types. DANCER (Differentiation Antagonizing Non-Protein Coding RNA) is mostly located in the cytoplasm and has potential to be a potential onco-lncRNA. Studies have shown that overexpression of DANCER may play a role in the formation of various cancers by affecting cell viability, cell proliferation, tumor development, stemness and metastasis. However, there are still issues that we do not know about DANCER's true roles in tumor progression.

We, previously, had treated human colorectal cancer (HCT116) and mouse embryo fibroblast (MEF) cells with doxorubicin and performed RNA-seq analysis. We had found that doxorubicin treatment deregulates DANCER expression depending on treatment durations. After that finding, DANCER gene locus had been deleted from HCT116 by CRISPR/Cas9 technology. In this Thesis, to examine DANCER's role in cellular process, DANCER overexpressing WT cells and DANCER expression restored KO cells were created and named OE and Rescue, respectively. Cellular assays were performed to analyze DANCER role. According to these findings, DANCER increases cell proliferation, 3D spheroid and anchorage independent features. Also, DANCER overexpression increases S phase but DANCER knock out increases G₁ phase. DANCER deficiency and redundancy cause resistant to doxorubicin compared to corresponding parental cells. RNA seq and bioinformatics analyses were done to investigate competing endogenous RNA roles of DANCER and candidate miRNAs and mRNAs targets of these miRNAs were identified in differentially expressed genes in DANCER deficiency. This Master's thesis study documents the roles of DANCER in cellular biological pathways in a colorectal cancer cell line.

KEYWORDS: Biological pathways, CeRNA, DANCER, Doxorubicin, lncRNA

ÖZET

Hücresel Biyolojik Yolaklarda Uzun Kodlanamayan RNA DANCER'ın Roller

İnsan genomunun %2'sinin proteini kodlayan genlerden oluştuğu gösterilmiştir. Bu genomun %80'i transkript edilse de bu RNA'lar proteine çevrilmemektedir. Uzun kodlamayan RNA'lar (lncRNAs) 200 nükleotidden daha uzundur ve genellikle doku veya hücre tipine spesifik ifade edilir. Çoğunlukla sitoplazmada bulunan DANCER (Farklılaşma Antagonize Edici Protein Kodlamayan RNA), onko-lncRNA olma potansiyeline sahiptir. Çalışmalar, DANCER'nin aşırı ifadesinin hücre proliferasyonunu, tümör gelişimini ve metastazını etkileyerek farklı kanser türlerinin oluşumunda rol oynayabileceğini göstermiştir. Bununla birlikte, tümör ilerlemesindeki potansiyel rolleri hakkında hala bilmediğimiz sorular var.

Önceden, insan kolorektal kanser (HCT116) ve fare embriyo fibroblast (MEF) hücrelerini doksorubisinle muamele edip RNA-dizileme yapmıştık. Hücrelerinin doksorubisin ile farklı zamanlar boyunca muamele edilmesinin DANCER ifadesini değiştirdiğini bulmuştuk. Bu bulgudan sonra, DANCER gen lokusunu, CRISPR/Cas9 teknolojisi ile HCT116'dan silmiştik. Bu tez çalışmasındaysa, hücresel süreçte DANCER rolünü araştırmak için, DANCER'ın aşırı ifade edildiği yabancı tip (WT) ve DANCER ifadesinin geri konduğu DANCER'ı silinmiş hücreleri (KO) oluşturularak sırasıyla OE ve Rescue olarak isimlendirildi. DANCER rolünü analiz etmek için çeşitli hücresel deneyler yapıldı. Bu bulgulara göre DANCER, hücre proliferasyonunu, 3D sferoid ve ankrajdan bağımsız özellikleri artırmaktadır. Ayrıca, DANCER aşırı ifadesi S fazını artırır, ancak DANCER'ın silinmesi G1 fazını artırır. DANCER eksikliği ve fazlalığı, parental hücelere kıyasla doksorubisine direnç geliştirir. DANCER'nin yarışan endojen RNA rolünü araştırmak için RNA-dizileme ve biyoinformatik analizi yapıldı ve aday miRNA'lar ve DANCER yokluğunda ifadesi değişen miRNA hedefli mRNA'lar bulundu. Bu yüksek lisans tez çalışması, DANCER'nin kolorektal kanser hücre hattındaki hücresel biyolojik yollardaki rollerini belgeler.

ANAHTAR KELİMELER: Biyolojik yollar, CeRNA, DANCER, Doksorubisin, LncRNA

1. AIM OF STUDY

LncRNAs are longer than arbitrarily defined 200 nucleotide and they are not translated into proteins. The total number of lncRNA genes in human genome was estimated to range from 20,000 to 100,000. They are able to affect gene expression in transcriptionally, post-transcriptionally, translationally, epigenetically, and through protein localization. DANCER, which is an onco-lncRNA, was found as a suppressor of epidermal cell differentiation. The level of DANCER was thought to be significant because overexpression was found in various cancers regulating malignant biological behaviors such as the abnormal proliferation of cells, invasion, metastasis, and chemoresistance. DANCER can sponge microRNAs or interact with different proteins, in this way, it can arrange components in the oncogene network. Other studies show that DANCER affects cell proliferation, cell motility, cell viability, and stemness. These properties of DANCER make a potential candidate in therapeutics.

In order to examine DANCER role in biological pathways, overexpressed DANCER and Rescue cells were created by mCAT mediated retroviral infection using a colorectal cancer cell line, HCT116 and its DANCER knock out derivative. Firstly, PCR genotyping was done to control whether DANCER gene locus was still intact or absent in the corresponding cells and RT-PCR and RT-qPCR were performed to assess DANCER expression at RNA level. Cell proliferation rates, cell cycle distributions, cell morphologies, and doxorubicin treatment responses for each cell type were examined. Also, the contribution of DANCER to several cancer hallmarks, which were anchorage independent growth and 3D spheroid features, were investigated. RNA sequencing and bioinformatics analysis were done to investigate the competing endogenous RNA roles of DANCER. This Master's thesis study contributes to the literature with the roles of DANCER in cellular biological pathways in colorectal cancer cell line, HCT116.

2. INTRODUCTION

2.1 Classification of non-coding RNAs

Genome sequences that are draft of the living organisms have pivotal roles in origination all life forms (1). The number of protein-coding genes had was found to be around 21.000 in human genome however this comprises almost only 2% of the genomic sequence (2). Genes in this class are first transcribed into messenger RNAs (mRNAs) and then translated into proteins. The rest of the genome, on the other hand, consist of non-protein-coding part, also sometimes referred to as junk DNA. With the rapid improvement in high-throughput sequencing technologies, recent information about the complexity of gene expression regulation has arisen. Deep sequencing of oligo-dT-primed cDNAs, also simply known as RNA-sequencing, followed by novel computational methods has identified novel non-coding transcripts across diverse cell types and tissues (3). These non-protein-coding gene does not encode for proteins and they lack evolutionary sequence conservation. Nonetheless, non-coding RNA (ncRNA) transcripts form a large class in the genome and demonstrate that RNAs have various roles in biological processes. Interestingly, the complexity of an organism was found to be associated with abundancy of ncRNA genes (4).

NcRNAs are roughly divided into three categories according to their corresponding lengths. These are referred to as small non-coding RNAs, medium non-coding, or long non-coding RNAs. The short non-coding RNAs (sncRNAs), which are the size between 20-50 nucleotides (nt) in length and contain microRNAs (miRNAs), piwi-interacting RNAs (piRNAs), small interfering RNAs (siRNAs), centromere repeat-associated small interacting RNA (crasiRNA) and telomere-specific small RNA (telsRNAs) (5). SncRNAs can arrange gene regulation by

interacting with other molecules, such as RNA, DNA, or protein, through RNA interference, RNA modification, or spliceosomal involvement (6). The medium non-coding RNAs (mncRNAs) are between 50-200 nucleotides (nt) in length and contain small nucleolar RNAs (snoRNA), transcription initiation RNAs (tiRNA), and small nuclear RNAs (snRNA). Long non-coding RNAs (lncRNAs) are longer than 200 nt and has the most maximum regulatory potency (7). Additionally, lncRNAs are classified as antisense, sense, intergenic, bidirectional intronic, and exonic genes depending on their locations and positions within the genome.

2.1.1 Long non-coding RNAs

lncRNAs are longer than arbitrarily defined 200 nt and they are not translated into proteins. The total number of lncRNA genes in human genome was estimated to range from 20,000 to 100,000 (8). They are able to affect gene expression in transcriptionally, post-transcriptionally, translationally, epigenetically, and through protein localization (4). lncRNAs compose convenient secondary and tertiary structures by interacting with proteins, DNA, or other RNAs, and the final structure gives cues about their function. Secondary structures of lncRNAs have significant recognition elements guiding their interactions. Among the most widespread structures are stem-loops (hairpins), internal loops, bulges, pseudoknots, kink-turns, complex multibranch loops, and G-quadruplexes (9). In such ways, chromatin organization, epigenetic regulation, gene transcription and translation may be regulated (10). They are additionally found to have vast variety of biological significances by playing roles in biological processes effecting development, differentiation, proliferation, invasion, metastasis, apoptosis, growth, drug resistance, and metabolism (11) (12). Although, most lncRNAs have poor primary sequence conservation among between different species, their overall stem-loop structures are usually conserved to maintain molecule functionality (13).

Even though proteins are the master regulators and executioners of cellular function, almost half of the changes in expression occur at the RNA level, especially via controlling of RNA stability (14). The advantage of using RNA as a medium suggests that potential regulatory functions may be performed quickly and efficiently without processing further into protein translation. LncRNAs have some unique features compared to mRNAs. They have lower expression levels relative to mRNA, and this makes difficult to capture and annotate lncRNAs from transcriptomic data, which are not deep enough to cover rare transcripts. LncRNAs do not have major open reading frames (ORFs) that would otherwise simplify prediction of structural domains, of final 3-dimensional structures, and of protein-protein interactions such as in protein-coding genes. Numerous lncRNAs are degraded within nucleus shortly after they are produced or by cytosolic nonsense-mediated decay (NMD) machinery during their transport into the cytosol, however, mRNAs are commonly degraded by cytoplasmic decapping enzymes and exonucleases (5' to 3') (15). On the other hand, lncRNAs share several features with mRNAs as well; such that both are transcribed by RNA polymerase II and undergo splice-processing and post-transcriptional modifications, which are 5'capping and poly(A)-tail.

2.1.2 Categories of lncRNA genes

Regulatory actions of lncRNAs may be related with their corresponding genomic locations. Although classifying lncRNAs based on a single criterion is difficult, according to the nearest protein-coding gene relative to their particular genomic locations or transcriptional origins, lncRNAs may be classified as bidirectional, intergenic, intronic, sense or antisense classes (**Figure 1**). Bidirectional or divergent lncRNAs share same promoter regions with their nearest protein-coding genes but are transcribed from the opposite strand (16). Intergenic lncRNAs are surrounded by two protein-coding genes on both end and are transcribed in the same direction with them (13). Intronic lncRNAs are located and

transcribed completely from introns of protein-coding genes. Sense lncRNAs are transcribed from the same strand, on which the nearest protein-coding gene lays, and they can be overlapped with one or more exons or introns of the surrounding protein-coding gene. Antisense lncRNAs also overlap similarly with exonic or intronic regions, however, they are transcribed from the opposite strand of these (17).

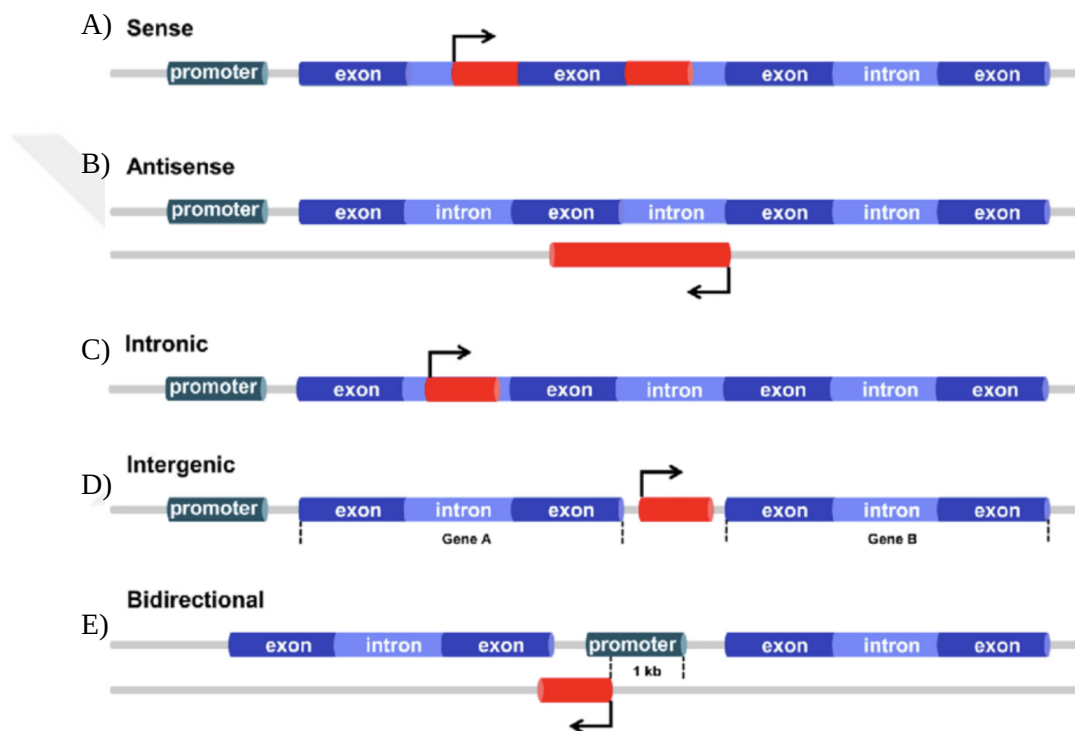


Figure 1: The classification of lncRNAs by their genomic location. **A)** Sense lncRNAs are transcribed from the same strand of protein-coding genes. **B)** Antisense lncRNAs are transcribed from the opposite strand of protein-coding genes. **C)** Intronic lncRNAs are located in protein-coding gene' intronic regions. **D)** Intergenic lncRNAs are transcribed from same direction but located between two protein-coding genes. **E)** Bidirectional lncRNAs are transcribed in the opposite direction, and located inside of 1 kb of the protein-coding gene' promoter region (18).

Ref (18): Hermans-Beijnsberger S, van Bilsen M, Schroen B. Long non-coding RNAs in the failing heart and vasculature. *Non-coding RNA Res.* 2018;3(3):118–30.

2.1.3 Actions of lncRNAs

Regulatory actions of lncRNAs can be classified as cis or trans regulation. The transcription of neighboring genes may be regulated via cis-acting lncRNAs located in the vicinity of the corresponding genes on the same chromosome, in nucleus. In this way, after lncRNAs are transcribed, they mediate transcription and splicing processes within the neighboring gene (**Figure 2A**) (19). The trans-acting function of lncRNAs can regulate chromatin states and gene expression at distant regions on the same chromosomes or at another chromosome by leaving their transcription sites (**Figure 2B**). Additionally, proteins and other RNA molecules, such as mRNAs and miRNAs, may be regulated by trans-acting lncRNAs after they are transported from the nucleus into the cytoplasm (**Figure 2C**).

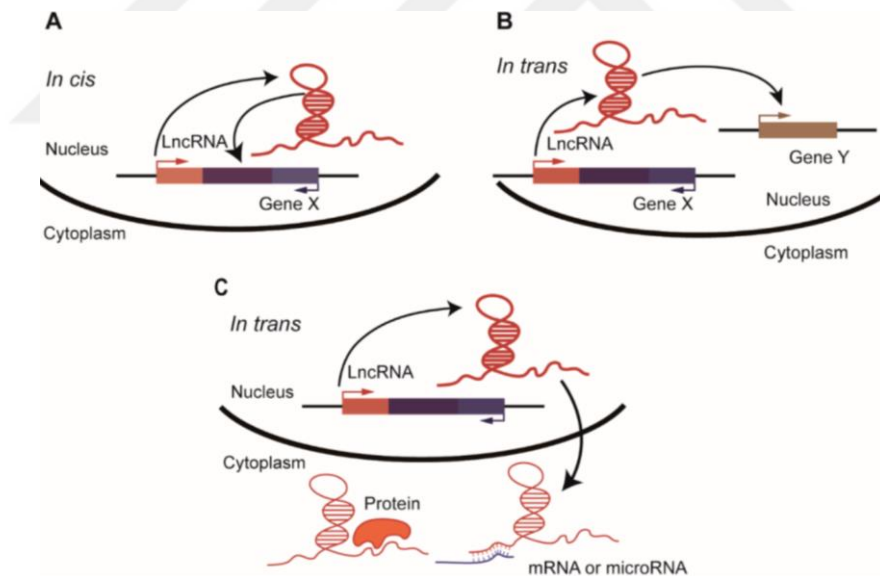


Figure 2: lncRNAs may act as cis or trans. **A)** Cis-acting lncRNAs arrange the transcription of their neighboring genes on the same chromosome. **B)** The trans-acting lncRNAs depart from their transcription sites to arrange genes' expression located in far regions in the nucleus. **C)** Trans-acting lncRNAs bind proteins or other RNAs after themselves are transported to the cytoplasm (19).

Ref (19): Zhao Y, Teng H, Yao F, Yap S, Sun Y, Ma L. Challenges and strategies in ascribing functions to long noncoding RNAs. *Cancers (Basel)*. 2020;12(6):1–21.

2.1.4 Subcellular locations of lncRNAs

lncRNAs occupy both nuclear and cytoplasmic compartments. Knowing their subcellular localizations provides valuable knowledge regarding their functions and mechanisms of action. lncRNAs playing roles in histone modifications and transcription factors recruitment localize in nucleus (**Figure 3**). Whereas, cytoplasmic lncRNAs affect post-transcriptional steps regulating ribosome recruitment, mRNA stability, miRNA interaction, and translation of mRNAs among many other processes in the cytoplasm (20). Additionally, some lncRNAs are capable of encoding short peptides, which are known as micropeptides, that take part in diverse biological processes. However, this is suspected to be a rare event and it demands more research to further elucidate the functional significance of micropeptides (4).

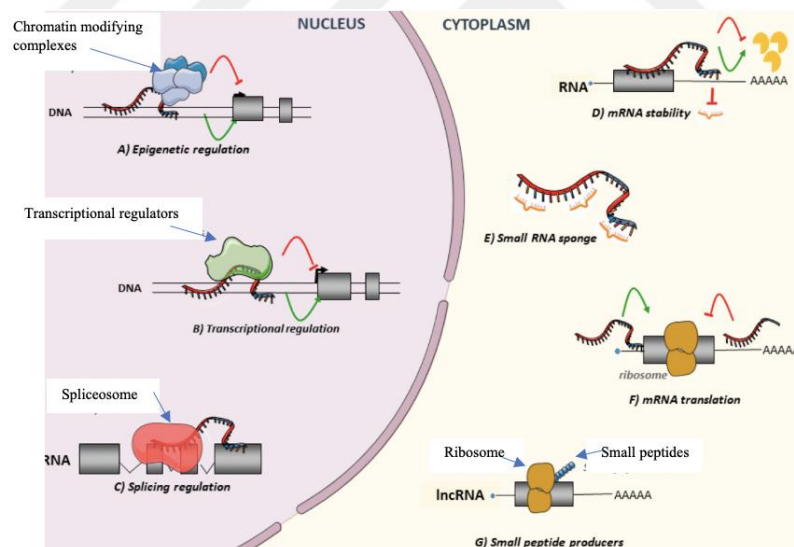


Figure 3: Roles of lncRNAs in nucleus and cytoplasm. **A)** Epigenetic regulations can act as activator or repressor chromatin modifying complexes, and recruited on target promoters. **B)** Transcriptional regulations can guide or prevent the recruitment of transcription factors on their targets' promoters or on other active chromatin sites. **C)** Splicing regulations can regulate recruitment of spliceosome partners. **D)** mRNA stability can modulate the degradation positively or negatively. **E)** Small regulatory RNAs can be sponged. **F)** mRNA translations can be regulated. **G)** Small peptides can be encoded (21).

Ref (21): Gourvest M, Brousset P, Bousquet M. Long noncoding RNAs in acute myeloid leukemia: Functional characterization and clinical relevance. *Cancers (Basel)*. 2019;11(11). 22.

2.1.5 Molecular mechanisms of lncRNAs

The molecular mechanisms of lncRNAs may be classified as signals, decoys, guides, scaffolds, enhancers, and competing endogenous RNAs (ceRNAs). These ensure beneficial clarifications of biological outcomes (**Figure 4**).

2.1.5.1 Signal lncRNAs

lncRNAs take part in cell signal regulation roles and respond to diverse stimuli to regulate transcription, so that gene expression is arranged specific to cell type (11). Xist is one of the earliest mammalian lncRNAs to be discovered. It plays an essential and defining role during X chromosome inactivation (XCI) process in females. The X chromosome to be inactivated expresses Xist and then that X chromosome is coated by it. This process causes chromosome-wide repression of gene expression and the repressive state on the chromosome is maintained the whole life of the cell. On the other hand, Xist expression is repressed by Tsix antisense lncRNA. Also, accumulated lncRNA Jpx during XCI activates Xist on the inactive X (22). A p53 tumor suppressor protein-controlled transcriptional response arises in a normal cell in response to a damaging assault on DNA. lincRNA-p21, which is located just upstream of CDKN1A gene, acts as a transcriptional repressor in the canonical p53 pathway that plays a crucial role in triggering apoptosis (23).

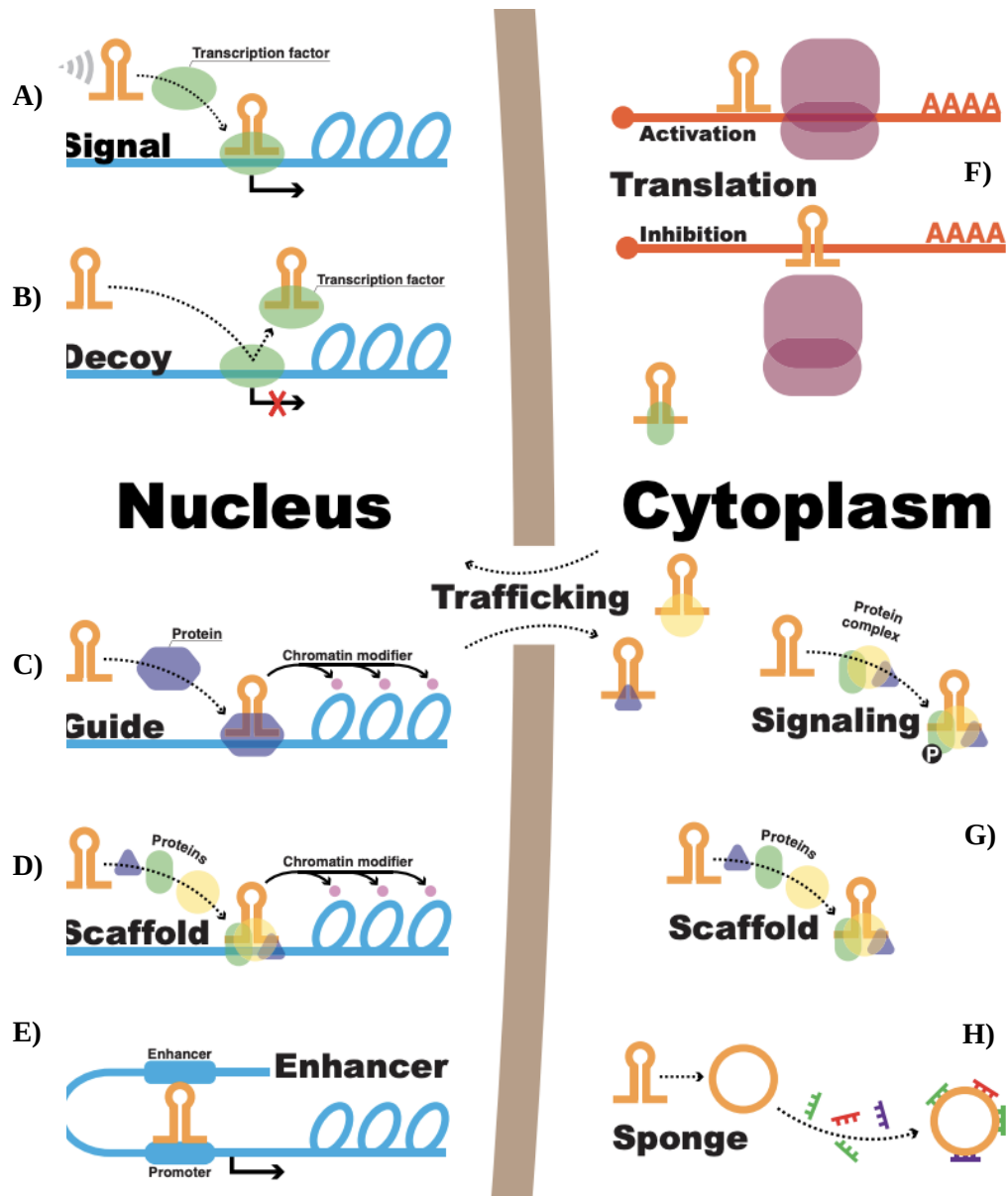


Figure 4: Molecular mechanisms of lncRNAs. **A)** LncRNAs can respond to specific stimuli. **B)** LncRNAs decoy transcription factors away from their target DNA. **C)** LncRNAs guide regulatory proteins to their target DNA. **D)** LncRNAs act as scaffold to bring different proteins together both in cytoplasm or in nucleus. **E)** LncRNAs act as enhancer-promoter bridging factors in nucleus. **F)** LncRNAs activate or inhibit translation by binding to target mRNAs. **G)** LncRNAs regulate protein trafficking, posttranslational modification, and signaling in cytoplasm. **H)** LncRNAs sponge miRNAs (24).

Ref (24): Gomes CPC, Spencer H, Ford KL, Michel LYM, Baker AH, Emanuelli C, et al. The Function and Therapeutic Potential of Long Non-coding RNAs in Cardiovascular Development and Disease. *Mol Ther- Nucleic Acids*. 2017;8(September):494–507.

2.1.5.2 Decoy lncRNAs

Decoy lncRNAs can arrange transcription factors, chromatin modifiers, or other regulatory factors by diverting them from their targets. They bind and titrate a protein away from its intended target without exerting additional functions on the protein. When such a decoy lncRNA is silenced, its protein partner will gain normal function on its particular target (11). PANDA lncRNA possesses decoy function on NF-YA (nuclear transcription factor). NF-YA activates the apoptotic program in response to DNA damage. PANDA, which is very sensitive to DNA damage, inhibits expression of apoptotic genes. In this regard, NF-YA is sequestered by PANDA to arrest cell-cycle. Intriguingly, PANDA was found overexpressed in human breast cancers. It is further elucidated that PANDA depletion sensitized cells to chemotherapeutic agents and made them more apoptotic (25).

2.1.5.3 Guide lncRNAs

Guide lncRNAs bind protein to create ribonucleoprotein complexes and orient them to specific targets. Gene regulatory components, which are repressive or activatory complexes and transcription factors, are controlled by lncRNAs to transmit regulatory instruction and check target gene expression. lncRNAs guide changes in co-transcriptional chromatin states in cis. AIR lncRNA silences transcription of its target gene on the paternal chromosome, helped by interactions with chromatin at the promoter. When AIR accumulated at the promoter, G9a was recruited. This causes targeted H3K9 methylation and allelic silencing (11). Also, lncRNAs assert their transcriptional effects across chromosomes in trans. HOTAIR lncRNA, which has been associated with cancer metastasis in several types, was found to be increased in breast cancer. When the levels of HOTAIR decreases, the level of polycomb proteins is increased, resulting in reduced cell invasiveness. This shows that HOTAIR can regulate epigenetic states through targeting chromatin-modifying complex polycomb proteins (26).

2.1.5.4 Scaffold lncRNAs

Scaffold lncRNAs consist of a functionally complex class. LncRNAs, which possess different domains, bind distinct effector molecules that may have transcriptional activating or repressive activities at the same time (27). INK4b/ARF/INK4a is a tumor suppressor locus and is controlled by H3K27me3 guided by the Polycomb-group (PcG) proteins, and by the antisense ANRIL lncRNA emanating from the same locus. In this regard, ANRIL, which serve as a molecular scaffold to dynamically modulate transcriptional activity, causes transcriptional repression of the target INK4b locus by binding components of Polycomb Repressive Complexes (PRC1, PRC2) (28).

2.1.5.5 Enhancer lncRNAs

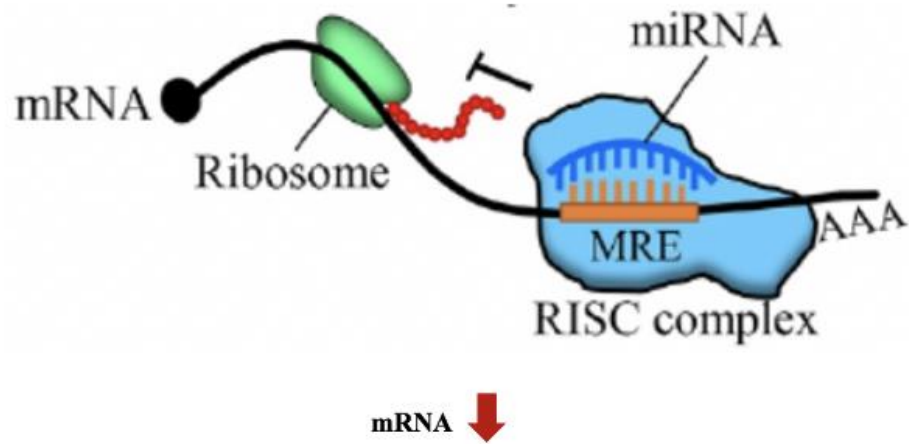
The emerging class of enhancer lncRNAs (eRNAs), which are transcribed from enhancers in a tissue-specific manner, contribute to gene control by altering chromatin environment. Dynamic stabilization of enhancer-promoter looping can be arranged by cis action of eRNAs. Also, chromatin-remodeling events that modulate transcription factor complex assembly can be regulated by a trans action of eRNAs. Post-translational modifications on histone tails are adjusted by eRNAs that are in complex with epigenetic modifying enzymes (29).

2.1.5.6 Competing Endogenous lncRNAs

The competing endogenous RNA (ceRNA) theory, which was first identified by Leonardo Salmena, has become a hotspot field in lncRNAs recently (30). CeRNAs bind miRNAs to sponge them out. Therefore, the translation inhibitory

effects of these miRNAs on target mRNA transcripts are ceased (**Figure 5A**). MiRNAs are about 20-22 nucleotides in length and cause RNA silencing at post-transcriptional level by creating RNA-induced silencing complex (RISC) (31). The mechanism of action in miRNA-directed RNA silencing depends on microRNA response elements (MREs) in a coding or non-coding target transcript and on miRNAs' seed sequence. MiRNAs recognize target transcripts through MREs with partial sequence complementarity. CeRNAs compete with mRNAs to bind corresponding miRNAs, suggesting a fine tuning of translation control mechanisms (30). This allows an indirect regulation of the inhibitory action of miRNAs on mRNA targets (**Figure 5B**). Also, mRNAs may contain more than one MREs targeted by different microRNAs (30). When the ceRNAs increase and bind to their miRNA targets, the miRNA targeted transcript will become free. However, lack of ceRNAs cause a decrease in the targeted transcripts because of increased miRNA availability. A perturbation of the ceRNA network may deregulation of protein expression levels hence may lead to cancer or other diseases. For example, 3' UTR shortening may be observed in some cancer-causing transcripts of human cancer cells. It affects microRNA-dependent mRNA regulation. Therefore, high or low levels of transcripts may perform increased oncogenic or decreased tumor-suppressor activities, respectively (30).

A)



B)

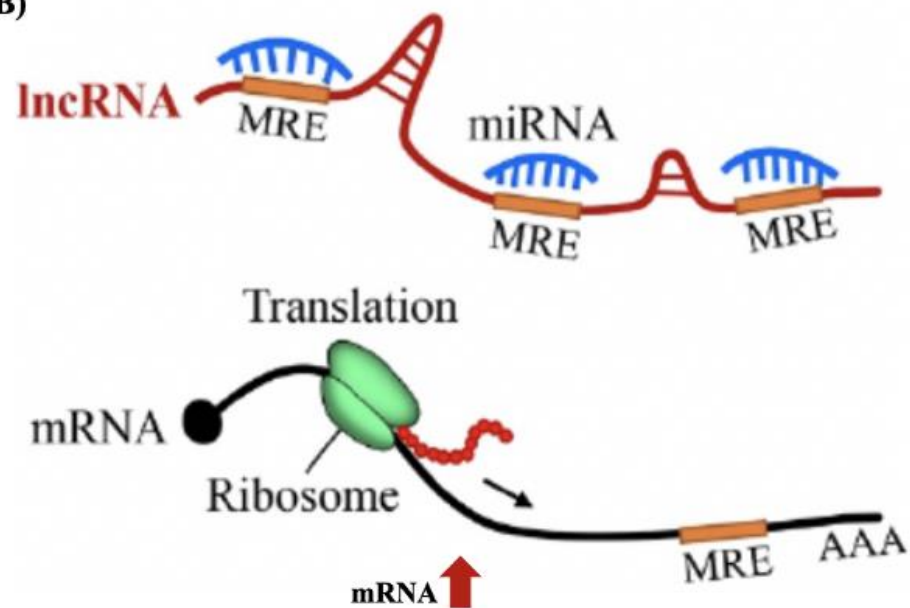


Figure 5: Molecular mechanisms of miRNAs and ceRNAs. **A)** miRNAs bind to target mRNAs inhibiting their translation or decreasing their stability. **B)** LncRNAs sponge miRNAs on themselves through their MRE sites and free target transcripts from their miRNAs (14).

Ref (14): Zhang Xiao-Zhen LH and CS-R. Mechanisms of Long Non-Coding RNAs in Cancers and Their Dynamic Regulations. *Cancers (Basel)*. 2020;12(5).

2.1.5 LncRNAs in cancer

Cancer can be defined as a multistep process in which normal cells acquire traits to become tumorigenic. When normal cells evolve progressively to a neoplastic state, they gain several properties, also known as cancer hallmarks, such as maintaining proliferative signaling, reprogramming of energy metabolism, escaping growth suppressors and immune destruction, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis (32). The nineteen million more new cases cancer patient cases were estimated worldwide in 2020 by International Agency for Research on Cancer (IARC) of World Health Organization (WHO) (33). Incidence rank of cancer is the highest in breast (58.5%), followed by prostate (36%), lung (28.3%), and colorectum (24.3%). Additionally, mortality rate is the highest in lung (23%), followed by colorectum (12%), liver (10.7), and stomach (9.9%). Determining the definitive reason of a cancer case is hard, but risk factors can be listed as radiation, obesity, infections, alcohol and tobacco usage, and less physical activity (34). Genetic background, on the other hand, is a significant reason for acceleration of cancer acquisition.

LncRNAs are highly tissue specific and have elevated efficiency and high stability. These properties make them good candidates as therapeutic targets. Moreover, they can also be exploited as biomarkers for diagnosis or for prognosis (12). Whole-transcriptome analyses have revealed new examples of lncRNAs and they are found as regulators of human cancer development largely by modifying gene expression at different levels, however, their molecular functions have not been characterized entirely (35). LncRNAs act as regulators of oncogene and cancer suppressor mRNA stabilities. In this way, they either decrease cancer cell proliferation and invasion or increase tumor growth and metastasis. When these mechanisms were dysregulated, lncRNAs can get involved in several pathological conditions such as in breasts, lungs, prostate, and liver (16). Additionally, lncRNAs

arrange cancer inducing factors such as DNA damage, metastasis, immune escape, drug resistance, and angiogenesis (36).

2.1.5.1 DNA damage

DNA damages and genomic instability can be arisen by environmental factors such as genotoxic drugs or by the result of endogenous carcinogenesis itself. Noncoding RNA activated by DNA damage (NORAD) is a cytoplasmic lncRNA and is essential for an accurate mitotic division, in which it plays roles in preserving chromosomal stability (37). Upon an insult on the DNA, NORAD was found to interact with a component of the DNA-damage response (RBMX). In this way, the assembly of the NARC1 complex is promoted to help establishing genome stability.

2.1.5.2 Metastasis

Metastasis is a process of spread of the tumor from its primary site of origin into distance tissues. Epithelial-mesenchymal transition (EMT) has a significant role in normal cell differentiation throughout early development, however, it is also a causative reason for metastasis to take place (38). The underlying mechanism of EMT is the loss of epithelial cell features in such a way that cells gain migratory, hence invasive, phenotypes of mesenchymal cells. When the activity of MALAT1 lncRNA in ovarian cancer is inhibited, EMT was prevented (39). The expression of E-cadherin, which is an epithelial cell marker, was restored to its normal levels, indicating that cells reversed EMT. In addition, mesenchymal cell markers, such as N-cadherin, snail, and vimentin, were reduced via downregulating PI3K/AKT signaling pathway.

2.1.5.3 Immune escape

Tumor growth can be both enhanced or inhibited by immune system, and this, in the end, affects disease outcomes. Tumors acquire some properties to escape from immune response elements such as regulatory T cell (Tregs), M2 macrophages, and cytotoxic T lymphocytes (CTLs) among others (35). For example, differentiation of Tregs and suppression of CTLs by lnc-epidermal growth factor receptor (lnc-EGFR) were found to be the causative reason of immune evasion in hepatocellular carcinoma. Another study shows that SNHG1 lncRNA raises Tregs differentiation and influences tumor volume in breast cancer (40).

2.1.5.4 Drug resistance

Resistance against therapeutic agents can arise right from the beginning of or in the course of cancer treatment. It is a major problem for patients. Recently, researches identified many lncRNAs that have roles in drug resistance. When the chemotherapeutic agent doxorubicin (DOX) was used in gallbladder cancer, GBCLRlnc1 was found to be upregulated, which caused autophagic activity enhancement that is associated with drug-resistance of cancer cells (41). In this process, GBCLRlnc1 interacts with phosphoglycerate kinase 1 (PGK1) and causes inhibition of ubiquitin-mediated degradation in cancer cells.

2.1.6 LncRNAs as biomarkers and therapeutic targets

LncRNAs are high tissue specificity, and they can be encapsulated in EVs, so they have the potential to end up in the bloodstream. These properties make lncRNAs ideal biomarker candidates. Some lncRNAs have already been found, but clinical trials haven't been done yet (42). SNGH1 can be a tumorigenesis biomarker for tissue biotypes such as brain, breast, lung, liver, and ovary. The level of SNGH1 increases in colorectal carcinoma compared to healthy individuals. When the SNGH1 level was decreased, cell proliferation was diminished, and apoptosis was enhanced in the cell line (43).

RNA therapeutics is a recently emerging and a speedily expanding field. Generally, small molecule drugs are used to attack RNA molecule, or anti-sense oligos (ASO) are utilized specifically to interfere with RNA function. ASOs, which are short DNA sequences and complementary to interested RNA-of-interest, hybridize to target RNA and blocks its molecular actions. Two ASOs, named as fomivirsen and mipomersen, have been approved as therapeutic drugs by the Food and Drug Administration (FDA), and they are used to treat cytomegalovirus retinitis and homozygous familial hypercholesterolemia, respectively (5). LNA-gapmeR antisense oligonucleotide triggers cellular RNase H activity to degrade the target lncRNA, in which elevated MALAT1 is lowered in multiple myeloma (44). H3K27Me3 was reduced at KEAP1 promoter by MALAT1; as a consequence KEAP1 mRNA is upregulated. Moreover, the targets of KEAP1, which are proteasome subunit genes (NRF1 and NRF2), were downregulated. Therefore, polyubiquitinated proteins were accumulated. When MALAT1 was targeted by the ASO, cell proliferation was decreased, and apoptosis is triggered in multiple myeloma cell lines (44).

MRNAs can be tailored with lncRNAs harboring tuneable sequence elements (SINE-UPs), and their translation can be increased in this way. This approach can be adaptable to diseases that disrupted protein levels in cells such as Friedreich's ataxia (42). Also, Abivax has started phase 2 clinical trials for ulcerative colitis and rheumatoid arthritis patients. When the lncRNA level is increased by Abivax, miR-124 will be upregulated. Therefore, pro-inflammatory cytokines were reduced, and adaptive immunity was initiated (42). If lncRNA function and disease mechanism are identified, they can be exploited as therapeutic targets.

2.2. Differentiation Antagonizing Non-protein Coding RNA (*DANCR*)

DANCR, which locates on human chromosome 4q12, is an 855 nucleotide lncRNA, and firstly identified as suppressor of epidermal cell differentiation by Kretz (45). The closest adjacent genes of *DANCR* are Ubiquitin Specific Peptidase 46 (*USP46*) and Endogenous Retrovirus Group MER34 Member 1 (*ERVMER34-1*) (**Figure 6**). Also, MicroRNA (MIR4449) and snoRNA (SNORA26) were harbored in first and second introns on *DANCR* locus, respectively.

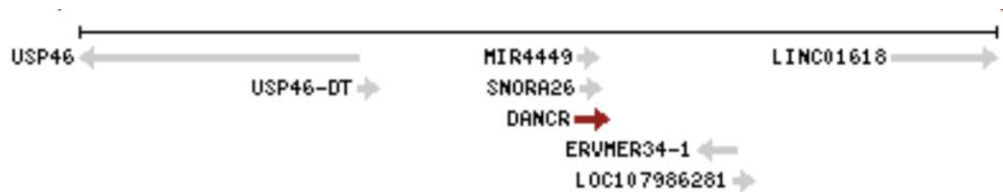


Figure 6: The annotation of *DANCR*. *USP46* and *ERVMER34-1*, which are protein-coding genes, surrounding *DANCR* (46).

Ref (45): *DANCR* differentiation antagonizing non-protein coding RNA [Homo sapiens (human)] NCBI. [Internet]. Retrieved from March 15, 2021.

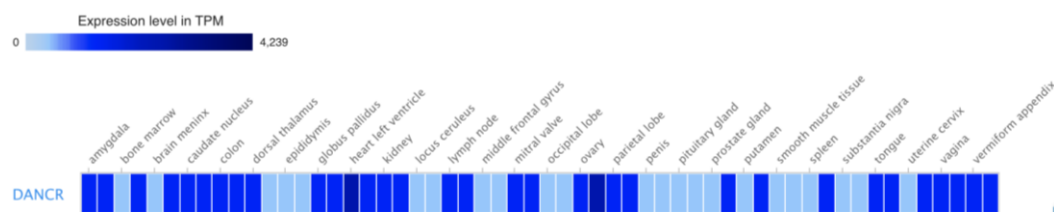


Figure 7: The expression level in transcript per million (TPM) of DANCER in different tissue samples from different age and sex (47).

Ref (46): RNA-Seq CAGE (Cap Analysis of Gene Expression) analysis of human tissues in RIKEN FANTOM5 project. Expression Atlas. [Internet]. Retrieved from March 15, 2021.

2.2.1 Function of DANCER in cancer

DANCER is mostly located in the cytoplasm and is widely expressed in various human tissues including but not limited to the heart, ovary, colon, etc. (**Figure 7**) (45). The overexpressed levels of DANCER were found in various cancers and it was associated with regulation of malignant biological behaviors such as abnormal proliferation of cells, invasion, metastasis, and chemoresistance (48). DANCER is also able to arrange oncogene network components by sponging microRNAs or by interacting with different proteins within the network. Additionally, numerous experiments showed that DANCER affects cell proliferation, cell motility, cell viability, and stemness. These properties of DANCER make it a good potential candidate for therapeutic targeting.

2.2.1.1 Cell Proliferation

Competing endogenous RNAs (ceRNA) competes with mRNAs for binding to miRNAs, therefore they regulate miRNA-targeted mRNAs. When DANCR was overexpressed in endometrial carcinoma, miR-214, which can act as tumor suppressor, was down-regulated (49). However, DANCR knockdown suppressed cell proliferation and induced cell apoptosis. Therefore, their hypothesis was DANCR act as a ceRNA on miR-214 so the inhibitory effect of miR-214 on target mRNAs was alleviated.

Dysregulation of cell cycle causes an increment in cell proliferation rate. DANCR is found to promote proliferation, migration, and invasion and is expressed at high levels in non-small-cell lung carcinoma (50). This process was linked to the silencing of cell cycle inhibitor p21 (CDKN1A) expression. When DANCR was knocked-down, p21 expression was restored and the cell cycle arrest followed by apoptosis as induced.

2.2.1.2 Cell Motility

Cell motility is related to metastasis and invasion features of cancer, and DANCR supports this process in different ways. DANCR was found as a regulator of invasion and metastasis in nasopharyngeal carcinoma cells by increasing Hypoxia-inducible factor 1 (HIF-1 α) mRNA stability through interacting with NF90/NF45 complex (51). Also, the patient survival rate is decreased with DANCR. DANCR promotes invasion and metastasis of prostate cancer as well. DANCR and enhancer of zeste homolog 2 (EZH2) may work synergistically, hence, the expression of tissue inhibitor of metalloproteinases (TIMP2/3), which are critical metastasis inhibitors of prostate cancer, are repressed (52). Moreover,

terminal differentiation of prostate epithelial cells was found to be related to the Androgen-AR signaling pathway. However, DANCER is a suppressor of epidermis differentiation, so they work as antagonists to each other. Although androgen-AR represses DANCER, DANCER suppresses androgen-AR because of the decrement of TIMP2/3 expression. This was shown when DANCER was knocked-down, leading to TIMP2/3 upregulation, and androgen-AR suppresses invasion and migration. DANCER was also found to be significantly up regulated in osteosarcoma. DANCER sponges miR-149 while acting as a ceRNA, and the level of MSI2 protein is increased. This causes osteosarcoma cell migration and invasion (53).

The extracellular matrix remodeling is a highly dynamic process, and it regulates cell differentiation. When this dynamic is corrupted, normal cell processes are changed and cancer may arise. The most important class of enzymes in ECM remodeling is matrix metalloproteinases (MMPs) (54). DANCER sponges miR-33b, resulting in an increase in the expression of MMP16s in pancreatic cancer cells (55). When DANCER was knocked-down in this setup, the level of E-cadherin as increased while N- cadherin expression was decreased.

DANCER targets also miR-216a/LGR5 axis and PI3K/AKT signaling pathway in glioma cells (56). LGR5 is a leucine-rich repeat-containing receptor and it involves in Wnt signaling pathway. Also, PI3K-AKT is the most known cancer related pathway and affects various cellular process. MiR-216a has an inhibitory role in tumor progression. It is sponged by DANCER, and hence, proliferation, migration, invasion and angiogenesis are increased in this way. Also, LGR5, PI3K, AKT, and p-AKT are accumulated in the cells as a response.

Tumor angiogenesis supplies necessary blood oxygen and nutrients to fast proliferating cancer cells. DANCER promotes VEGF expression and modulates tumor angiogenesis in ovarian cancers by sponging miR-145. When tumor suppressor and inhibitor of tumor angiogenesis miR-145 is bound by DANCER, the level of VEGF increases, further indicating that DANCER has much potential to act as ceRNA (57).

MiR-335 was found as a tumor suppressor and it negatively regulates Rho-associated coiled-coil containing protein kinase 1 (ROCK1), which is a metastatic-related gene. However, DANCER sponges miR-335-5p and miR-1972, hence, it promotes proliferation and metastasis by indirectly increasing ROCK1.

Table 1: Complex ceRNA mechanism of DANCER. Various miRNAs can be sponged by DANCER and miRNA-targeted mRNAs can be regulated by this mechanism (58).

DANCER targeted miRNAs	miRNA targeted genes	Tissue	Function
miR-2214 miR-320a miR-199a miR-135a-5p	CTNNB1 BMI1	Hepatocellular carcinoma Glioma	Increase stemness (59) Proliferation, migration and invasion
miR-149	MSI2	Osteosarcoma	Migration and invasion (53)
miR-216a-5p	LGR5	Glioma	Proliferation, migration, invasion, angiogenesis (56)
	KLF12	Hepatocellular carcinoma	Tumor growth (60)
miR-216a miR-33a-5p miR-335-5p miR-1972 miR-33b	C-CBL AXL ROCK1 MMP16	HDPCs Glioma Osteosarcoma Pancreatic cancer	Inhibit differentiation (61) Cell proliferation (62) proliferation and metastasis (63) ECM remodeling (55)

2.2.1.3 Cell Viability

One of the major problem of cancer therapy in the clinics is that tumor cells may develop resistances to chemotherapeutic drugs. In this regard, deregulated expression of lncRNAs may contribute chemotherapy resistance. DANCER can cause docetaxel (DTX) resistance in prostate cancer (PC) by acting as a ceRNA for miR-34a-5p and as result by upregulating JAG1 (64). When DANCER expression is silenced, levels of JAG1 and other drug-resistant associated proteins re decreased, including P-gp, LRPP-gp, MRP1, and LRP. Hence, DTX efficacy is increased in resistant PC cells by silencing DANCER (64).

DANCER expression can also cause cisplatin resistance in glioma cells. DANCER competitively binds to miR-33a-5p, miR-33b-5p, miR-1-3p, miR-206, and miR-613 (62). When AXL, which a receptor tyrosine kinase, is upregulated, PI3K/Akt/NF- κ B signaling pathway is activated in glioma cells. When DANCER is knocked-down, cell proliferation was decreased, and cisplatin sensitivity is restored leading to cell apoptosis.

2.2.1.4 Stemness

DANCER was found to increase stemness properties of hepatocellular carcinoma (HCC) cells, and it causes promotion of tumorigenesis. B-catenin encoded by Catenin Beta-1 (*CTNNB1*), which activates T-cell factor/lymphoid enhancer factor (TCF/LEF), is a regulator of stem cell self-renewal and tumorigenicity, and this mechanism is a significant reason for HCC. DANCER sponges miR-2214, miR-320a, and miR-199a, which are suppressors of *CTNNB1*, and hence, the level of *CTNNB1* is increased (59).

2.2.2 Function of DANCER in diseases other than cancer

Cell differentiation is a significant process in which cells become more specialized. Pluripotency is the potential to become any cell type. It is observed in stem or progenitor cells at the preliminary stage of the differentiation process. When cells differentiate, pluripotency potential is decreased in parallel, and in return cells gain specialized functions (65). For example, mesenchymal stem cells (MSCs) have potential to differentiate into osteogenic, chondrogenic, and adipogenic lineages. DANCER reduces osteogenic differentiation of bone marrow-derived MSCs (BMSCs). P38 mitogen-activated protein kinase (MAPK), which is a critical trigger of osteogenic differentiation, can be arranged by DANCER, and this may be a reason of the osteogenic differentiation of HBMSCs was reduced (66). When DANCER was knockdown, the alkaline phosphatase activity (ALP), osteogenic marker genes, and the mineralized matrix depositions were increased. These collectively indicate an increment of osteogenic differentiation of HBMSCs (66).

Human dental pulp cells (hDPCs) are a heterogeneous population in dental pulp and are capable of differentiating into odontoblast-like cells, osteoblast-like cells and adipocytes (61). The odontoblast-like differentiation is arranged by Wnt/ β -catenin signaling pathway. DANCER can inhibit this pathway throughout the odontoblast-like differentiation of human dental pulp cells (hDPCs) via suppressing the expression of p-GSK-3 β and β -catenin (67). While the hDPCs differentiation continues, C-CBL, which encodes a RING finger E3 ubiquitin ligase, is downregulated by miR-216a. DANCER sponges miR-216a, and hence, the level of c-Cbl is increased in this way, and odontoblast differentiation of hDPCs is inhibited. When DANCER is knocked-down, calcified nodule formation is raised, DSPP and DMP1, which are well-known differentiation markers, are upregulated (61).

2.3. Doxorubicin as a chemotherapeutic agent

Doxorubicin (DOX), also called as adriamycin, Rubex, or Myocet, is an anthracycline antibiotic and agenotoxic chemotherapeutic agent. DOX is used on different cancer treatments such as breast cancer, bladder cancer, Kaposi's sarcoma, lymphoma, and acute lymphocytic leukemia (68). DNA damage is induced by DOX, and significant proteomic changes were arisen in the cancer cells (69). However, drawback of DOX treatment is drug resistance that is related with changes mainly in DNA repair, aberrant gene expression, and epigenetics (70).

2.3.1 Action mechanism of doxorubicin

Human DNA topoisomerases in humans are categorized into two classes. Monomeric type enzymes (Top1) create DNA single-strand breaks. Dimeric type II (Top2), which is an ATP-dependent enzyme, binds DNA and creates DNA double-strand breaks (DSBs) (71). It helps releasing torsional stress created along with molecular biological processes such as DNA replication and transcription. Cancer cells divide rapidly, so they need to replicate DNA continuously, and topoisomerases are major form of enzymes assisting this process. Top2 α is an isoform of Top2 that is considered a marker of cell proliferation, and it is often overexpressed in aggressive or rapidly proliferating tumors (71). DOX intercalates between DNA strands with covalent bond mediated by formaldehyde, and this interaction causes stabilization of Top2 (70) (**Figure 8A**). This process locks the enzyme within the cleavage complex and prevents the enzyme turnover. Also, the re-ligation of DNA is prevented (71). As the DNA is broken, DNA cuts become irreversible at genomic regions, and this causes cell apoptosis. DOX specifically increases irreversible DNA damage in proliferating cancer cells rather than in post-mitotic normal cells.

Another action mechanism of DOX includes changes in oxidative metabolism. NAD(P)H-oxidoreductases can oxidize the quinone structure of doxorubicin to semiquinone by adding one electron (**Figure 8B**). When the semiquinone radicals are converted again into quinone structure, reactive oxygen species (ROS) are generated. This causes inducing oxidative stress, DNA damage and damaged lipid peroxidation in membrane. In this way, cancer cells were killed (72).



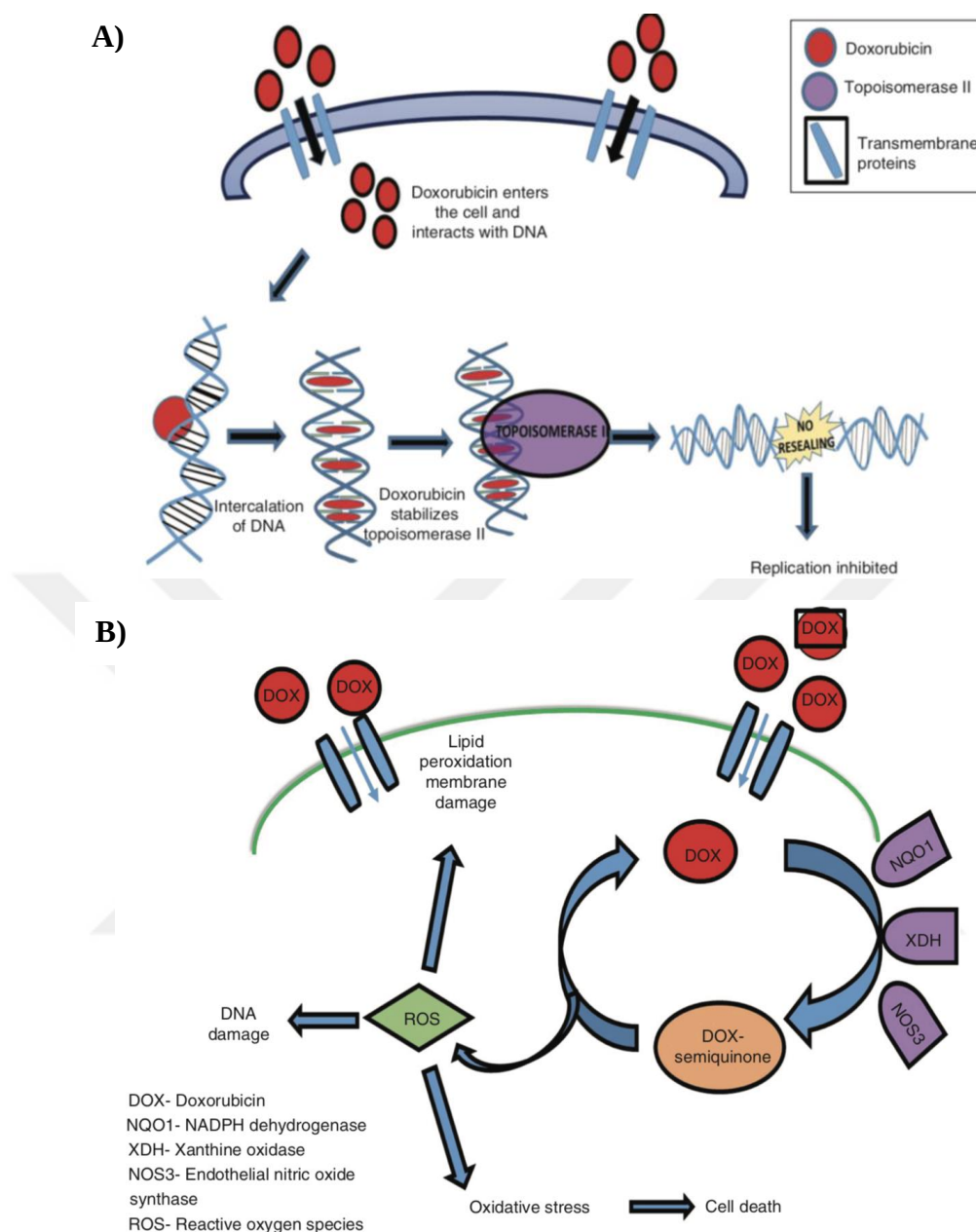


Figure 8: Action mechanisms of DOX. **A)** DOX enters the cell owing to transmembrane proteins and then intercalates DNA. Therefore, the activity of Topoisomerase II is inhibited, and this causes blockage of the DNA replication. **B)** When the quinone structure of doxorubicin is oxidized, ROS is created, and this toxicity helps to cancer cells death (68).

Ref (67): Roychoudhury S, Kumar A, Bhatkar D, Sharma NK. Molecular avenues in targeted doxorubicin cancer therapy. *Futur Oncol.* 2020;16(11):687–700.

2.3.2 DOX resistant lncRNAs

Chemotherapy is a routine treatment for malignant tumors, but drug resistancy may be arisen during the treatment, negatively affecting the efficacy of chemotherapy (73). Drug resistance against DOX may be conferred following several treatments and this may be related with disfunctional lncRNAs (74). For example, lncRNA MALAT1 can sponge miR-3129-5p out and the target of this miRNA, which is Nova1, is upregulated in hepatocellular carcinoma, and this cause doxorubicin resistance (73). Nova1 is a neuron-specific RNA-binding protein and was found that it is upregulated in different carcinogenesis (75). The miR- 3129-5p/Nova1 axis may serve as a novel candidate for Dox-resistant HCC therapy. Moreover, lncRNA-HOX transcript antisense RNA (HOTAIR) was found to be overexpressed in various cancers and might be related to drug resistance (74). When HOTAIR was silenced, DOX resistancy of breast cancer cells were decreased by suppressing the PI3K/AKT/mTOR pathway (74). Also, expression of multidrug-resistant proteins was decreased, and cell proliferation was inhibited, promoting apoptosis in breast cancer cell lines. Furthermore, FOXC2-AS1, which is a single antisense oligonucleotide RNA, was found to be upregulated in DOX-resistant osteosarcoma (76). FOXC2-AS1 forms RNA-RNA duplex with FOXC2, in this way mRNA and protein levels are increased. This duplex, in turn, increases ABCB1 expression that is a multi-drug resistance gene. When FOXC2-AS1 is knockdown, osteosarcoma cells become more sensitive because of reduction in ABCB1 levels.

3. MATERIALS AND METHODS

3.1. Buffers and Other Chemicals

EDTA (Ethylene Diamine Tetraacetic Acid) (0.5M, pH 8.00): 372,24 g EDTA dihydrate powder were dissolved in 60 mL ddH₂O for stock solution. NaOH pellets were added to fix pH to 8.00. Then, the volume of the solution was completed to 100 mL with ddH₂O and the solution was stored at room temperature (RT).

50X TAE Buffer: 242 g Tris Base, 57.1 mL glacial acetic acid, 100 mL 0.5M EDTA (pH 8.00), and ddH₂O were mixed. Then, buffer volume was completed to 1 L with ddH₂O.

6X Loading Dye: 36 mg bromophenol blue, 4.8 mL 0.5M EDTA, and 27.6 mL of 87% glycerol were mixed. Then, ddH₂O was added up to 40 mL.

Tail Lysis Buffer: 5 mL 1M Tris (pH 8.00), 10 mL 5M NaCl, 10 mL 0.5M EDTA (pH 8.00), and 25 mL 10% SDS were mixed. ddH₂O was added until volume reach 500 mL and stored at RT.

Proteinase K: 50 mg Proteinase K was dissolved in 2.5 mL proteinase buffer to make 20 mg/ml proteinase K stock, and aliquotes were stored at -20 °C.

Doxorubicin (10 mM): 10 mg doxorubicin (DOX) was dissolved in 1.724 mL ddH₂O, and filtered through 0.22 µm pore size filter. The solution was aliquoted and stored at -20 °C.

Gelatin (0.1%): 1 g gelatin was dissolved in 1 L ddH₂O. After it was autoclaved for 15 min at 121°C, solution was stored at +4 °C.

Complete Mammalian Cell Culture Medium (cDMEM): 50 mL heat-inactivated fetal bovine serum (FBS) and 5 mL penicillin-streptomycin mix were added in 500 mL high glucose Dulbecco's Modified Eagle Medium, and the final solution was stored at +4 °C.

Mammalian Cell Freezing Medium (FM): 5 mL DMSO and 45 mL FBS were mixed, and stored at -20 °C.

LB (Luria Bertani) Broth: 20 g LB broth powder was dissolved in 1L ddH₂O. Then, solution was autoclaved at 121 °C for 15 min, and stored at RT.

LB Broth/Agar: 20 g LB broth powder and 15 g agar powder were dissolved in 1L ddH₂O. Solution was autoclaved at 121 °C for 15 min. When the solution temperature was decreased to almost RT, 1 mL ampicillin (100 mg/mL) was added. Solution was poured into bacterial petri dishes, and the dishes were stored at +4 °C.

Bacterial Glycerol Stock: 885 µL of overnight grown bacterial suspension was mixed with 115 µL of autoclaved 87% glycerol, and stored at -80 °C.

Alkaline Lysis Solution I: Final concentrations of 50mM glucose, 25mM Tris-Cl (pH 8.00), and 10mM EDTA (pH 8.00) were mixed. ddH₂O was added until volume reached 100 mL. Then, the solution was autoclaved at 121°C for 15 min and stored +4 °C.

Alkaline Lysis Solution II: 40 µL NaOH (5N), 100 µL SDS (10%) and 860 µL ddH₂O were mixed, and stored at RT.

Alkaline Lysis Solution III: 60 mL potassium acetate (5M) and 11.5 mL glacial acetic acid were mixed. ddH₂O was added until volume reached 100 mL, and the solution was stored at +4°C.

GeneRuler DNA Ladder Mix: 100 µL GeneRuler DNA Ladder Mix (Fermantas SM0331) was mixed with 100 µl 6X DNA loading dye and 400 µL ddH₂O. The solution was stored at -20 °C.

3.2. Methods

3.2.1 DANCER knock-out cell line created by the previous study

DANCER gene has 14 transcripts as annotated on ENSEMBL release 103 (February 2021). Among these DANCER-201 transcript (ENST00000411630.7) was selected to be investigated in the current thesis. DANCER-201 has three exons (**Table 2**). This thesis used DANCER^{-/-} (DANCER KO) cell lines created by a previous study that was done by Gülin Baran, MSc. (27). In brief, two sgRNAs encompassing DANCER gene from the upstream of the first exon of DANCER-201

and from the downstream of the last exon of DANCER-201 were designed and cloned into CRISPR/Cas9 gene-editing plasmids to delete DANCER gene locus in human colorectal cancer cell line, HCT116 (**Figure 9**). A pair of PCR primers (DANCER screen fwd and rev) surrounding the DANCER gene locus from outside the sgRNA targeting sites were used for genotyping PCR (**Table 3**). Wild type cells are expected to produce a PCR product of 2551 bp, whereas, DANCER KO cells produce approximately 300 bp.

Table 2: Introns and exons of the DANCER-201 transcript (ENST00000411630.7) (77).

No. Exon / Intron	Start	End	Start Phase	End Phase	Length	Sequence
5' upstream sequence						cgcgccgccccatccgggattggctgtgcgtgcgcggccctccacccc
1 ENSE00003899391	52,712,325	52,712,527	-	-	203	GCCCCGCTCAGGATCTTCCCGGGATTGGCTGAGCTACCGACCTCTCCAGGGGATCGGCTGCGCGCCCCGCCCTTGCCAGAGTCTTCCCGGATTGGCTGCGGGCCTCGCGACCTCCTGCTTCCCTCCCCGCCCGCGCCCTCTCTGGTTGTGCGCCGTCGCAGGTCGCAGGCCCTTTGTGAGCTGGAGTTGCGCG
Intron 1-2	52,712,528	52,712,823			296	gtgagtgcacatccagtccttcggct.....acaattttgcgctttgcttccccag
2 ENSE00001622615	52,712,824	52,712,959	-	-	136	GGCTGACGCGCCACTATGTAGCGGGTTTCGGCGGGCCACGCTGCGGGACAGGAACCCAACCCAGCCGACCTTGAGCTCCAGGAGTTCGTCTCTACGTCTGCGGAAGTGCAGCTGCCTCAGTTCTTAGCGCAG
Intron 2-3	52,712,960	52,713,493			534	gtatgttcctagccttgagcgggt.....catttttaaaacttttctttctatag
3 ENSE00003903732	52,713,494	52,714,141	-	-	648	GTTGACAACTACAGGCACAAGCCATTGAAGCTGGAATGTCTGTGCTGGTATTCAATTGACTTAAGCCAACTATCCCTTCAGTTACAATAGGAAAGTGCCTCTAATAAGGCCAAATATGCGTACTAATTGTAGCAACCAGTGTCCGTGCAGTGCCACAGGAGCTAGAGCAGTGACAATGCTGGTGCAACAGGCGAGTGTAGCAGGTGCTTCATGTTCACTTTTCAACCTTTTCAATTTAATTGTACAACTCGAGGTGGATTCTGTAGGGACAGGCTGCCAGGACCACTCCGCCCGCGCTAACTCAATGACGTGACCTTACCTCTGAATCTCTGACGTGCATTCTCTGAACCGTTATCTAGGCGCTATAGCAAGGTCACCACTTGTACACCAAGGCCCTCTGGGTGGCAGGGGAGGTCATGAGAAAGCTGGATTACACCCCTTGTAATTCCTATTTCACAAATAATATATTGTAAGCCGTCATGAGATTATGTGGTAAAGTTAATTGACTAACAACCCAGGGTCTCTCTCCCCATATAAACCCCTCATTGTGTAAGCTCAGGGCTGCCACCTCCGACTGGTGAGAAAGCTGGCAGGTTAATAAACTTACTTGGCTGACCTT
3' downstream sequence						gggtctccttgcctttctctcgcgctaaccctacagattctattctcccat.....

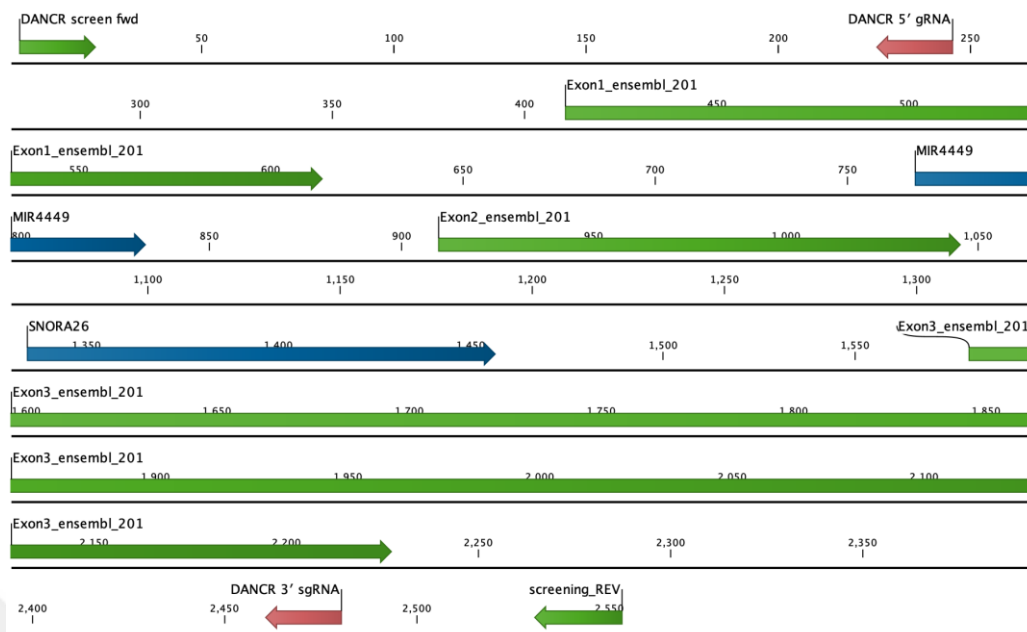


Figure 9: Annotations of gRNAs and genotyping primers on human DANCR-201 ENST00000411630.7 (CLC Main Workbench). DANCR screen fwd and rev are genotyping primers, DANCR 5'- DANCR 3' gRNA target regions. Genotyping PCR product length is 2551 bp in wild type cells. If sgRNAs deleted DANCR locus with CRISPR/Cas9, PCR length is approximately 300 bp.

Table 3: The sequences and the functions of gRNAs and primers for DANCR gene locus.

Oligos Name	Oligo Sequence	Purpose
DANCR screen		
Fwd	CTTTAGGAGAGGCCACGGGA	Genotyping of wild type and
Rev	ACAGATACTCCTTCTGTGCCAAA	DANCR KO cells
DANCR 5' gRNA	TACCTACCAATCCCCGGCGT	Guide RNA sequence for the upstream of DANCR
DANCR 3' sgRNA	TATTCTTAACAGCTCGACTG	Guide RNA sequence for the downstream of DANCR

3.2.2 Creating DANCER overexpressing cells created by mCAT mediated retroviral infection

3.2.2.1 PCR-amplification of DANCER cDNA

Total RNA was isolated from HCT116 cells by following the protocol of Macherey-Nagel (MN) RNA Isolation Kit. Poly-A tailed enriched cDNA was synthesized from total RNA by using Protoscript II first-strand cDNA synthesis kit. DANCER 201 fwd and rev primers were used to amplify DANCER cDNA (**Table 4**). PCR protocol was performed as in **Table 5** with thermocycling conditions as in **Table 6**.

It is important to note that when cloning primers were designed for DANCER 201 cDNA, ENST00000411630.6 from ENSEMBL was used as a reference sequence. This cDNA is supposed to be 904 nt long. This also resembles NR_024031.2 from NCBI. In the latter, there is an additional 10 nt in the beginning of the cDNA and 1 in the end. However, later on ENSEMBL updated the sequence of DANCER 201 transcript and named it ENST00000411630.7. This newer version is 987 nt long and has additional 79 nt in the beginning and 4 nt in the end compared to the previous ENSEMBL version. Therefore, as a summary, this thesis used ENST00000411630.6 in the overexpression plasmid.

Table 4: The sequences and purposes of DANCER cDNA cloning primers.

Oligo Name	Oligo Sequence	Purpose
DANCER 201 Fwd	aaagaattcAGAGTCTTCCCGG GATTGGC	For PCR-amplifying and cloning DANCER cDNA
DANCER 201 Rev	aaagaattcTCAGGCCAAGTAA GTTTATTAAACCTGCC	For PCR-amplifying and cloning DANCER cDNA

Table 5: PCR protocol to amplify DANCER 201 cDNA.

Components	Volume (μL)	Final
Phusion GC Buffer (5 X)	4 μL	1 X
dNTP (10 μM)	0.4 μL	0,2 μM
DANCER 201 fwd primer (2.5 μM)	4 μL	0,5 μM
DANCER 201 rev primer (2.5 μM)	4 μL	0,5 μM
Phusion Enzyme (2U/ μL)	0.2 μL	0.1 U/μL
cDNA template	1 μL	
Nuclease free water	6.4 μL	
Total	20 μL	

Table 6: Thermocycle conditions to amplify DANCER 201 cDNA.

STEP	TEMPERATURE	TIME
Initial Denaturation	98 °C	30 sec
	98 °C	10 sec
35 Cycles	62 °C	20 sec
	72 °C	30 sec
Final Extension	72 °C	5 min
Hold	4 °C	∞

3.2.2.2 Agarose Gel Electrophoresis

Throughout this thesis, samples (PCR products) were visualized by EtBr (Ethidium Bromide) in agarose gel electrophoresis. 50X TAE buffer was diluted into 1X by using ddH₂O. The percentage of the agarose gel depends on the product length. 1X TAE buffer volume (50-150 mL) and agarose amount (0.3-3 g) were prepared according to agarose gel percentage. The mixture was boiled, while mixing occasionally, in a microwave oven for 2-5 min to dissolve agarose. Then, the flask was cooled down under cold tap water. 0.3-1 µL EtBr was added depending on the total volume and mixed by swirling. The gel comb was located in the casting tray, and agarose gel solution was poured. After the gel solidified, the gel comb was taken. 2 µL of the Gene Ruler DNA Ladder Mix was loaded to be used as a ladder to identify DNA fragment sizes. Then, samples were mixed with 6X loading dye to final concentration of 1X and loaded into the gel. The power supply was set between 80-130 V (volt) for 45-60 min to run samples. For sample visualization, agarose gel was imaged under UV by using the ChemiDoc Imaging System (Bio-Rad).

3.2.2.3 EcoRI Digestion

After the confirmation of the cDNA band on agarose gel, PCR-amplified DANCER cDNA was extracted from the 2% agarose gel using an Invitrogen gel extraction kit according to the manufacturer's protocol. Then, PCR-amplified DANCER cDNA and pBABEpuro empty plasmid were digested with EcoRI restriction endonuclease as in **Table 7** with thermocycling conditions as in **Table 8** to create complementary sticky ends.

Table 7: Protocol for EcoRI digestion reaction.

PCR Product		Volume	Plasmid Digestion		Volume
Digestion		(μL)			(μL)
EcoRI HF		3 μL	EcoRI HF		1.5 μL
PCR Product	(DANCR cDNA)	35 μL	Plasmid (pBABEpuro Empty)	(0.1 $\mu\text{g}/\mu\text{L}$)	3 μL
Cut Smart (10X)		7 μL	Cut Smart (10X)		3 μL
ddH ₂ O		25 μL	ddH ₂ O		22.5 μL
Total		70 μL	Total		30 μL

Table 8: Thermocycle conditions to EcoRI digestion.

Condition	
Temperature	Time
37 °C	2 h
65 °C	20 min
4 °C	∞

3.2.2.4 CIAP treatment and ligation

CIAP enzyme was used, as in **Table 9**, to prevent re-circularization and re-ligation of linearized *DANCR* cDNA by removing phosphate groups from both 5' termini (78). CIAP treated plasmid was run on 1% agarose gel and the expected band was extracted using Invitrogen gel extraction kit. After the extraction, different volumes from the samples were re-run in a 1% agarose gel to determine the approximate concentration by comparing the intensities of band under Chemidoc UV.

Table 9: The reaction and condition for CIAP treatment on plasmid.

Components	Volume (μL)
Digestion reaction of plasmid	30 μL
CIAP (20U/ μL)	1.5 μL
Dephosphorylation Buffer (10X)	5 μL
ddH ₂ O	13.5 μL
Total	50 μL
Condition	
Temperature	Time
37 °C	45 min

1:1, 1:3, or 1:6 molar ratio of vector: insert DNA was used for the purpose of ligation reaction. Insert (*DANCR* cDNA) and vector (pBABEpuro) were ligated (**Figure 11**). Vector without insert was also used to assess self re-ligation of the CIAP treated-vector as negative control. The ligation was done with T4 DNA ligase and in the presence of excess ATP in addition to ATP already supplied within the enzyme's buffer and the reaction was incubated at 16 °C for 16 h. Molar ratio calculations were done according to **Figure 10**.

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}} = \text{ng of insert}$$

Figure 10: Calculation formula of the insert for ligation (79).

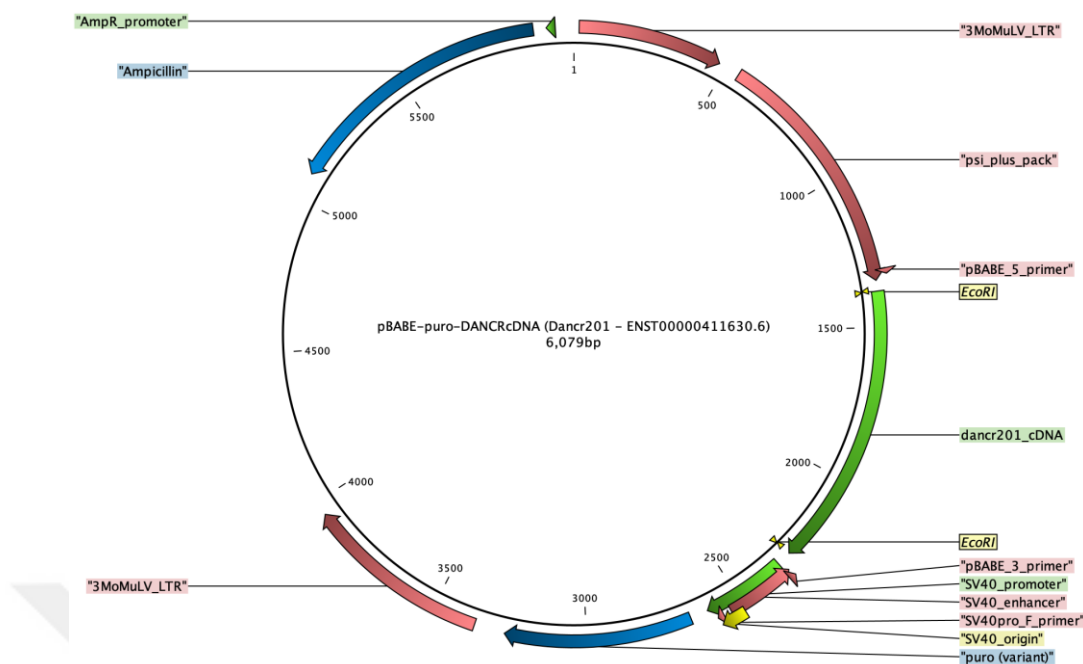


Figure 11: DANCR cDNA cloned pBABEpuro retroviral plasmid map. The plasmid construct has 6,079 bp that includes DANCR 201 cDNA, ampicillin resistance cDNA, 2 EcoRI enzyme restriction sites, bacterial promoter sequence (SV40), and 2 moloney murine leukemia virus long terminal repeats (5' and 3').

3.2.2.5 Plasmid Transformation into DH5 α

10 μ l DANCR cDNA cloned pBABEpuro plasmid reaction was added into 200 μ l thawed DH5 α competent cells. Mixtures were incubated for 30 min on ice. Cells were heat-shocked at 42 $^{\circ}$ C for 90 sec and again immediately put back on ice. Then, 800 μ l LB broth was added to cells and they were incubated at 37 $^{\circ}$ C for 45 min for recovery. Followingly, cells were centrifuged at 13000 rpm for 30 sec. 900 μ l supernatant was discarded and the pellet was resuspended in the rest of the 100 μ l supernatant. Later, suspension, which contains the transformed bacteria, was spread on LB agar with ampicillin by the help of glass beads. The plate was incubated for 16 h at 37 $^{\circ}$ C.

3.2.2.6 Alkaline Lysis Miniprep for Plasmid DNA Isolation

1.5 mL overnight-grown bacteria culture was centrifuged at max speed for 10 min at RT and supernatants were discarded. For each microcentrifuge tube, an ice-cold 100 µL alkaline lysis solution I was added, and tubes were vortexed. 200 µL freshly prepared alkaline lysis solution II was added. tubes were mixed by inversion. Next, ice-cold 150 µL alkaline lysis solution III was next added and tubes were gently inverted again a few times. After 4 min incubation on ice, tubes were centrifuged at max speed for 5 min. 450 µL supernatant was transferred into a fresh tube and 900 µL absolute EtOH was added. Tubes were inverted harshly and centrifuged at max speed at +4 °C for 5 min. After the supernatant had been discarded, tubes were blotted on tissue paper to remove residual supernatant on the necks of the tubes. 1 mL 70% EtOH was added and centrifuged at max speed at +4 °C for 2 min. After the supernatant had been discarded, again tubes were blotted on paper and left opened for air dry for 15 min. Pellets were resuspended in 50 µL TE buffer with RNase A (20 µg/µL) to eliminate RNA contamination. Extracted DNAs were stored at -20 °C. pBABEpuro-DANCR plasmids were controlled with restriction digestion reactions using SacI and SmaI enzymes. Finally, the cDNA sequence in the plasmid was assessed with Sanger sequencing.

3.2.2.7 mCAT mediated retroviral infection

Human cells were pseudotyped with mouse cationic amino acid transporter (mCAT) encoding pIRES-eGFP-mCAT plasmid transfection to make them susceptible to retroviral infections as depicted in **Figure 12** (80). For this purpose, firstly, Phoenix cells, which are second-generation retrovirus producer cell line, were seeded onto 10 cm plates at day 0. Next day, Phoenix cells were co-transfected with pCL-ECO plasmid carrying the virus packaging factors and pBABEpuro-DANCR plasmid using Pei-Max mix. Transfection suspensions were

prepared as in **Table 10**, and vortexed gently. The ratio of $\mu\text{g DNA}:\mu\text{g PEI}$ was determined as 1:3 in previous optimization transfections. The transfection mixture was incubated at RT for 30 min and transferred drop by drop onto the 6-well plate. 24th and 48th hours post-transfection, virus containing cell supernatants were collected and filtered with 0.45 μm pore size filters. Viral supernatants were aliquoted and stored at -80 °C.

Table 10: PEI-Max transfection mixture.

	Plate 1	Plate 2	Plate 3
Components	pBABEpuro Empty (1 $\mu\text{g}/\mu\text{L}$)	pBABEpuro DANCRC clone (1 $\mu\text{g}/\mu\text{L}$)	LMP-dsRED (1 $\mu\text{g}/\mu\text{L}$)
Serum Free DMEM	200 μL	200 μL	200 μL
Plasmid	5 μL	5 μL	5 μL
PCI-ECO plasmid (1 $\mu\text{g}/\mu\text{L}$)	3 μL	3 μL	3 μL
PeiMAX mix (800 μL SF-DMEM + 96 μL peiMAX (1 $\mu\text{g}/\mu\text{L}$))	224 μL	224 μL	224 μL

Secondly, HCT116 WT and DANCRC KO HCT116 cells were seeded as 1.000.000 cells per / well of a 6-well plate at day 0. Next day, cells were transfected with either 3 μg of pires2eGFP-mCAT plasmid for pseudotyping or with 3 μg of pires2eGFP as negative control. The ratio of $\mu\text{g DNA}:\mu\text{g PEI}$ was determined as 1:4 in previous optimization transfections. On days 2 and 3, cells were infected with collected viruses for 3 hours. Followingly, cells were selected in the presence of puromycin.

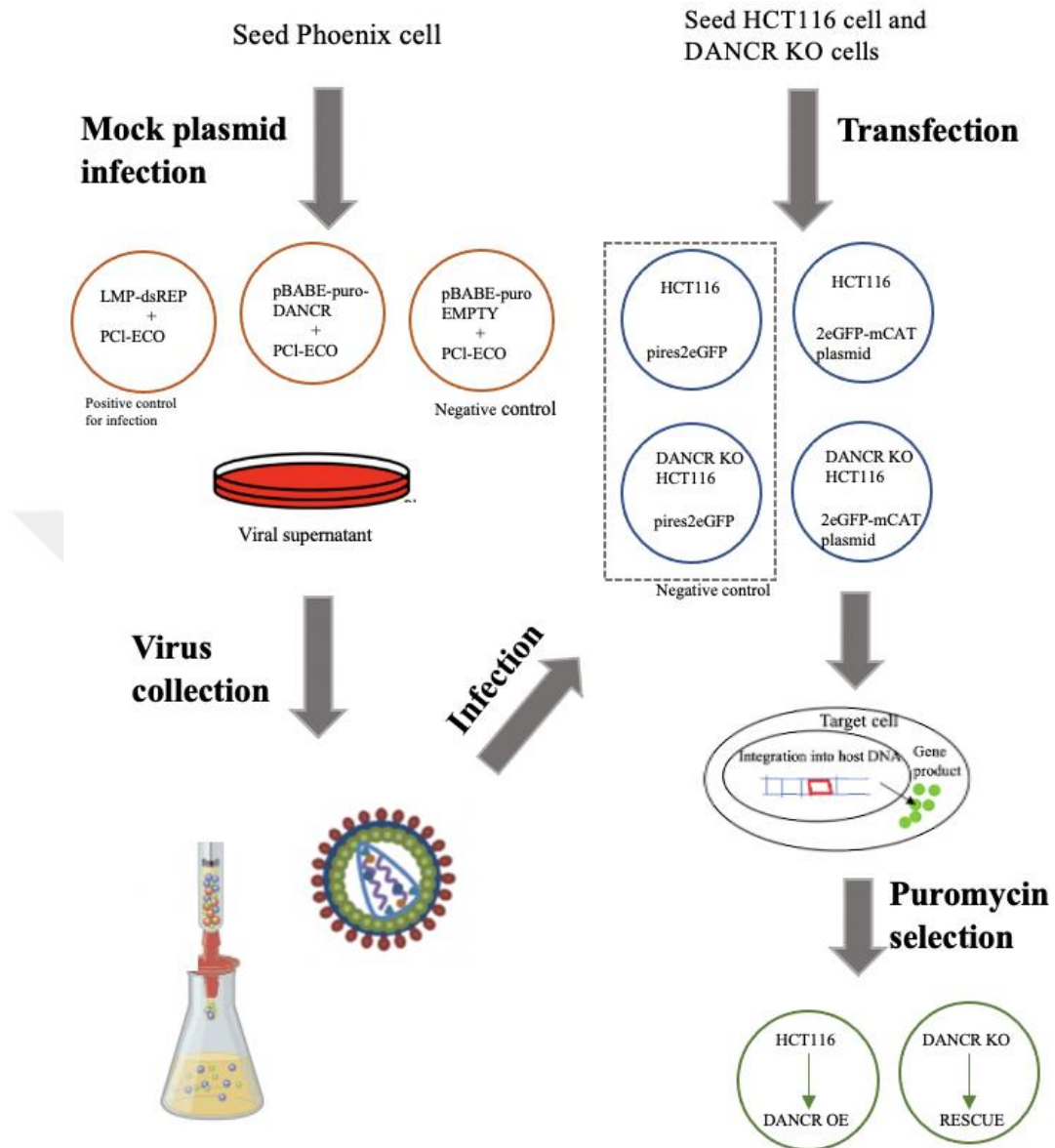


Figure 12: Steps of mCAT mediated retroviral infection. Phoenix cells are first co-transfected with packaging and the retroviral vector along with appropriate controls. Virus is collected from the supernatant and filtered to be later used for the infection. In the meantime, target cells are transfected with mCAT carrying plasmid for pseudotyping again along with appropriate controls. Collected virus is used to infect target cells and infected cells were selected with and appropriate antibiotic.

3.2.4 Mammalian Cell Culture

3.2.4.1 Maintenance

Matthias Dobblesstein of Georg August Universität Göttingen had shared HCT116 (Human Colorectal Cancer) cell line previously (80). Cells were maintained in a tissue culture incubator with a 5% CO₂ humidified atmosphere at 37 °C. 50 mL heat-inactivated FBS (10%) and 5 mL pen/strep (1%) were added in 500 mL DMEM to make it complete DMEM (cDMEM) and this was used to support cell growth. When cells reach almost 80-90% confluency, cells were sub-passaged with 1:10-1:20 split ratios in sterile cell culture plates every 3-4 days.

3.2.4.2 Passaging

Growth medium was aspirated by gentle suction and cells were washed with 2 mL of Dulbecco's Phosphate Buffered Saline (1X). After PBS was removed, 2 mL Trypsin-EDTA solution (1X) was added. Until cells dissociated from the surface, the plate was left in the incubator, for about 2-3 min. Then, 8 mL of pre-warmed cDMEM was added to the plates to inactivate trypsin. Homogenous cell suspensions were obtained by numerous pipetting and they were transferred to 15 mL Falcon tubes. Cells were collected at 300 g for 3 min at RT. After the supernatant was discarded, cells were resuspended with 10 mL fresh DMEM. According to the split ratio, cells were split into sterile cell culture plates and put back in the cell culture incubator.

3.2.4.3 Cryopreservation and thawing of cells

For long term storage of mammalian cells in liquid nitrogen, cells were collected into a falcon tube as described in the passaging protocol and centrifuged at 300g for 3 minutes. After the supernatant was removed, a previously prepared 1 ml mammalian cell freezing medium (FBS with 10 % DMSO) was used per 1-2 million cells to resuspend the cell pellet and the new suspension was transferred into a cryovial. Vials were cooled down in Coolcell LX freezing container at -80 °C for 48-72 h. Later, cryovials were transferred into liquid nitrogen tanks for long term storage.

For thawing mammalian cells, cryovials were quickly transferred from nitrogen tank in a 37°C water bath and kept there for 1-2 min. When almost half of the ice in the vial melted, the mixture was resuspended into pre-warmed 9 mL cDMEM to remove residual DMSO. Cells were collected by centrifugation at 300 g for 3 min at RT. Supernatant was removed and the pellet was resuspended in 10 mL fresh cDMEM. Total volume was used to seed a 10 cm cell culture dish and the dish was maintained in a cell culture incubator.

3.2.5 Genotyping of cell lines

3.2.5.1. Genomic DNA isolation

Genomic DNA was manually extracted following the Tails-Proteinase K method (3). 1.000.000 cells per extraction were harvested from cell culture plate as described in the passaging protocol and they were treated with 500 µL Tail Lysis Buffer supplemented with 10 µL Proteinase K (20 mg/ µL). Samples were

incubated in a 55 °C heat block for 90 min. After 250 µL NaCl (5M) was added, tubes were harshly inverted approximately 20 times and incubated on ice for 10 min. Next, samples were centrifuged at 3000 RPM at 4 °C for 15-30 min and supernatants were transferred to new tubes. 650 µL isopropanol was added and mixed gently. After 15 min incubation step performed at RT, samples were centrifuged at max speed for 10 min in RT. The supernatant was discarded, and pellets were washed with 70% EtOH, two times. pellets were recovered with the same centrifugation step each time. Tubes were inverted on a tissue paper while their caps were left open, and they were left for air-dry for 10 min to remove residual EtOH from gDNA pellets. Finally, 200 µL TE Buffer (7.5 pH) with RNase A (20 µg/mL) was added on pellets and gDNA was stored at +4 °C.

3.2.5.2. Genotyping PCR

After DANCER OE (overexpressed) and DANCER Rescue cell lines was created, genotyping PCR was performed for cell line confirmation. DANCER screen fwd and rev primers, given in **Table 3**, were used to screen DANCER gene locus. The protocol of PCR was performed as in **Table 11** with thermocycling conditions as in **Table 12**. If the DANCER gene exists, the length of the PCR products is around 2550 bp. Although DANCER Rescue cells had DANCER cDNA in them, the primers were not able to bind within this cDNA Therefore, they were expected to generate a band similar to what was observed in DANCER KO cells, as around 300 bp.

Table 11: PCR protocol to amplify DANCER locus.

Components	Volume (μ L)	Final Concentration
gDNA	1-2 μ L	< 1,000 ng
MyTaq Reaction Buffer (5X)	5 μ L	1 X
DANCER screen fwd primer (2.5 μ M)	2 μ L	0.2 μ M
DANCER screen rev primer (2.5 μ M)	2 μ L	0.2 μ M
MyTaq DNA Polymerase (5U/ μ L)	0.5 μ L	0.1 U/ μ L
ddH ₂ O	13.5-14.5 μ L	-
Total	25 μ L	

Table 12: Thermocycling conditions to amplify DANCER locus.

STEP	TEMPERATURE	TIME
Initial Denaturation	95 °C	3 min
40 Cycles	95 °C	15 sec
	62 °C	15 sec
	72 °C	90 sec
Final Extension	72 °C	10 min
Hold	4 °C	∞

3.2.5.3. RNA isolation

Cells were collected as described in the passaging protocol and centrifuged at 300 g for 5 min at RT. RNA was isolated according to Macherey-Nagel (MN) RNA Isolation Kit's protocol. Later, RNA concentrations were estimated via NanoDrop and the integrity of total RNA was checked on an agarose gel. RNA samples were aliquoted and stored at -80 °C.

3.2.5.4. RT-PCR

cDNA was synthesized from total RNA by using ProtoScript® II First Strand cDNA Synthesis Kit as in **Table 13**. According to RNA concentration, template RNA volume was estimated for KO, OE, WT, and Rescue cell lines. Firstly, oligo d(T)₂₃, RNA, and nuclease-free water were mixed on ice and the mixtures were incubated at 65°C for 5 min. Then, the rest of the kit's components were added. No-RT mix negative control did not have any reverse transcriptase added to it. Samples were incubated at 42°C for 1 h and later at 80 °C for 5 min. Single stranded cDNAs were stored at -20 °C.

Table 13: cDNA synthesis protocol from total RNA.

Components	Volume (µL) (1X)	Final Concentration
Total RNA	up to 1 µg	50 ng/µL
Oligo d(T) ₂₃ (50µM)	2 µL	2.5 µM
ProtoScript II Reaction Mix (2X)	10 µL	1X
ProtoScript II Enzyme Mix (10X)	2 µL	1X
Nuclease-free H ₂ O	up to 20 µL	
Total	20 µL	

For RT-PCR, primer sequences for DANCER and PGK1 targets were as in **Table 14**. PGK1 (Phosphoglycerate Kinase 1) is used as housekeeping gene. Also, samples without any cDNA template were utilized to check the purity of the RT-PCR components. Reactions were prepared as in **Table 15**, and conditions were set as in **Table 16**. Then, for visualization of RT-PCR products, they were run in 2% agarose gel at 90 V for 45 min, and images were obtained from ChemiDoc Imaging System.

Table 14: The sequences of RT-PCR primers.

Primer	Sequences
DANCR-RT	
Fwd	TTTGTCTAGCTGGAGTTGCGCGG
Rev	TTGTCAACCTGCGCTAAGAACTG
PGK1-RT	
Fwd	ATTAGCCGAGCCAGCCAAAATAG
Rev	TCATCAAAAACCCACCAGCCTTCT

Table 15: RT-PCR protocol.

Component	Volume (μL)	Final Concentration
cDNA	1 μL	
MyTaq Reaction Buffer (5X)	5 μL	1X
Fwd primer (2.5 μM)	2 μL	0.2 μM
Rev primer (2.5 μM)	2 μL	0.2 μM
MyTaq DNA Polymerase (5U/μL)	0.5 μL	0.1U/μL
ddH₂O	13.5-14.5 μL	
TOTAL	25 μL	

Table 16: Thermocycling conditions for RT-PCR.

STEP	TEMPERATURE	TIME
Initial Denaturation	95 °C	3 min
30 Cycles	95 °C	15 sec
	62 °C	15 sec
	72 °C	6 sec
Final Extension	72 °C	1 min
Hold	4 °C	∞

3.2.5.5. qRT-PCR

To quantify DANCER gene expression, qRT-PCR was set up in duplicate for each sample, and no template reactions were also prepared for each primer pair. Primer efficiency had been determined via Pfaffl method previously, and 1:10 diluted cDNA was selected for both primer pairs (81). Mix was prepared for KO, OE, Rescue, and WT as in **Table 17**. PGK1 primers were selected for normalization, and DANCER primers were used to analyze the expression level of DANCER in each cell line. The condition of RT-qPCR was started with Uracil-DNA Glycosylase (UDG) activation at 50°C for 2 min. Then, dual-Lock™ DNA Polymerase activation was kept at 95°C for 2 min. Denaturation step proceeded for 40 cycles for 2 sec at 95°C, and annealing-extension steps were continued for 30 sec at 60°C. Then, the plate was read out during the melting curve while temperature was being raised from 55°C to 95°C by ramping up 0.5°C for every 5 sec. Later, Bio-Rad CFX Maestro 1.0 and GraphPad Prism 7 were used to analyze results.

Table 17: qRT-PCR protocol.

Component	Volume (μL)	Final Concentration
Template cDNA (1:10)	1 μL	1:100
PowerUp™ SYBR™ Green Master Mix (2X)	5 μL	1X
Fwd primer (2.5 μM)	2 μL	0.5 μM
Rev primer (2.5 μM)	2 μL	0.5 μM
TOTAL	10 μL	

3.2.6. Cellular assays

3.2.6.1. MTT assay

MTT assay was performed to determine half-maximal inhibitory concentration (IC_{50}) values for doxorubicin on HCT116 WT, DANCER KO, DANCER OE, and Rescue cells. Cells were seeded into 96-well cell culture plate in 100 μ l medium as 5000 cells per well in duplicates. Additionally, 2 only cDMEM wells (without any cell) were also included in plates for background measurements. Next day, 0 nM, 10 nM, 25 nM, 50 nM, 150 nM, 200 nM, 400 nM, 600 nM, 800 nM, 1000 nM, 1200 nM, 1500 nM, 2000 nM, 5000 nM and 10000 nM final concentrations of doxorubicin were prepared from stock (10 mM). Media was discarded by gentle suction and cDMEM with different doxorubicin concentrations was added into wells. After cells were incubated for 2 days in incubator, 10 μ l MTT reagent was added into each well for 4 hours. Followingly, 100 μ l solubilization buffer was added to dissolve crystals. After 16 hours, formazan concentration in wells was measured spectrophotometrically at 570nm and 690nm wavelengths by using VarioScan (Thermo Scientific). Raw read data was analyzed and IC_{50} values were calculated by GraphPad Prism 7. This was repeated 5 times for analyzing standard deviations.

3.2.6.2 Trypan blue exclusion assay

Cells were seeded into 6 cm cell culture plates in triplicates in 4 ml medium as 200,000 cells per plate. For determining the exact cell, cells were harvested at 24th, 48th, and 72nd hours post-seeding as in the passaging protocol. Then, cell suspension was mixed with 0.4% trypan blue solution at a 1:1 ratio to assess cell

viability. 10 μ l of the mix was loaded into hemocytometer and alive cells were counted for each time points. Results were analyzed by using GraphPad Prism 7.

3.2.6.3 Colony formation assay

Firstly, 6 cm tissue culture plates were pre-coated with 1 mL gelatin solution (0.1%) to promote cell attachment. After the plate was kept in incubator for 15 min, gelatin solution was aspired. Cells were seeded in gelatin coated plates in 4 mL medium as 1,000, 2,000, 3,000, or 4,000 cells per plate in triplicates. Cell media was refreshed every 2-3 days throughout 11-13 days. When colonies exceeded 45-50 cells, estimated under microscope, plates were prepared for staining. Initially, plates were washed twice with 1mL of PBS (1X). For visualization, 2.5 mL ready-made Crystal Violet was added onto each plate. After 10 min incubation at RT, staining solution was removed, and plates were washed by submerging them into a dH₂O filled box until excessive crystal violet was removed. Then, plates were let to air-dry. Dried plates were covered by heavy cream and scanned by a generic brand document scanner at a resolution of 600 dpi. Then, images were analyzed by using ImageJ.

To examine DOX on long term colony formation assay, cell lines were seeded as 500 cells per plate in triplicate. Next day, cells were treated with the different concentration of DOX (0nM, 250nM, 500nM or 1000nM) for 2 hours. The protocol was performed as indicated above.

3.2.6.4 Hematoxylin and eosin staining

1.000.000 cells were seeded into a 6 cm plate one day earlier. Next day, cells were washed 3 times with 1 mL PBS (1X). Cells were fixed with ice-cold methanol for 10 min. Then, cells were washed 3 times with ddH₂O. 1,5 mL Harris hematoxylin stain (1%) was applied for 130 sec and plates were washed 3 times with ddH₂O. 1 mL Scott's step water was added and incubated for 30 sec. After 3 times of doing this, plates were washed with ddH₂O and 1,5 mL eosin y stain 1% (alcoholic) was added for 15 sec. Cells were washed again 3 times with ddH₂O. Cells were imaged at 40X magnification with bright field option in fluorescence microscopy.

3.2.6.5 Propidium iodide staining for cell cycle

500.000 cells were seeded into 6 cm plates in duplicate. After overnight growing, cells were harvested and centrifuged at 300g for 3 min. Supernatant was removed and 300 µl ice-cold PBS (1X) was added to resuspend the pellet. For fixing cells, 700 µl ice-cold absolute EtOH was added to cell suspension dropwise while vortexing and the mixture was incubated for a minimum of 30 min on ice. Cells were centrifuged at 300g for 10 min and washed with 1 ml PBS, this step was repeated twice. After the supernatant had been removed, RNase A-Triton X-100 mix was prepared. Solution contains 5 µl RNase A (100 µg/ml), 25 µl Triton X (1%), and 475 µl PBS (1X). Then, the pellet was resuspended in this solution and incubated at 37°C for 30 min. Later, 2.5 µl propodium iodide (PI) (2.5 µg/ml), 25 µl Triton X (1%), and 472.5 µl PBS (1X) were added as PI staining solution. Also, 2.5 µl PI (2.5 µg/ml) and 477.5 µl PBS (1X) were prepared for negative control. Cells were incubated at 37 °C for 15 min. The quantification of DNA contents of stained and unstained cells was done via flow cytometry. Data was examined by FlowJo.

3.2.6.6 3D spheroid culture

To examine the spheroid properties of cells, harvested cells were counted with a hemacytometer. 2500 cells in every 10 μ l droplets that contain cDMEM were placed in each lid of a 6 cm plate. 2 ml PBS was added into plates to ensure humidity. Then, images were taken with a fluorescence microscope at 24, 48 and 72 hours. Dimensions of spheroids were calculated by Image J.

3.2.6.7 Semi solid soft agar colony formation assay

4% agarose was boiled with occasional mixing in a microwave oven for 2-5 min to dissolve agarose. It was kept at 60 °C until being used. Pre-warmed 4.1 mL cDMEM containing 10% FBS and 0.9 mL of 4% agarose were mixed (final agarose: 0.75%) and poured into a 6 cm cell culture dish. It was left to cool for 2-3 minutes. DANCER OE, KO, Rescue and WT cells were resuspended as 3,000 cells in 3ml (1000cells/ml). Then, prewarmed 2.73 mL cell suspension was mixed with 270 μ l of 4% agarose (final:0,36%) and this mixture was poured into plate. Cells were grown for 21 days and colonies were visualized with an inverted microscope.

3.2.7. RNA-Sequencing

1 million cells were seeded per well of a 6-well cell culture plate in duplicates, left for attachment for 24 h, and then treated with 1 μ M doxorubicin for 16 hours or left untreated. After 16 hours, cells were harvested, and total RNA was isolated by using MN RNA purification kit. RNA samples from treated and untreated DANCER OE and HCT116 cells were sequenced on a NovaSeq 6000 platform with 40 million reads of Illumina TruSeq Stranded mRNA 100 bp paired-end (PE) library (Macrogen Europe, The Netherlands). RNA samples from untreated DANCER KO and HCT116 cells were sequenced on a DNBseq platform with 40 million reads of BGISEQ-500 Transcriptome 150 PE library (BGI, Hong Kong).

Differential gene expression analyses based on the RNA sequencing were assessed by utilizing the "RNA-Seq Analysis" pipeline of CLC Genomics Workbench 20 software (Qiagen) on Homo sapiens hg38 reference genome. Differentially expressed genes with absolute fold change of > 2 and with a false discovery rate (FDR) value of $< 0,05$ were used for further analysis.

3.2.8. Bioinformatics analysis

Different RNA transcripts may share miRNA response elements (MREs) to communicate with and to coregulate each other, so they may act as competing endogenous RNAs (ceRNAs) or natural miRNA sponges (82). The sequences of exons from "DANCER-201" were obtained through the Ensembl database to examine possible ceRNA features of DANCER. This transcript was knocked out with CRISPR/Cas9 previously and overexpressed with the retroviral system. Prediction of MREs on DANCER-201 sequence was performed on miRDB online database (83). Identified candidate miRNAs were then used to find their possible

mRNA targets. Selected miRNAs were analyzed via microT-CDS software of the DIANA tool for target genes with a 0.7 threshold (84) (85). Also, 2D structure was obtained from CLC, and 3D structure of DANCER was created by HNADOCK Server (86).

The common genes between the miRNA-targeted mRNAs identified in silico above and differentially expressed genes (DEGs) identified in our RNA-Seq analysis were filtered out and listed. These genes were examined via gene set analysis mode of ConsensusPathDB (CPDB) with a 0.01 p-value cutoff for pathway analysis (87) (88). Later, genes from selected pathways were visualized with induced network module analysis at CPDB. Additionally, gene ontology (GO) enrichment analysis was done with PANTHER tool to analyze a biological processes, molecular functions, and cellular components of defined gene sets (89).

Gene Set Enrichment Analysis (GSEA), which determines whether a previously characterized set of genes demonstrates statistically significant, concordant distinctions among two biological states, was applied to our RNA seq data (90) (91). DNA damage and p53 related gene sets were selected as datasets for GSEA analysis (92) (93). File formats, which are required for GSEA, were prepared accordingly. Then, gene sets were derived from upregulated and downregulated DEGs in DANCER KO vs HCT116, HCT116 treated vs untreated, and OE treated vs untreated comparisons. Enrichment score (ES), normalized enrichment score (NES), and FDR p-values were estimated, and enriched genes were listed.

4. RESULT

In our previous experiment, HCT116 human colorectal cells and mouse embryo fibroblasts (MEF) were treated with 1 μ M Doxorubicin (DOX) or left untreated for 8 hours. Then, total RNA was extracted from the samples and poly-A tail-enriched RNA sequencing was performed. After bioinformatic analysis of the RNAseq readings, differentially expressed genes were listed and among them DANCER was identified as a significantly downregulated gene in response to doxorubicin-induced DNA damage (80). In order to identify biological significance of DANCER in DNA damage response, the DANCER gene locus was deleted from HCT116 genome by using CRISPR/Cas9 gene editing technology by Gulin Baran, Msc., and DANCER^{-/-} HCT116 cells were simply named as KO cells (27). For the generation of KO cell lines, briefly, two CRISPR/Cas9 constructs had been created for targeting the upstream and downstream regions flanking the DANCER locus. pSpCas9(BB)-2a-Puro plasmid vector had been used for cloning. After that, HCT116 cells had been co-transfected with DANCER 5' and DANCER 3' constructs by using PeiMAX, and transfected cells were selected in the presence of puromycin. In this current Master's thesis study, DANCER cDNA was cloned into a mammalian expression plasmid and transfected into WT HCT116 and KO cells to overexpress and to restore DANCER expression and the cell lines were named as OE and Rescue, respectively. By these, we comprehensively investigated cellular biological pathways in which DANCER plays roles.

4.1 Characterizing *DANCR* expression in cells

Genotyping by PCR was performed in order to document whether the whole *DANCR* gene locus had been deleted from the genome of KO cells and but had remained intact in WT cells. For this, genomic DNAs were isolated from sample groups and genotyping PCR was performed using primers encompassing the *DANCR* gene locus. PCR product lengths for WT and OE samples were around 2550 bps, meaning that *DANCR* gene remained intact (**Figure 13A**). Because Rescue cells had been generated from KO cells by transfecting *DANCR* cDNA, the genotyping primers were not able to generate PCR yield from them, therefore, Rescue cells generated a PCR yield similar to that of KO cells, which was around 300 bps. To explore *DANCR* expression in *DANCR* OE, KO, Rescue, and WT cells, total RNA was isolated from the cells, and cDNAs were synthesized. Firstly, RT-PCR was performed with primers specific for PGK1 housekeeping transcript or *DANCR* transcript. As shown in **Figure 13C**, the PCR band that shows PGK1 expression was 152 bps in length and had similar intensities for all cells. The PCR band that indicates *DANCR* expression was 165 bps in length (**Figure 13D**). As expected, the band intensity of WT is lower than OE cells but higher than Rescue cells. Moreover, this band was absent in KO cells. No-RT samples, which include all components in RT reaction mixture except for the reverse transcriptase enzyme, were used as negative controls. To quantitatively compare *DANCR* expression across the cell groups, RT-qPCR was performed. As shown in **Figure 13B**, the result of RT-qPCR illustrated that *DANCR* expression was about 1.5-fold more in OE than in WT and 0.2-fold in Rescue, up from nothing in KO. These evidences indicated that *DANCR* expression level was highest in OE cells, while KO cells had none.

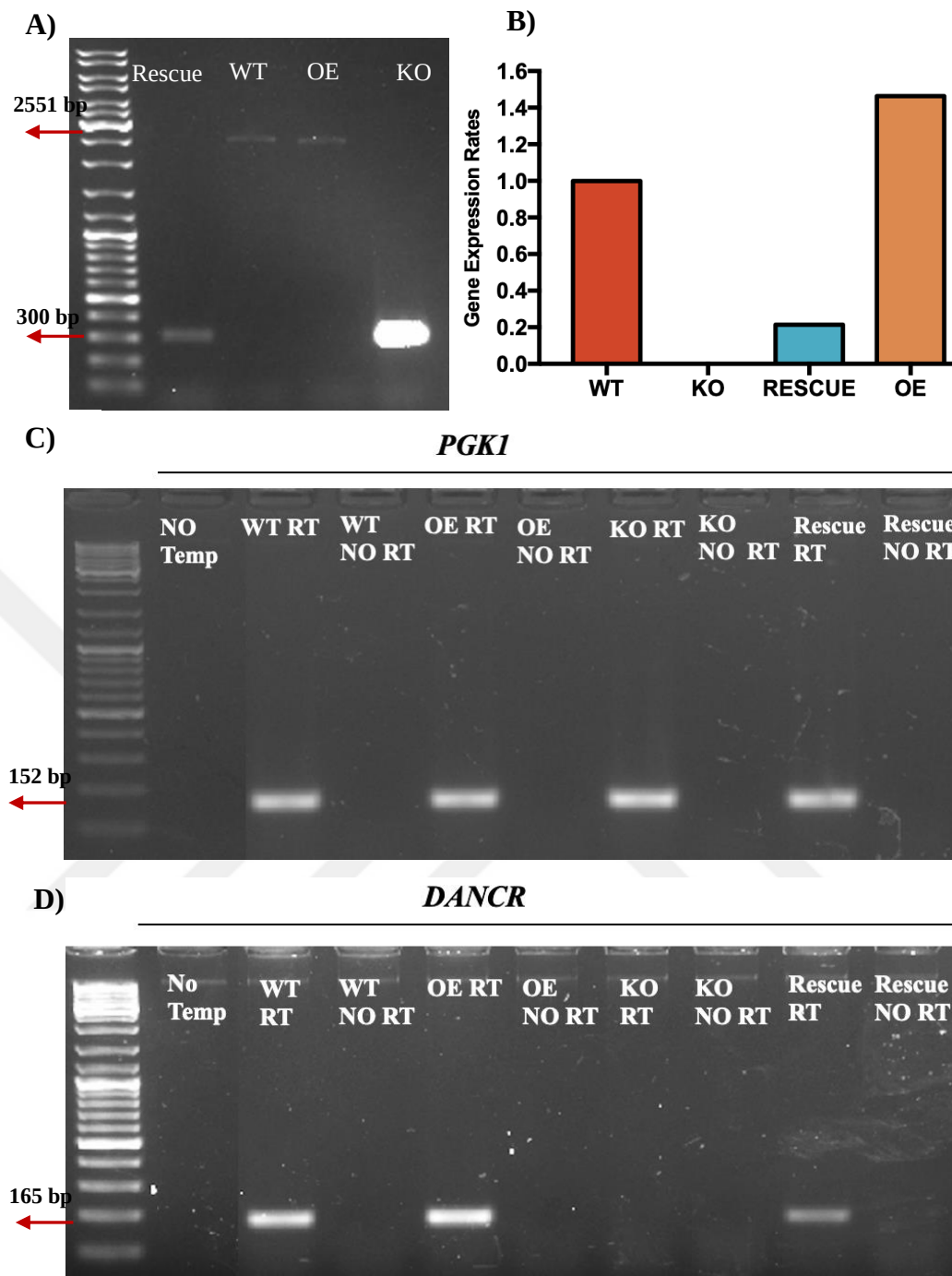


Figure 13: HCT116, OE, and Rescue cells express DANCR, but KO cells does not. **A)** Genomic DNAs were isolated from target cells and genotyping PCR was performed for the DANCR gene locus. Gene expression was visualized on agarose gel. **B)** The expression of DANCR was measured by RT-qPCR. This was done only for one time. PGK1 expression was used for normalization. RT-PCR was performed **C)** with PGK1 primers and **D)** with DANCR primers.

4.2 *DANCR* controls cellular proliferation rates in short and in long term

Trypan blue exclusion assay was implemented to analyze the growth rates of the cells at different time points. WT, KO, Rescue and OE cells were seeded in triplicates as 200,000 cells per 6 cm tissue culture plate. Cells were harvested with trypsinization, stained with trypan blue and counted on hemocytometer at 24th, 48th, and 72nd hours post-seeding (**Figure 14**). According to these results, the proliferation rate of WT was significantly higher than both KO (**** $p < 0.0001$) and Rescue cells (** $p < 0.0002$) at all 3 time points of growth, however, OE finally caught up WT and even proliferated significantly faster (** $p = 0.0073$) than WT at 72nd hour. Expectedly, the proliferation rate of OE was significantly higher than KO and Rescue cells at 48th and 72nd hours. In parallel to these results, Rescue cells proliferated faster than KO (**** $p < 0.0001$) at 48th and 72nd hours. Therefore, in general, KO cells' growth rate was lowest, whereas, Rescue cells' growth rate was significantly higher than KO but lower than WT and OE cells. Collectively, these show that *DANCR* expression positively regulates cellular proliferation.

To further analyze the long-term proliferation and colony formation capacities of the corresponding cell types, colony formation assay was performed. WT, KO, Rescue, and OE cells were seeded in triplicates as 1000, 2000, 3000, and 4000 cells per 6 cm tissue culture plate and grown for approximately 2 weeks. Growth medium was refreshed every 3-4 days during this time. At the end of the growth period, the colonies were fixed and stained with crystal violet for straightforward visualization. After the plates had been scanned by a scanner, colony numbers and average colony sizes in pixel were estimated by Image J (**Figure 15A**). As seen in **Figure 15B** and **Figure 15C**, the colony number of WT and OE were significantly higher than KO and Rescue cells (**** $p < 0.0001$) in all initially different seeded cell number plates. Also, the average sizes of colonies of WT and OE cells were significantly higher than KO and Rescue cells in 4000 seeded cell number. Therefore, difference of long-term proliferation and colony formation capacities of

endogenous-DANCR expression efficient cells over endogenous-DANCR expression deficient cells had similar pattern to that of short-term proliferation difference. Besides, exogenous DANCR expression in Rescue and OE cells did not yield a significant difference among cell groups.

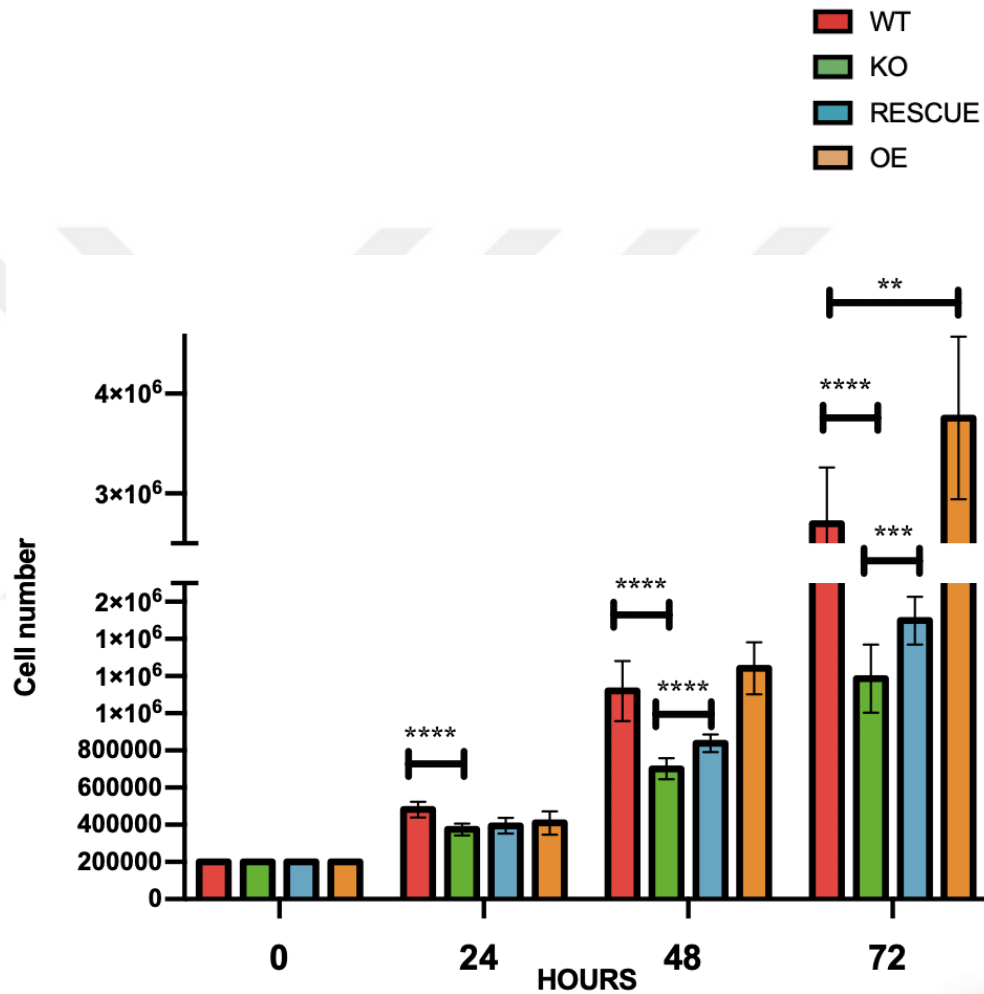


Figure 14: DANCR increases cell proliferation rate. Trypan blue exclusion assay was performed to estimate cell numbers at 24th, 48th, and 72nd hours post-seeding. Experiment was repeated for 4 times with triplicates and performed in triplicate. Bar graph with a mean $\pm$ standard deviation of 4 independent experiments is obtained by using GraphPad Prism 5 version 5.01 utilizing two-way ANOVA analysis was performed.

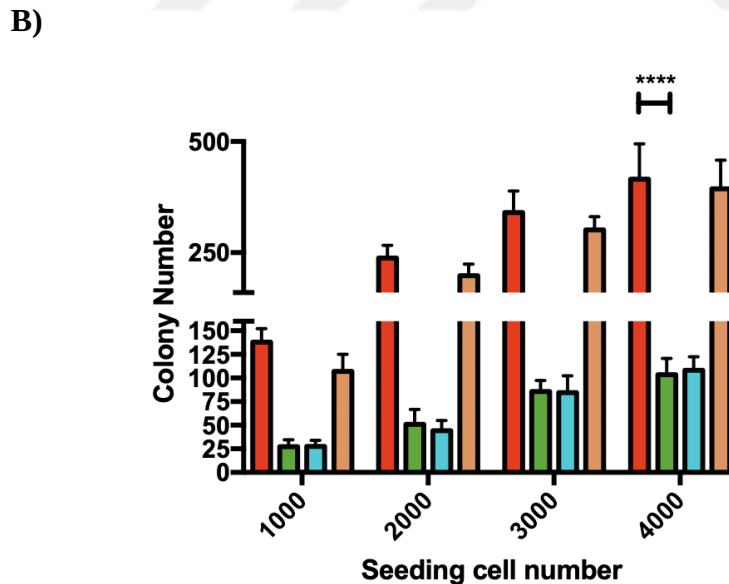
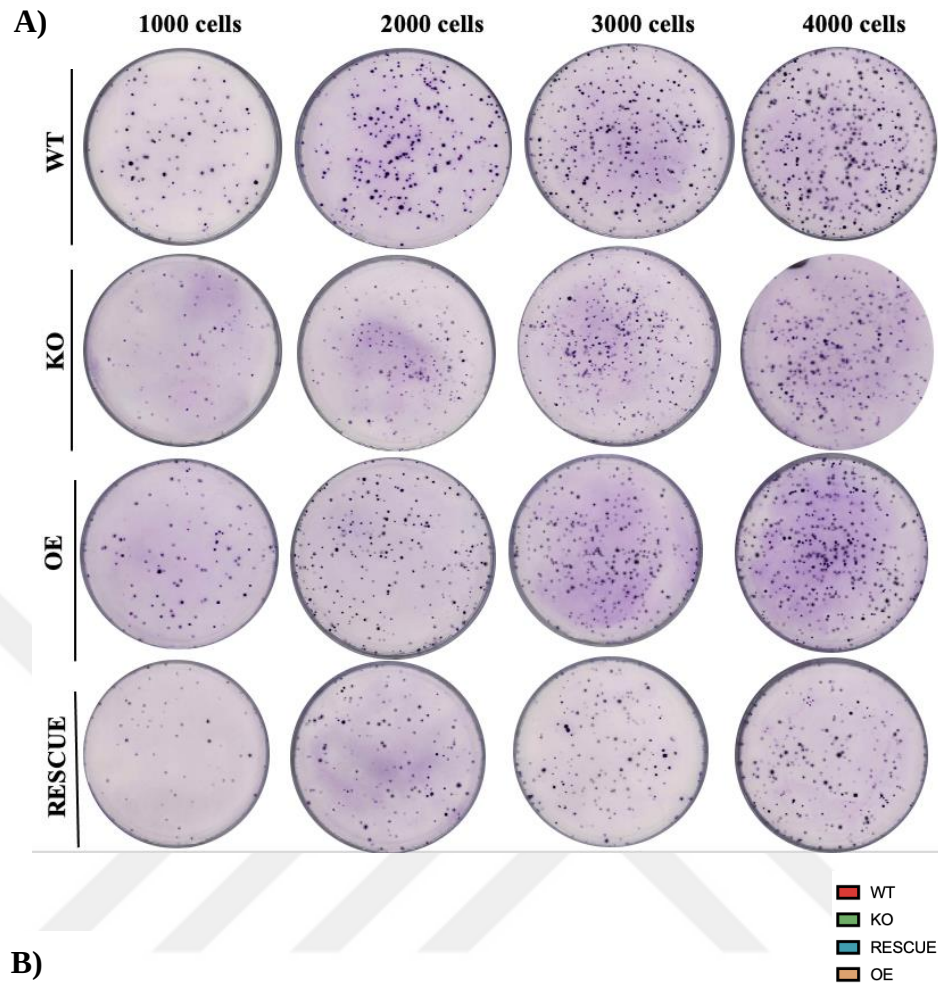


Figure 15: DANCER controls long term proliferation and colony formation capacities. **A)** The representative image of DANCER OE, KO, RESCUE and WT colonies seeded initially with different cell numbers at the end of approximately 2 weeks. Colonies were visualized with crystal violet and plates were scanned. Experiment was repeated at 2 times with triplicates. **B)** The total colony numbers for each cell line. **C)** The total colony sizes for each cell line in pixel unit. Two-way ANOVA analysis was performed with GraphPad Prism 5 version 5.01.

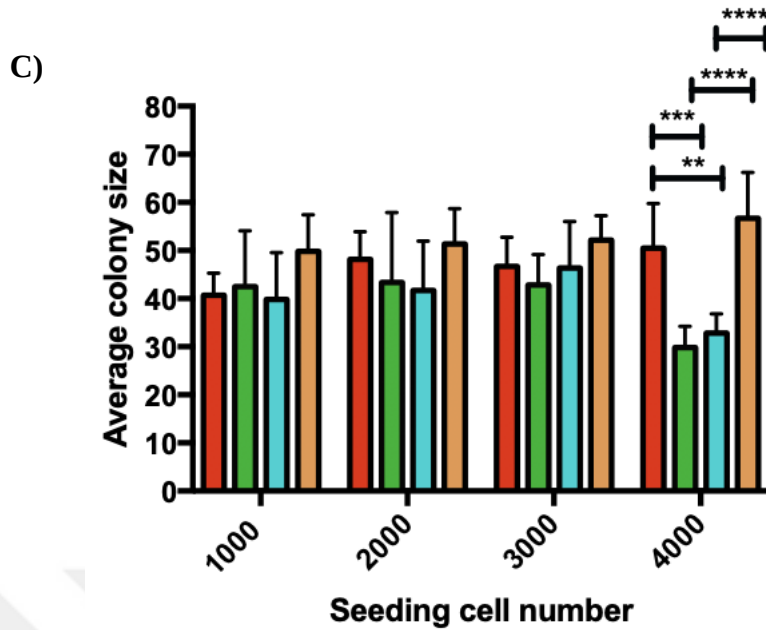


Figure 15 (continued): DANCER controls long-term proliferation and colony formation capacities.

4.3 DANCER affects doxorubicin treatment

To explore the physiological effects of DANCER in DOX-treated cells, MTT cell proliferation assay was performed under different DOX concentrations (0nM-10000nM). Firstly, 5000 cells were seeded per well of a 96-well cell culture plate in duplicates for each cell line. Next day, the cells were treated with DOX for 48h and MTT assay was utilized to assess relative live cell counts. According to that, IC_{50} against doxorubicin was calculated by using GraphPad Prism (**Figure 16**). The highest IC_{50} value was 426.5nM in OE, and the second highest IC_{50} value was 357.1nM in KO cells. Also, the lowest IC_{50} was found as 245.2nM in WT. The IC_{50} of Rescue cells was 314.2nM. As a result, we observed that exogenous DANCER expression in addition to the endogenous DANCER in OE cells and loss of endogenous DANCER expression in KO cells conferred resistance to DOX in them compared to WT cells. However, exogenous DANCER expression in an endogenous

DANCR deficient background sensitized Rescue cells to DOX compared to KO cells.

To further investigate the DANCR response to DOX, a long-term colony formation assay was performed. 500 cells were seeded from WT, KO, Rescue, and OE cells in triplicates. The next day, they were treated with 250nM, 500nM, or 1000nM DOX or left untreated for 2 hours. Later, cells were left for recovery. At the end of the recovery period, the colonies were fixed and stained with crystal violet (**Figure 17A**). Colonies from scanned plates were analyzed via Image J. As seen in **Figure 17B** and **Figure 17C**, without any DOX treatment, colony numbers of WT and OE cells were similar to each other but were together significantly higher than both KO and Rescue colonies (**** $p < 0.0001$), as expected. Under treatment with 250nM or with 500nM DOX, WT and OE cells colony numbers were significantly higher than KO and Rescue colonies (**** $p < 0.0001$). Under the 1000nM DOX treatment, no significant difference between cells was observed. Average size of OE cells were higher than other cells in all DOX treated and untreated conditions but not strikingly significant.

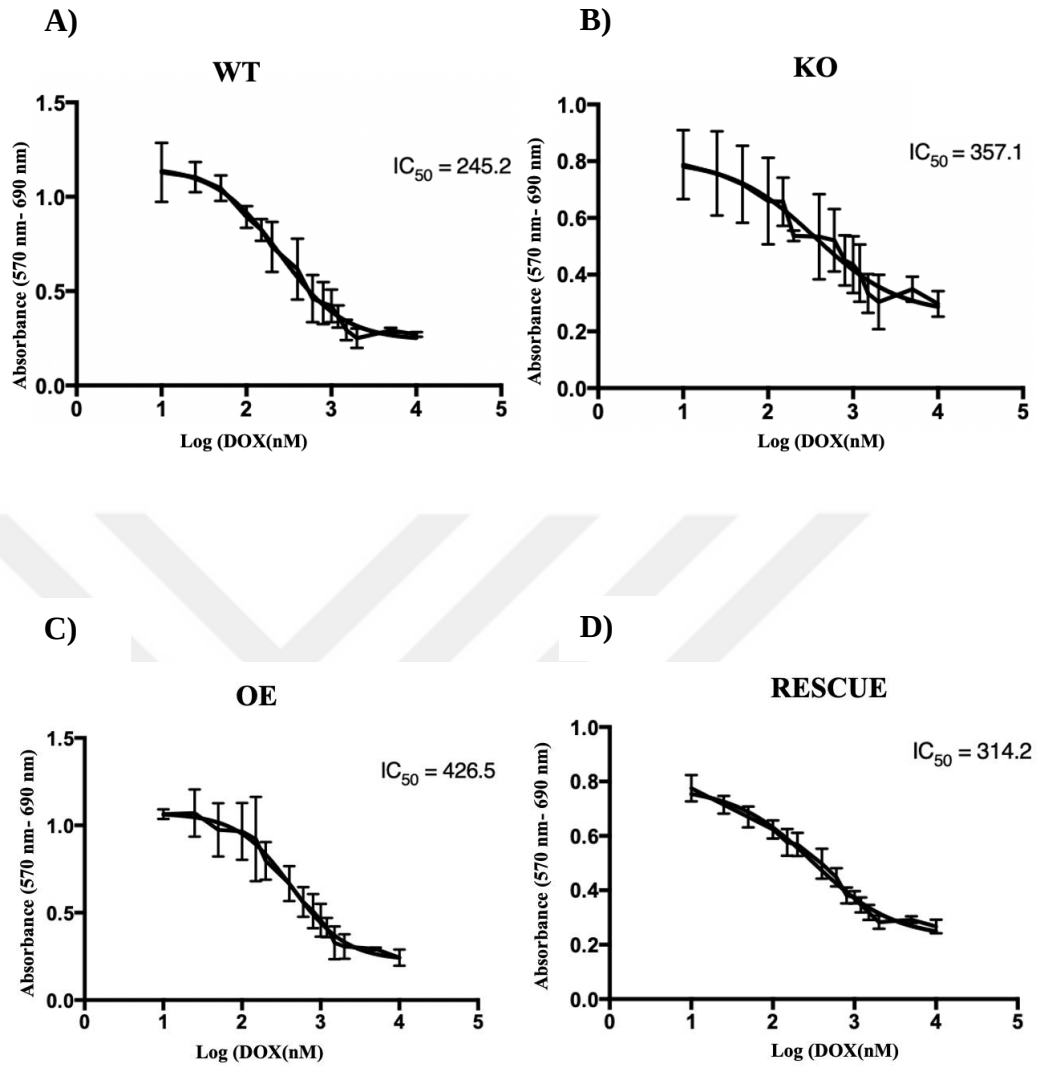


Figure 16: DANCER controls cell's response to doxorubicin treatment. IC_{50} values of WT, KO, Rescue and OE cells against doxorubicin were assessed. Doxorubicin resistance of the cells were evaluated by MTT proliferation assay. After the assay, IC_{50} values were calculated according to absorbance measures at 570 and 690 nm using GraphPad Prism 5 version 5.01. Data were shown as mean $\pm$ SD from four independent experiments.

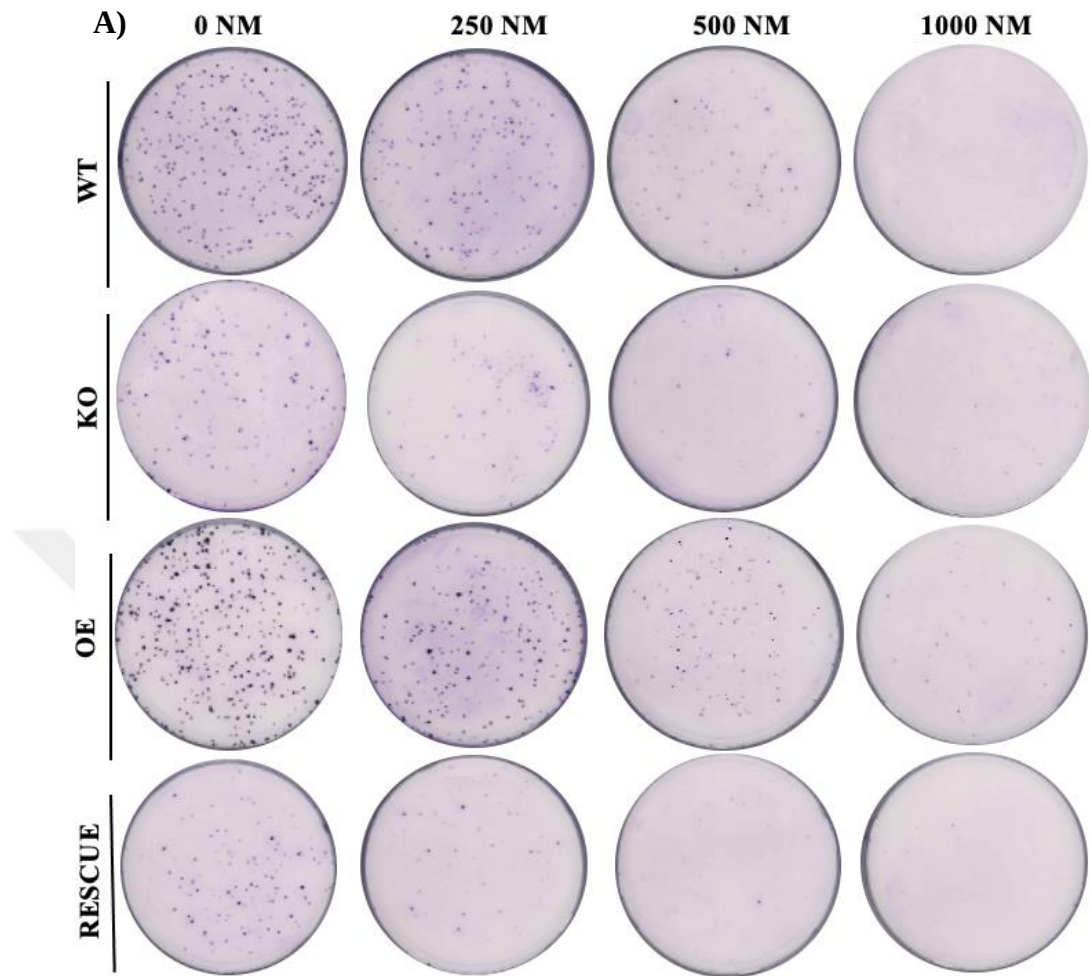


Figure 17: DANCER controls long term proliferation and colony formation capacities of doxorubicin treated cells. **A)** The representative images of WT, KO, Rescue and OE cells treated with different DOX concentrations. Colonies were visualized with crystal violet and plates were scanned. Experiment was repeated 2 times each in triplicates. **B)** The total colony numbers for each cell line. **C)** The total colony sizes for each cell line in pixel unit. Two-way ANOVA analysis was performed with GraphPad.

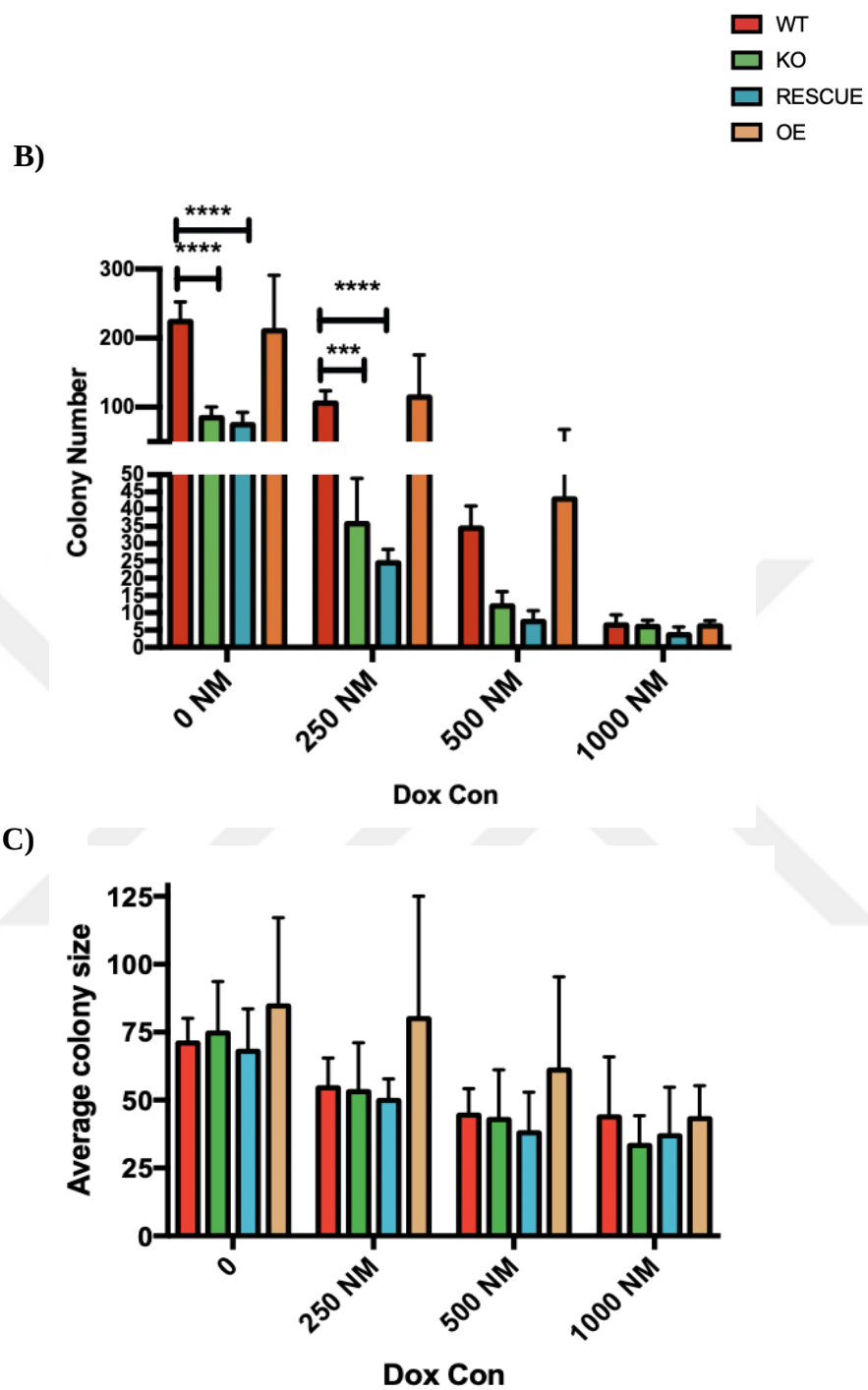


Figure 17 (continued): DANCER controls long term proliferation and colony formation capacities of doxorubicin treated cells.

4.4 DANCER affects cell cycle distribution

To uncover the effects of DANCER role on cell cycle, WT, KO, Rescue and OE cells were stained with propidium iodide (PI) and analyzed using flow cytometry. As seen in **Figure 18**, DANCER efficient and deficient cells were found with differentially regulated cell cycle distributions compared to WT cells. Sub- G_1 phase, which may be the identifier of apoptotic cells, was slightly bigger in KO cells compared to the others albeit not strikingly significantly. G_1 phase that is for cellular growth and is a check point for cell cycle arrest was significantly highest in Rescue cells (**** $p < 0.0001$) and was higher in KO cells than WT and OE cells (** $p = 0.0084$). S phase, which is the DNA replication period, was significantly highest in OE cells and higher in WT cells than KO (** $p = 0.0037$) and Rescue cells (**** $p < 0.0001$). S phases of KO cells and Rescue were not significantly different from each other. At G_2 phase, which accounts for another cell cycle arrest check point, percent of WT was significantly higher than Rescue cells (* $p = 0.0120$) but no significant change was observed between WT, KO and OE cells. Moreover, at late G_2 phase, Rescue cells were significantly higher than KO (* $p = 0.0216$), WT (* $p = 0.0123$) and OE (**** $p < 0.0001$) cells. The overall cell cycle distribution is parallel to cellular proliferation rates of the corresponding cell types, in which WT and OE cells are more proliferative, hence they were found more in S phase, however, in which KO and Rescue cells are less proliferative, hence they were found more in G_1 phase.

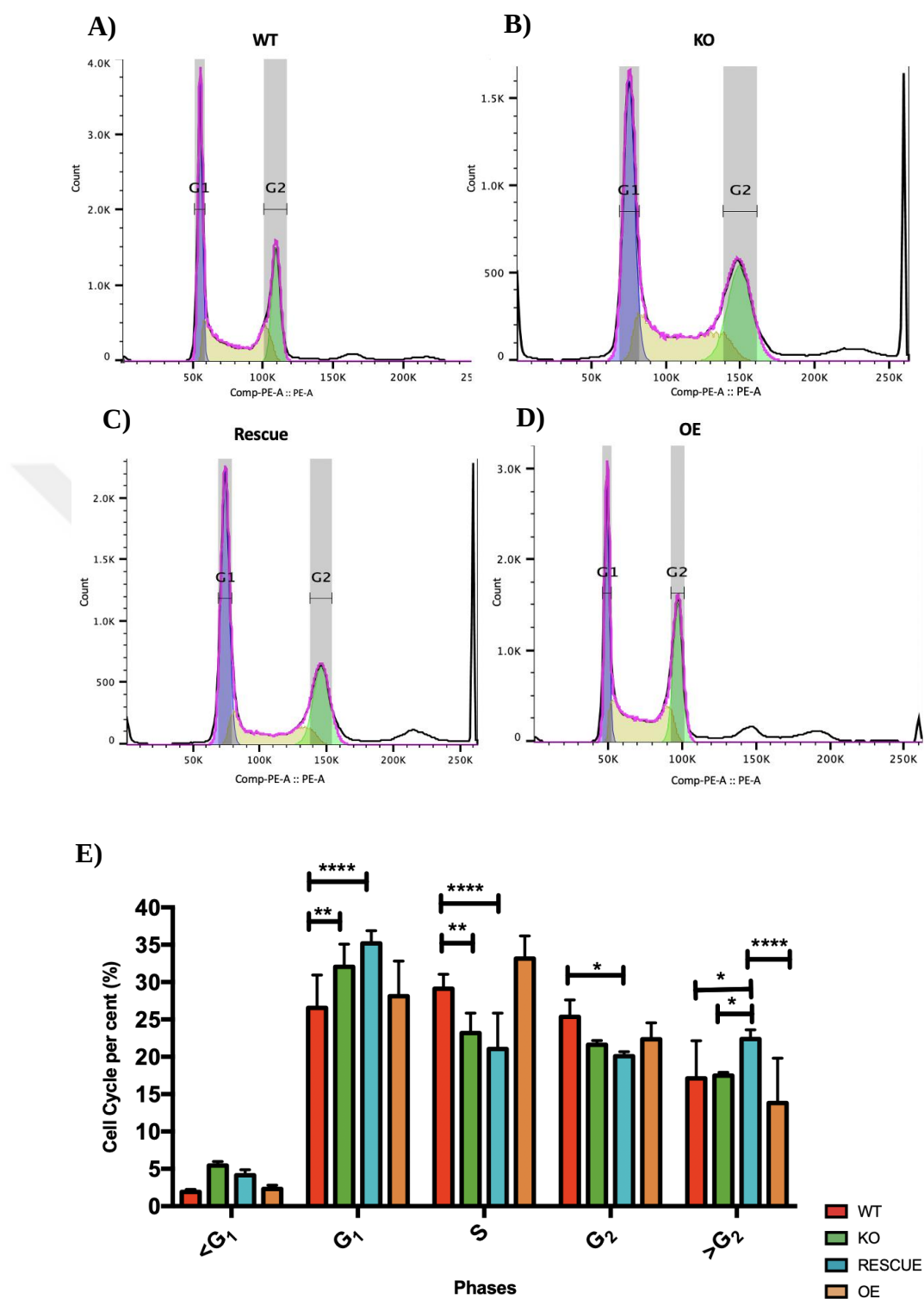


Figure 18: DANCR affects cell cycle distribution. **A-D)** The representative images of PI-stained cell cycle phases were obtained by FlowJo 10.6.1. Experiment was repeated for 3 times in duplicates. **E)** Cell cycle distribution quantification analysis was performed with two-way ANOVA by GraphPad Prism 5 version 5.01.

4.5 DANCER affects cell size and multi-nucleation morphology

To analyze morphologic changes between WT, KO, Rescue and OE cells, cells were stained with hematoxylin and eosin (H&E) and analyzed under microscope. As seen in **Figure 19**, nuclei, which are stained with hematoxylin, have darker color relative to the cytoplasm, which is stained with eosin. The size of WT and OE cells were smaller than KO and Rescue cells. Generally, KO cells had multi-nucleated morphology such as enlarged cytoplasm, multiple centric nuclei and indistinct cell boundary (94), but mononucleated cells were also frequently observed. Moreover, Rescue cells resembled more KO cells but they were not multi-nucleated. However, there was not a striking difference between WT and OE cells.

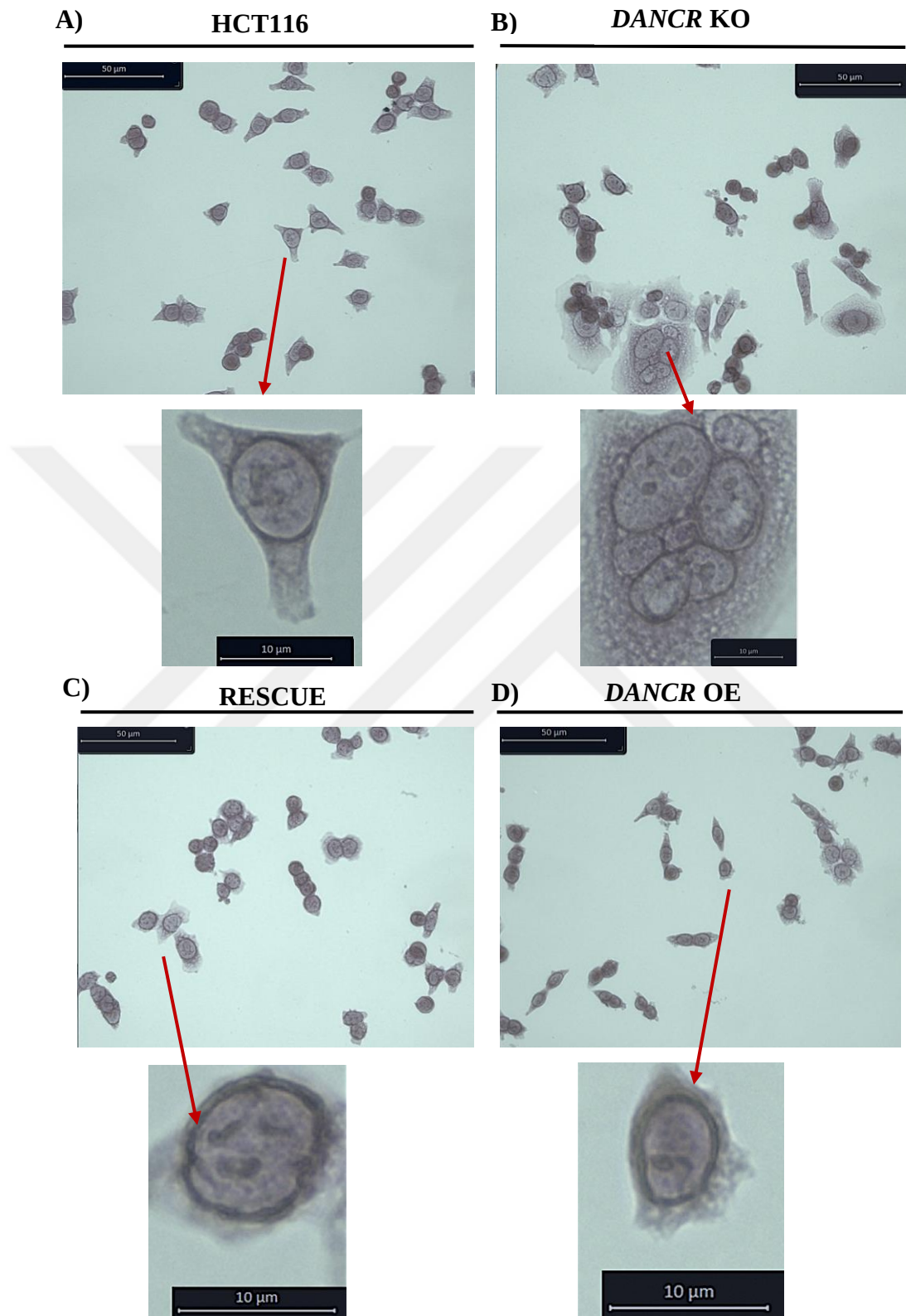


Figure 19: DANCER KO cells are larger in size and multi-nucleated. H&E staining was used to assess morphology of the cells. Images were generated from Fluorescence microscopy under 40X magnification.

4.6 3D spheroid formation capacities of DANCER WT, KO, Rescue and OE cells

Spheroids, which are 3D cultures, are composed of a living assembly of cells that generate cell-to-cell interactions (95). The aggregated cells create a high densely packed 3D tumor-like structures and are organized in proliferative, senescent and necrotic layers through outside to inside, respectively (96). They imitate a solid tumor because of diffusion-caused-restraints on glucose and oxygen transports, which generate a hypoxia-like environment in the center of the spheroids (97). To examine the spheroid properties of WT, KO, Rescue and OE cells, a hanging drop assay was performed. Cells were initially treated with 250 and 500 nM DOX for 2 hours or left untreated, and 2500 cells per sample were placed in a 10 μ l droplet each on the lid of a 6 cm plate. Then the lid was carefully inverted, and the droplets kept their adhesion to the surface because of surface tension. Cells settled and concentrated at the bottom of the drop due to gravity, so only one spheroid was created (95). Fluorescence microscopy was utilized to follow spheroid formation in the droplets from Day 1 through Day 3. Therefore, spheroid forming capacity and speeds were evaluated for each group (**Figure 20 and Figure 21 A**).

Determination of integrity and growth volume of spheroids is significant to identify the tumor role of cells (98). First, spheroids of cells without any initial DOX treatment were analyzed and compared. Cells were becoming more aggregated at day 1, and aggregates happened more spheroidal, compact, intense and dark at day 3. The integrity and circularity of KO spheroids were the least among 4 different cell groups at day 3, followed by Rescue spheroids (**Figure 20**). Moreover, the average size of KO spheroids were the smallest (**** $p < 0.0001$ for OE and Rescue; *** $p = 0.0002$ for WT) on day 3. The spheroid sizes in pixel² decreased gradually for all cell groups, except OE, after 24h through 72 h. Also, the decrease was highly significant for KO spheroids from day 1 to day 3 (****

$p < 0.0001$) (**Figure 21B**). WT and OE spheroid sizes did not change that significantly but OE spheroid sizes increased probably because cells started to proliferate and increased the overall spheroid size. Proliferating cells of spheroids are expected to be very light green and are usually located on the outside boundaries of spheroids (99). In this regard, the proliferating cell area was highest in OE and in WT cells (**Figure 20**). Quiescent cells are expected to accommodate in the middle of the spheroid and are medium green colored (99). Accordingly, quiescent cells were seen clearly in WT and OE cells but absent in KO and Rescue cells. Necrotic cells are expected to be located in the center and are dark green. Necrotic cells observed more in WT and OE but were almost absent in KO and Rescue cells. These are probably due to higher proliferative rates of WT and OE cells, meaning that they already proliferated, and the inner parts of the spheroids of WT and OE cells had already fulfilled their proliferation capacity

To observe DOX efficacy against spheroids, cells were treated with 250 or with 500 nM DOX (**Figure 20**). When cells were treated with 250 nM DOX, the integrity of OE spheroids were the highest, and the number of dead cells were the lowest. However, the circularity of all spheroids in all groups decayed progressively albeit at different rates, OE being the most intact one, WT is the second, Rescue is the third, and KO is the last. However, it is also important to remember the lack-of-perfect-circularity of KO spheroids in untreated condition in the first place. Moreover, spheroids were almost not formed in KO and Rescue cells under DOX treatment. Cell colonies disaggregated and dispersed around. When cells were treated with 500 nM DOX, spheroids of WT and OE cells also did not form perfectly.

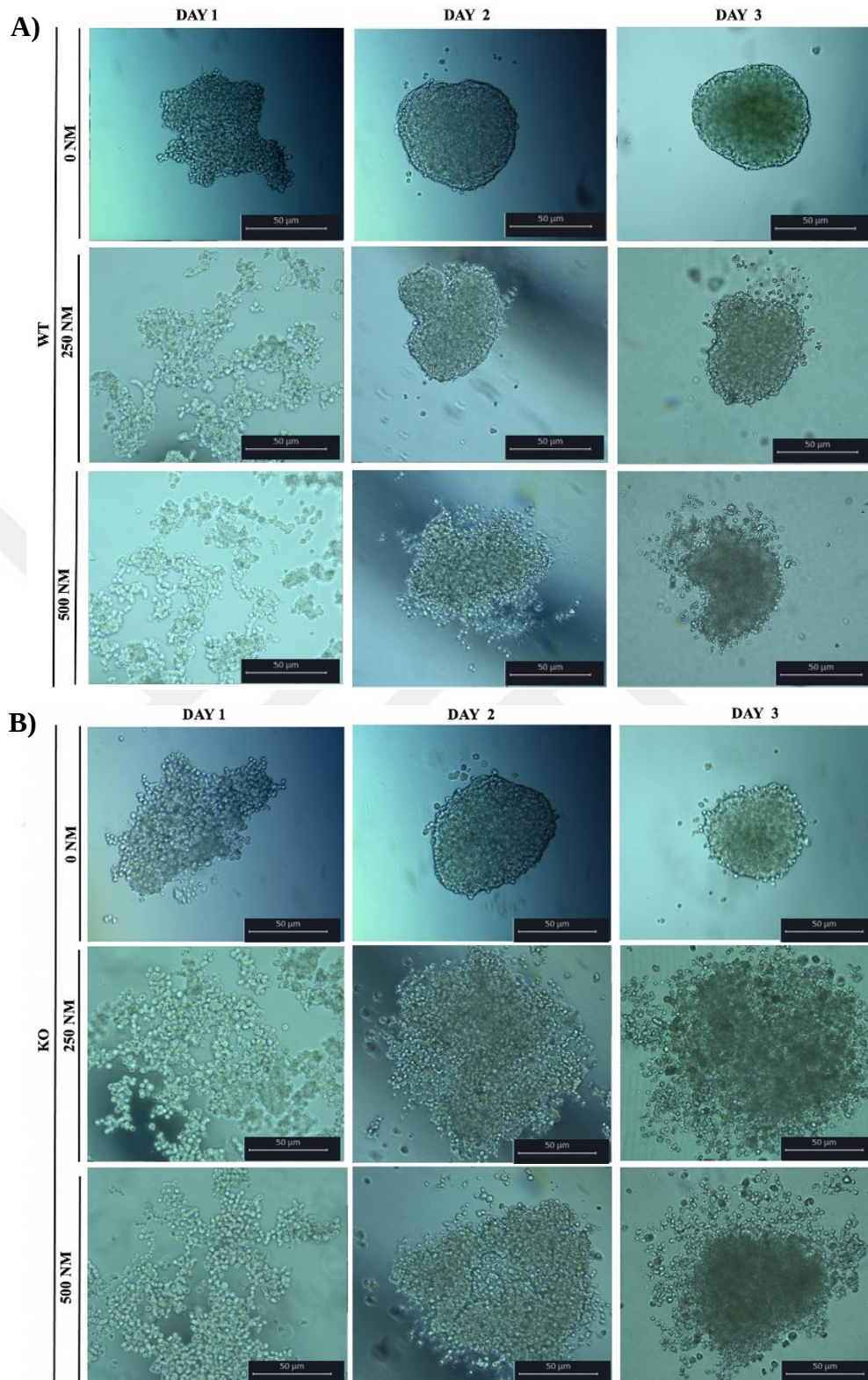


Figure 20: DANCER expression can regulate spheroid size. WT, KO, RESCUE and OE cells were seeded in hanging droplets after being treated with 250 and 500 NM DOX for two hours or not treated. Random selected spheroids were photographed at first, second and third days by fluorescence microscope under 20X.

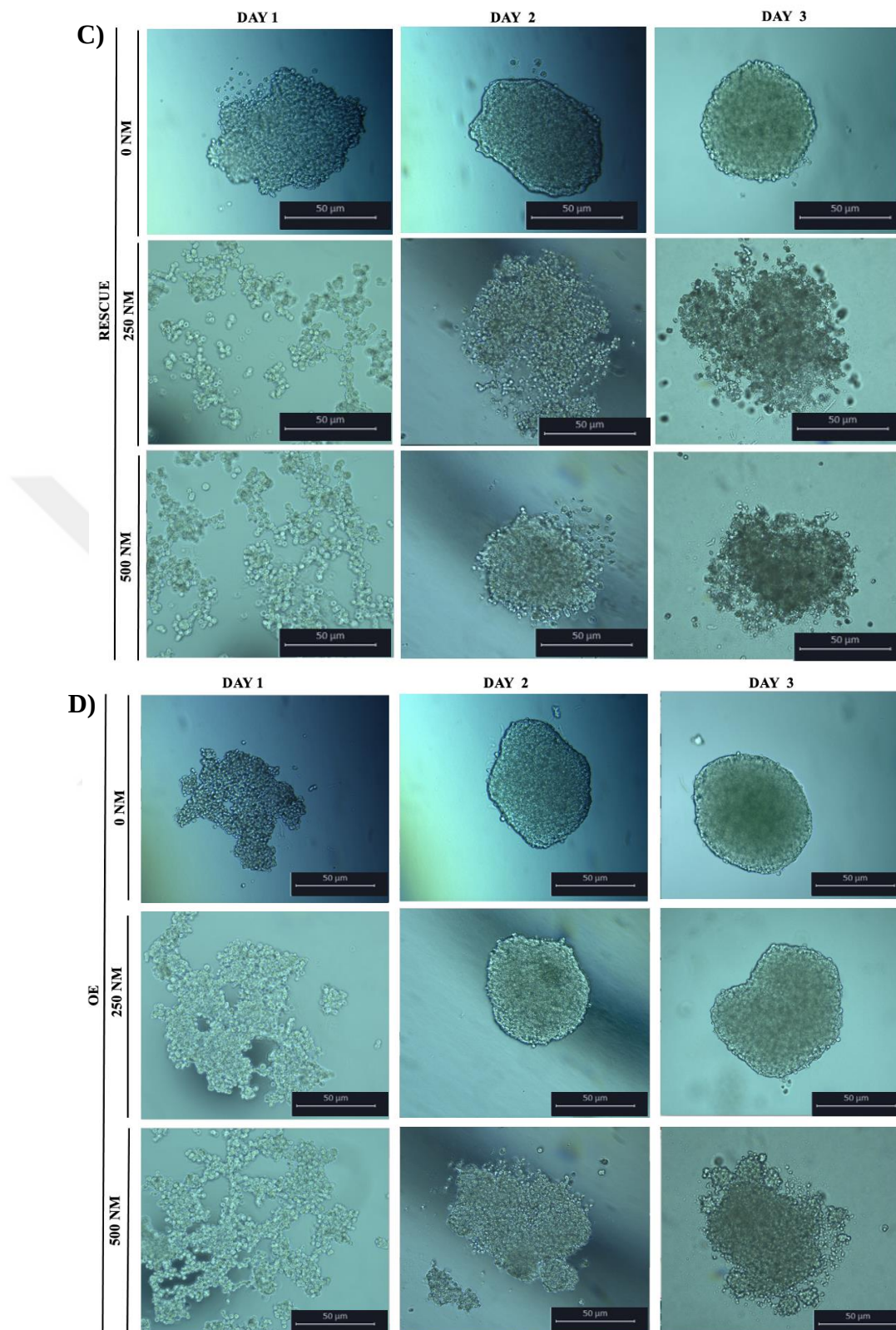


Figure 20 (continued): DANCER expression can regulate spheroid size.

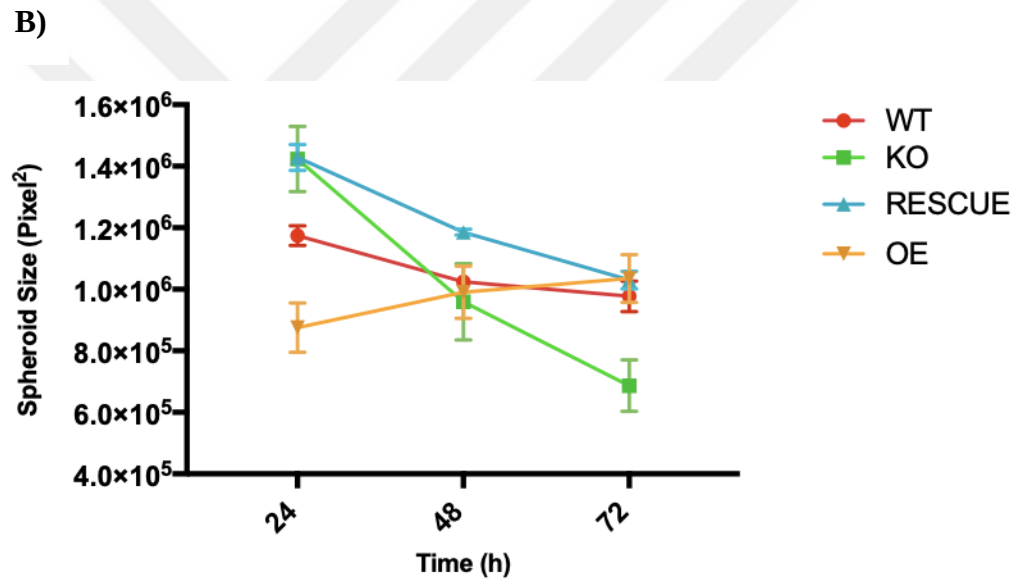
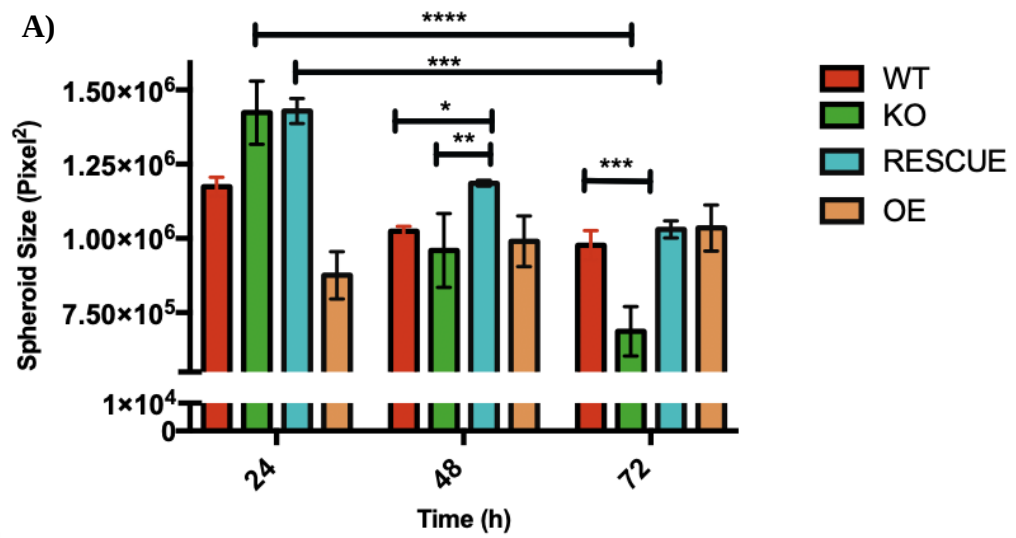


Figure 21: Statistical comparisons of 3D spheroids sizes in pixel². **A)** and **B)** Spheroid's size in different time points shown by different graphs. Random 5 spheroids were selected and measured in pixel² with Image J.

4.7 Anchorage independent growth capacities of DANCER WT, KO, Rescue and OE cells

Tumor cells can easily grow under anchorage-independent conditions. However, normal-like or untransformed cells can not successfully grow without attachment to a solid surface. Therefore, this assay was performed to differentiate between the malignant-like from non-neoplastic-like cells. For this, semi-solid soft agar colony formation assay was done, in which, 1000 cells/mL cells were encapsulated within 4% agarose and anchorage-independent colony formation of WT, KO, Rescue and OE cells was monitor for 21 days. Agarose medium allows uniform diffusion of nutrients into the colony and equal expansion in all directions (100).

All type of colonies became more aggregated, spheroidal, and compact by day 7 (**Figure 22**). Sizes of KO and Rescue colonies were nearly the same, WT and OE colonies were significantly 2-fold and 4-fold higher (**** $p < 0.0001$), respectively at day 14 (**Figure 23A, B**). WT and OE colony shapes were more spheroidal and necrotic cores were easily visible in their centers. Although, both KO and Rescue colonies were relatively unshaped, Rescue colonies seemed to have more defined boundaries compared to that of KO. Also, cell-cell physical interactions were lost in KO colonies, and holes were composed in the spheroid surface because of cellular death. At day 18, OE colonies were significantly the largest in size (**** $p < 0.0001$) and the increment rate was highest. The size of WT colonies increased a little bit in this period (**Figure 23A**).

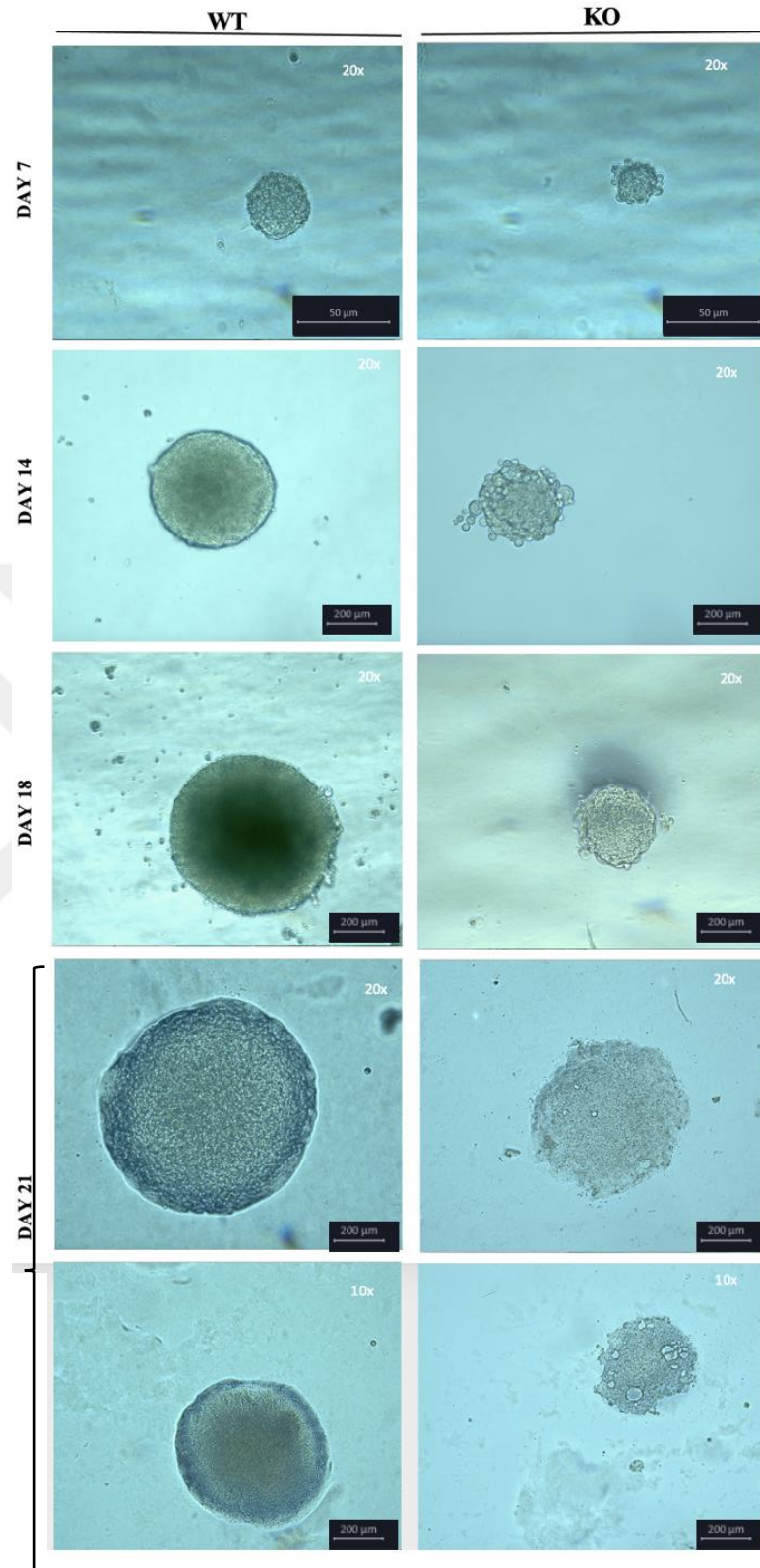


Figure 22: DANCER expression increases anchorage-independent property. WT, KO, Rescue, and OE cells were seeded within agarose medium and grown for 21 days. Colonies were visualized with an inverted microscope. Randomly selected colonies were photographed at 7th, 14th, 18th and 21st days by fluorescence microscope under 20X and 10X.

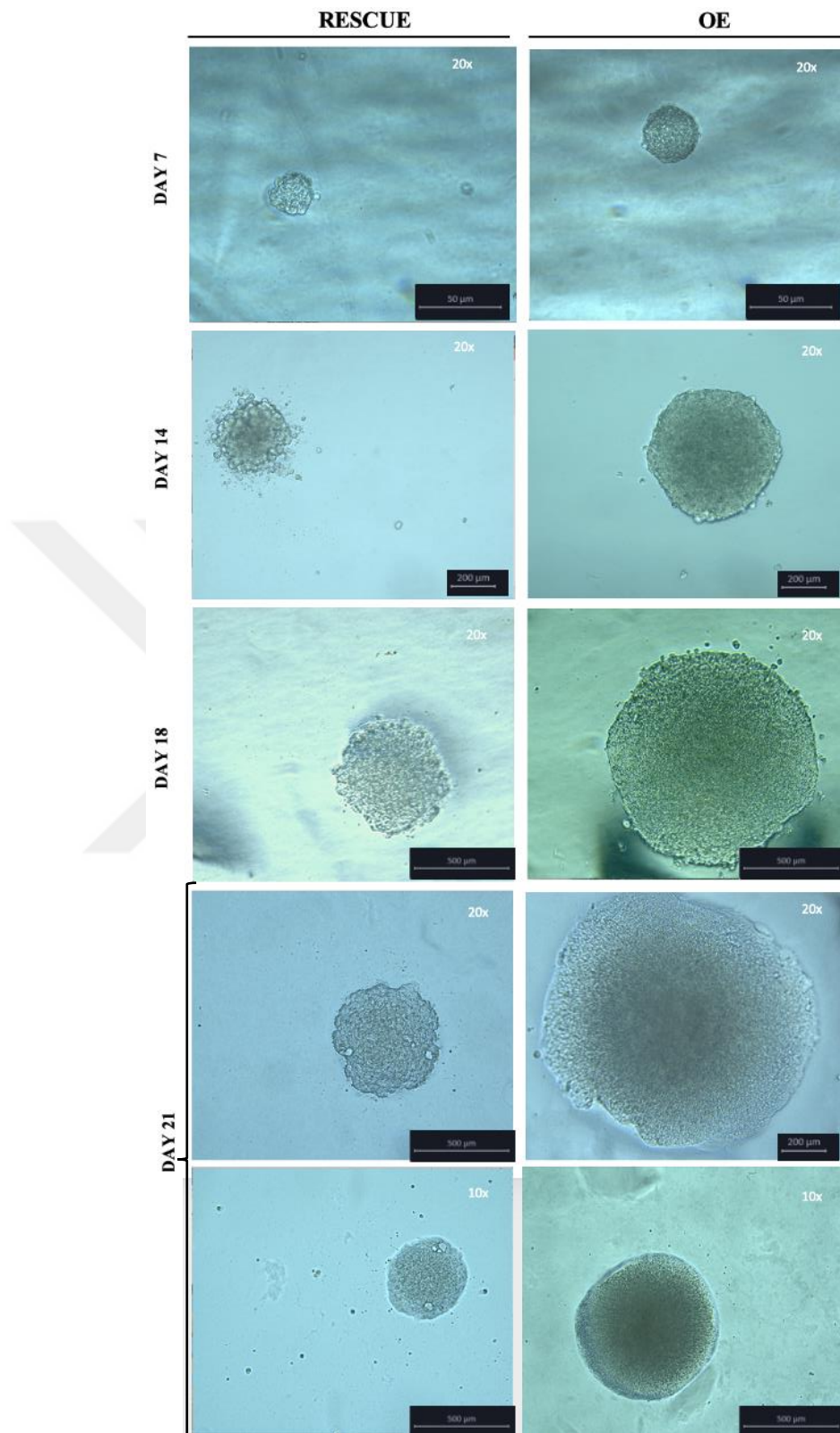


Figure 22 (continued): DANCER expression increases anchorage-independent property.

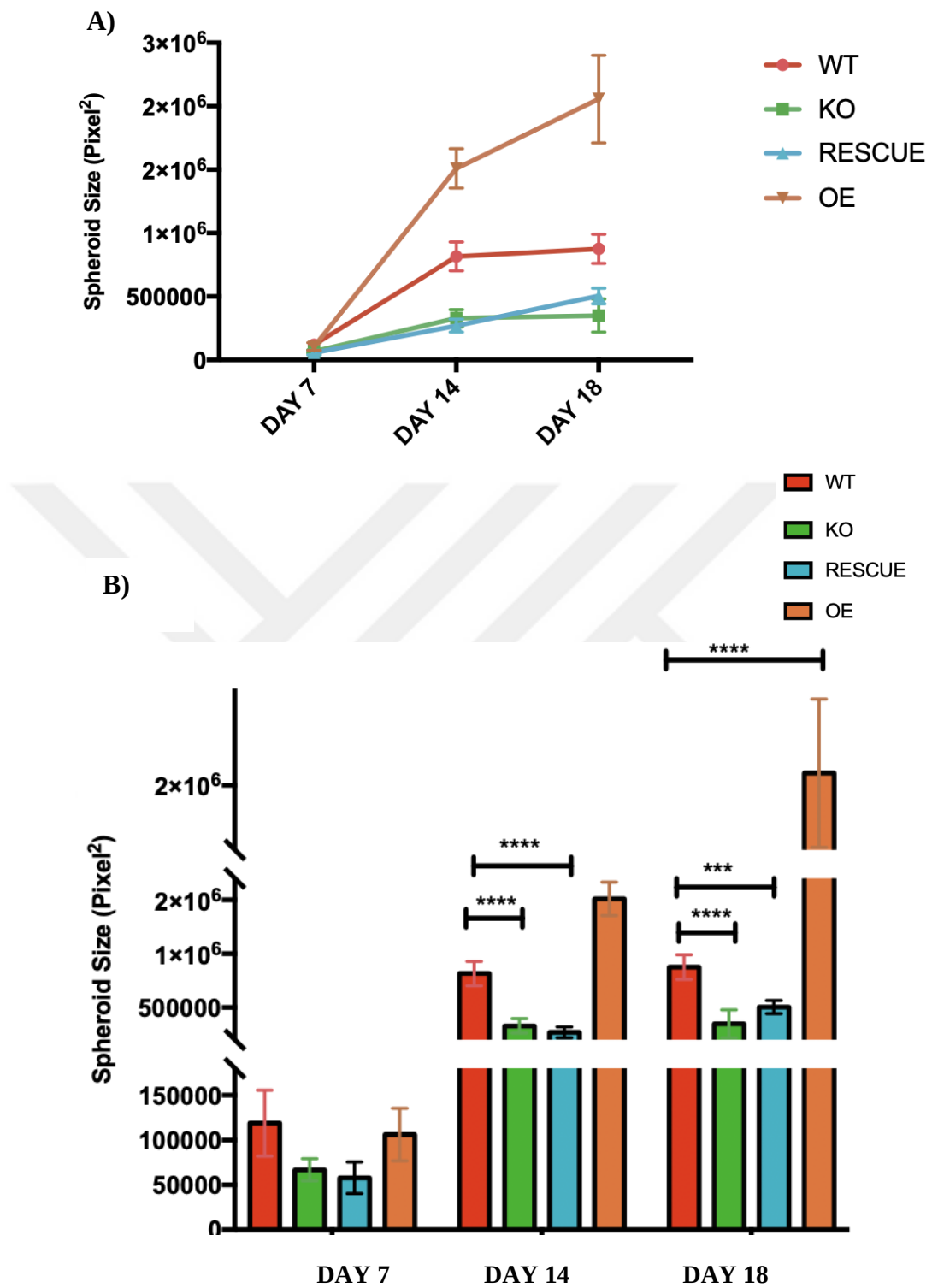


Figure 23: Statistical comparisons of anchorage-independent colony sizes in pixel². **A)** and **B)** Colony sizes in different time points shown by different graphs. Random colonies were selected and measured in pixel² with Image J.

4.8 DANCER can act as a competing endogenous RNA

Micro RNAs (miRNAs) bind to miRNA response elements (MREs) on target mRNAs, causing their degradation or inhibiting their translation. Therefore, they are important in regulation of gene expression at translational level. In addition to their vital importance for a healthy cellular homeostasis, they also have great functionally to induce diseases, such as cancer in which they may act as oncogenic or tumor suppressive genes. Same or different kind of RNAs may compete for binding to shared miRNAs, leading to regulation of each other's levels. These RNAs are referred to as competing endogenous RNAs (ceRNAs). To investigate whether DANCER transcripts have potential to act as ceRNA, a bioinformatics pipeline was followed. For this, the strategy was developed as seen in **Figure 24**. When DANCER binds to specific miRNAs, inhibitory action of the miRNAs can be prevented on target mRNAs (**Figure 25**). However, DANCER absent condition may cause the accumulation of the miRNAs and mRNAs may be degraded by these. In order to list differentially expressed genes in the absence of DANCER, RNA seq was performed for WT and KO cells. Therefore, we hypothesized that DANCER KO vs WT RNA seq results may give clues about downregulated mRNAs by these now accumulated miRNAs.

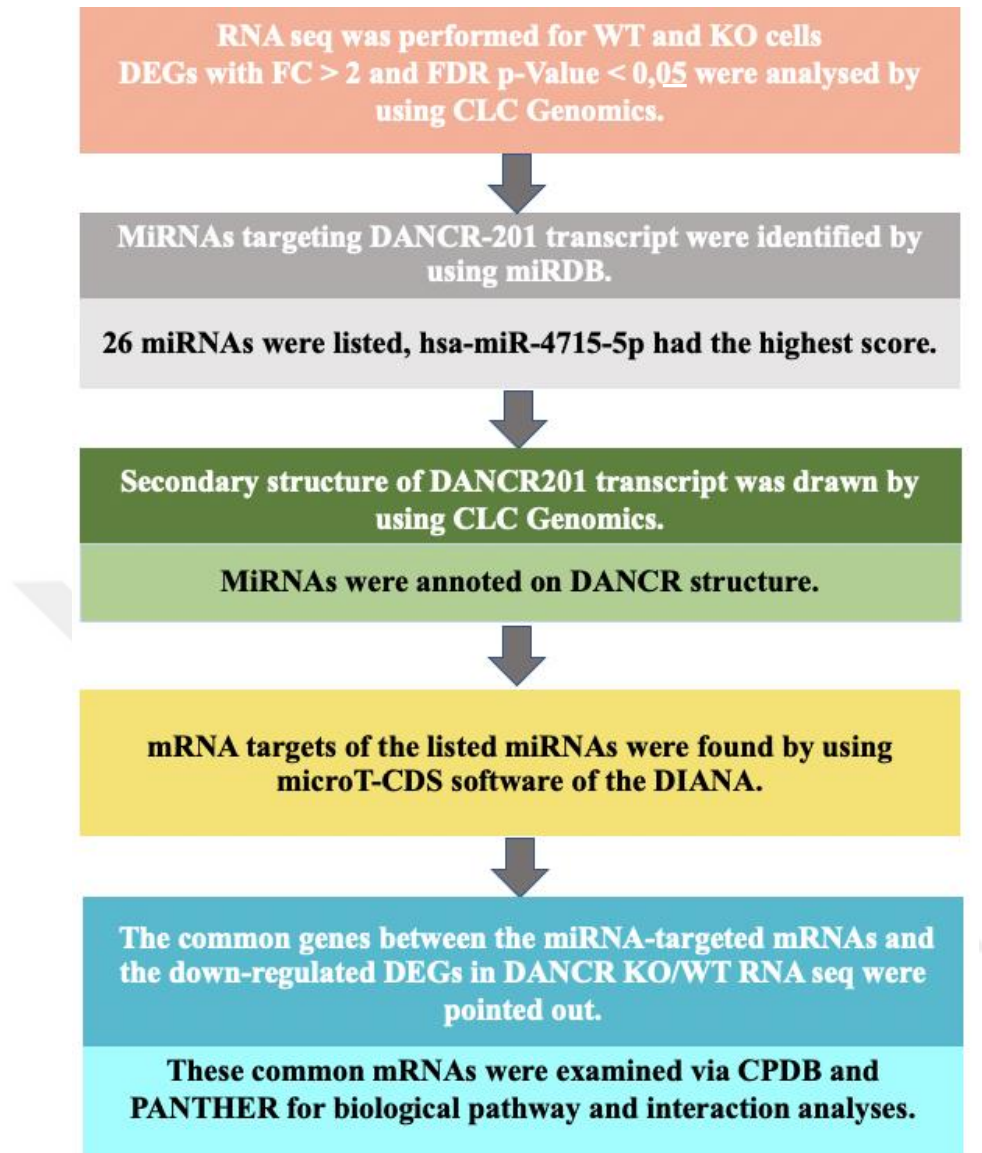


Figure 24: The workflow for analyzing ceRNA function of DANCER. RNA seq: RNA Sequencing, DEG: Differentially Expressed Gene, FC: Fold Change, FDR: False Discovery Rate, miRNA: microRNA, miRDB: MicroRNA Target Prediction Database, mRNA: messenger RNA, CPDB: ConsensusPathDB.

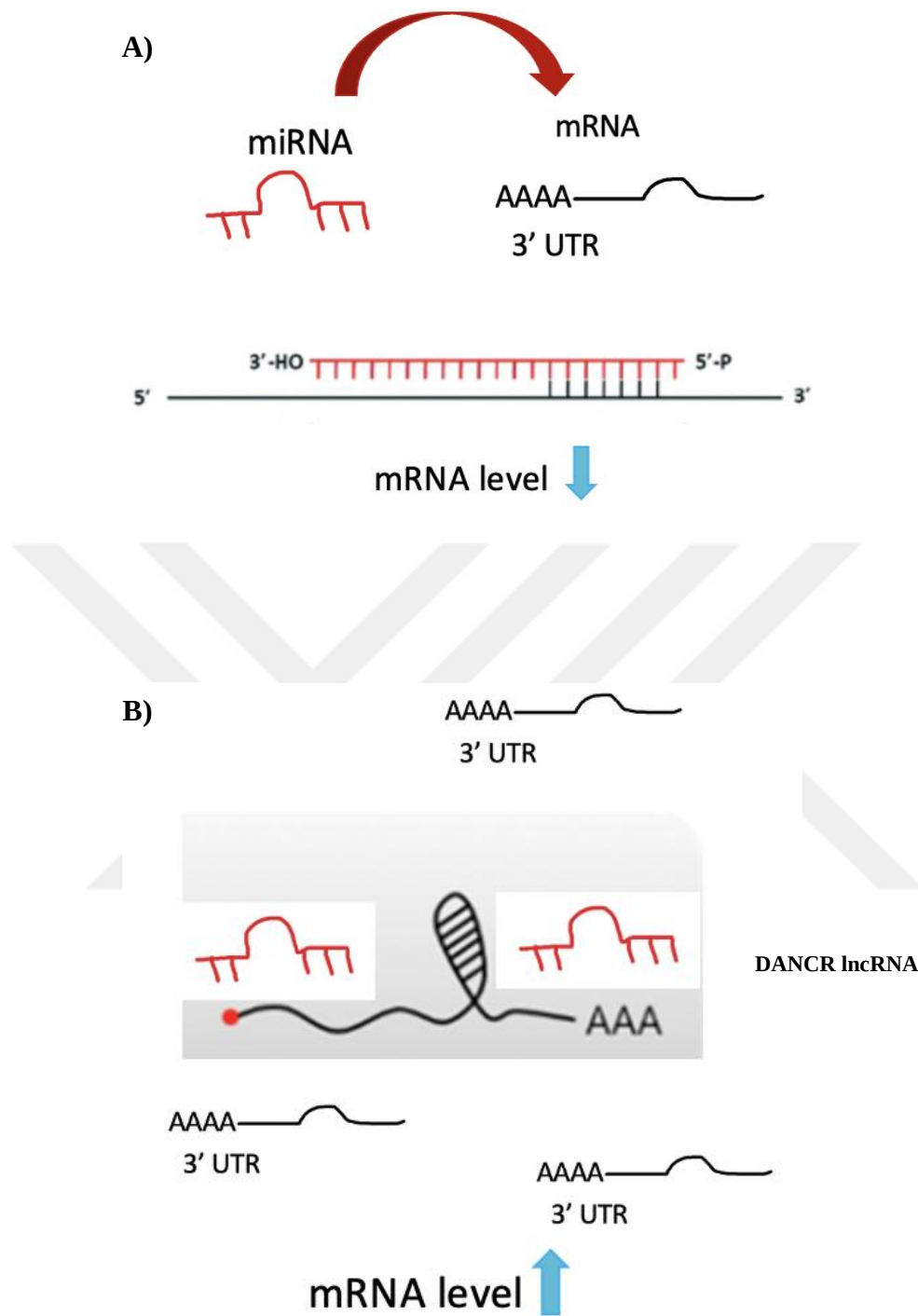


Figure 25: Representative image of ceRNA function of DANCER. **A)** MiRNAs bind to microRNA response element (MRE) on mRNAs, and they cause mRNA degradation. **B)** DANCER can compete with miRNAs and prevent inhibitory action of miRNAs on mRNA targets.

Firstly, total RNA was extracted from WT, KO and OE cells that are either treated with 1 μ M DOX for 16 h or left untreated and RNA sequencing was performed for poly-A tail enriched RNA from all samples. RNA seq data from DANCER KO/WT and DANCER OE/WT groups were filtered as absolute fold change value > 2 and FDR p-value $\leq$ 0,05. According to these DEGs, in the absence of DANCER, 934 genes were upregulated while 819 genes were downregulated (**Figure 26**). When DANCER was overexpressed, only 21 genes were upregulated while 19 genes were downregulated. Also, when WT and OE cells were separately treated with DOX and compared to their untreated control groups, 2259 DEGs were upregulated and 1626 DEGs were downregulated in WT treated/untreated comparison, whereas, 2237 DEGs were upregulated and 1470 DEGs were downregulated in OE treated/untreated comparison.

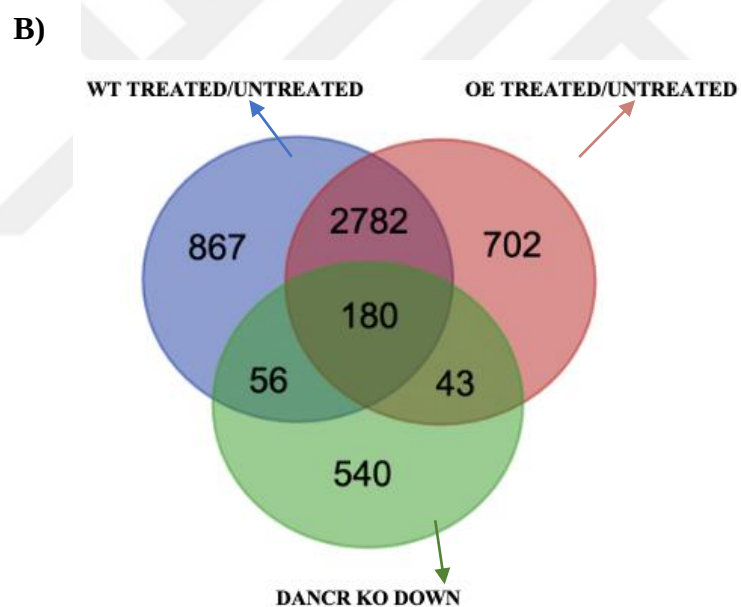
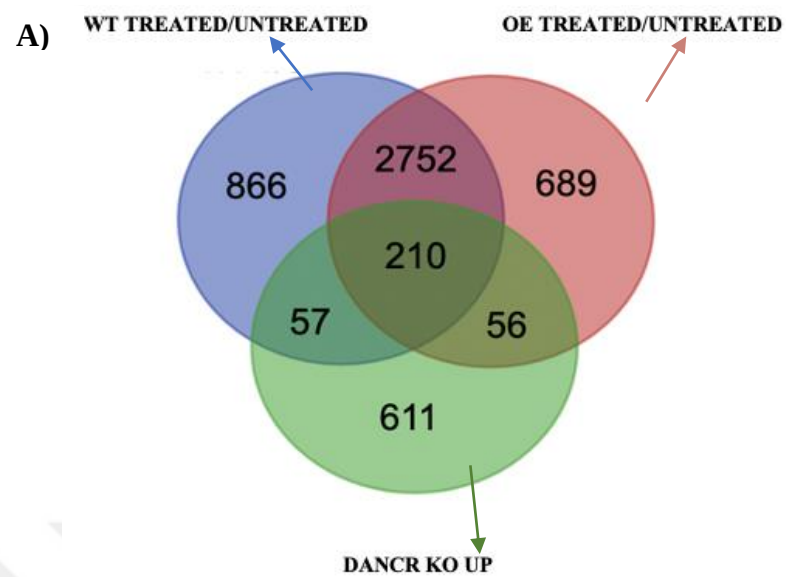


Figure 26: Venn diagrams for common DEGs of DOX treated WT and OE cells and **A)** upregulated DEGs of untreated DANCER KO and **B)** downregulated DEGs of untreated DANCER KO.

Table 18: The list of DANCER 201 transcript targeted 26 miRNAs obtained by miRDB database (83).

miRNAs Names	Target Score	Sequence	Seed Sequence	Length	Seed Location
hsa-miR-4715-5p	89	5' - aaguuggcugcaguuaa ggugg - 3'	gcc aacta	22	338
hsa-miR-4504	88	5' - ugugacaauagagauga acaug - 3'	ttgtc aca	22	516
hsa-miR-5194	84	5' - ugagggguuuggaaug ggaugg - 3'	accctca	22	835
hsa-miR-338-5p	83	5' - aacaauauccuggugcu gagug - 3'	atat tgta	22	757
hsa-miR-193b-5p	82	5' - cggggguuuugagggcg agauga - 3'	aaccca aacc cca aaacccc	22	194 807 833
hsa-miR-656-3p	82	5' - aaauuuuacagucaac cucu - 3'	ataatata	21	752
hsa-miR-634	79	5' - aaccagcaccccaacu ggac - 3'	tgctgg ta tgctggt	22	315 452
hsa-miR-7111-5p	79	5' - ugggggaggaaggacag gccau - 3'	ctcccca	22	822
hsa-miR-6870-5p	79	5' - ugggggagauugggggu uga - 3'	ctcccca	19	822
hsa-miR-5698	79	5' - ugggggagugcaguga uugugg - 3'	ctcccca	22	822
hsa-miR-4723-5p	79	5' - ugggggagccaugagau aagagca - 3'	ctcccca	24	822
hsa-miR-3646	76	5' - aaaauaaaugagccca gccca - 3'	ttca ttt	22	507
hsa-miR-1972	75	5' - ucaggccaggcacagug gcuca - 3'	tggc ctga	22	907
hsa-miR-6738-5p	71	5' - cgagggguagaagagca cagggg - 3'	accctca	23	835
hsa-miR-1914-3p	71	5' - ggagggguccgcacug ggagg - 3'	accctca	22	835
hsa-miR-3124-3p	66	5' - acuuuccucacuccgu gaagu - 3'	aggaaag	22	362
hsa-miR-187-5p	65	5' - ggcuacaacacaggacc cgggc - 3'	ttgtagca tgtagca	22	401 472
hsa-miR-629-5p	64	5' - uggguuuacguuggga gaacu - 3'	taaacc	21	832
hsa-miR-6866-5p	62	5' - uuagaggcuggaauaga	g cctctaa	23	370

		gauucu - 3'				
hsa-miR-550a-5p	61	5'	-	caggcaca	23	282
		agugccugagggaguaa				
hsa-miR-1271-3p	61	5'	-	caggcaca	22	282
		agugccugcuaugugcc				
hsa-miR-1539	56	5'	-	gcgcag g	21	265
		uccugcgcguccagau				
hsa-miR-216a-5p	53	5'	-	tgagatta	22	774
		uaaucucagcuggcaac				
hsa-miR-4480	52	5'	-	acttggc	21	904
		agccaaguggaaguuac				
hsa-miR-6816-5p	52	5'	-	ccgcccc	21	62
		uggggcggggcaggucc		cc gcccc		569
hsa-miR-27b-5p	50	5'	-	taa gctca	22	848
		agagcuuagcugauugg				
		ugaac - 3'				

Secondly, DANCER-201 targeted miRNAs were analyzed by using miRDB database, and 26 miRNAs were found (**Table 18**). This result shows that which location on DANCER binds to miRNAs. To examine DANCER function, secondary structure of DANCER RNA folding was created. Seed locations of miRNAs were aligned to this structure by using CLC Genomics and CLC Main Workbench softwares (**Figure 27**). These miRNAs bound different structural subdomains of DANCER such as hairpin loop, multiloop, interior loop, and bulge secondary structures. Hsa-miR-7111-5p, hsa-miR-5698, hsa-miR-4723-5p, and hsa-miR-6870-5p were located on multiloop structure. Hsa-miR-193b-5p, hsa-miR-629-5p, hsa-miR-6738-5p, hsa-miR-5194 and hsa-miR-1914-3p were accommodated on bulge structure. Also, several miRNAs had shared the same or overlapping seeding sequences hence they aligned very close proximities on the secondary structure of DANCER. Moreover, several miRNAs bound more than one region. For example, hsa-miR-193b-5p bound to both a bulge and an interior loop of secondary structure of DANCER. Moreover, the 3D structure of DANCER was visualized in VMD by using a PDB file which was created by HNADOCK Server (86) (**Figure 28**). This tertiary structure may later give clues for a possible catalytic activity for DANCER or a DANCER-Protein physical interaction.

After that, 26 candidate miRNAs were then further investigated to find their possible mRNA targets. Selected miRNAs were analyzed via microT-CDS software of the DIANA tool for target genes with a 0.7 threshold setting (Data not shown). (84) (85). These were cross-checked with the DEGs identified in our RNA-Seq analysis and the common genes between the miRNA-targeted mRNAs and DANCER regulated mRNAs were filtered out and listed (**Table 19**).

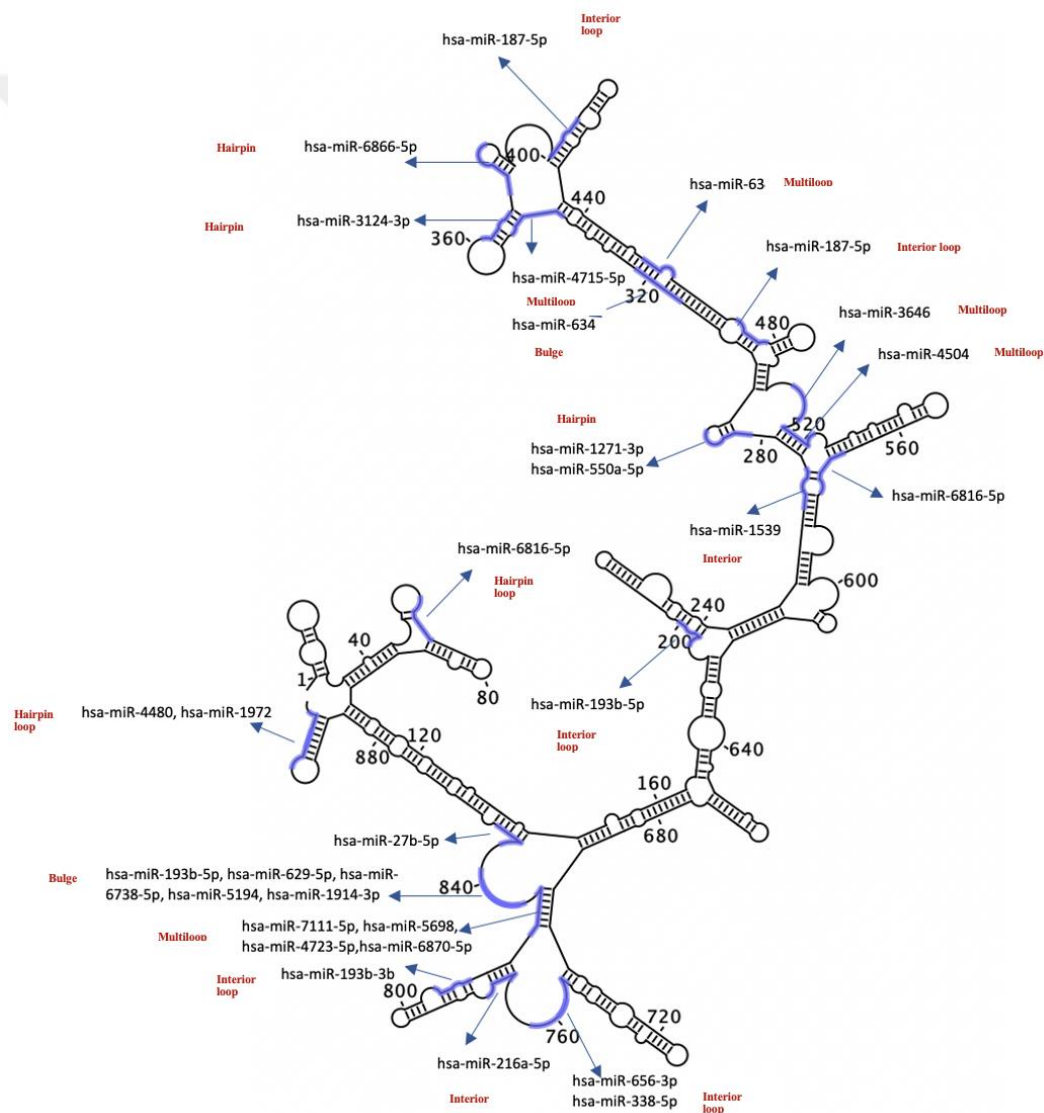


Figure 27: MiRNAs were aligned according to their seed sequences and locations on the secondary structure of the DANCR 201 by CLC Genomics and CLC Main Workbenches.



Figure 28: The visualization of the 3D structure of DANCR in VMD. The PDB file was created from HNADOCK Server (85). Red:A, green:C, pink: G, blue: U.

Table 19: The list of common genes between the miRNA-targeted mRNAs identified in silico and DEGs identified in DANCR KO/WT RNA Seq comparison.

miRNAs	UP-REGULATED GENES	DOWN-REGULATED GENES
hsa-miR-4715-5p	CSRNP3,FUNDC2,HEY2,IFI44L,KCNB1,MUC19,NCAM1,PIK3R3SCN3A,SCN3B,TCN2,UFM1,WDR63	ARL5B,C2,CBX5,KCNQ3,MTAP,N4BP2,NFIA,PCDH7,SLC5A5,THSD4,VSIG10,WWC2
hsa-miR-4504	AHR,COL3A1,COLGALT2,CSRNP3,GLIS3,GREM2,HIF1A,IFI44L,ITGB8,JUN,KIAA0408,LBH,MMP24,NAV3,NHLRC3,PARP12,PDE4D,PIK3R1,SCN2A,SPTLC3,STC1,SUCNR1,SULF2,SYTL5	AREG,CBX5,CHPT1,CKAP2L,DCBLD2,EFNB2,FSBP,HLF,LIN7A,MOB3B,MTSS1,MYZAP,NOS1AP,PALLD,PDZD2,RASA3,RBM46,SBSN,TEX19,SYNPO2,TUBGCP3,ZFP36L1,MFHAS1
hsa-miR-5194	APOBEC3C,ARL4D,CCL5,CFB,CHMP1B,CPLX2,FGF14,KRT13,MAP1B,MSTN,SYP,PLEKHS1,PPP1R1A,PRAF2	C2,CBX5,CKAP2L,DDIT4,DPF3,FOXP2,KCNQ3,LIN7A,NLRP4,PRDM1,RNASE1,SCN4A,SLC44A2,WNK1,SPECC1L-ADORA2A,SYNPO2,SYPL2,ZFP36L1
hsa-miR-338-5p	ABCA1,ACTBL2,ADAM28,ADM,AHR,AMOTL2,DSC3,ANKRD1,ANKRD26,CDK6,ATP6V0A4,B3GALT1,ETS2,CREB5,CSRNP3,EIF2AK2,ERAP2,EYA4,FMN2,FRMD3,FSTL5,GLI3,GPR137C,HIF1A,ITGA1,LONRF2,LRMP,LRP1B,MAGED2,MAN1A1,MAP2,MSTN,NFIB,NR4A3,NRP1,PCDH19,PDE4D,PDGFB,RGS4,PTCH1,RGS7,RIMKLB,RND3,RUNX2,SCML1,SCN2A,SCN9A,SLC25A5	ARHGAP21,ARHGEF7,CA2,CACNA2D1,CAMK1D,CAMK2D,CREG2,CYP24A1,DAAM2,DCBLD2,DLGAP1,EBF3,EEA1,FBXO36,FGF2,FKBP5,FOXP2,FRMD4B,GATA3,HNF4G,HS3ST1,HSPA4L,IPO5,ITCH,KLF10,LDLRAD4,LIN7A,LTBP1,MARK1,MCM10,MOB3B,MSRB3,MTAP,MTSS1,NFIA,NPY1R,NR5A2,NRIP1,NUDT5,PALLD,PCDH7,PPARG,PRKG1,PTPRZ1,PYGO1,RAD21,RBM46,RPP30,RTKN2,RUNDC3B,SEMA6A,

	3,SOX6,STC1,UFM1,SPOCK1,TCF4,TENM3,ULBP2,TNFSF15,VEZF1,YY2,ZNF396	SGIP1,SLC1A2,SLC40A1,SLC4A7,TAF3,TIMP3,TLL1,UTRN,UTS2B,WWC2,ZNF488,ZNF615
hsa-miR-193b-5p	B3GALT1,CACNB4,CATSPER1,CCND1,CD22,DDR2,DRAXIN,LOXL4,MAGIX,OAS2,SLC25A53,SP100,CTA,TEAD2,TRANK1,XAF1	DNAH2,ANKRD13A,CKAP2L,DACT3,DDX25,ETV1,KIAA1257,LIN7A,MEIOB,N4BP2,PGBD5,RFC5,NKX61,PTAFR,RORC,ZFP36L1
hsa-miR-656-3p	SEMA3A,ACTBL2,AHR,AMOTL2,ANKRD26,B3GALT1,C18orf32,CACNB4,CCDC148,CCDC89,CDK6,CMMPK2,CREB5,CSRNP3,DSC2,DSC3,DUSP10,EIF2AK2,ERAP1,ETS2,EYA4,FGF14,FKBP7,FRMD3,FSTL5,GIP2,GPRASP1,GRIK2,HEPH,HPGD,IFI44,ITGA1,ITGB8,KIAA0408,KMO,LINC01118,LONRF2,LRP1B,MAMDC2,MAML2,MAN1A1,MAP2,MAP4K5,MSTN,NFIB,NR4A2,NR4A3,OLFML2B,PCDH19,PDGFB,PIK3R1,PLSCR1,PTCH1,PTCHD4,PTPN22,RGS4,RIMKLB,RUNX2,SAMD4A,SCN2A,SCN3A,SCN9A,SERPINE1,SLCO1B3,SOX4,SOX6,STAT1,SUCNR1,TBX5,TCF4,TLR3,TLR4,TNC,TNFSF11,TNFSF15,TNFSF18,TNFSF4,TRIM38,UFM1,WNT5A,WNT7A,ZFP30,ZNF804A	ACVR1C,ALDH1L2,AP1AR,ARHGAP26,ARL5B,ATG12,BEND7,BMP2,C8orf37,CA8,CACNA2D1,CACNB2,CCBE1,CD73,CHPT1,CKAP2L,CPED1,CTH,DLGAP1,DPP4,DYPD,EBF3,EEA1,EFNB2,ERI1,ETV1,FAM107B,FBXO36,FOXP2,GATA3,GNAI1,GNNG2,GPR37,GPSM2,GREB1,HNF4G,ITCH,KCNQ3,KIF14,KIF1A,KIN,KLF10,KLF4,LIN7A,LIN9,LMO7,MAP2K6,MFHAS1,MLLT10,MTAP,N4BP2,NEBL,NPR3,NPY1R,NRIP1,PAG1,PALLD,PCDH7,PER2,PPARG,PRDM5,PRKG1,PRR11,PRUNE2,PTER,PYGO1,RACGAP1,RAD21,RAP2A,RBM17,RFC5,RGS2,RUNDC3B,SGIP1,SHH,SLC1A2,SLC4A7,SMC4,SPATA18,ST8SIA6,STK32A,SUV39H2,SYNPO2,THRB,TIMP3,TRDMT1,UBE2E2,USP6NL,WNNT16,WWC2,ZBTB8B,ZNF615,ZNF850
hsa-miR-634	ATOX1,CCND1,COL3A1,COLGALT2,EYA4,FUNDC2,HSEF1,IFI27L1,IFIT2,ITGB8,KCNB1,MACROD2,NAV3,NFIB,NRP1,OLFML2B,PIK3R1,PPP1R1A,RBKS,STX16,NPEPL1,TCN2,THBS1,TM7SF2,VASN	AKAP12,CBX5,CCNF,CYP24A1,DPF3,EEA1,HRK,IGF2BP3,KSR2,MTAP,NFIA,PALLD,PBX1,PKDCC,PTDSS1,RBM46,SKIDA1,TLL1TRPC3,ZBTB8B
hsa-miR-7111-5p	ABCD1,CD7,CLDN2,CLSTN2,CPLX2,DCLK1,DLG4,DUSP10,EYA1,FBLIM1,FKBP10,FN3K,FUNDC2,GLI3,HOXB4,KRT23,LINC01124,MMMP24,NAT16,PARP9,PCDH19,PCDHGA9,PDE4D,PLEKHB1,RERG,SLC25A45,SOX4,STAT2,STC1,TUBAL3	BHLHE41,BMP6,CAMK1D,CBX5,CCBE1,CGREF1,CNBD2,DAAM2,EFHD1,FAM50B,GAL3ST4,GRHL3,HR,HRK,KIF1A,KSR2,LAMC3LIN7A,MTAP,MYRF,NEBL,PAG1,PRKCA,PROM2,SLC25A42,SLC25A48,SYP L2,TGFBR3,WNK4,ZFP36L1
hsa-miR-6870-5p	ABCD1,ANKRD26,CCDC103,CD7,CLDN2,CLSTN2,COL9A2,CPLX2,DCLK1,DLG4,DUSP10,FKBP10,FN3K,FUNDC2,HOXB4,HOXC11,KLK7,LINC01124,MMP24,MYL2,NAT16,OAS3,PADI1,PARP9,PCDH19,PCDHGA9,PDE4D,PLEKHB1,PTPN22,RERG,ROBO4,RYR2,SCN3B,SLC25A45,SOX4,SPARC,SPHK2,SPTLC3,STAT2,TEAD2,TUBAL3	APOD,AVPR2,BAIAP2L2,BHLHE41,BMP6,CAMK1D,CASP10,CBX5,CCBE1,CGREF1,CNBD2,DAAM2,FAM50B,FZD8,GRAMD1B,GRHL3,HR,HRK,HS3ST1,HS6ST3,KIF1A,KIN,KSR2,LAMC3,LARP4B,LIN7A,MTAP,MYRF,NEBL,PAG1,PBX1,PRKCA,PROM2,SLC25A42,SLC25A48,SULT1A4,SYPL2,THSD4,T RIM29,WNK1,WNK4,ZFP36L1
hsa-miR-5698	ABCD1,CD7,CLDN2,CLSTN2,CPLX2,DCLK1,DLG4,DUSP10,FAT2,FBLIM1,FN3K,FUNDC2,GLI3,HOXB4,KCNF1,KLK10,LINC01124,MMMP24,NAT16,PARP9,PCDH19,PCDHGA9,PDE4D,RERG,SLC25A45,STAT2,STC1,TUBAL3	BHLHE41,BMP6,C8orf37,CAMK1D,CBX5,CCBE1,CGREF1,CNBD2,DNAH2,GAL3ST4,GRHL3,HNF4A,HR,HRK,KIF1A,KSR2,LAMC3,LARP4B,LIN7A,MAP2K6,METTL7A,MTAP,MYRF,NEBL,P2RX7,PAG1,PRKCA,PROM2,SLC25A42,SLC25A48,SYPL2,TGFBR3,WNK4,ZFP36L1
hsa-miR-4723-5p	ABCD1,CD7,CLDN2,CLSTN2,CPLX2,DCLK1,DLG4,FN3K,FUNDC2,HOXB4,LINC01124,MMP24,NAT16,PADI1,PARP9,PCDH19,PCDHGA	BHLHE41,BMP6,CAMK1D,CBX5,CCBE1,CGREF1,CNBD2,EFHD1,FAM50B,GRHL3,HNF4A,HR,HRK,KIF1A,KSR2,LAMC3,MYRF,NEBL,PAG1,PRKCA,P

	9,PDE4D,PDGFB,PLEKHB1,RERG,SLC25A45,STAT2,STC1,TEAD2,TUBAL3	ROM2,SLC25A42,SLC25A48,SYPL2,TGFR3,WNK4,ZFP36L1
hsa-miR-3646	SEMA3A,AKT3,ANKRD26,C18orf32,C2orf15,CDKN1A,CREB5,DDR2,EPSTI1,ETS2,EYA1,FUNDC2,MAML2,MAP1B,MAP2,MTUS2,NAALADL2,NWD1,PIK3R3,RGS7,SAMD4A,SCN2A,STAMBPL1,SVEP1,TLR4,TNFSF15,ZFP30,ZNF525	ARHGAP26,BCAS1,BIVM,BUB1,CA8,CAMK1D,CAMK2D,CERKL,CRY1,CTH,DCBLD2,EMP2,ETV1,FAT4,FBN1,FKBP5,FOXP2,GNAI1,GNG2,HRK,HSPA4L,ITCH,KIN,KSR2,LIN28B,NAV2,NET1,NPR3,NR5A2,PBX1,PCDH7,PGBD5,PRDM5,PROSER2,RPP30,SNTG2,SRR,STXBP5L,THRB,TIMP3,TMEM150C,TPP2,TRABD2A,UTRN,ZNF493
hsa-miR-1972	ADAMTS14,CDKN1A,CPA4,CUEDC1,FGF14,FGF9,HEPH,LGALS3BP,MAP2,PLEKHG4B,PTPRR,ROBO4,SCN3B,SLCO1B3,SYP,TMEM198,TWIST1	C6orf141,CACNB2,CASP10,CBX5,CCNF,CREB3L3,ESPL1,FXND4,HAS3,HOGA1,KIAA1549L,KIF18B,LRR32,MYBPC3,NPNT,PCDH7,PCED1B,PLEKHA6,PRKACB,PRKCA,PROM2RNF157,RSP04,SCN4A,SLC12A5,TEX19,TLL1,TPX2
hsa-miR-6738-5p	ARL4D,CHMP1B,EDA2R,PLEKHS1,SYP	C2,DPF3,FOXP2,KCNQ3,NLRP4,SPEC1L-ADORA2A,WNK1
hsa-miR-1914-3p	APOBEC3C,ARL4D,CEND1,CHMP1B,IFI44L,KRT80,LGALS3BP,MAP1B,NAGK,PLEKHG4B,PLEKHS1,RIMKLB,SDK1,SYP,TGFB1,THTPA	BEND7,C2,DPF3,DUOX2,ESPL1,FOXP2,HR,KIAA1217,KSR2,LIN7A,NLRP4,PTK2B,RNASE1,SCN4A,SPECC1L-ADORA2A,TIMM17B,WNK2
hsa-miR-3124-3p	DDR2,HLA-F,HOXC4,IL7R,NR4A3,SAMD4A,ZFP30	AGPAT4,CA8,CBX5,CHAMP1,FBN1,FOX2,KCNJ1,KIN,KSR2,NMUR1,NOS1AP,SLC44A2,SYPL2,TTC28,UTRN
hsa-miR-187-5p	ABCA1,CLSTN2,CUEDC1,DCLK1,FGF14,KRT23,KRT81,MEX3B,NFIB,RGS4,RGS7,RUNX2,SETD9	APOLD1,CBX5,CRY1,EBF3,KDRMAR1,PTPRZ1,TTC28
hsa-miR-629-5p	AKT3,BTN3A3,MAML2,NAALADL2	ARHGAP26,KIAA1257,KSR2,MYH11,PRDM16,SRR,ZFP36L1
hsa-miR-6866-5p	AKT3,AL627309.1,COL13A1,CSF1,FUNDC2,GALNT5,ITGB8,KLK10,NEB,NAALADL2,NFIB,RUNX2,STC1,SPAN1	ARHGAP26,CCNA2,CDKN2C,CHPT1,DHTKD1,FAM27E3,FAM90A1,KIN,KSR2,NFIA,P4HA3,PER2,RTTN,SLC1A2,SLC4A7,UBE2E2
hsa-miR-550a-5p	CLSTN2,CSRNP3,DRAXIN,EHF,FAT2,FUNDC2,MAN1A1,NCAM1,NWD1,PSME1,PTCH1,SOX4,TNFSF4,TRIM22,VWA5A	ARHGAP26,CCDC3,CSGALNACT1,DNAH2,GJC1,GPSM2,HOGA1,HSPA12B,IBTK,KCNMB4,LARP4B,PAG1,SAMD5,SKIDA1,ZBTB8B
hsa-miR-1271-3p	CLSTN2,CSRNP3,DRAXIN,FAT2,FUNDC2,KLK10,MAN1A1,MUC19,NCAM1,NWD1,PSME1,SOX4,TNFSF4	ARHGAP26,CCDC3,CHPT1,CSGALNACT1,DNAH2,GJC1,GPSM2,HOGA1,HSPA12B,INHBE,KCNMB4,LARP4B,MKI67,PAG1,SAMD5,SKIDA1,TRPC3,ZBTB8B
hsa-miR-1539	-	-
hsa-miR-216a-5p	AFAP1L2,BATF2,CHMP1B,CSRNP3,DCLK1,DDR2,DSC3,EIF2AK2,FKBP7,GPR137C,KCNB1,KCNE2,KRT39,PLSCR1,RBKS,SERPINB8,SH3BGL,STAMBPL1,TRIM38,WDR63	ALDH1L2,ARHGAP26,CAMK1D,CBX5,CCDC65,CDC73,CLGN,CREG2,CTH,FOXP2,GPR19,HNF4G,IDI1,IGF2BP3,KIF15,LIN28B,LIN9,MCM4,N4BP2,NCAPG,NEBL,NEK2,NOS1AP,NPR3,NR5A2,NSUN6,PRDM16,RBM46SLC16A7,SLC1A2,TRDMT1
hsa-miR-4480	ANKRD26,CACNB4,CSRNP3,EYA1,FOXA2,FUNDC2,SLC30A10	AP1AR,ARHGAP26,C6orf141,CBX5,DRP2,FAM47E,GRAMD1B,KSR2,NEBL,PAG1,PPARG,PRDM5,PYGO1,SAMD5,TFDP1,TTC28,ZBTB8B

hsa-miR-6816-5p	ATOH8, GLI3, KRT17, NAT16, NUPR1, TGFB111	AK7, C2, CPLX1, NR5A1, SPECC1L-ADORA2A
hsa-miR-27b-5p	FOSB, BDH2, CREB5, CSRN3, IFIT5, NAALADL2, PCDH19, SLC30A10, S	ACVR1C, DNAH2, KIF14, PER2, TGFB3, THRB, TRABD2A
	TC1	

In order to point out biological pathways in which common genes between DANCER regulated miRNAs found in RNAseq and miRNA-target genes found by in silico analysis, the gene set analysis mode of ConsensusPathDB (CPDB) was utilized with a 0.01 p-value cutoff (87) (88). The number of DEGs in DANCER OE/WT RNAseq comparison was low, therefore, pathway analysis was not performed in this group. Instead, the number of common genes between up-regulated DEGs in DANCER KO/WT comparison and miRNA target genes was 296. These were related with different pathways including cytokine signaling in immune system, extracellular matrix organization, TGF-beta signaling, Notch signaling, JAK-STAT signaling, osteoclast differentiation, keratinization, EMT in colorectal cancer, adipogenesis, DNA Damage Response, ESC pluripotency pathways, collagen formation, senescence and autophagy in cancer, cellular senescence, and differentiation pathway (**Table 20**).

The number of common genes between down-regulated DEGs in DANCER KO/WT comparison and miRNA target genes was 322. These were associated with circadian entrainment, melanogenesis, proteoglycans in cancer, Aurora B signaling, differentiation of white and brown adipocyte, platelet homeostasis, transcriptional regulation of pluripotent stem cells, aldosterone synthesis and secretion, regulation of sister chromatid separation at the metaphase-anaphase transition, transport of small molecules, angiogenesis, integration of energy metabolism, G0 and Early G1 and mitotic G1-G1/S phases and hedgehog signaling events mediated by Gli proteins (**Table 21**).

When DANCER is overexpressed, not many genes were altered in their expression levels, however, there was a prompt difference of OE cells in response to DOX treatment compared to that of WT. Therefore, to examine DOX response of DANCER OE and WT cells and the effect of DANCER bound miRNAs on these responses, common DEGs between each comparison and miRNA target genes were found (data not shown) and the numbers were overlapped with each comparisons. As seen in **Figure 29**, 876 DEGs were found in DOX treated WT group, and 1385 DEGs were found in DOX treated DANCER OE group. More importantly, there are common DEGs shared between upregulated or downregulated DEGs in DANCER KO/WT comparison.



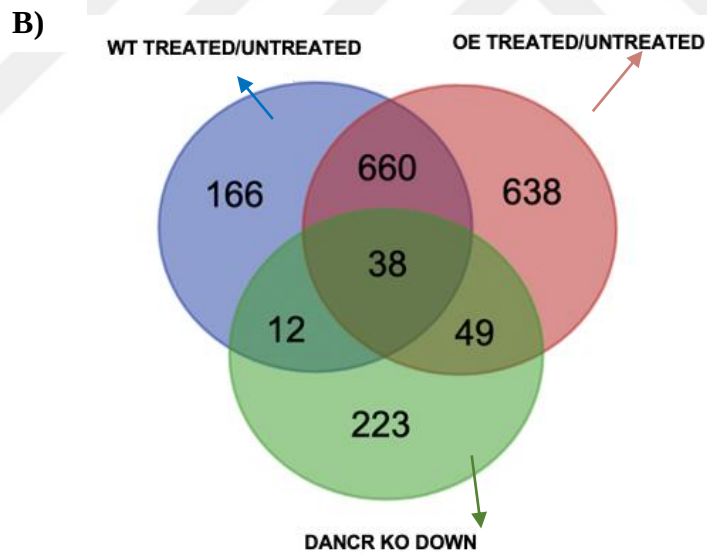
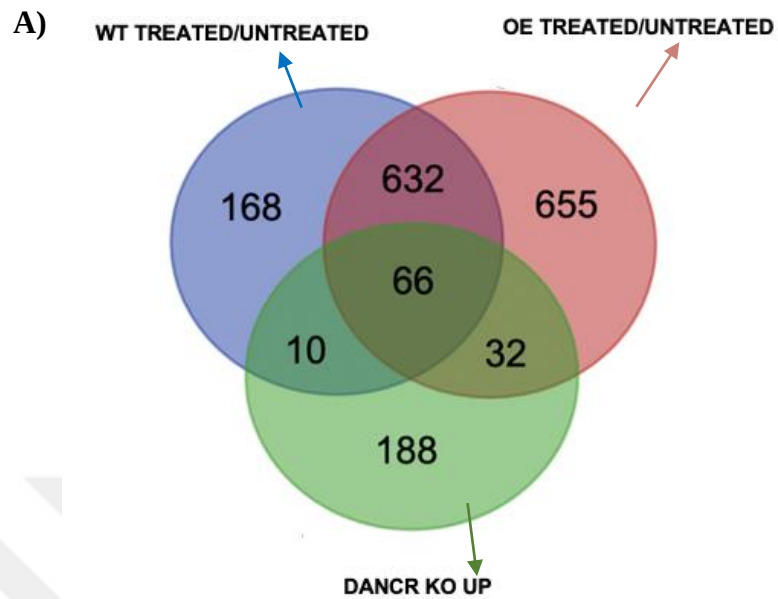


Figure 29: The Venn diagram of DEGs that are miRNA targets and identified in RNA seq of DOX treated WT and OE, and untreated DANCER KO cells.

Table 20: Pathway analysis of common genes between *up*-regulated DEGs in DANCRCO/WT comparison and miRNA target genes by using CPDB.

PATHWAYS	SET SIZE	CANDIDATE CONTAINED	P-VALUE	GENES
Cytokine Signaling in Immune system	458	31	6.01e-12	LIF,HLAF,TNFSF11,SP100,OAS3,CSF1,TRIM22,PIK3R3,HERC5,IFI6,IFIT2,STAT1,OASL,GBP3,NCAM1,JUN,IFI35,MX2,CTF1,STAT2,TRIM38,TNFSF15,EIF2AK2,XAF1,PIK3R1,EDA2R,TNFSF13,TNFSF4,IL7R,TNFSF18,OAS2
Interferon Signaling	158	18	5.38e-11	STAT2,TRIM38,HLAF,IFIT2,XAF,STAT1,IFI35,MX2,OASL,SP100,OAS3,GBP3,TRIM22,EIF2AK2,HERC5,IFI6,NCAM1,OAS2
Extracellular matrix organization	294	19	2.02e-07	COL9A3,LOXL4,ITGA1,THBS1,SERPINE1,PDGFB,COL3A1,COL9A2,ITGB8,TNC,COL13A1,MMP24,SPARC,COLGALT2,DDR2,HSPG2,ADAMTS14,NCAM1,KLK7
Focal adhesion	199	14	3.22e-06	MYL2,PDGFB,CCND1,ITGA1,ITGB8,JUN,PIK3R1,AKT3,COL9A2,COL9A3,RASGRF1,TNC,PIK3R3,THBS1
Integrin cell surface interactions	67	8	9.84e-06	THBS1,ITGB8,COL13A1,HSPG2,ITGA1,COL9A3,TNC,COL9A2
PI3K-Akt Signaling Pathway	340	21	1.53e-05	THBS1,CCND1,FGF14,FGF9,PDGFB,TNC,ITGA1,IL7R,CSF1,CDKN1A,CHRM1,PIK3R3,ITGB8,TLR4,CDK6,AKT3,PIK3R1,G6PC3,COL9A2,COL9A3,CREB5
Proteoglycans in cancer	201	13	1.8e-05	CDKN1A,HIF1A,CCND1,PTCH1,AKT3,TWIST1,PIK3R1,WNT5A,HSPG2,WNT7A,PIK3R3,THBS1,TLR4
Transcriptional regulation by RUNX2	75	8	2.28e-05	GLI3,RUNX2,AKT3,TWIST1,CDKN1A,STAT1,HEY2,CCND1
Vitamin D Receptor Pathway	184	12	3.5e-05	COL13A1,CLDN2,ABCD1,CCND1,TNFSF11,S100A2,CDKN1A,HIF1A,DUSP10,TNFSF4,MX2,KRT13
TGF-beta Signaling Pathway	132	10	4.5e-05	THBS1,TGFB1I1,CCND1,STAMBP1,JUN,CDKN1A,RUNX2,TNC,FOSB,PIK3R1
Notch Signaling Pathway	61	7	4.66e-05	CCND1,MAML2,PIK3R1,HEY2,CDKN1A,HIF1A,NOTCH4
JAK-STAT signaling pathway	162	11	5.16e-05	PDGFB,CCND1,CDKN1A,STAT1,STAT2,PIK3R1,AKT3,PIK3R3,LIF,CTF1,IL7R
Phase 0- rapid depolarisation	44	6	6.14e-05	FGF14,SCN9A,CACNB4,SCN2A,SCN3B,SCN3A
Osteoclast differentiation	128	9	0.000181	STAT1,STAT2,JUN,PIK3R1,AKT3,FOSB,PIK3R3,TNFSF11,CSF1
Keratinization	129	9	0.000203	KRT80,KRT81,DSC3,KRT17,KRT23,KRT39,DSC2,KRT13,KLK5
Epithelial to mesenchymal transition in colorectal cancer	160	10	0.000224	CLDN2,WNT5A,SPARC,HIF1A,PIK3R3,TWIST1,PIK3R1,AKT3,WNT7A,NOTCH4

Adipogenesis	131	9	0.000228	STAT1,STAT2,HIF1A,TWIST1,AHR,CDKN1A,SPOCK1,SERPINE1,LIF
DNA Damage Response (only ATM dependent)	110	8	0.000347	CCND1,PIK3R3,JUN,CDKN1A,AKT3,PIK3R1,WNT5A,WNT7A
Endochondral Ossification	63	6	0.000461	GLI3,DDR2,PTCH1,SOX6,RUNX2,STAT1
ESC Pluripotency Pathways	116	8	0.000496	FGF14,FGF9,PDGFB,JUN,AKT3,LIF,WNT5A,WNT7A
IL-7 Signaling Pathway	25	4	0.000587	CCND1, PIK3R1, IL7R, STAT1
Collagen formation	92	7	0.00062	COL9A3,LOXL4,COL3A1,COL9A2,COL13A1,COLGALT2,ADAMTS14
Senescence and Autophagy in Cancer	106	7	0.00143	THBS1,SPARC,TNFSF15,JUN,CDKN1A,SERPINE1,COL3A1
Cellular senescence	160	8	0.00387	CDKN1A,HLA-F, CCND1,PIK3R1,AKT3,PIK3R3,SERPINE1,CDK6
Differentiation Pathway	48	4	0.00681	TNFSF11,PDGFB,WNT5A,CSF1
Cell cycle: g1/s check point	25	3	0.00689	CCND1,CDK6,CDKN1A

Table 21: Pathway analysis of common genes between *down*-regulated DEGs in DANCRCO/WT comparison and miRNA target genes by using CPDB.

PATHWAYS	SET SIZE	CANDIDATE CONTAINED	P-VALUE	GENES
Circadian entrainment	96	11	7.63e-07	GNG2,PRKACB,PER2,KCNJ,CACNA1G,GNG,GNAI1,CAMK2D,PRKC,GNG3,PRKACB
Melanogenesis	101	9	6.05e-05	WNT4,WNT16,CREB3L,GNAI1,FZD8,CAMK2D,WNT8B,PRKACB, PRKCA
Proteoglycans in cancer	201	12	0.000191	WNT4,HPSE2,FZD8,WNT16,FGF2,KDR,SHH,PRKACB,CAMK2D,WNT8B,TIMP3,PRKCA
Oxytocin signaling pathway	152	10	0.000302	KCNJ5,GNAI1,CACNA2D1,CACNB2,CAMK1D,CAMK2D,RGS2,MYLK2,PRKACB,PRKCA
Factors involved in megakaryocyte development and platelet production	131	9	0.000439	PRKACB,GATA3,KIF1A,DOCK9,CBX5,KIF15,KIF13B,RACGAP1,KIF18B
Aurora B signaling	41	5	0.000658	BUB1,SMC4,CBX5,NCAPG,RACGAP1
Differentiation of white and	25	4	0.000818	BMP2,PRDM16,PPARG,EB

brown adipocyte				F3
Platelet homeostasis	89	7	0.000857	GNG2,KCNMB4,PRKG1,TRPC3,P2RX7,ATP2A1,GNG4
Transcriptional regulation of pluripotent stem cells	28	4	0.00127	POU6F1,KLF4,PBX1,FGF2
Aldosterone synthesis and secretion	96	7	0.00134	KCNJ5,CACNA1G,CREB3L3,CAMK1D,CAMK2D,PRKACB,PRKCA
Regulation of sister chromatid separation at the metaphase-anaphase transition	15	3	0.00197	BUB1, RAD21, ESPL1
Transport of small molecules	666	22	0.00253	WNK2,FXYP4,GNG4,CA2,SLC16A7,SLC40A1,PRKACB,AVPR2,CREB3L3,TFR2,SLC12A5,WNK4,WNK1,APOD,CAMK2D,TRPC3,SLC1A2,ATP2A1,SLC44A2,GNG2,SLC4A7,SLC5A5
Angiogenesis	20	3	0.00464	KDR, TIMP3,FGF2
Integration of energy metabolism	94	6	0.00567	GNG2,PRKACB,PRKCA,GNAI1,CACNB2,GNG4
Signaling by WNT	278	11	0.00845	GNG2,PYGO1,PRKG1,RSP04,PRKCA,WNT16,WNT8B,FZD8,CDC73, GNG4,WNT4
G0 and Early G1	25	3	0.00879	TFDP1,LIN9, CCNA2
Mitotic G1-S phases	104	6	0.00918	MCM10,CCNA2,CDKN2C,TFDP1,LIN9,MCM4
Hedgehog signaling events mediated by Gli proteins	48	4	0.00925	GNG2,GNAI1,SHH,MTSS1

Next, gene ontology (GO) enrichment analysis of common DEGs between DANCER KO/WT comparison and miRNA target genes was done with the PANTHER tool in three GO categories: molecular functions, biological processes, and cellular components (**Table 22-27**) (89).

Molecular functions refer to the processes that are performed by a single molecule or a macromolecular complex via direct physical interactions with other molecules (101). Molecular functions of up-regulated DEGs were found as sodium channel activity, cytokine activity, transcription factor binding, calcium ion

binding, etc. **(Table 22)**. Molecular functions of down-regulated DEGs were found as protein serine/threonine kinase activity, DNA-binding transcription activator activity, and different binding molecules, etc. **(Table 25)**.

Cellular component is defined as the subcellular location where the molecular process occurs, therefore, this term can identify cellular locations of gene products (102). Cellular components of up-regulated DEGs were found as channel complexes, plasma membrane, cytoplasm, ER lumen, ECM, etc. **(Table 23)**. However, down-regulated DEGs were found in only plasma membrane protein complex **(Table 26)**.

Biological process describes biological programs formed by organized multiple molecular processes (102). The biological processes of up-regulated DEGs were related to differentiation, angiogenesis, apoptosis, cornification, cell migration and proliferation, etc. **(Table 24)**. The biological processes of down-regulated DEGs were associated with regulation of ion transport, cell migration and proliferation, cell-matrix adhesion, Wnt signaling, mitotic sister chromatid segregation, etc. **(Table 27)**.

Table 22: GO molecular function of common genes between *up*-regulated DEGs in DANCER KO/WT comparison and miRNA target genes, analyzed via PANTHER.

GO MOLECULAR FUNCTION	SET SIZE	CANDIDATE SET	P-VALUE
Binding	16539	278	2.97E-09
Protein binding	14359	247	6.54E-07
Signaling receptor binding	1700	49	2.38E-06
Transcription factor binding	663	25	9.05E-06
Calcium ion binding	733	26	5.21E-05
Cytokine activity	233	13	5.85E-05
Sodium channel activity	45	6	8.56E-05
2'-5'-oligoadenylate synthetase activity	4	3	9.73E-05

Table 23: GO cellular component of common genes between *up*-regulated DEGs in DANCER KO/WT comparison and miRNA target genes, analyzed via PANTHER.

GO CELLULAR COMPONENT	SET SIZE	CANDIDATE SET	P- VALUE
Cytoplasm	11873	218	6.46E-08
Extracellular matrix	566	27	1.34E-07
Axon	641	28	4.30E-07
Collagen-containing extracellular matrix	424	21	1.98E-06
Sodium channel complex	26	6	5.41E-06
Endoplasmic reticulum lumen	313	17	6.09E-06
Extracellular region	4402	98	6.85E-06
Cation channel complex	227	15	1.06E-05
Extracellular space	3389	79	1.45E-05
Intracellular anatomical structure	14785	246	4.03E-05
Perinuclear region of cytoplasm	733	25	1.15E-04
Voltage-gated sodium channel complex	17	4	2.03E-04
Ion channel complex	307	14	2.34E-04
Cell periphery	5930	116	2.38E-04
Plasma membrane	5813	114	2.80E-04

Table 24: GO biological process of common genes between *up*-regulated DEGs in DANCER KO/WT comparison and miRNA target genes, analyzed via PANTHER.

GO BIOLOGICAL PROCESS	SET SIZE	CANDIDATE SET	P- VALUE
Type I interferon signaling pathway	68	13	1.28E-10
Interferon-gamma-mediated signaling pathway	72	10	2.67E-07
Collagen fibril organization	103	10	5.25E-06
Positive regulation of heart rate	26	6	5.41E-06
Positive regulation of macrophage chemotaxis	17	5	1.25E-05
Negative regulation of epithelial cell proliferation	131	10	3.67E-05
Positive regulation of endothelial cell migration	108	9	4.77E-05
Cornification	113	9	6.62E-05
Membrane depolarization	52	6	1.77E-04
Endothelial cell development	53	6	1.95E-04
Positive regulation of vascular associated smooth muscle cell proliferation	34	5	2.24E-04
Negative regulation of cellular senescence	20	4	3.48E-04
Positive regulation of smooth muscle cell migration	38	5	3.58E-04
Positive regulation of chemokine production	61	6	3.92E-04
Negative regulation of vascular associated smooth muscle cell differentiation	8	3	4.40E-04
Primitive streak formation	9	3	5.80E-04
Regulation of ribonuclease activity	9	3	5.80E-04
Positive regulation of interferon-gamma production	67	6	6.22E-04
Positive regulation of angiogenesis	156	9	6.38E-04
Positive regulation of interferon-alpha production	24	4	6.42E-04
Chondrocyte differentiation	68	6	6.69E-04
Negative regulation of epithelial cell migration	70	6	7.71E-04
Apoptotic signaling pathway	276	12	9.62E-04
Positive regulation of fibroblast proliferation	49	5	1.03E-03
Cell differentiation in spinal cord	50	5	1.12E-03
Mammary gland epithelial cell proliferation	12	3	1.16E-03
Negative regulation of osteoclast differentiation	29	4	1.21E-03

Table 25: GO molecular function of common genes between *down-regulated* DEGs in DANCER KO/WT comparison and miRNA target genes, analyzed via PANTHER.

GO MOLECULAR FUNCTION	SET SIZE	CANDIDATE SET	P-VALUE
Ion binding	6354	144	1.40E-06
Protein binding	14359	268	2.88E-06
Nuclear receptor activity	54	8	5.67E-06
Ligand-activated transcription factor activity	54	8	5.67E-06
Binding	16539	296	6.54E-06
Anion binding	2874	76	1.01E-05
Metal ion transmembrane transporter activity	431	20	3.56E-05
Adenyl ribonucleotide binding	1560	47	3.85E-05
Protein serine/threonine kinase activity	435	20	4.03E-05
Adenyl nucleotide binding	1572	47	4.32E-05
Enzyme binding	2289	62	4.58E-05
ATP binding	1497	45	6.73E-05
Cation transmembrane transporter activity	635	25	7.65E-05
Carbohydrate derivative binding	2272	60	1.33E-04
DNA-binding transcription activator activity, RNA polymerase II-specific	442	19	1.45E-04
Protein kinase activity	581	23	1.57E-04
DNA-binding transcription activator activity	446	19	1.63E-04
Phosphotransferase activity, alcohol group as acceptor	687	25	1.84E-04
Purine ribonucleotide binding	1912	52	1.88E-04
Kinase binding	766	27	1.96E-04
Voltage-gated calcium channel activity involved in cardiac muscle cell action potential	5	3	2.05E-04
Integrin binding	148	10	2.12E-04

Table 26: GO cellular component of common genes between *down*-regulated DEGs in DANCER KO/WT comparison and miRNA target genes, analyzed via PANTHER.

GO CELLULAR COMPONENT	SET SIZE	CANDIDATE SET	P-VALUE
Plasma membrane protein complex	717	31	1.05E-06

Table 27: GO biological process of common genes between *down*-regulated DEGs in DANCER KO/WT comparison and miRNA target genes, analyzed via PANTHER.

GO BIOLOGICAL PROCESS	SET SIZE	CANDIDATE SET	P- VALUE
Negative regulation of cell differentiation	638	25	7.94E-05
Regulation of calcium ion transmembrane transport via high voltage-gated calcium channel	12	4	9.52E-05
Cardiac muscle cell contraction	42	6	1.03E-04
Canonical Wnt signaling pathway	84	4	1.04E-04
Cell-cell signaling involved in cardiac conduction	26	5	1.15E-04
Somatic stem cell population maintenance	65	7	1.39E-04
Regulation of glial cell differentiation	68	7	1.80E-04
Blood vessel endothelial cell migration	30	5	2.08E-04
Mitotic sister chromatid segregation	121	9	2.24E-04
Mesenchymal cell proliferation	17	4	2.94E-04
Membrane depolarization	52	6	2.98E-04
Regulation of cell migration	885	29	3.44E-04
Cellular response to fluid shear stress	18	4	3.55E-04
Regulation of ERK1 and ERK2 cascade	291	14	3.73E-04
Regulation of sodium ion transmembrane transporter activity	55	6	3.94E-04
Negative regulation of sodium ion transport	19	4	4.24E-04
Positive regulation of cell-matrix adhesion	56	6	4.30E-04
Positive regulation of potassium ion import across plasma membrane	7	3	4.30E-04
Regulation of aldosterone biosynthetic process	7	3	4.30E-04

Gene set enrichment analysis (GSEA) is performed to identify whether a previously defined set of genes indicates statistically significant. The top to the bottom of the ranked list of genes are identified via GSEA. The ES score is calculated as the running sum max value (90) (103). Also, normalized enrichment score (NES) is obtained by normalizing relative to pathway size. If enrichment score (ES) is increased, this means gene is part of the pathway otherwise it is decreased. P53 is the most famous tumor suppressor protein, and it controls vast variety of cellular processes including but not limited to senescence, DNA repair, and cell death (104). Therefore, in order to figure out whether DANCER regulated genes are also correlated with p53 pathway, p53 regulated gene dataset was obtained from GSEA database and DANCER KO up and down genes were separately analysed in p53 pathway via GSEA. 145 genes out of 295 upregulated DEGs in DANCER KO/WT comparison gave a 0.186 ES, whereas, 148 genes out of 319 downregulated DEGs gave 0.238 ES (**Figure 30**). P-value was more significant in DANCER KO down (0.367) than DANCER KO up genes (0.796).

UV radiation causes DNA damage, and it activates different cellular mechanisms such as DNA damage response (DDR) pathway, cell cycle arrest and apoptosis (92). Similar to above, UV irradiation responsive gene dataset was obtained from GSEA database. 45 genes out of 295 upregulated DEGs in DANCER KO gave -0.198 ES, whereas, 39 genes out of 319 downregulated DEGs in DANCER KO gave -0.330 ES. Additionally, p-value is more significant in DANCER KO down (0.451) than DANCER KO up genes (0.938). Here, low UV dose induces transient cellular replicative arrest, however, high UV dose induces apoptosis (92). Therefore, it may be deduced from the GSEA that cellular replicative arrest related genes were more enriched in DANCER KO cells than apoptosis related genes.

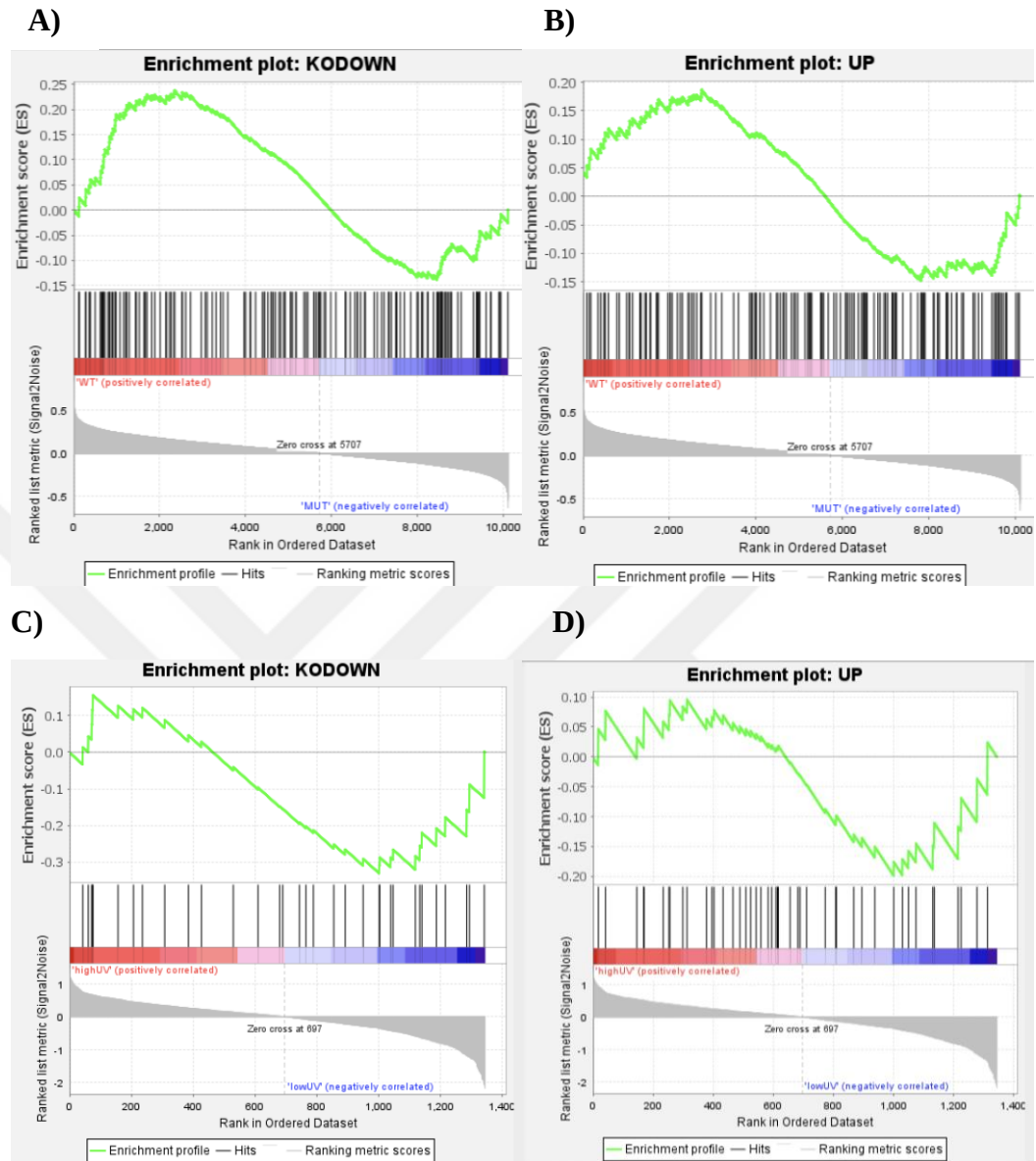


Figure 30: Gene Set Enrichment Analysis for DEGs in DANCER KO/WT RNA seq comparison in different gene datasets. **A-B)** P53 dataset includes transcriptional profiles from p53 wildtype and p53 mutant cancer cell lines and it was obtained from GSEA (89). **C-D)** UV irradiation dataset includes temporal analysis of differences in WS1 human skin fibroblast gene expressions in response to low or high doses of UV radiation (92). **E)** The result of GSEA analysis.

E)

	Original Size	Enriched Size	Enrichment Score	Normalized Enrichment Score	Nominal p-value
P53					
DANCR KO UP	295	145	0.18637	0.83917	0.79658
DANCR KO DOWN	319	148	0.23776	1.05914	0.36687
DNA DAMAGE					
DANCR KO UP	295	45	-0.19812	-0.63870	0.93803
DANCR KO DOWN	319	39	-0.33048	-1.02196	0.45088

Figure 30 (continued): Gene Set Enrichment Analysis for DEGs in DANCR KO/WT RNAseq comparison in different gene datasets.

5. DISCUSSION AND CONCLUSION

Differentiation Antagonizing Non-Protein Coding RNA (DANCR) has potential to be an onco-lncRNA. Studies have shown that overexpression of DANCR may play a role in the formation of different types of cancer and affects cell proliferation, cell viability, stemness, tumor development, and metastasis. These properties of DANCR make it a good candidate for therapeutic targeting. However, there are still missing parts in DANCR's potential roles in tumor progression. In this thesis, the role of DANCR was investigated in different biological pathways. In this regard, enlightening the biological pathways in which DANCR has roles may shed light on providing an opportunity for developing effective treatment methods.

In our previous experiment, in order to assess gene expression changes of cells in response to DNA damage induced by doxorubicin (DOX), human colorectal cancer cells (HCT116) and mouse embryo fibroblasts (MEFs) were treated with 1 μ M DOX or left untreated for 8 hours. Then, total RNA was extracted from the samples and poly-A tail-enriched RNAs were sequenced via next generation RNA sequencing platforms. After bioinformatic analysis of the RNAseq readings, differentially expressed genes were listed and among them DANCR was identified as a significantly downregulated gene in response to doxorubicin induced DNA damage. DANCR was interesting in these results because although it is a lncRNA, it was conserved among human and mouse. In order to identify biological significance of DANCR in DNA damage and cell death responses, DANCR gene locus was deleted from HCT116 genome by using CRISPR/Cas9 gene editing technology by Gulin Baran, Msc., and DANCR^{-/-} HCT116 cells were simply named as KO cells (27). In this current Master's thesis study, DANCR cDNA was amplified from HCT116 cells and cloned into a mammalian expression plasmid to be transfected back into WT HCT116 and KO cells to overexpress and to restore DANCR expression and the cell lines were named as OE and Rescue, respectively.

By these, we comprehensively investigated cellular biological pathways in which DANCER plays roles.

After *DANCER* gene at its genomic locus and *DANCER* expression at RNA level were confirmed by appropriate PCR techniques in all four cell lines, various phenotypic analyses were performed by these mutant cell lines. First, cell proliferation rates were determined by short-term and long-term analyses. According to Trypan blue exclusion assay, we found that exogenous *DANCER* expression in addition to endogenous *DANCER* in OE cells or only endogenous *DANCER* by itself in WT cells increased cell proliferation rate, in contrast, exogenous *DANCER* expression in endogenous *DANCER* deficient cells (Rescue) and loss of endogenous *DANCER* expression in KO cells decreased cell proliferation rate (**Figure 14**). Also, Rescue's growth rate was significantly higher than KO's and OE's growth rate was higher than WT's, meaning that these additional *DANCER* expressions supplemented exogenously affected growth rate in short term assessment. After that, colony formation assay was performed to assess long-term cell proliferation rates of the corresponding cells. We found that OE and WT cells had more colonies covering more area on the plate but long-term cell proliferation was significantly suppressed in KO and Rescue cells (**Figure 15**). Cumulatively, it was shown that *DANCER* expression correlated with the proliferation rate of cells especially significantly for shorter time windows.

To explore the physiological effects of *DANCER* under DOX treatment, MTT assay was performed with different DOX concentrations ranging from no DOX to 10000nM and the experiment was repeated for four times. The highest IC_{50} value was of OE with 426.5nM, and the second highest was 357.1nM in KO cells, so they were DOX-resistant cells (**Figure 16**). The IC_{50} of Rescue cells was 314.2nM and the lowest IC_{50} was found as 245.2nM in WT. This means that WT was the most sensitive cell against DOX. To further investigate *DANCER* in response to DOX, a long-term colony formation assay was performed (**Figure 17**). Without any DOX

treatment, OE and WT cells had more colonies as the previous experiment, but were together significantly higher than both Rescue and KO cells. After 2 hours treatment with 250 nM DOX, the colony numbers of WT and OE cells, at the end of about 2 weeks of recovery period, were still the highest and nearly the same. When 1000nM DOX was applied, no significant difference between the cells was observed because this high concentration was probably excessively fatal. Therefore, we concluded that loss of endogenous DANCER affected cell's recovery after DOX treatment and that the restoration of DANCER with exogenous expression was not enough to increase cell viability during the recovery for prolonged times. Here, it is important to account the proliferation rate differences of the cells. It is possible that the initial 2 hours of DOX treatment was not long enough to point out the cells' capacities for recovery or it is also possible that the 2-week recovery period afterward was too long during which cell proliferation rate differences shadow the differences of cells for recovery capacity. Furthermore, this may be related to different biological mechanisms such as alteration in membrane transport proteins to raise drug efflux, enhancing DNA repair, modifying cell cycle regulation to block apoptosis, and detoxification (105). According to the literature, DANCER deficiency increases p21 expression and this may cause cell cycle arrest and senescence (50). This may clarify the slow cell proliferation rate of KO cells. Additionally, we previously had shown that DANCER is downregulated at the end of 24-hour DOX treatment but later is upregulated even in longer DOX treatment (27). Therefore, it is possible that DANCER gave a response to DOX only during the first 2 days of treatment and then the response was lost. Lastly, it is always important to keep in mind that lncRNAs may act in *cis* or in *trans* or sometimes both (19). Here in this Thesis study, we created exogenous DANCER expression with expression plasmid carrying the cDNA of DANCER. Therefore, if DANCER acts in *cis*, it may not be modelled here, and this fact may alter the true cellular responses in the presence of overexpressed DANCER. It would be good to induce DANCER expression from endogenous genomic locus by altering the epigenetic statues of the locus by using CRISPR/dCas9 activators. As a result, deregulated DANCER expression may give cells an advantage for a prompt response to DOX

treatment, however for longer periods, it may be difficult to see DANCER's role in response to DOX treatment.

To uncover the cell cycle effect of DANCER's role in cells, cells were stained with PI and analyzed using FACS. Apoptotic cells include fractional DNA content and therefore they can be identified as cells with fractional "sub- G₁" DNA content in this type of assay (106). The sub- G₁ phase was highest in KO cells and lowest in WT cells but not highly significantly (**Figure 18**). It may be related to slowest cell proliferation rate of KO cells because they may undergo apoptosis. G₁, which is a growth phase, was significantly high in Rescue and KO cells compared to WT and OE cells. This may cause large cell size and cells may be arrested at G₁/S transition, so, cells cannot rapidly divided, explaining slower proliferation rates of endogenous DANCER deficient cells. Also, endogenous DANCER expression with or without additionally supplemented exogenous DANCER may regulate G₁/S transition by arranging the restriction point and may aid cells in rapidly passing it. S phase, which is a DNA replication step, was highest in OE cells, so exogenous DANCER expression in addition to the endogenous DANCER may activate S phase genes or suppress S phase inhibitor genes. Additionally, S phase of KO cells was nearly equal with Rescue cells but significantly lower than WT and OE cells. p21 (CDKN1A), which blocks the activity of CDKs required for S phase entry, was found as increased in DANCER KO previously (50). Therefore, in this Thesis study, we did not check it but the low S phase of DANCER KO cells may be related to p21 expression. In the end, the cell cycle distributions of the cells were consistent with the cell proliferation rates such that KO proliferated slower than WT and that there were slight differences between OE vs. WT and KO vs. Rescue.

During regular cell culture maintenance of the 4 different cell lines we created and because of our previous studies (27), cells were observed differently under microscope. Therefore, cellular morphologic changes were investigated by using hematoxylin and eosin staining (H&E). KO cells were bigger in cell size and

exogenous DANCER expression in Rescue cells did not shrink them back (**Figure 19**). This may be related to higher percentage of KO and Rescue cells in G₁ phase, in which, cellular growth takes place. Additionally, KO cells had multi-nucleated morphology and enlarged irregularly shaped cells. Interestingly, Rescue cells were more like KO cells except for being multi-nucleated. Multinucleation may be associated with the cell-cell fusion process or acytokinetic cell division (107). Besides, mitotic slippage, also known as endomitosis, inhibits the activity of checkpoint by prolonging the time of mitotic arrest (108). Cyclin B1 does not get rid of proteasomal degradation, and cells are forced by this reason to exit from the M phase. However, multiple nuclei are formed by reconstitution of the nuclear envelope without undergoing correct segregation of chromosomes or cytokinesis, and cell size is increased (108) (109). The underlying reason for the multinucleated cells in KO may be related to mitotic slippage. This issue definitely deserves further investigation.

2D cell culture limits functions of cells because cells adhere to substrate more than to each other, and this does not imitate complex microenvironment of cells in a tissue (95) (110). However, 3D cultures are more reflective *in vivo* environments because of improved cell-to-cell interactions. They exert biomechanical forces that change the shape, cytoskeleton, and function of the cells and they secrete biochemical signals that alter gene expression and cell function (95). The aggregated cells create a high densely packed 3D tumor-like structure, and this imitates a solid tumor causing diffusion restraint for glucose and oxygen. Tumor spheroids are generally used in basic research, drug screening, and preclinical studies (111) (97). To examine the spheroid properties of cells, a hanging drop assay was performed. We found that OE cells created the biggest and the most intact spheroids, indicating that OE cells were the most successful in imitating a solid tumor. Also, consistent with previous results, spheroids from OE cells were more resistant to DOX. (**Figure 20**). The loss of endogenous DANCER expression in KO cells caused a significant decrement in spheroid morphological characteristics.

Semi-solid soft agar colony formation assay gives clues about the tumorigenicity potential of the cells and that was performed to monitor anchorage-independent colony formation of mutant cells that we created. Although sizes of KO and Rescue colonies were nearly the same, WT and OE colonies were higher on day 14 (**Figure 23A, B**). WT and OE colonies had a regular circular shape, whereas KO and Rescue colonies were usually irregularly shaped at the end of 21 days. Also, cell-cell physical interactions were lost in KO colonies, assumed from the holes composed in the spheroid surfaces. According to these results, exogenous DANCER expression in addition to the endogenous DANCER in OE cells and also endogenous DANCER in WT cells increase anchorage-independent properties and contribute to malignant tumor cells but the loss of endogenous DANCER expression in KO cells caused a non-neoplastic cell's properties and the exogenous DANCER supplement in Rescue cells did not rescue this phenotype.

DANCER-201 targeted miRNAs were identified via online miRDB database and their corresponding seed sequences on DANCER RNA were annotated on 2D structure of DANCER. Secondary structures of RNAs may give clues about catalytic, regulatory, ligand binding, and scaffolding functions. These miRNAs bound different regions of DANCER RNA structure such as hairpin loop, multiloop, interior loop, and bulge (**Figure 21**). Multiloop structures may be catalytically active and may sponge these miRNAs out. Bulges may act as molecular handles within helical regions, disrupting the RNA backbone, so base pairs in the deep groove can be accessible, providing easy accessibility for miRNAs binding (112). Therefore, DANCER may sponge those miRNAs binding to bulge regions on it. Moreover, there were several miRNAs sharing the same or overlapping seeding sequences hence they aligned very close proximities on the secondary structure of DANCER. The roles of secondary structure regions are pivotal to identify miRNAs' interaction mechanism. Although 2D and 3D prediction algorithms provide insights for a possible catalytic activity for DANCER or for a DANCER-Protein physical interaction, physical and experimental methods should be applied to obtain accurate

analysis such as NMR and X-ray crystallography, followed by in vitro functional validation assays.

After identifying miRNAs that may bind to DANCER RNA by using miRDB online database, their target mRNAs were further investigated by using DIANA online tool. We hypothesized that our RNAseq results of DANCER KO cells may indicate differential expressions for these mRNAs. According to our hypothesis, DANCER is a competing endogenous RNA (ceRNA) and when DANCER sponges miRNAs out, the inhibitory action of these miRNAs may be prevented on their actual mRNA targets (**Figure 25**). However, DANCER-absent condition causes these mRNAs degradation by the miRNAs. Therefore, we thought that loss of endogenous DANCER expression in KO cells gives clues about downregulated genes by these miRNAs. 322 down-regulated DEGs in DANCER KO group, which were associated with melanogenesis, proteoglycans in cancer, Aurora B signaling, platelet homeostasis, transcriptional regulation of pluripotent stem cells, aldosterone synthesis and secretion, regulation of sister chromatid separation at the metaphase-anaphase transition, transport of small molecules, angiogenesis, integration of energy metabolism, G₀ and Early G₁ and mitotic G₁-G₁/S phases and hedgehog signaling events mediated by Gli proteins (**Table 21**), may be related with the ceRNA function of DANCER. Additionally, GO molecular function of this group was related to DNA-binding transcription activator activity, etc. GO cellular component was associated with plasma membrane protein complex, and GO biological process was correlated with mitotic sister chromatid segregation, negative regulation of cell differentiation and mesenchymal cell proliferation, etc. Therefore, the loss of endogenous DANCER may increase the availability of tumor suppressor miRNAs, and this may attenuate cancer-related processes such as elevated cell proliferation, stemness, and angiogenesis.

The ceRNA role of DANCER was identified previously as decoying miR-335-5p and miR-1972, and this promotes proliferation and metastasis by increasing ROCK1 (63). Although we could not find miR-335-5p in our miRDB analysis as a binding miRNA partner of DANCER, we did find miR-1972. miR-1972 target mRNAs were identified by using DIANA and the common ones with our down-regulated DEGs in KO vs WT RNA seq result were listed for further research (**Figure 31**). KIF18B may be a candidate target of miR-1972 in KO cells. One previous study showed that KIF18B decreases the length of the microtubule by arranging microtubule catastrophe and regulate mitotic spindle positioning (113). However, when KIF18B knocked-out, astral microtubule length is increased, and it causes spindle mispositioning. In our RNAseq, we found that KIF18B expression was decreased in DANCER KO cells (fold change (fc)=-2,14, false discovery rate (fdr)=0). This may be interesting because KIF18B downregulation may explain the morphology of KO cells. The other target of miR-1972 was found as RSPO4, and its level decreased in KO cells (fc=-2,37, fdr=3,09E-3). RSPOs were found as gene expression regulators in Wnt signaling pathway and they cause cancer growth (114). Therefore, miR-1972 may have potential to cause tumor formation and it may be sponged by DANCER.

Another study showed the ceRNA role of DANCER while it is sponging miR-216a-5p and therefore indirectly increasing SOX5 expression. They found that cell progression and autophagy in osteosarcoma cells is inhibited via silencing DANCER but apoptosis is increased (115). We also identified miR-216a-5p as a binding partner of DANCER, and we found that miR-216a-5p may target NSUN6 in KO cells. NSUN6 is a 5-methylcytosine (m5C) methyltransferase and it localizes to cytoplasm. RNA m5C modification plays essential roles in regulating RNAs alternative splicing, stabilization, localization, transportation, and translation but its dysregulation causes formation of different cancers such as hepatocellular carcinoma and bladder cancer (116) (117). The level of NSUN6 was found to be decreased in our KO cells (fc=-2,49, fdr=1,52E-15). Therefore, DANCER and NSUN6 levels may be positively correlated, and the target of NSUN6 may be

tumor suppressor gene so this may contribute to cancer. Another target of miR-216a-5p may be MCM4. MCM4 is a member of minichromosomal maintenance protein family and it has roles in the initiation of DNA replication, and its' deregulation causes cell proliferation and tumorigenesis (118). The level of MCM4 was found to be decreased in KO cells (fc=-2,23, fdr=0). It may clarify the reason varied cell proliferation rates of DANCER KO, Rescue and OE cells compared to WT cell.

We also identified miR-656-3p as a binding partner of DANCER and this interaction was not indicated previously. The target of miR-656-3p may be STK32A in our KO cells. STK32A (Serine/Threonine Kinase 32A) has roles in cellular homeostasis, transcription factor phosphorylation and cell-cycle regulation (119). The level of STK32A was found to be decreased in KO cells (fc=-24,57, fdr=8,17E-7). Again, this gene might be a candidate gene for further investigation of proliferation rate discrepancies among our mutant cells. It is noteworthy that our OE vs WT RNAseq result did not yield excessively high number of DEGs as compared to that of KO vs WT. Therefore, we could not identify target genes of DANCER binding miRNAs in OE cells as DEGs. However, this does not diminish ceRNA roles of DANCER on these miRNAs and their target mRNAs identified in our KO cells because it is possible that OE cells do not exhibit physiological levels of DANCER that is acting rightly.

Other than the role of ceRNA, DANCER can also act as scaffold, guide, signal, or decoy RNA. 296 upregulated DEGs may be associated with the direct target of DANCER because they were increased at the lack of endogenous DANCER expression. They were related with different pathways such as cytokine signaling in immune system, extracellular matrix organization, osteoclast differentiation, keratinization, adipogenesis, DNA Damage Response, collagen formation, senescence and autophagy in cancer, cellular senescence, and differentiation pathway (**Table 20**). Additionally, GO molecular function of this group was related

to transcription factor binding, GO cellular component was correlated with cytoplasm, channel complexes, and ER lumen. GO biological process was associated with cell differentiation, apoptotic signaling pathway, and immune response. Therefore, the loss of endogenous DANCER condition may identify the DANCER-targeted genes. DANCER was found as a suppressor of differentiation and we expected that knocking out DANCER increases cell differentiation (45). Also, the loss of endogenous DANCER may regulate senescence and autophagy. After that, RNA Seq data from DANCER OE and DANCER KO groups was investigated in depth.

SOX2 is as regulator of different cancer hallmarks such as promoting cellular proliferation, invasion and metastasis, and evading apoptotic signals while enhancing cancer stemness properties. Previous studies showed that DANCER forms a complex with RBM3 to stabilize SOX2 mRNA and contributes cellular proliferation in nasopharyngeal carcinoma (NPC) (120). According to our RNA seq results, the level of SOX2 was decreased in KO cells ($fc=-9,07$, $fdr=0$). Additionally, the anchorage independent properties as indicated by the spheroid size and its internal structure were the highest in DANCER OE cells and the lowest in KO cells. It is probably that DANCER may exert decoy or guide functions on SOX2 and contribute to stemness, therefore, it does its differentiation antagonizing functions partly through SOX2. C-Fos is a proto-oncogene and is found as a regulator of epithelial mesenchymal transition (EMT) and cancer stem cell (CSC) reprogramming in head and neck squamous cell carcinoma (HNSCC) cells, and contributes to spheroid formation (121). The relationship with DANCER was not indicated previously but the level of FOS expression was found to be decreased in our KO cells ($fc=-1,54$, $fdr=5,96E-3$) but raised in DANCER OE cells ($fc=2,48$, $fdr=0$). Therefore, DANCER may regulate and recruit transcription factor oncogenes such as SOX2 and FOS, in this way, their level may increase.

The tumor-suppressive role of Sterile Alpha Motif Domain-containing 9-like (SAMD9L) was found in human hepatitis B virus-associated HCC (122). According to our RNA Seq data, the level of SAMD9L was highly significantly upregulated in DANCER KO cells (fc=197,56, fdr=7,19E-3). The secreted protein acidic and rich in cysteine (SPARC) was found to have different roles in differentiation, tissue remodeling, embryogenesis and angiogenesis (123). SPARC induces apoptosis in neuroblastoma through inducing ER stress leading to autophagy. The level of SPARC increased in our KO cells (fc=10,92, fdr=0). This may indicate the reason for slower cell proliferation rate of KO cells because maybe autophagy is induced in them. We did not check any autophagy signatures in these cells; however, it would be worth investigating this.

DOX intercalates Topoisomerase 2 enzyme and blocks DNA synthesis and transcription. As DNA is broken in double strands because of this blockage, DNA breaks become irreversible at genomic regions causing cellular apoptosis. If alterations induced in genomic DNA cause modifications, drug resistance may be acquired (124). In order to investigate the effects of DANCER on DOX treatment, we compared DOX treated WT and OE cells to their individual corresponding untreated controls in RNA seq data, i.e., WT-treated vs WT-untreated and OE-treated vs OE-untreated. Our data revealed several DOX-response-related genes that were differently differentially expressed in OE cells treated with DOX compared to WT cells treated with DOX. Several genes identified with this criteria are as the followings; *TTL10* (fc=677,02, fdr=1,84E-3 in OE treated, fc=69,18, fdr=1,29E-5 in WT treated), *POU3F1* (fc=517,31, fdr=2,91E-3 in OE treated, fc=133,35, fdr=3,36E-7 in WT treated), *CHRNA6* (fc=497,46, fdr=3,12E-3 in OE treated, fc=387,23, fdr=6,73E-3 in WT treated), *AMIGO3* (fc=298,45, fdr=7,71E-3 in OE treated, fc=36,96, fdr=0 in WT treated), *MAFB* (fc=277,64, fdr=1,60E-9 in OE treated, fc=78,13, fdr=0 in WT treated), *STPG3* (fc=227,74, fdr=0,01 in OE treated, fc=27,69, fdr=1,54E-3 in WT treated), *PADI1* (fc=163,76, fdr=0,02 in OE treated, fc=98,44, fdr=0,05 in WT treated), *CLCA2* (fc=131,98, fdr=0,03 in OE treated, fc=83,38, fdr=0,06 in WT treated), and *PAX5* (fc=108,15, fdr=0,03 in OE

treated, $fc=60,89$, $fdr=0,08$ in WT treated). We had found that the IC_{50} value of OE cells were the highest and these DEGs may be the reason for DOX resistance in OE cells. Further work should definitely be performed to answer the question about DOX-resistance genes.

Lastly, GSEA was performed to identify whether a priori defined set of genes indicates statistically significance (90). According to this analysis, both up- and down-regulated genes in DANCER KO cells were enriched with p53 and DNA damage datasets. P53, which is a tumor suppressor, controls various cellular process such as senescence, DNA repair, cell death, and it is mutated in nearly half of human cancers (104). UV radiation causes DNA damage, and it activates different mechanisms such as DDR pathway, cell cycle arrest and apoptosis (91). We found that the downregulated genes in DANCER KO cells were more significant in p53 WT and cell cycle arrest condition. Therefore, our data showed that the loss of endogenous DANCER may activate p53 and cell cycle arrest, so DANCER may work as inhibitor of p53 and exert tumorigenic properties. These are consistent with our cell cycle and bioinformatic data analyses. However, further studies are necessary to examine the significances of p53 pathway in this association.

In conclusion, this Master's thesis study documented the roles of DANCER in cellular biological pathways in a colorectal cancer cell line. Accordingly, DANCER increases cell proliferation, 3D spheroid and anchorage independent features. DANCER overexpression increases S phase, however, DANCER knock out increases G_1 phase. DANCER deficiency and redundancy confer resistance to doxorubicin. RNA seq and bioinformatics analyses were done to investigate the ceRNA role of DANCER, and candidate miRNAs were found as miR-1972, miR-216a-5p and miR-656-3p. miR-1972 targeted mRNAs may be Kif18b and RSPO4; miR-216a-5p targeted mRNAs may be SOX5, MCM4 and NSUN6; and miR-656-3p targeted mRNA may be STK32A.

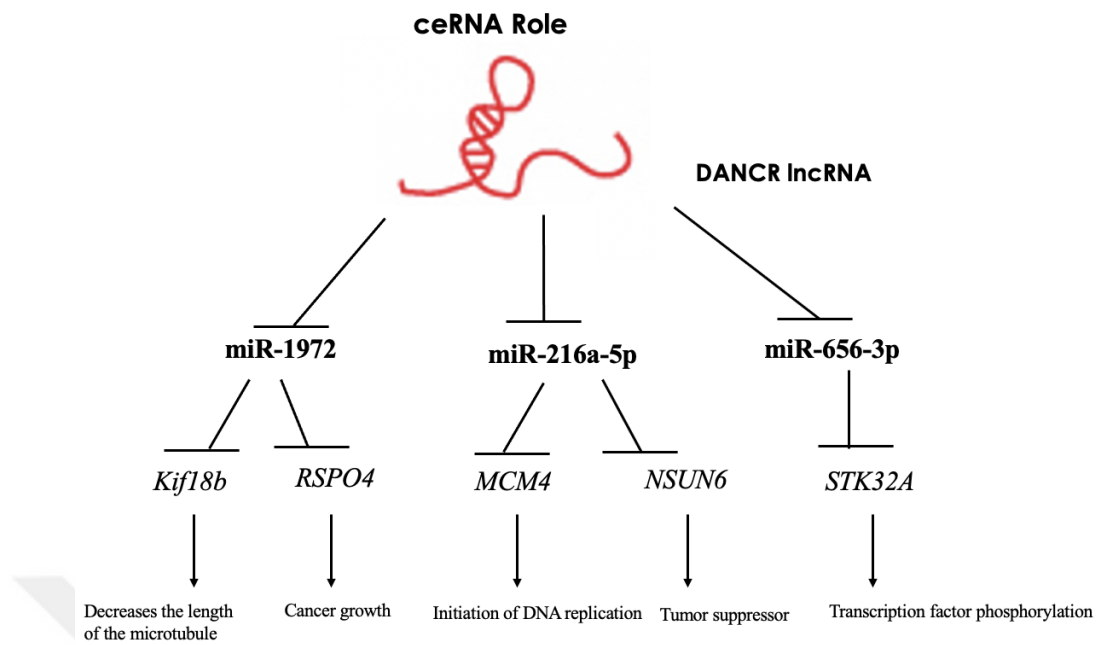


Figure 31: The representation of ceRNA role of DANCR.

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7. APPENDIX

APPENDIX A: Chemicals Used in This Project

Chemicals and Other Components	Number	Supplier Company, Catalog
Absolute Ethanol		Sigma, #32221-2.5L
Agar		Sigma, #05039
Agarose Universal		Boga, #35-1010
Ampicillin		Neofroxx, #1728GR010
Adenosine 5'-Triphosphate (ATP)		NEB, #P0756S
Bromophenol Blue		Sigma, #B0126
Cell Proliferation Kit I (MTT)		Roche, #11465007001
Crystal Violet		Norateks, #106092
DH5 α competent cells		NEB, # C1010S
Deoxynucleotide Solution Set		NEB, #N0446S
Dimethyl Sulfoxide		Biomatik, #A2424
Doxorubicin Hydrochloride		Sigma, #D1515-10MG
Dulbecco's Phosphate-Buffered Saline		Gibco, #14190094
EcoRI-HF		NEB, # R3195S
Eosin Y stain 1% (alcoholic)		HistoPlus #33101761
Ethlyenediamine Tetraacetic Acid		Gibco, #25300054
Etidium Bromide		Sigma #e1510-10
Fetal Bovine Serum, heat inactivated, E.U.-approved, South America Origin 500 Ml		Gibco #10500064

Gelatin	Sigma, #G9391
Gene Ruler DNA Ladder Mix SM0331	Thermo Scientific, #LSG-
Glacial Acetic Acid	Merck, #1.0005602500
Glucose	Sigma, #16301-1KG
Harris Haematoxylin Stain	HistoPlus, #32101781
Heat Inactivated Fetal Bovine Serum	Gibco, #10500064
High Glucose Dulbecco's Modified Eagle Medium	Gibco, #41965039
Isopropanol	Sigma, #24137
Luria-Bertani Broth	Sigma, #L7659
Magnesium Chloride	NEB, #B9021S
Mg ²⁺ Free Standard Taq Buffer	NEB, #B9015S
MyTaq TM DNA Polymerase	BIOLINE, #BIO-21105
NucleoSpin RNA Isolation Kit	Macherey-Nagel (MN), #740955.5
Oligos/Primers	Sentegen Biotechnology
pBABE-puro	Addgene #1764
Penicillin-Streptomycin	Gibco, #15140122
Polyethylenimine Hydrochloride MAX Potassium Acetate	Polysciences, #24765-1
Potassium Acetate	Merck, #104820
PowerUp TM SYBR TM Green Master Mix.	Thermo Scientific, #A25741
Proteinase K	Macherey-Nagel, #740506
ProtoScript® II First Strand cDNA Synthesis Kit	NEB, #E6560L

PureLink™ Quick Gel Extraction Kit	Invitrogen # K210012
PureLink™ PCR Purification Kit	Invitrogen #K310001
Puromycin	Sigma, #P9620
RNase A	Macherey-Nagel, #740505
SmaI	NEB #R0141S
Sodium Chloride	Sigma, #13423
Sodium dodecyl sulfate	Sigma, #151-21-3
Sodium Hydroxide	Sigma, #06203
T4 DNA Ligase	NEB, #M0202S
T4 DNA Ligase Buffer	NEB, #M0202S
T4 Polynucleotide Kinase	NEB, #M0201S
Taq DNA Polymerase	NEB, #M0320S
TE Buffer	Invitrogen, #00431008
Tris Base	BioRad, #1610716
Tris-Cl	Molekula
Trypan Blue	Sigma-Aldrich, #T8154
Trypsin-EDTA Solution	Gibco, #25300054

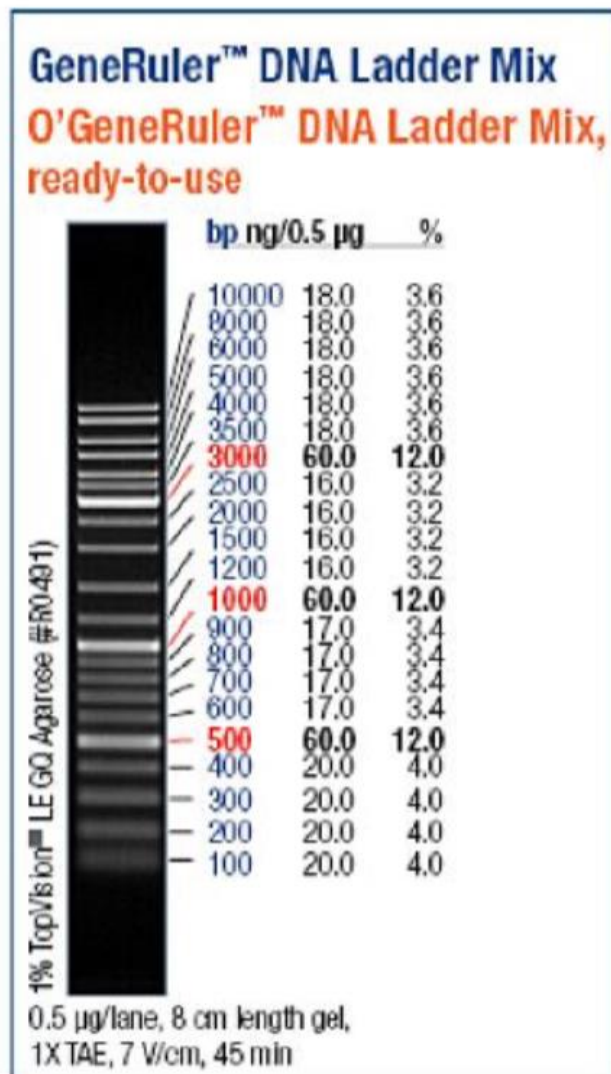
APPENDIX B

Equipment Used in This Project

Equipment Number	Supplier Company, Catalog
T100™ Thermal Cycler PCR Machine	BioRad, #1861096
0.2 mL PCR Tube	PCR-02-C Axygen
1.5 mL Microcentrifuge Tube	CAPP, #5101500C
100 mm Tissue Culture Plate	TPP, #93100
60 mm Tissue Culture Plate	TPP, #93060
96-well qPCR Plate	BioRad, #HSP9601
96-well Tissue Culture Plate	TPP, #92096
Bacterial Cell Culture Incubator	Forma Steri-Cycle CO2; Thermo Scientific, #521030301
Centrifuge Machine	Thermo Scientific, #SL16R
ChemiDoc Imaging System Cryovial Tube	BioRad, #1708265
Cryovial Tube	TPP, #89012
Flow Cytometry	BD FACSVerser™, #651155
Florescent Microscope	ZEIS AXIO Vert.A1
Freezing container	Coolcell LX, # BCS-405
Gel Electrophoresis Casts	Major Science, #1406200009; VWR Mini Gel II, #95043-688
Digital Shaking Dry Bath	Thermo Scientific, #88880028
Light Microscope	ZEIS PrimoVert
Microcentrifuge Machine	Thermo Scientific, #MicroCL21R

Microwave Oven	Samsung, #MW71E
Neubauer Chamber	Marienfeld, #C964110
qPCR	Bio-Rad CFX96
Shaker	Biosan, #ES-20/60
Spectrophotometer	Hitachi Spectrophotometer, #U-1900
Tissue Culture Incubator	Thermo #51030301
VarioSkan Flash	Thermo MIB#5250030
Vortex	VWR, #VV3

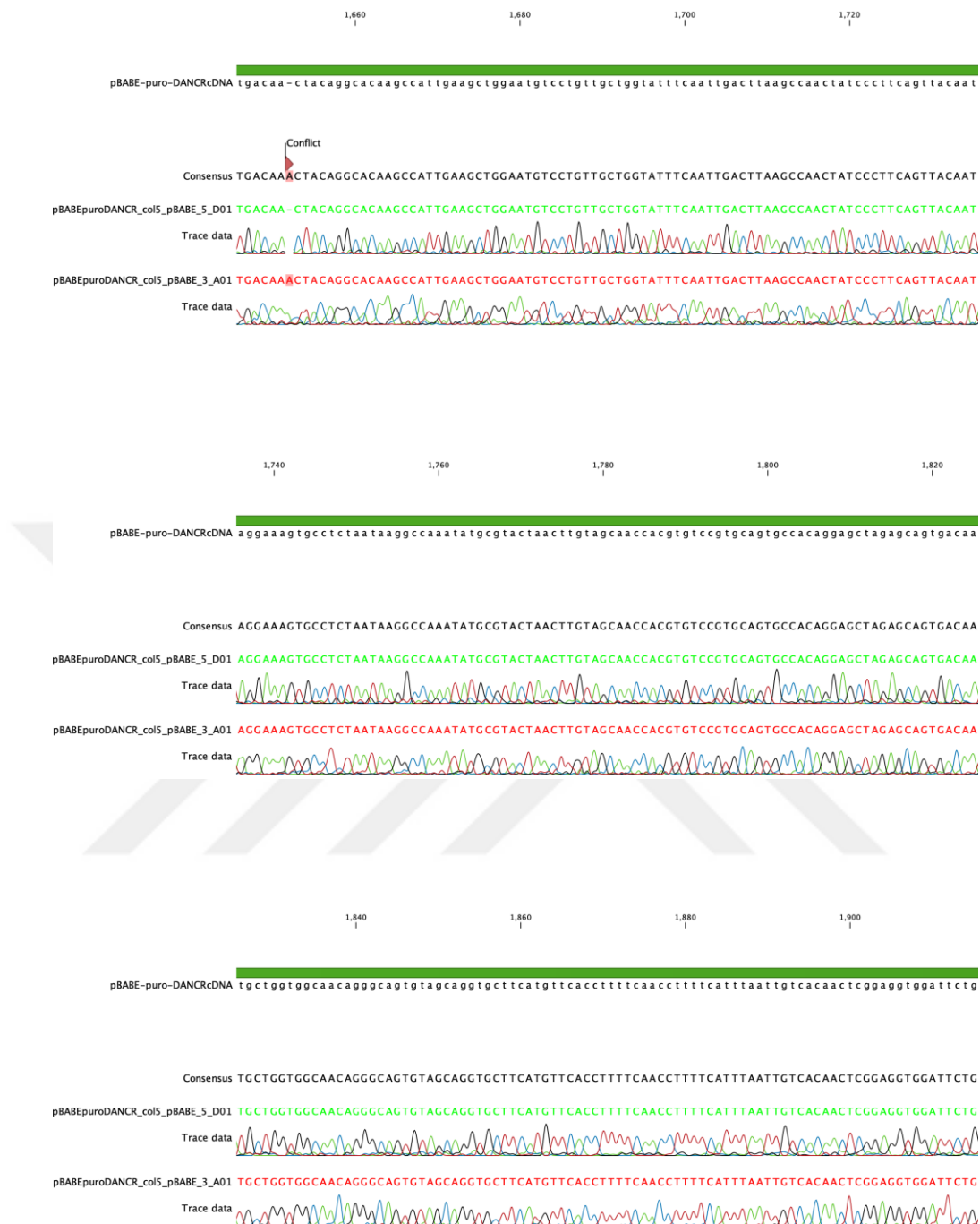
APPENDIX C: DNA Ladder

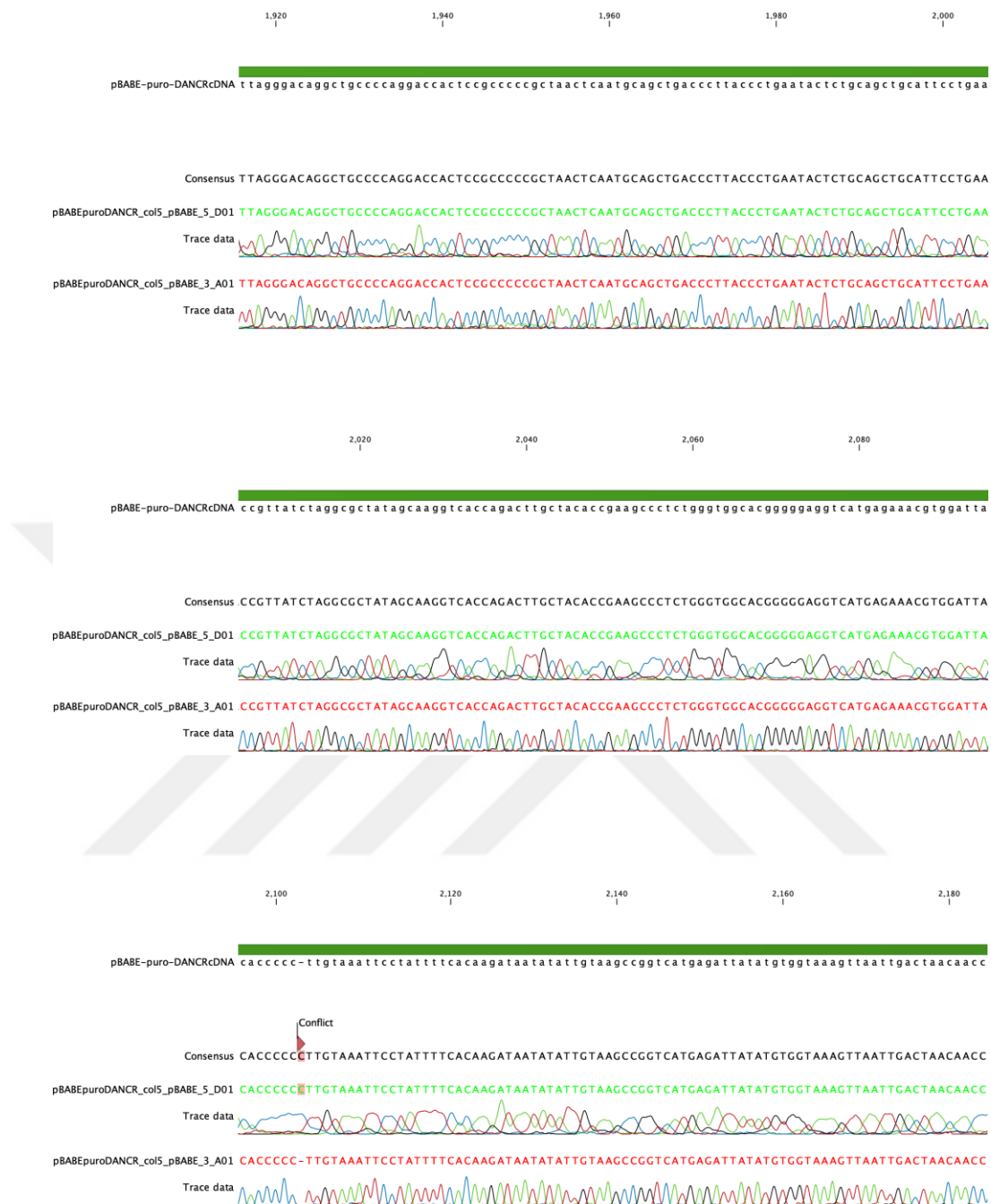


APPENDIX D

Sanger sequencing analysis of DANCER cDNA cloning into pBABEpuro plasmid.







CURRICULUM VITAE

Personal Information

Name	Nurdeniz	Surname	Nalbant
Birth of place		Date of birth	
Nationality		Telephone Number	
E-mail			

Educational Level

	Institution Name	Graduate Year
Master	Acıbadem Mehmet Ali Aydınlar University	2021
Undergraduate	Kadir Has University	2019
High-School	Bursa Final High School	2014

Work Experience

	Position	Enterprise	Duration
1.	Medical Genetic Department. Bursa Uludağ University Hospital	Internship	07/2017-08/2017
2.	Betül- Ziya Eren Genome ve Stem Cell Center, GENKÖK	Internship	07/2018-08/2018

Foreign Languages	Reading*	Speaking*	Writing*
English	Fluent	Fluent	Fluent

Foreign Languages Exams*

YDS	UDS	IELTS	TOEFL IBT	TOEFL PBT	TOEFL CBT	FCE	CAE	CPE
65/100								

	Quantitative	Equally Weighted	Verbal
ALES Exam	69,809	61,392	66,409

Presentations & Awards		
Organization	Presentation Type	Title of Presentation
8 th Multidiciplinary Cancer Research Congress, 2021	Poater / 1 st place award	DANCR Act as a Competing Endogenous RNA to Regulate Gene Expression

