INTEGRATED ANALYSES OF EMODIN IN BREAST CANCER CELL LINES

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

ELİF SAKALLI TECİM

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

JUNE 2023

Approval of the thesis:

INTEGRATED ANALYSES OF EMODIN IN BREAST CANCER CELL LINES

submitted by **ELİF SAKALLI TECİM** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** i**n Biotechnology, Middle East Technical University** by,

Prof. Dr. Halil Kalıpçılar
Dean, Graduate School of **Natural and Applied Sciences** _____

Prof. Dr. Yeşim Soyer Küçükşenel
Head of the Department, **Biotechnology** _____

Prof. Dr. N. Tülün Güray
Supervisor, **Biotechnology, METU** _____

Assoc. Prof. Dr. Bala Gür Dedeoğlu
Co-Supervisor, **Biotechnology Inst, Ankara University** _____

Examining Committee Members:

Prof. Dr. Orhan Adalı
Biological Sciences, METU _____

Prof. Dr. N. Tülün Güray
Biological Sciences, METU _____

Prof. Dr. Özlem Yıldırım
Biology, Ankara University _____

Assoc. Prof. Dr. Çağdaş Devrim Son
Biological Sciences, METU _____

Prof. Dr. Leyla Açık
Biology, Gazi University _____

Date: 20.06.2023

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name Last name : Elif Sakallı Tecim

Signature :

ABSTRACT

INTEGRATED ANALYSES OF EMODIN IN BREAST CANCER CELL LINES

Sakallı Tecim, Elif
Doctor of Philosophy, Biotechnology
Supervisor : Prof. Dr. N. Tülün Güray
Co-Supervisor: Assoc. Prof. Dr. Bala Gür Dedeoğlu

June 2023, 132 pages

Breast cancer has been among the most prominent cancers with high incidence rates and mortality. Currently most of the offered therapeutics for treatment are toxic; hence, less toxic therapeutic intervention is required. Here, we studied the molecular mechanisms of the effect of a phytoestrogen Emodin on estrogen receptor positive MCF-7 and negative MDA-MB-231 cells by carrying out a comprehensive network assessment. Differentially expressed microRNAs along with their identified differentially expressed mRNAs were analyzed through microarrays by using integrative systems biology approach. For each cell line miRNA-target gene networks were built, gene ontology and pathway enrichment analyses were performed, enrichment maps were constructed and the potential key genes, microRNAs and microRNA-gene interactions were studied.

Keywords: Breast Cancer, Phytoestrogen, Emodin, miRNA-mRNA Integrated Analysis, Systems Biology

ÖZ

MEME KANSERİ HÜCRE HATLARINDA EMODİN'İN ENTEGRE ANALİZİ

Sakallı Tecim, Elif
Doktora, Biyoteknoloji
Tez Yöneticisi: Prof. Dr. N. Tülün Güray
Ortak Tez Yöneticisi: Doç. Dr. Bala Gür Dedeoğlu

Haziran 2023, 132 sayfa

Meme kanseri, yüksek insidans ve mortalite oranları ile en önde gelen kanserler arasında yer almaktadır. Şu anda tedavi için sunulan terapötiklerin çoğunun toksik olması nedeniyle, daha az toksik terapötik müdahale gerekmektedir. Bu çalışmada, kapsamlı bir ağ değerlendirmesi yaparak bir fitoöstrojen olan Emodin'in östrojen reseptör pozitif MCF-7 ve negatif MDA-MB-231 hücreleri üzerindeki etkisinin moleküler mekanizmalarını inceledik. Diferansiyel olarak eksprese edilen mikroRNA'lar, diferansiyel olarak eksprese edilen tanımlanmış mRNA'ları ile birlikte, entegre sistem biyolojisi yaklaşımı kullanılarak mikrodiziler aracılığıyla analiz edildi. Her hücre dizisi için miRNA-hedef gen ağları oluşturuldu, gen ontolojisi ve yolak zenginleşme analizleri yapıldı, zenginleşme haritaları oluşturuldu ve potansiyel anahtar genler, mikroRNA'lar ve mikroRNA-gen etkileşimleri incelendi.

Anahtar Kelimeler: Meme Kanseri, Fitoöstrojen, Emodin, miRNA-mRNA Entegre Analizi, Sistem Biyolojisi

To My Family

ACKNOWLEDGMENTS

First of all, I would like to express my deepest gratitude to my supervisor Prof. Dr. N. Tülün Güray for her excellent academic guidance, continued support, critical discussions throughout the research. I am grateful her for accepting me as her student and letting me benefit from her experience.

I am also grateful to my co-supervisor Assoc. Prof. Dr. Bala Gür Dedeoğlu for her great support and positive attitude from the beginning of the study.

I would like to thank to Assoc. Prof. Çağdaş Devrim Son for his valuable criticism and contributions to this study during the whole period. I would also like to thank to the rest of my examining committee members; Prof. Dr. Özlem Yıldırım, Prof. Dr. Leyla Açık and Prof. Dr. Orhan Adalı for their valuable comments.

I would also like to express my special thanks to Assoc. Prof. Yeşim Aydın Son for her valuable criticism and comments during this research.

I also would like to thank to Prof. Dr. Mesude İşcan who initiated this study and is no longer with us, with all my respect and longing.

I would like to thank to all my lab colleagues and especially to Eda for all her support. I would also like to thank to the students of my co-supervisor, Alp and Senem for their technical support. I also wish to thank to my colleagues at work; Aysun, Asiye, Seda, Başak, Nurci, Ayşe for their support throughout this process.

I also thank to my dear cats Patiş and Miko for making the process easier.

Finally, my deepest gratitude to my family; to my mother Fatma Funda, to my father Hüseyin İhsan, to my sister Fulya and to my uncle Lütfü, to my aunt İnci and to my grandmothers Neziha and Nurefşan for their endless love and support in every moment of my life. And special thanks to my lovely husband Şafak for his patience, support and love.

This work is partially funded by Scientific and Technological Research Council of Türkiye under grant number TUBITAK 117Z561 and by funds from METU, BAP-07-02-2017-004-054.

TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

LIST OF ABBREVIATIONS

ABBREVIATIONS

AKT1	AKT Serine/Threonine Kinase 1
CCND1	CyclinD1
CDKN1A	Cyclin Dependent Kinase Inhibitor 1A (p21)
ER	Estrogen Receptor
ER+	Estrogen Receptor Positive
ER-	Estrogen Receptor Negative
FOXO1	Forkhead Box O1
GADD45A	Growth Arrest And DNA Damage Inducible Alpha
GO	Gene Ontology
IGF1R	Insulin Like Growth Factor 1 Receptor
KEGG	Kyoto Encyclopedia of Genes and Genomes
miRNA	MicroRNA
PI3K	Phosphoinositide 3-kinase
SAC	Spindle Attachment Checkpoint
TNBC	Triple Negative Breast Cancer

CHAPTER 1

INTRODUCTION

1.1 Phytoestrogens

Plant derived estrogens, which are referred as phytoestrogens have structural similarities to estrogen and are able to bind to estrogen receptors (ERs) (Harris et al., 2005). Phytoestrogens show anti-carcinogenic properties (Huang et al. 2013) and have been investigated for their growth inhibitory effects on breast cancer widely as drug candidates which have less side effects (Tanwar et al., 2021).

Based on their chemical structure, they are classified into several groups like flavanols, isoflavones, lignans, stilbenes, antraquinones. For example, Genistein is an isoflavone present in soybean and has anticancer activities in several types of cancer cells like leukemia, pancreatic cancer, ovarian cancer, cervical cancer through inducing cell cycle arrest, apoptosis and inhibiting proliferation (Bhat et al., 2021). Quercetin is a flavanol and found in foods like nuts, teas, vegetables. Quercetin repressed proliferation of several cancers like liver cancer, thyroid cancer and breast cancer and ovarian cancer (Vafadar et al., 2020). Resveratrol is a stilbene and is found in berries, pines, grapes. For many cancer cases like colorectal, liver, breast, skin cancer, it has various anti-carcinogenic effects from initiation to progression of cancer like DNA repair, inhibition of ROS, cell cycle arrest, apoptosis, inhibition of invasion and metastasis (Ko et al., 2017).

1.1.1 Emodin

Emodin (6-methyl-1,3,8-trihydroxyanthraquinone) as shown in Figure 1.1 belongs to anthraquinone class of phytoestrogens. Rheum ribes is one of the rhubarb species found in Türkiye (Fazeli, 2016; Tosun and Akyüz-Kızılay, 2003) and

Emodin is one of its compounds. It is used as laxatives in traditional Chinese medicine and has beneficial effects in many diseases including diuretic, anti-bacterial, anti-viral, anti-inflammation and anti-cancer effects (Shrimali et al., 2013).

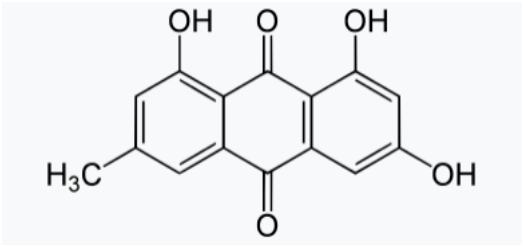

Figure 1.1 Emodin (6-methyl-1,3,8-trihydroxyanthraquinone).

Emodin shows its anti-carcinogenic potential in different cell signaling pathways through inhibition of cell proliferation (Huang et al., 2013), invasion, metastasis and apoptosis induction (Wei et al., 2013). Furthermore, it can inhibit the proliferation of cancer cells as a tyrosine kinase inhibitor (Jayasuriya et al., 1992; Zhang et al., 1998; Zhang et al., 1999).

In human tongue squamous cancer SCC-4 cells, Emodin induced apoptosis through reactive oxygen species and mitochondrial cytochrome c release dependent caspase cascade activation (Lin et al., 2009) and inhibited migration and invasion through inhibiting MMP-9 (Chen et al., 2010). In gastric carcinoma cells, Emodin induced the generation of reactive oxygen species and inhibited RhoA activation, which in turn sensitized cells to a form of programmed cell death called anoikis (Cai et al., 2008). In liver HepG2 cells, Emodin induced apoptosis and arrested cells in G1 phase (Yu et al., 2012). It also suppressed proliferation and induced apoptosis in colorectal cancer (Wang et al., 2018). In multiple myeloma cells, a combination therapy with carfilzomib, Emodin increased ROS formation, induced apoptosis (Hsu et al., 2022). In ovarian cells, Emodin inhibited growth and furthermore inhibited migration and invasion by inhibiting epithelial mesenchymal-transition (EMT) (Long et al., 2022).

Emodin shows its antitumor activity in both estrogen receptor positive (ER+) and estrogen receptor negative (ER-) breast cancer cells. According to the study of Huang et al., Emodin suppressed MCF-7 cell proliferation by decreasing both nuclear and cytosolic ERα protein levels and inhibiting ERα activation via proteasomal degradation. Furthermore, CCND1 protein expression, which is one of the ERα-regulated proteins, was also decreased by Emodin treatment. Furthermore, having ability to act as a tyrosine kinase inhibitor Emodin suppressed proliferation of ER- MDA-MB-453 cell, which is a HER-2 overexpressing cell suggesting Emodin could act both ER+ and ER- breast cancer cell lines (Huang et al., 2013). In addition, as it was previously shown, Emodin inhibited invasion of MDA-MB-231 cell line through suppressing AP-1 and NFKB (Huang et al., 2004).

Similarly, Sui et al. (2014) stated in their study that, Emodin inhibited growth of both ER+ MCF-7 and ER- MDA-MB-231 cells when induced with estrogen. The arrest was observed in MCF-7 cell cycle at G0/G1 phase, with induced apoptosis, decreased transcription and protein levels of ERα and consequently decreased expressions of ERα target genes, namely CCND1 and BCL2. Beside this genomic pathway, Emodin also inhibited MCF-7 non-genomic pathway by decreasing protein expressions of phosphorylated MAPK and AKT genes.

In another study, Emodin inhibited proliferation, induced apoptosis and further suppressed transcriptions of angiogenesis related genes in MCF-7 (Cheshmi et al., 2017). Again in other studies with MCF-7, Emodin showed growth inhibitory effects and apoptosis induction through regulation of apoptosis related gene expressions (Li et al., 2013a) and induced cellular senescence and apoptosis by increasing the effect of chemotherapy (Zu et al., 2018).

Emodin also inhibited proliferation, suppressed invasion and metastasis in TNBC (triple negative breast cancer) cell lines, which do not possess estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2. According to the study of Guo et al. (2013), Curcumin and Emodin synergistically inhibited the growth of MDA-MB-231 and MDA-MB-435 cells, their survival and invasion

through upregulation of miR-34a. Similarly, Emodin inhibited angiogenesis and metastasis (Ma et al., 2015) and migration and invasion (Sun et al., 2015) in both MDA-MB-231 cell line and MDA-MB-231 xenografts. In another study, Emodin inhibited proliferation and invasion of MDA-MB-231 and MDA-MB-453 cell lines and further suppressed tumor growth and inhibited lung and liver metastasis in mice (Song et al., 2018).

Most recent studies reported the anti-carcinogenic effects of Emodin in breast cancer cells. Emodin treatment decreased AKT1 protein levels and suppressed tumor growth in MCF-7 injected mice (Li et al., 2021). Another study reported that, Emodin inhibited the MCF-7 growth through activating aryl hydrocarbon receptor-CYP1A1 signaling pathway (Zhang et al. 2021). According to the study of Liu et al. (2020), Emodin suppressed EMT and cancer stem cell formation in breast cancer cell lines in which MCF-7 and MDA-MB-231 were included. Furthermore, Emodin administration into mice with breast cancer before surgery inhibited metastatic recurrence of breast cancer after the surgery. When Berberine and Emodin given together, it suppressed MCF-7 and MDA-MB-231 cell proliferation and induced cell cycle arrest and apoptosis through suppressing SIK3 induced mTOR-AKT signaling pathway (Ponnusamy et al., 2020). Similarly with Thymoquinone, Emodin induced apoptosis, inhibited cell migration in MCF-7 cells (Bhattacharjee et al., 2020).

The effect of Emodin with other drugs were also studied. Li et al. (2021), showed in their study, the increased effect of Doxorubicin in breast cancer cells by decreasing the resistance of cells. The growth of both MCF-7 and MDA-MB-231 cells were also inhibited but more significantly in MCF-7. However, Kim et al. (2019) reported an opposing effect, showing the decreased effect of Tamoxifen in MCF-7 cells.

1.2 Breast Cancer

Cancer which is characterized by uncontrolled growth and spread of cells (National Cancer Institute, 2021) is the leading cause of death worldwide according to "Global Cancer Statistics 2020" and by 2040 death percentages are expected to increase by 47% with respect to 2020. Furthermore, according to another study, female breast cancer has been reported to be the most commonly diagnosed cancer and the fifth leading cause of cancer deaths in women (Sung et al., 2021).

Breast cancer tumors are biologically variable. They are distinguished by their genetic variability, clinical behaviors, responses to treatment and risk factors (Tamimi et al., 2012; Yang et al., 2011). Obesity, physical inactivity, menopausal hormone therapy, environmental risk factors, smoking, alcohol consumption are factors related with breast cancer. For each individual treatment, radiotherapy, chemotherapy, hormonal therapy combinations are coordinately applied as systemic therapy (American Cancer Society, 2015).

In 2000, 6 hallmarks of breast cancer were determined as self-sufficiency in growth signals, insensitivity to antigrowth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis (Hanahan and Weinberg, 2000). Since estrogen has roles in breast cancer initiation by stimulating cell growth through estrogen receptor (ER) (Russo and Russo, 2006), targeting ER is an effective way for treatment (Burstein, 2020).

Estrogen signaling pathway primarily proceeds in the nucleus. Estrogen diffuses through the membrane and cytosol to nucleus where it binds to ER leading to transcription. Majority of ER resides in the nucleus but a small portion localized at or near cell membrane. The signaling pathway is also mediated by other growth factor receptors. In order to repress ER signaling, Tamoxifen, a selective estrogen receptor modulator, is largely used for ER inhibition. However, Fulvestran is found to be more effective than Tamoxifen which degrades the ER (Bai and Gust, 2009). Nevertheless, besides having side effects, these treatments could be inadequate due

to other growth receptors and signal transduction pathways and can lead to drug resistance. Another challenge to overcome is the highly invasive tumors, which do not have ER due to the lesser amount of therapy alternatives (Feng et al., 2018; Massarweh and Schiff, 2006). Therefore, the alternative and combinational treatment strategies have been under study.

1.3 microRNAs and Their Functions

microRNAs (miRNAs) are small non protein coding RNAs composed of approximately 22 nucleotides and have important roles in gene regulation (Bartel, 2004). They are first discovered in 1993 when it was seen that C. elegans lin-4 gene which has roles in developmental events was found to regulate LIN-14 protein levels by being transcribed but not translated (Lee et al., 1993; Wightman et al., 1993). miRNAs show their mechanism of action and regulate gene expressions through their target mRNAs. miRNAs consequently inhibit translation or degrade their target mRNAs (Perron and Provost, 2008). One miRNA can target mRNAs of many genes and similarly mRNA of gene can be regulated by more than one miRNA due to imperfect matching (Dong et al., 2013; Xu et al., 2020). Furthermore, it is estimated that over 60% of human protein coding mRNA have miRNA binding parts (Friedman et al., 2009). According to the latest version of microRNA database (miRBase v.22) 1917 annotated hairpin precursors, and 2654 mature sequences have been identified in human genome (Kozomara et al., 2019).

As summarized in Figure 1.2, initially, miRNAs are transcribed as primary miRNAs having length of 1000-3000 bases. These miRNAs are then cleaved in the nucleus by enzyme Drosha to precursor miRNAs, which are 70-100 nucleotide long, stem-loop structures. These precursor miRNAs are then exported to cytoplasm. In the cytoplasm, with a second cleavage process by enzyme Dicer microRNA duplex (miRNA:miRNA*) forms comprised of mature miRNA and passenger miRNA represented with an asterisk. After strand separation, passenger miRNA is often degraded or plays a functional role in miRNA regulation process.

Whereas, mature miRNA assembles into RNA-induced silencing complex (RISC) to be able to base pair with target mRNA and consequently suppress mRNAs. Complementarity between miRNA and the target mRNA sequences determines this suppression to occur either through translation inhibition or mRNA degradation. Furthermore, it is indicated that most animal miRNAs pair imperfectly with their target mRNAs, thus inhibition mechanism can cover multiple mRNAs (Perron and Provost, 2008; MacFarlane and Murphy, 2010; Dong et al., 2013; Di Leva et al., 2014).

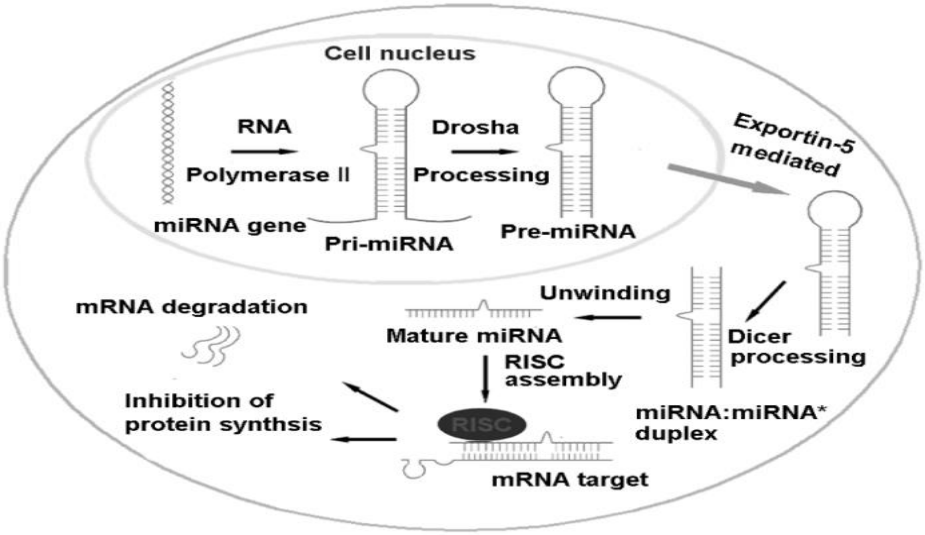

Figure 1.2 Biogenesis and function of miRNA (Dong et al., 2013).

miRNAs have a wide variety of regulatory roles in biological processes. They mediate early cellular differentiation and cell proliferation in embryonic stem cells (Wang et al., 2009), regulate apoptotic signaling and fat metabolism (Xu et al., 2003), developmental timing (Ambros, 2003), regulate immune system (Lodish et al., 2008; O'Connell et al., 2010), insulin release (Poy et al., 2004), neurotransmitter synthesis (Greco and Rameshwar, 2007), circadian rhythm (Cheng et al., 2007) and also regulate photoreceptors (Pawlick et al., 2021).

Due to their important regulatory roles in wide range of systems, their dysregulation lead to diseases like bone diseases (Moore and Xiao, 2013),

neurodegenerative diseases (Sharma and Lu, 2018), cardiovascular diseases (Zhou et al., 2018), autoimmune diseases (Long et al., 2018), infectious diseases (Tribolet et al., 2020) and cancer (Tan et al., 2018).

1.3.1 miRNAs and Cancer

Since miRNAs have important regulatory roles in cell cycle, differentiation, stress response, apoptosis, inflammation and migration; with their deregulation normal cells can be converted into cancer cells progressively (Di Leva et al., 2014). There are many studies on miRNA regulation in different cancer types as lung cancer (Wu et al., 2019), colon cancer (Zhu et al., 2020), lymphoma (Jørgensen et al., 2020), glioblastoma (Buruiană et al., 2020) and breast cancer (Loh et al., 2019). Different breast cancer subtypes showed discrete miRNA expression profiles (Singh and Mo, 2013; Denkiewicz et al., 2019). Loh et al. (2019), in their study, reported the involvement of the regulatory miRNAs in hallmarks of breast cancer.

miRNAs can act both as tumor suppressors and oncogenes depending on circumstances (Stahlhut Espinosa and Slack, 2006; MacFarlane and Murphy, 2010); further their expressions can be regulated by tumor suppressors and oncogenes which are transcription factors (Lee and Dutta, 2009). Previously it was also shown that, miRNAs can bind and regulate the other miRNAs and subsequently regulate mRNA expressions. This interaction with other miRNAs was found to regulate the cancer pathways (Hill and Tran, 2018).

miRNA expression profiles could be used as biomarkers for diagnosis (Condrat et al., 2020), prognosis (Zografos et al., 2019), metastasis (Papadaki et al., 2019), surgical treatment response (Filipów and Łaczmański, 2019), drug response and personalized medicine (Latini et al., 2019), diagnosis and therapy of genetic disorders (Finotti et al., 2019). There are numerous of studies on miRNAs for breast cancer diagnosis (Adhami et al., 2018; Lagendijk et al., 2018) or treatment (Grimaldi et al., 2021; Normann et al., 2021).

The difference between miRNA profiles of normal and tumor tissues revealed the therapy approach of bringing the expression levels of miRNAs back to normal levels (Di Leva et al., 2014; Loh et al., 2019). In addition, different breast cancer types having distinct miRNA profiles can be used for diagnosis and for personalized treatment (Di Leva and Croce 2013, Liu, 2012; Kurozumi et al., 2017). For reaching normal miRNA levels, there are some therapy approaches. In miRNA inhibition therapy; miRNA inhibitor, miRNA antagomir, miRNA mask and miRNA sponge are used for suppression of miRNA actions. In miRNA replacement therapy; miRNA mimics, miRNA agomir, miRNA precursor and miRNA-expressing plasmids are used to enhance miRNA actions. Furthermore to increase the application efficiency, appropriate miRNA delivery systems are important for the stability of miRNAs in vivo (Fu et al., 2019).

It was shown that, miRNA levels can be regulated by natural compounds through altering miRNA expressions and consequently affecting their target genes and downstream pathways in healthy people with dietary intake (Ferrero et al., 2021) as well as in cancer cases (Lin et al., 2017). Natural compounds show their effects on miRNAs through modulating their transcription, their processing or maturation and indirectly through regulating long noncoding RNAs which act as sponges to inhibit miRNAs (Zhang et al., 2020). Resveratrol (Wang et al., 2015), Curcumin (Saini et al., 2011), Genistein (Xu et al., 2013) are some of the natural compounds, that have been investigated for their anti-carcinogenic effects through miRNA regulations.

1.3.2 Emodin and miRNA Regulation

Studies on miRNA regulation of phytoestrogen Emodin in cancer cases have been accelerated recently but are still limited. Emodin, via miRNA regulations, increased erythroid differentiation in leukemia (Ma et al., 2013); inhibited angiogenesis (Lin et al., 2015), EMT and invasion (Li et al., 2018) in pancreatic cancer; induced apoptosis in colorectal cancer (Ceylan, 2018); suppressed proliferation in lung cancer (Ren et al., 2016); and synergistically with Curcumin,

suppressed proliferation and invasion in MDA-MB-231 and MDA-MB-435 TNBC cell lines (Guo et al., 2013).

1.3.3 miRNA Detection Assays and Microarray

Having high specificity, Northern Blot in which molecules are identified according to their molecular weight via running under electrical field force, is the golden standard for the known miRNA detection. Further, reverse transcription-quantitative polymerase chain reaction (RT-qPCR), in which miRNAs are reversely transcribed into cDNAs and these templates are used for real-time fluorescence qPCR, is another widely established miRNA detection technique having high sensitivity and specificity and especially used for validation of other methods for known miRNAs (Ouyang et al., 2019). In addition to these, screening methods are used in order to determine natural products which have regulatory effects on miRNA expressions (Zhang et al. 2020). One screening approach is 'luciferase reporter assay' in which complementary sequence of the interested miRNA is introduced into the downstream of luciferase reporter gene in a reporter plasmid. While the presence of miRNA suppresses luciferase signal, the presence of miRNA inhibitor increases the signal (Connelly et al., 2012). Fluorescence-polarization-based assay is another method in which presence of interested natural agents decrease miRNA-Ago2 (a RISC component) complex and as a result fluorescence polarization signal declines (Tan et al., 2012). Furthermore, in silico high-throughput screening is used to predict miRNA regulatory molecules. In their study, using three-dimensional structure of miRNA processing enzyme Dicer, Shi et al. (2013), identified a miRNA inhibitor molecule which blocked Dicer binding site on its target miRNA, thus ceased miRNA maturation. In the study, EMT and tumor growth suppression effects of the identified compound also were shown in vitro and in vivo. Another alternative method for miRNA detection is next generation high-throughput sequencing in which the output of sequencing experiment is aligned to the reference genome of the sequenced organism, as a

result known and novel miRNAs are identified and quantified (Motameny et al., 2010; Ferrero et al., 2021).

Microarray is another high-throughput technology for miRNA detection. In this assay, biotin labeled cDNA targets of interested RNAs hybridize with sense strand of miRNA gene specific oligonucleotide probes which have previously printed on miRNA chips. Hybridized probes are stained and resultant signal is detected by laser scanning. Measure of signal intensities of hybridizations corresponds to low and high expression of miRNAs. Identified miRNAs are finally confirmed with real time PCR experiments (Liu et al., 2008). Microarray approach has been used for miRNA analyzes in different organisms (Takahashi et al., 2021; Shriram et al., 2016). Also, in breast cancer tissues, microarray data has been used for miRNA identification for diagnosis (Hamam et al., 2016), treatment (Liang et al., 2018), and natural compound studies (Cansaran-Duman et al., 2021).

Since mRNAs can be regulated with multiple miRNAs and one miRNA can regulate more than one mRNA (Xu et al., 2020), following microarray performances, it is important to integrate miRNA and mRNA profiles rather than just focusing on individual miRNA-mRNA interactions in order to have comprehensive and meaningful results and further to understand their functions. These integration analysis results are achieved with network systems biology approach (Guzzi et al., 2015; Licursi et al., 2019) and has been used extensively in different tissues like cardiac muscle (Yao et al., 2019) or brain (Genovesi et al., 2011) and also in breast tissues (Enerly et al., 2011; Evangelista et al., 2021) in order to reveal miRNA-mRNA interactions.

1.4 Scope of the Study

Breast cancer is one of the malignant types of cancer mostly seen in women. Since estrogen plays important roles in breast cancer initiation and progression, estrogen receptor is an important therapy target. While anti-estrogenic agents are successful

in treatment of estrogen receptor positive cells, different treatment approaches are needed for estrogen receptor negative cells.

Phytoestrogen Emodin has shown its anti-carcinogenic affects in a variety of different cancers including breast cancer. In addition, Emodin has shown its anti-tumorigenic activities in both estrogen receptor positive and negative breast cancer cells, which makes it a promising compound for therapy investigations.

miRNAs regulate important biological processes in normal cells and also, they have roles in different stages of cancer processes. miRNAs show their mechanism of action by degrading target mRNAs or suppressing translation. Currently, they are being investigated for cancer diagnosis, classification, prognosis and treatment and it is a popular area to investigate the initiation and progression of cancer and advance therapeutic treatments.

Since one miRNA can suppresses more than one mRNA and one mRNA can be the target of more than one miRNA, it is important to study miRNA and mRNA profiles together in order to understand their functions. Network biology approach, in which computational tools, bioinformatics is used to interpret huge amount of data from different sources, in order to understand the molecular basis of such kind of interactions from integrated analysis of the information collected will be more accurate than a separate study of any data source. Therefore it is important to analyze miRNA and mRNA profiles together using bioinformatics methods.

The scope of this study is to investigate the molecular mechanism of anti-tumorigenic effects of Emodin in both ER+ MCF-7 and ER- MDA-MB-231 cell lines by analyzing microarray-based mRNA and miRNA expression profiles which were further integrated by using network biology approach. As long as miRNA-target interactions of Emodin in breast cancer cells are well understood, better treatment strategies can be achieved for both ER+ and ER- breast cancer cell lines.

Some of the results of this study were published in 2022 in Nutrition and Cancer 74(2):592-604. doi: 10.1080/01635581.2021.1889622.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Cell Lines

MCF-7 and MDA-MB-231 breast cancer cell lines were obtained previously from ATCC.

2.1.2 RNA Isolation

Total RNA was isolated by using 'Total RNA isolation solution (RiboEx, GeneAll®)'. RNA concentration was determined by using spectrophotometer (BioDrop, U.K.). For RNA integrity analysis, 2100 Bioanalyzer (Agilent) and RNA 6000 Nano Kit (Agilent) compatible with this device were used.

2.1.3 miRNA Microarray Analysis

The microarray studies were carried out with GeneChip® miRNA 4.0 (Applied Biosystems, USA) chips. FlashTag™ HSR Biotin RNA Labeling Kit (Applied Biosystems, USA) was used for poly A tailing and biotin labeling. Wash Buffer A (Applied Biosystems, USA), Wash Buffer B (Applied Biosystems, USA) solutions and GeneChip® Hybridization, Wash, and Stain Kit (Applied Biosystems, USA) were used for the hybridization, washing and dyeing steps. Hybridization of the chips was carried out in the GeneChip® Hybridization Oven 640 (Applied Biosystems, USA). Washing and dyeing steps were performed in the GeneChip® Fluidics Station 450 (Applied Biosystems, USA) wash station. Scanning of the

chips was performed with the GeneChip® Scanner 3000 (Applied Biosystems, USA).

2.1.4 mRNA Microarray Analysis

mRNA microarray data which was obtained from previous MS thesis (Sakallı, 2010) was reanalyzed and used for the integration analysis. The data is available in EBI's ArrayExpress database with an accession number of E-MTAB-8310, and is composed of Emodin treated and Emodin non-treated sample data for MCF-7 and MDA-MB-231 cell lines with two biological replicates.

2.2 Methods

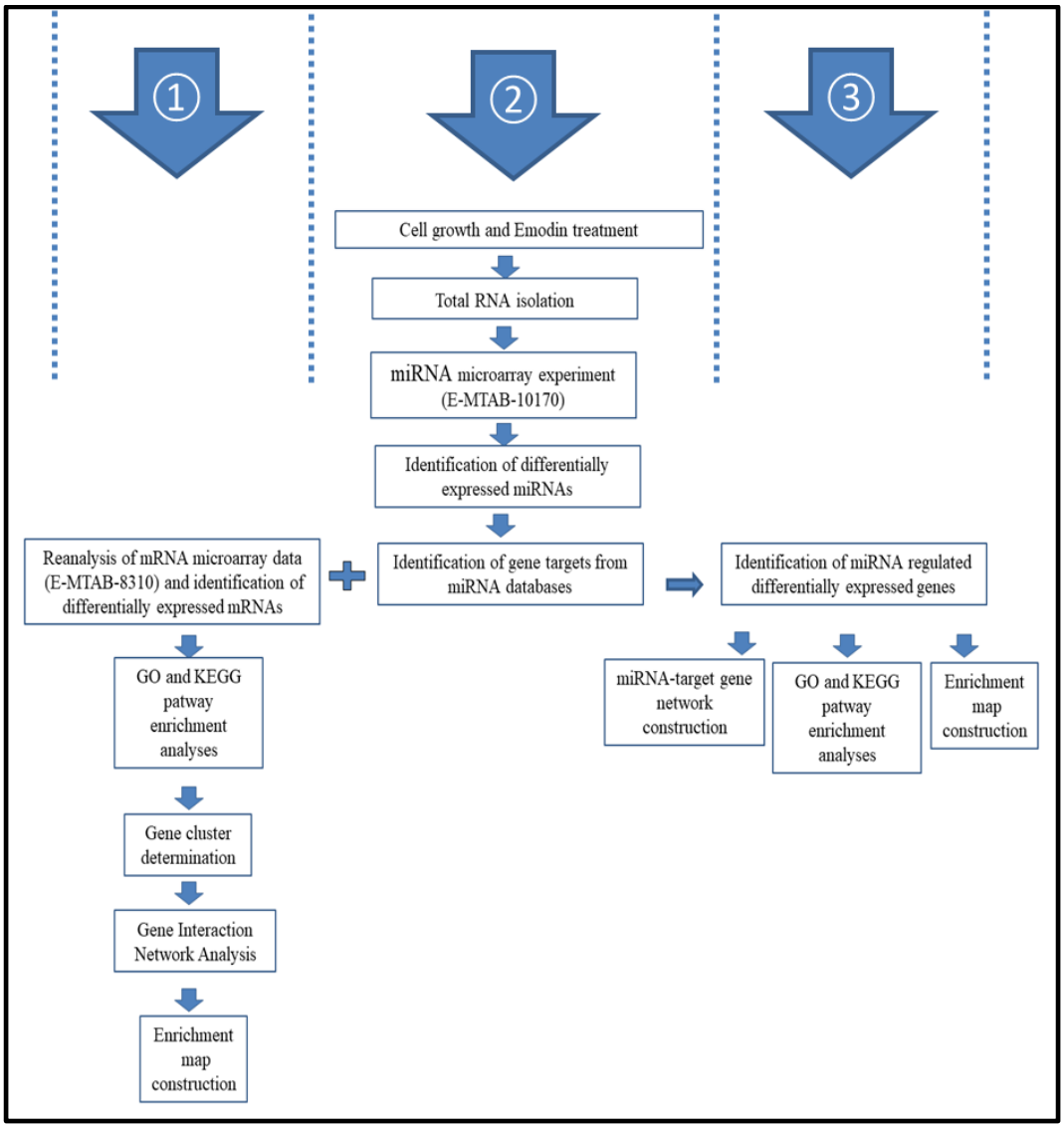

Figure 2.1 General workflow of the study which consists of 3 stages.

2.2.1 Cell Culture

ER+ MCF-7 and ER- MDA-MB-231 monolayer breast cancer cell lines which were obtained previously from ATCC (American Type Culture Collection) were grown in complete medium containing RPMI-1640 cell culture medium with phenol red (Biochrom AG) containing 10% heat inactivated serum fetal bovine

serum (Biochrom AG) and 1% penicillin streptomycin solution (Biowest) and incubated at 37°C in a 95% humidified atmosphere of 5% CO2. Cell passaging, freezing and thawing were performed as described previously (Sakallı, 2010).

2.2.2 Emodin Treatment

After the cells were grown, medium was discarded and cells were washed with Dulbecco's phosphate buffered saline (Biowest) in order to get rid of medium, and in order to detach cells 1-2ml Trypsin/EDTA (Biochrom AG) was added and incubated at 37 ºC for 2-5 minutes. After the cells were detached, trypsinization was stopped by complete medium. 'Tryphan Blue' dye, which is dependent on the metabolic activities of living cells, was used to determine the cell number. The number of viable cells was determined by visualizing the dead cells in blue, while the living cells disposed the dye out and appeared bright. Cell counts were performed using the cell counter device (Invitrogen Thermo Fisher Scientific Life Technologies Countess II FL Cell Counter). For cell counting, the number of cells per ml was determined by adding 10 µl of the cell suspension (prepared by mixing 10µl cells + 10µl Tryphan Blue dye) into a chamber of "Invitrogen Slides". After determining the cell number, detached cells were diluted to be placed into 6-well plates as 500,000 cells per well. After 24 hours, cells were treated with Emodin.

For Emodin treatment, Emodin (1,3,8-trihydroxy-6-methylanthraquinone) dissolved in DMSO (Applichem) was diluted in RPMI-1640 medium containing 10% FBS without phenol red, to have a final Emodin concentration of 20 µg/ml containing 2% DMSO. The growth medium of the cells was discarded from wells and wells were washed with PBS. Emodin with a final concentration of 10µg/ml was added to the wells by adding 1 ml of RPMI-1640 complete growth medium and 1 ml of 20µg/ml Emodin to the washed wells. For control wells, 1 ml of RPMI-1640 complete growth medium and 1 ml RPMI-1640 medium containing 10% FBS without phenol red containing 2% DMSO concentration were added to the washed

wells as a result, control cells were incubated in a medium with a final concentration of 1% DMSO. Incubations were lasted for 48 hours.

An experimental setup was created with one Emodin and one Control applications for both cell lines, together with one biological replica for each application.

2.2.3 Total RNA Isolation

Total RNA was isolated by using Trizol (RiboExTM, GeneAll®). One ml of RiboExTM was added to each well of 6 well plates and pipetted several times. After incubation for 5 minutes at room temperature, 0.2 ml of chloroform was added into the tubes. Tubes were inverted vigorously for 15 seconds and then incubated for 2 more minutes at room temperature. Then tubes were centrifuged at 12,000g for 15 minutes at 4°C and the aqueous phase was transferred to a clean tube. 0.5 ml of isopropyl alcohol was added and the tubes were inverted 3-5 times. The samples were incubated for 10 minutes at room temperature. Then tubes were centrifuged at 12,000g for 10 minutes at 4°C and the supernatant was discarded. One ml of 75% ethanol was added onto the pellet and centrifuged at 7,500g for 5 minutes at 4°C. The supernatant was discarded and the RNA pellet was allowed to dry for 5 minutes. After the pellet was dissolved in 30-50 µl of water, it was incubated at 56°C for 10 minutes. RNA concentration was determined using by using BioDrop and the samples were stored at -80°C.

RNA concentration was calculated as nanograms (ng) in 1 µl. Nucleic acids (DNA and RNA) give absorbance at 260 nm, proteins at 280 nm, and organic compounds like carbohydrates give absorbance at 230 nm. A260/A280 and A260/A230 ratios show the purity of RNA. A260/A280 ratio should be ~2 and A260/A230 ratio should between 2.0 and 2.2 for RNA purity.

RNA quality was determined using Agilent 2100 Bioanalyzer (Agilent Technologies, U.S.A.) at Ankara University Biotechnology Institute Central Laboratory. The assay was performed as described previously (Sakallı, 2010).

2.2.4 Preparation of Microarrays

Microarray experiment was performed using the facilities of Ankara University Biotechnology Institute Central Laboratory. miRNA profiles of MCF-7 and MDA-MB-231 cell lines were obtained by using 8 arrays containing 10µg/ml Emodin and 1% DMSO Control treatments for each cell line with one biological replica for each application. The data is available in EBI's ArrayExpress database with an accession number of E-MTAB-10170. miRNA microarray procedure overview is represented in Figure 2.2.

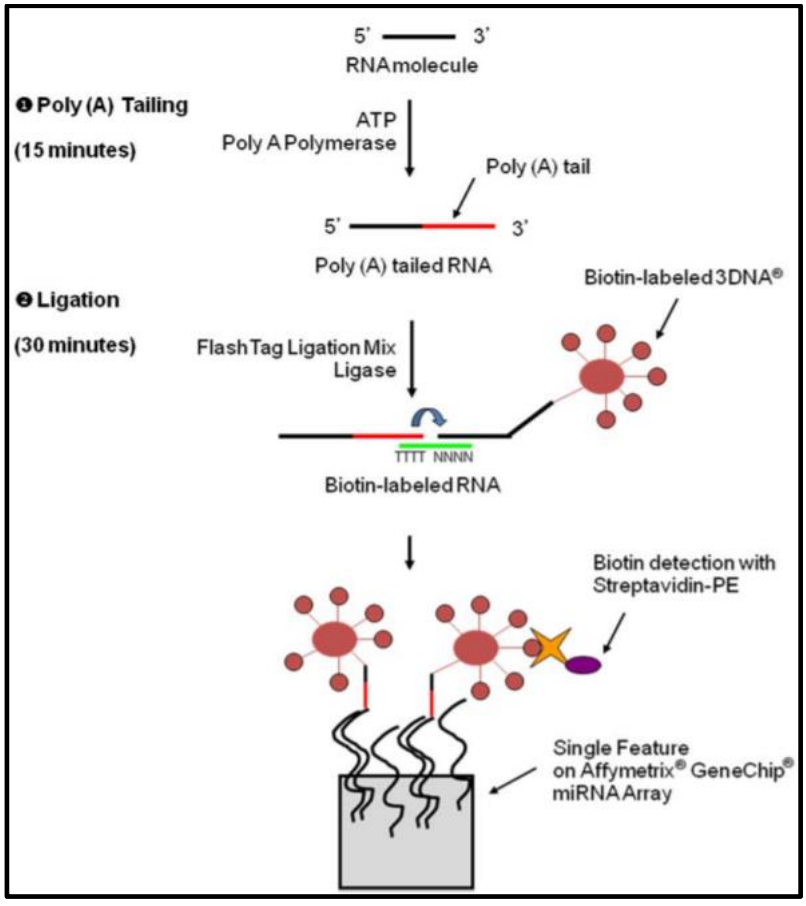

Figure 2.2 Microarray procedure overview.

(https://media.affymetrix.com/support/downloads/manuals/mirna_flashtag_manual.pdf)

2.2.4.1 Poly(A) Tailing

For Poly (A) tailing, first of all, 2 µl of RNA Spike Control Oligos were added into 500 ng/8 µl total RNA sample on ice to have a final volume of 10 µl. ATP mix was diluted as 1:500 in 1 mM Tris. Poly A Tailing Master Mix was prepared for single reaction by adding 1.5 µL 10X Reaction Buffer, 1.5 µL 25mM MnCl2, 1.0 µL diluted ATP Mix, 1.0 µL PAP Enzyme. Five µL of prepared Master Mix was added onto 10 µL RNA/Spike Control Oligos for a volume of 15 µl. Tubes were incubated at 37°C for 15 minutes.

2.2.4.2 Biotin Ligation

Fifteen µL of tailed RNA was placed on ice. Four µL 5X FlashTag Biotin HSR Ligation Mix was added to each sample. Two µL of T4 DNA Ligase was added to each sample and incubated at 25°C for 30 minutes for biotin ligation. The reaction was stopped by addition 2.5 µL HSR Stop Solution.

2.2.4.3 Hybridization

20X Eukaryotic Hybridization Controls (bioB, bioC, bioD, cre from GeneChip® Eukaryotic Hybridization Control Kit) were heated for 5 minutes at 65°C. Hybridization Master Mix was prepared by addition the following components as in order, 66 µL 2X Hybridization Mix, 19.2 µL 27.5% Formamide, 12.8 µL DMSO, 6.6 µL 20X Hybridization Controls, 2.2 µL Control Oligo B2, 3nM, 3.7 µL Nuclease-free Water. 110.5 µL Hybridization Master Mix was added to 21.5 µL of biotin-labeled sample and incubated at 99°C for 5 minutes, then 45°C for 5 minutes. 130 µL (100 format array) was injected into an array from an opening called septa. Both septa were closed to minimize evaporation and/or prevent leaks. Arrays were placed into hybridization oven trays. The trays were loaded into the hybridization oven. The arrays were incubated at 48°C and 60 rpm for 18 hours.

2.2.4.4 Wash, Staining and Scanning

After 18 hours of hybridization, the arrays were removed from the oven, septa were opened. The hybridization cocktail was extracted and each array was completely filled with Array Holding Buffer. The arrays were equilibrated to room temperature before washing and staining. Then arrays were placed into the fluidics station. FS450_0002 Fluidics Protocol was chosen for washing and staining.

After these, arrays were scanned and the data were obtained in .DAT scan format. Proper hybridization was confirmed with opening data files with AGCC program in AGCC 4.0 (Affymetrix® GeneChip Command & Console Software). Furthermore, Hybridization Controls and array name were also controlled using .DAT file to confirm proper hybridization, wash, staining and scanning steps. Later the data was obtained in .CEL file which was the conversion of .DAT file into numeric values.

2.2.4.4.1 Microarray Data Normalization

Microarray miRNA expression data was normalized in Affymetrix® Expression Console (v1.4.0) using RMA (Robust Multiarray Average) method. This normalization provides elimination of the differences due to non-specific binding, washing and staining step or background noise and reveals real differences on biological data. RMA normalization method is a package that includes background correction, quantile normalization, and conversion of data to base 2 logarithms.

The data normalized on the Expression Console was pulled from the program in the 'Tab Delimited Text' format and loaded to the BRB-ArrayTools (Simon et al., 2007) as microarray data.

2.2.5 Identification of Differentially Expressed Genes and miRNAs

Statistical analyses were performed with BRB-Array Tools v4.4 for gene expression profile analysis and with BRB-Array Tools Version 4.6.0-Stable for

miRNA expression profile analysis. Differentially expressed genes and miRNAs were revealed through univariate two sample t-test with random variance model using Class Comparison command of BRB-Array Tools Program. Nominal significance level of each univariate test was adjusted to 0.001 and 0.05 for differential expressions of genes and miRNAs respectively. For both analyses cutoff value was determined as 2 fold.

After statistical analyses were completed for differentially expressed genes, multiple gene symbols corresponding to the same ENTREZ Gene ID were removed from duplications and final gene number for differentially expressed genes was obtained.

For differentially expressed miRNAs, hierarchical clusters were determined by Cluster 3.0 program using correlation (uncentered) similarity metric and average linkage clustering method. For their visualization, Java TreeView Version 1.2.0 program (Saldanha, 2004) was used.

2.2.6 Target Gene Determination of Differentially Expressed miRNAs from Databases

In order to increase reliability, validated gene target databases were used together with predicted gene target databases. Further to increase the reliability even more, predicted gene targets of TargetScan (Agarwal et al., 2015) and miRDB (Chen and Wang, 2020) databases were intersected and validated gene targets of miRTarBase (Hsu, et al., 2011) and DIANA-TarBase v8 (Karagkouni et al., 2018) databases were added. For each differentially expressed miRNA, these miRNA-gene target sets were generated (Figure 2.3).

2.2.7 miRNA-Regulated Gene Determination for Integrated Analysis

After determining miRNA-gene targets from databases, ‘miRNA-regulated genes’ were determined by intersecting miRNA-gene targets with differentially expressed genes identified previously. These miRNA-regulated genes were further used for integrated analysis. Venny 2.1.0 [Oliveros, J.C. (2007-2015) Venny. An interactive tool for comparing lists with Venn's diagrams. https://bioinfogp.cnb.csic.es/tools/venny/index.html] was used for determination of intersected clusters. miRNAs show their action mechanism through suppressing gene expressions. Therefore, for the upregulated miRNAs, the downregulated genes were selected as potential targets and for the downregulated miRNAs, the upregulated genes were selected as potential targets (Figure 2.3).

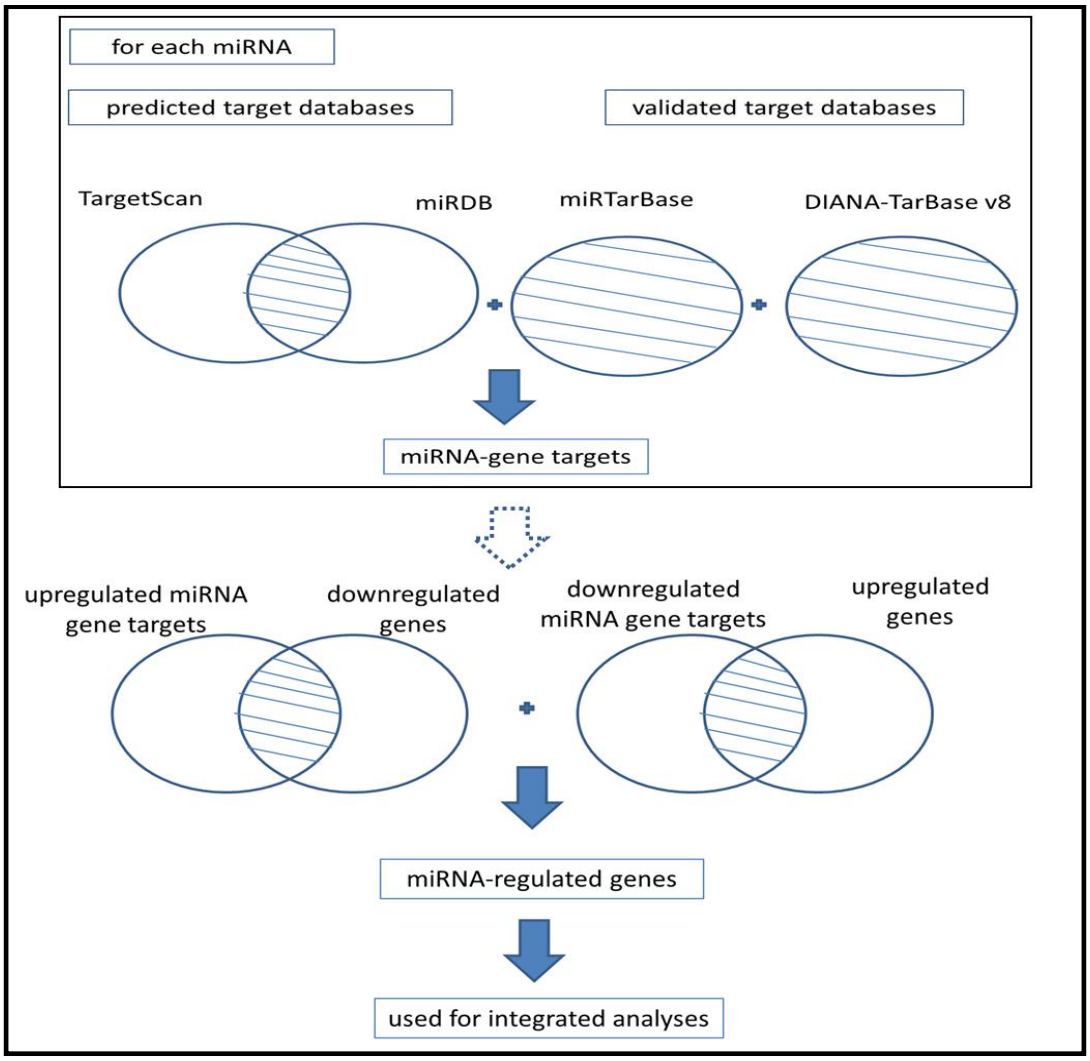

Figure 2.3 Determination of miRNA-gene targets and miRNA-regulated genes.

miRNA and target gene interaction networks were constructed with Cytoscape v.3.8.2 (Shannon et al., 2003). In cases one miRNA-gene pair had targets from both validated and predicted target databases, validated miRNA-gene relations were represented in the networks.

2.2.8 Functional Enrichment Analysis

Both for differentially expressed genes and miRNA-regulated genes; Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG)

functional pathway enrichment analyses were performed using g:Profiler version e98_eg45_p14_ce5b097 (Raudvere et al., 2019) with g:SCS multiple testing correction method applying significance threshold of 0.05 and choosing 'GO cellular component', 'GO biological function', 'GO molecular process' and KEGG biological pathway data sources.

2.2.9 Gene Cluster Determination

Venny 2.1.0 was used to determine genes that are regulated in more than one pathway by intersecting gene sets of breast cancer related pathways with one another. After determining the genes that are regulated by multiple pathways, they were studied together with literature and finally a gene cluster was generated for each cell line.

2.2.10 Gene Interaction Network Analysis

The interactions of the genes within gene clusters were constructed by using GeneMANIA (Warde-Farley et al., 2010) tool (latest version accessed on June 2023), which predicts the function of gene sets, through automatically selected weighting method and STRING v11 (Szklarczyk et al., 2019) tool, which studies protein-protein interactions, by using defined defaults. Layouts of the nodes were adjusted according to the literature studies.

Following miRNA analysis, gene interaction network was reconstructed for MCF-7 by using GeneMANIA 3.5.2 application of Cytoscape v.3.8.2 through automatically selected weighting method with addition of related miRNAs.

2.2.11 Visualization of Enrichment Analysis Results

Enrichment maps were constructed for both differentially expressed genes and miRNA-regulated genes in order to represent overlaps among results of enrichment

analysis and their relationship with each other. EnrichmentMap software 3.2.1 (Merico et al., 2010) of Cytoscape v.3.7.2 was used for differentially expressed genes and EnrichmentMap software 3.3.2 of Cytoscape v.3.8.2 was used for miRNA-regulated genes. For each gene set, the protocol by (Reimand et al., 2019) was followed. In this protocol, enrichment analysis results composed of KEGG biological pathway and GO biological function data were downloaded as GEM format. In addition, GMT file containing all the gene sets in data sources were also loaded to the software.

CHAPTER 3

RESULTS

As it is shown in workflow chart of study in Figure 2.1, there were three stages of the study. In the first stage, mRNA microarray data of Emodin treated MCF-7 and MDA-MB-231 cells, which were obtained previously, were reanalyzed. Their differentially expressed genes were identified and further analyzed. In the second stage, miRNA microarrays of Emodin treated MCF-7 and MDA-MB-231 cells were prepared; differentially expressed miRNAs were identified and their gene targets were determined from databases. In the third stage, mRNA and miRNA results obtained in previous stages were integrated and further analyzed.

3.1 Identification of Differentially Expressed MCF-7 and MDA-MB-231 Genes upon Emodin Treatment and Their Analysis

3.1.1 Differentially Expressed Genes of MCF-7 and MDA-MB-231 Cell Lines

In order to see the effect of Emodin on the expression of genes, differentially expressed genes were determined through univariate two sample t-test with a p value of 0.001 and a cutoff value of 2 fold. After this, unknown probes were subtracted and probe numbers were reduced to one gene symbol representing ENTREZ Gene ID. As a result, 1300 genes were found to be differentially expressed in MCF-7 cells. Among them, 514 genes were upregulated and 786 genes were downregulated. In MDA-MB-231 cells, 283 genes were differentially expressed upon Emodin treatment, in which 51 genes were upregulated and 232 genes were downregulated. Top ten most significant results are given in Table 3.1 and Table 3.2 for MCF-7 and MDA-MB-231 cell lines respectively. All the other

results were also given in Supplementary Table S1 and Supplementary Table S2 in Sakalli-Tecim et al. (2022) for MCF-7 and MDA-MB-231 cell lines respectively.

Table 3.1 Differentially regulated MCF-7 genes upon Emodin treatment

#	UP Regulated MCF-7 Gene Symbols	Gene Names	Probe Set	Fold Change	FDR Corrected pvalue
1	MAFF	v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog F	36711_at	37,04	1,26E-04
2	FAM129A	family with sequence similarity 129, member A	217966_s_at	29,41	< E-07
3	GEM	GTP binding protein overexpressed in skeletal muscle	204472_at	25,00	5,60E-05
4	ATF3	activating transcription factor 3	202672_s_at	25,00	< E-07
5	CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1	205749_at	21,28	< E-07
6	DDIT3	DNA-damage-inducible transcript 3	209383_at	18,52	< E-07
7	NUPR1	nuclear protein, transcriptional regulator, 1	209230_s_at	17,54	< E-07
8	GDF15	growth differentiation factor 15	221577_x_at	17,24	< E-07
9	CLGN	calmegin	205830_at	13,89	1,45E-04
10	MAP1B	microtubule-associated protein 1B	226084_at	12,99	5,60E-05
#	**DOWN Regulated MCF-7 Gene Symbols**	**Gene Names**	**Probe Set**	**Fold Change**	**FDR Corrected pvalue**
1	UHRF1	ubiquitin-like with PHD and ring finger domains 1	225655_at	30,61	1,18E-04
2	EHF	ets homologous factor	225645_at	21,23	5,60E-05
3	TFPI	tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)	213258_at	20,71	7,05E-05
4	RRM2	ribonucleotide reductase M2	201890_at	19,23	1,26E-04
5	ABAT	4-aminobutyrate aminotransferase	209459_s_at	13,85	2,35E-04
6	CDCA7	cell division cycle associated 7	224428_s_at	10,96	1,41E-04
7	MCM10	minichromosome maintenance complex component 10	220651_s_at	10,75	7,05E-05
8	DTL	denticleless E3 ubiquitin protein ligase homolog (Drosophila)	218585_s_at	10,43	2,57E-04
9	CCNE2	cyclin E2	211814_s_at	10,33	1,34E-04
10	GINS2	GINS complex subunit 2 (Psf2 homolog)	221521_s_at	10,11	8,60E-05

Table 3.2 Differentially regulated MDA-MB-231 genes upon Emodin treatment

#	UP Regulated MDA-MB-231 Gene Symbols	Gene Names	Probe Set	Fold Change	FDR Corrected pvalue
1	HSPA6	heat shock 70kDa protein 6 (HSP70B')	213418_at	11,36	< E-07
2	IL24	interleukin 24	206569_at	5,56	2,74E-04
3	HSPA1A	heat shock protein family A (Hsp70) member 1A(HSPA1A)	200799_at	5,00	2,74E-04
4	HMOX1	heme oxygenase (decycling) 1	203665_at	3,85	5,04E-04
5	GDF15	growth differentiation factor 15	221577_x_at	3,57	5,04E-04
6	KYNU	kynureninase	217388_s_at	3,45	6,62E-04
7	SQSTM1	sequestosome 1	213112_s_at	3,13	5,28E-04
8	NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	223218_s_at	3,03	6,00E-04
8	CXCL8	chemokine (C-X-C motif) ligand 8	202859_x_at	3,03	5,57E-04
8	PTGES	prostaglandin E synthase	210367_s_at	3,03	5,28E-04
9	FZD8	frizzled class receptor 8	227405_s_at	2,78	6,99E-04
10	CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1	202437_s_at	2,70	6,38E-03
#	DOWN Regulated MDA-MB-231 Gene Symbols	Gene Names	Probe Set	Fold Change	FDR Corrected pvalue
1	RRM2	ribonucleotide reductase M2	209773_s_at	6,59	5,57E-04
2	TOP2A	topoisomerase (DNA) II alpha 170kDa	201291_s_at	6,33	6,99E-04
3	DEPDC1B	DEP domain containing 1B	226980_at	5,78	2,74E-04
4	TTK	TTK protein kinase	204822_at	5,7	1,25E-03
5	KIF14	kinesin family member 14	206364_at	5,4	4,11E-04
6	CENPA	centromer protein A	204962_s_at	5,26	6,00E-04
7	DLGAP5	discs, large (Drosophila) homolog-associated protein 5	203764_at	5,19	7,05E-04
8	APOBEC3B	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3B	206632_s_at	5,16	5,04E-04
9	KIF23	kinesin family member 23	204709_s_at	5,14	2,74E-04
9	NEIL3	nei endonuclease VIII-like 3 (E. coli)	219502_at	5,14	6,70E-04
10	BUB1	BUB1 mitotic checkpoint serine/threonine kinase	215509_s_at	5,07	1,90E-03

Emodin affected MCF-7 (ER+) cells more than MDA-MB-231 (ER-) cells according to the differentially regulated gene numbers and also the fold changes of both up- and downregulations which were calculated as 37,04-fold increase/30,61-fold decrease for MCF-7 cells and 11,36-fold increase/6,59-fold decrease for MDA-MB-231 cells. Furthermore, these numbers showed that, Emodin predominantly regulated gene expressions through downregulating genes in both cell lines. Overall, 142 genes were found to be differentially regulated in both cell lines.

3.1.2 Functional Enrichment Analyses of Differentially Regulated MCF-7 and MDA-MB-231 Genes

3.1.2.1 Gene Ontology Enrichment Analysis of Differentially Regulated Genes

In order to reveal significant terms relating biological processes, cellular components, and molecular functions; GO enrichment analyses were performed. Top five most significant results and differentially expressed genes in both cell lines are given in Table 3.3 for MCF-7 and MDA-MB-231. All the other results were shown in Supplementary Table S3, S4 and S5 in Sakalli-Tecim et al. (2022).

In MCF-7 cells; cell cycle, cellular response to stress, DNA replication were the most significant biological processes; intracellular and nuclear parts were the most significant cellular components and protein binding, DNA helicase activity and nucleotide binding were the most significant molecular functions (Table 3.3).

In MDA-MB-231 cells; cell cycle, mitotic cell cycle, cell cycle process were the most significant biological processes; condensed chromosome, spindle, chromosome centromeric region were the most significant cellular components and ATPase activity, microtubule binding, tubulin binding were the most significant molecular functions (Table 3.3).

Table 3.3 Gene ontology enrichment analysis of differentially expressed genes

GO Enrichment Analysis	GO biological process			GO cellular component			GO molecular function		
Cell line	Term name	Gene#	Adjusted pvalue	Term name	Gene#	Adjusted pvalue	Term name	Gene#	Adjusted pvalue
Differentially expressed MCF-7 genes	cell cycle	252	1.8xE-26	intracellular part	1105	1.2xE-31	protein binding	971	8.1xE-16
	cellular response to stress	268	1.4xE-25	intracellular	1106	3.8xE-31	DNA helicase activity	28	2.7xE-12
	DNA replication	70	7.1xE-20	nuclear part	499	5.8xE-29	nucleotide binding	229	4.4xE-11
	mitotic cell cycle	156	1.4xE-19	nucleus	677	3.2xE-28	nucleoside phosphate binding	229	4.6xE-11
	cellular response to DNA damage stimulus	138	2.6xE-19	nucleoplasm	390	5.3xE-27	DNA-dependent ATPase activity	33	5.3xE-11
Differentially expressed MDA-MB-231 genes	cell cycle	145	4.4xE-71	condensed chromosome	43	5.6xE-34	ATPase activity	31	9.1xE-11
	mitotic cell cycle	111	3.8xE-65	spindle	49	1.2xE-31	microtubule binding	24	1.1xE-10
	cell cycle process	124	1.3xE-64	chromosome centromeric region	39	7.7xE-31	tubulin binding	26	1.7xE-09
	mitotic cell cycle process	99	1.7xE-58	condensed chromosome centromeric region	30	1.1xE-26	ATPase activity, coupled	24	2.3xE-09
	chromosome segregation	63	3.7xE-50	chromosomal region	44	2.9xE-26	protein binding	226	7.0xE-08
Differentially expressed genes in both MCF-7 and MDA-MB-231	cell cycle	75	1.6xE-35	chromosome	51	6.8xE-17	DNA helicase activity	11	3.1xE-09
	cell cycle process	62	5.9xE-30	nucleus	104	1.9xE-16	ATPase activity	21	4.1xE-09
	mitotic cell cycle	54	1.1xE-28	condensed chromosome	21	2.1xE-15	DNA-dependent ATPase activity	12	1.8xE-08
	DNA metabolic process	47	1.5xE-23	nuclear part	80	9.1xE-14	ATPase activity, coupled	17	2.3xE-08
	mitotic cell cycle process	46	1.9xE-23	nuclear lumen	75	9.6xE-13	drug binding	39	7.1xE-08

According to the results, cell cycle related genes were the most significantly regulated ones in both cell lines, but the adjusted p value was much more significant in MDA-MB-231 cells. In MCF-7, these regulations were most significantly in cellular components like intracellular and nuclear parts whereas in MDA-MB-231, they were most significantly in cellular components like chromosome and spindle.

Regarding GO enrichment analysis of differentially regulated genes in both cell lines; cell cycle, cell cycle process, mitotic cell cycle were the most significant biological processes; chromosome, nucleus, condensed chromosome were the most significant cellular components and DNA helicase activity, ATPase activity, DNA-dependent ATPase activity were the most significant molecular functions (Table 3.3).

3.1.2.2 KEGG Pathway Enrichment Analysis of Differentialy Regulated Genes

KEGG pathway enrichment analyses were performed using g:Profiler. Table 3.4 shows the pathways related to ER+ and ER- breast cancers. All the results were presented in the Appendix (Table 4.1).

Table 3.4 KEGG pathway enrichment analysis of differentially expressed genes

Cell line	Pathway name (KEGG pathways)	Gene #	Adjusted pvalue
Differentially expressed MCF-7 genes	Cell cycle	38	3.1xE-12
	DNA replication	17	2.1xE-8
	p53 signaling pathway	22	9.9xE-7
	Cellular senescence	33	8.4xE-6
	Apoptosis	25	3.4xE-3
	FoxO signaling pathway	24	3.5xE-3
	mTOR signaling pathway	25	2.2xE-2
	Pathways in cancer	63	2.3xE-2
	Longevity regulating pathway	17	3.0xE-2
Differentially expressed MDA-MB-231 genes	Cell cycle	20	6.7xE-14
	DNA replication	7	6.4xE-5
	p53 signaling pathway	7	7.3xE-3
	Cellular senescence	10	9.2xE-3
	Homologous recombination	5	2.4xE-2
Differentially expressed genes in both MCF-7 and MDA-MB-231	Cell cycle	10	3.1xE-6
	DNA replication	5	6.6xE-4
	p53 signaling pathway	5	2.0xE-2
	Homologous recombination	4	2.1xE-2

According to the results, among differentially regulated genes in either cell type or both, cell cycle was the most significantly regulated pathway with having MDA-MB-231 the most significant adjusted p value. The following most significant pathways regulated with these gene sets were DNA replication and the p53 signaling pathway.

The other common pathway differentially regulated in both MCF-7 and MDA-MB-231 was cellular senescence and apoptosis, FOXO signaling pathway, longevity regulating pathway, mTOR signaling pathway, pathways in cancer were the pathways differentially regulated only in MCF-7 cells.

Though homologous recombination was enriched in MDA-MB-231 cells but in MCF-7 cells, however, it was enriched in common regulated gene set. This result of the analysis was actually expected.

3.1.3 Gene Clusters of MCF-7 and MDA-MB-231 Cell Lines

Gene sets of breast cancer related pathways were intersected with each other in order to determine genes regulated in multiple pathways. In addition to these intersects, genes regulated in most of the pathways were determined and studied with literature.

In MCF-7 cells, at first, the genes of the most significantly regulated three pathways which were cell cycle, p53 signaling pathway, and cellular senescence pathways were intersected with each other. CCND1, CCNE1, CCNE2, CDK1, CDK2, CDK6, CDKN1A, CHEK1, GADD45A, GADD45B, GADD45G genes were found at the intersection point. After this, genes of the remaining pathways which were apoptosis, FOXO signaling pathway, mTOR signaling pathway, longevity regulating pathway were intersected with each other. AKT1, PIK3R1, PIK3R2, PIK3R3 were the genes found at the intersection. Figure 3.1 shows these intersections, which was given as Figure 1 in Sakalli-Tecim et al. (2022). ‘DNA replication’ pathway was omitted from this analysis because this pathway had common genes only with ‘Cell cycle’ pathway. ‘Pathways in cancer’ was also omitted, because it gives various kinds of different pathways.

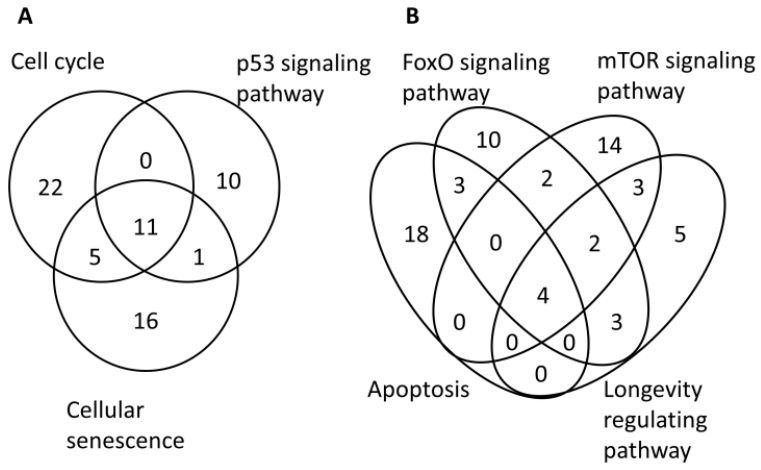

Figure 3.1 Venn diagram representations of the genes in the enriched MCF-7 pathways

A. Intersections of genes in cell cycle, p53 signaling pathway, cellular senescence. B. Intersections of genes in apoptosis, FOXO signaling pathway, mTOR signaling pathway, longevity regulating pathway.

In addition to intersection analyses, MCF-7 genes regulated with most of the pathways were determined. One hundred and forty-one genes were differentially regulated in all the pathways as given in Table 3.4. Among these, 61 genes were regulated in more than one pathway. Out of these 61 genes, 33 genes were regulated in 2 pathways, 11 genes were regulated in 3 pathways, 7 genes were regulated in 4 pathways, 3 genes were regulated in 5 pathways and 7 genes were regulated in 6 pathways. The genes regulated in 4- 6 pathways were examined and further studied regarding the genes which were regulated in most of the pathways. Seven genes that were regulated in 4 pathways were CCNE1, CCNE2, CDK6, FOXO1, IGF1R, SKP2, TGFB2; 3 genes that were regulated in 5 pathways were CCND1, CDK2, CDKN1A; 7 genes that were regulated in 6 pathways were AKT1, GADD45A, GADD45B, GADD45G, PIK3R1, PIK3R2, PIK3R3.

Genes that were found out by intersection analysis and by choosing the regulated ones in most of the pathways were further studied with literature which were

principally related to Emodin and MCF-7. As a result, it was seen that most of the genes above mentioned had roles in the regulation of FOXO1 expression or being regulated by FOXO1 itself, directly or indirectly as it was also revealed by literature listed in Table 3.5.

Table 3.5 Literature studies and regulated KEGG pathways of MCF-7 gene cluster

Genes of MCF-7 gene cluster	Up/Down regulated upon Emodin	Regulated KEGG pathway	References
AKT1	Down	A, CS, F, L, M, PC	Li et al., 2021; Reagan-Shaw and Ahmad, 2006; Sui et al., 2014
CCND1	Down	CC, CS, F, P, PC	Carroll et al., 2000; Huang et al., 2013; Li et al., 2013; Reagan-Shaw and Ahmad, 2006; Sui et al., 2014
CCNE1, CCNE2	Down	CC, CS, P, PC	Carroll et al., 2000
CDK1	Down	CC, CS, P	Katagi et al., 2016
CDK2	Down	CC, CS, F, P, PC	Carroll et al., 2000
CDKN1A	Up	CC, CS, F, P, PC	Carroll et al., 2000; Farhan et al., 2017; Huang and Tindall, 2007; Katagi et al., 2016
FOXO1	Up	CS, F, L, PC	Farhan et al., 2017; Guttilla and White, 2009; Huang and Tindall, 2007; Reagan-Shaw and Ahmad, 2006
GADD45A	Up	A, CC, CS, F, P, PC	Katagi et al., 2016
GADD45B, GADD45G	Up	A, CC, CS, F, P, PC	Farhan et al., 2017
IGF1R	Down	F, L, M, PC	Hartog et al., 2012; Jayasuriya et al., 1992; Zhang et al., 1999; Zhang et al., 2005
PIK3R1, PIK3R2, PIK3R3	Down	A, CS, F, L, M, PC	Massarweh and Schiff, 2006; Reagan-Shaw and Ahmad, 2006; Sui et al., 2014; Sun et al., 2001
SKP2	Down	CC, F, M, PC	Huang and Tindall, 2007; Wang et al., 2012

The genes which were used in the cluster are also represented on 'KEGG FOXO Signaling Pathway' and given in Figure 3.2.

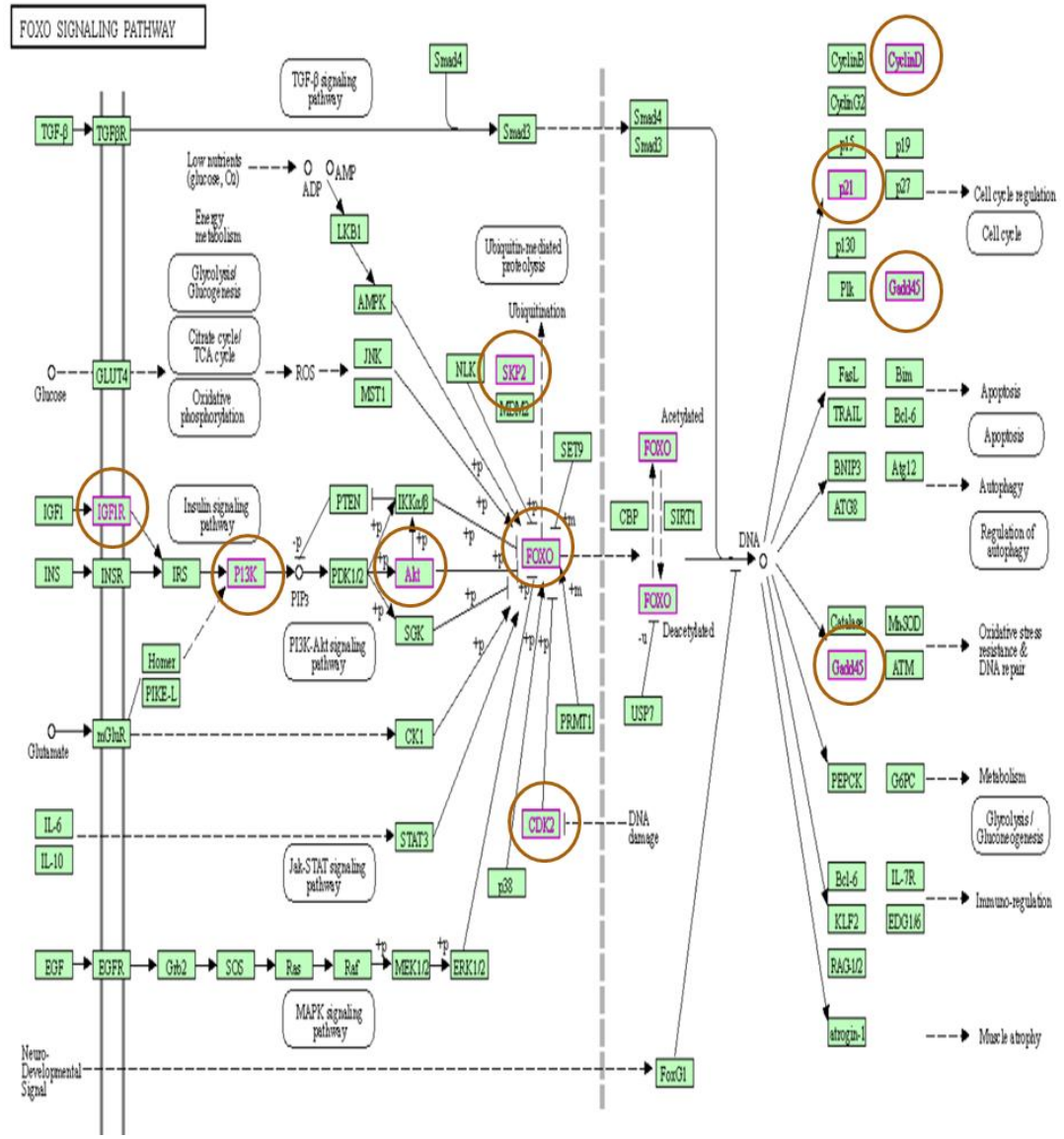

Figure 3.2 Representation of differentially expressed MCF-7 genes on KEGG FOXO Signaling Pathway

As a result, AKT1, CCND1, CCNE1, CCNE2, CDK1, CDK2, CDKN1A, FOXO1, GADD45A, GADD45B, GADD45G, IGF1R, PIK3R1, PIK3R2, PIK3R3, SKP2 genes were selected as gene cluster of MCF-7 cells. According to these results, we proposed that the upregulation of FOXO1 with Emodin treatment could have

important anti-carcinogenic consequences on ER+ breast cancer cells as FOXO1 is defined to play tumor suppressive role in various malignancies including breast cancer.

In addition to this gene cluster, downregulation of BCL-2 which is an apoptosis indicator (Sui et al., 2014) also drew attention in MCF-7 cells.

In MDA-MB-231 cells, 36 genes were differentially regulated in all the pathways as shown in Table 3.4. Among these, 8 genes were regulated in multiple pathways. Five genes, which were CDK1, CCNB1, CCNB2, CCNE2, and CHEK1, were regulated in cell cycle, p53 signaling pathway and cellular senescence pathways. CCNA2 and RBL1 were regulated in cell cycle and cellular senescence and MCM5 was regulated in cell cycle and DNA replication pathways.

Among enriched pathways cell cycle drew the most attention with the highest significance value and the highest number of gene counts. Emodin regulated MDA-MB-231 cell cycle genes through downregulation of expression. Downregulated genes in this cluster were BUB1, BUB1B, CCNA2, CCNB1, CCNB2, CCNE2, CDC20, CDC23, CDC25C, CDC45, CDC7, CDK1, CHEK1, MAD2L1, MCM5, PTTG1, RBL1, SKP2, TFDP1, TTK genes. Although no specific one gene for Emodin regulation in MDA-MB-231 cell could be identified, cell cycle pathway suppression was observed as an important action of Emodin and Spindle Attachment Checkpoint (SAC) mechanism (Marques et al., 2015; Bolanos-Garcia and Blundell, 2011; Ji et al., 2017) related genes inside cell cycle pathway were found to be regulated in a noticeable way. SAC mechanism related genes were namely BUB1, BUB1B, CCNB1, CCNB2, CDC20, CDC23, CDK1, MAD2L1, PTTG1, TTK genes. CENPE gene which is not found in SAC mechanism however, was found to be involved in kinetochore microtubule attachment and its suppression further contributing to the mitotic arrest. In addition to CENPE; CENPF, CENPA and several other centromere proteins responsible for the chromosome segregation (CENPN, CENPW, CENPU, CENPL) were also down regulated with Emodin treatment.

Furthermore, according to GO enrichment analyses; most significantly regulated biological processes were cell cycle related, and most significantly regulated cellular components were chromosome related, and most significantly regulated molecular functions were microtubule binding related terms. These results are also pointing out the importance of SAC mechanism in regulation. For all these reasons, SAC mechanism related genes were selected as gene cluster of MDA-MB-231 cells.

Differentially expressed MDA-MB-231 genes are also represented on SAC mechanism part of 'KEGG Cell Cycle' and given in Figure 3.3.

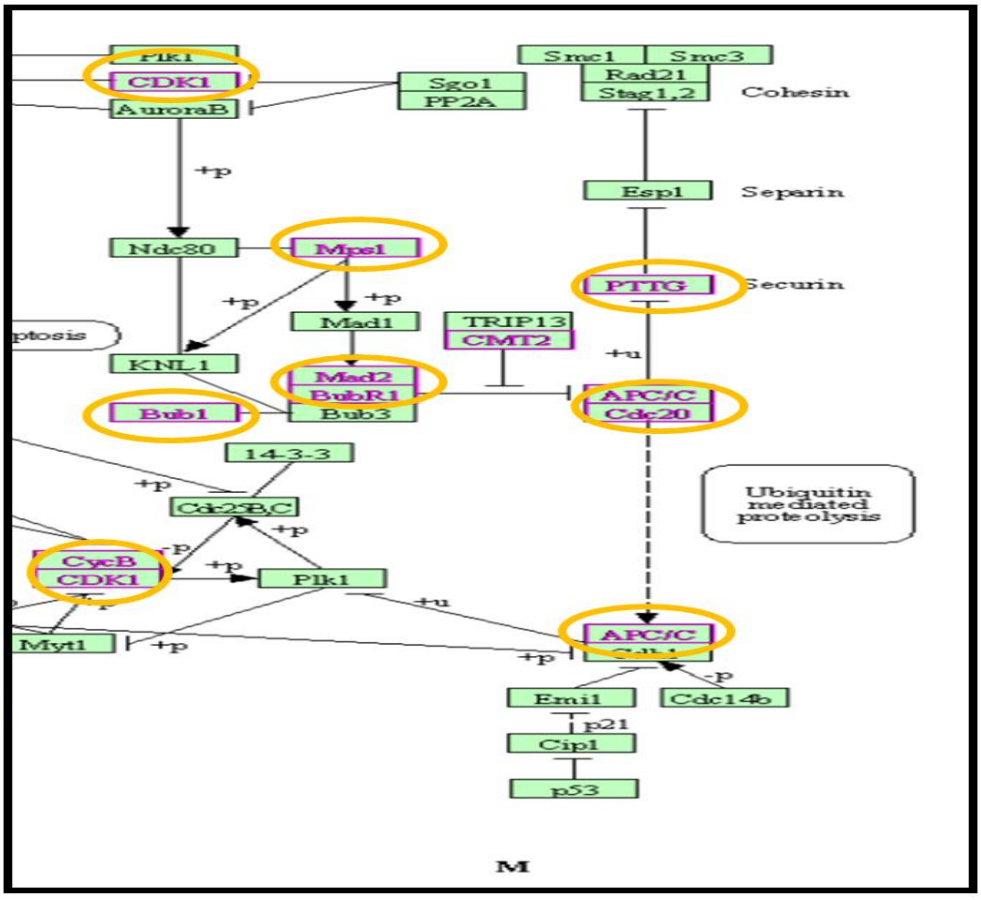

Figure 3.3 Representation of differentially expressed MDA-MB-231 genes on SAC mechanism part of KEGG Cell Cycle

3.1.4 Gene Interaction Network Analyses of MCF-7 and MDA-MB-231 Gene Clusters

Gene interaction network analyses of gene clusters were performed by using GeneMANIA 3.6.0 and STRING v11 tools. Gene clusters determined with enrichment analysis, intersection analysis and literature studies were used for the construction of gene interaction networks. Layouts of the nodes were adjusted according to literature studies given in Table 3.5. Network interaction results were in the same direction with literature studies for both cell lines with both interaction tools. GeneMANIA which represents gene interactions and STRING which represents protein interactions were also confirmed reciprocally.

In MCF-7 cells, AKT1, CCND1, CCNE1, CCNE2, CDK1, CDK2, CDKN1A, FOXO1, GADD45A, IGF1R, PIK3R1, PIK3R2, PIK3R3, SKP2 genes were selected within gene cluster for the network analyses. According to literature, mentioned genes were shown to have relation with each other directly or indirectly within FOXO1 pathway and have roles in FOXO1 pathway regulation. Gene interaction networks for MCF-7 were shown in Figure 3.4.

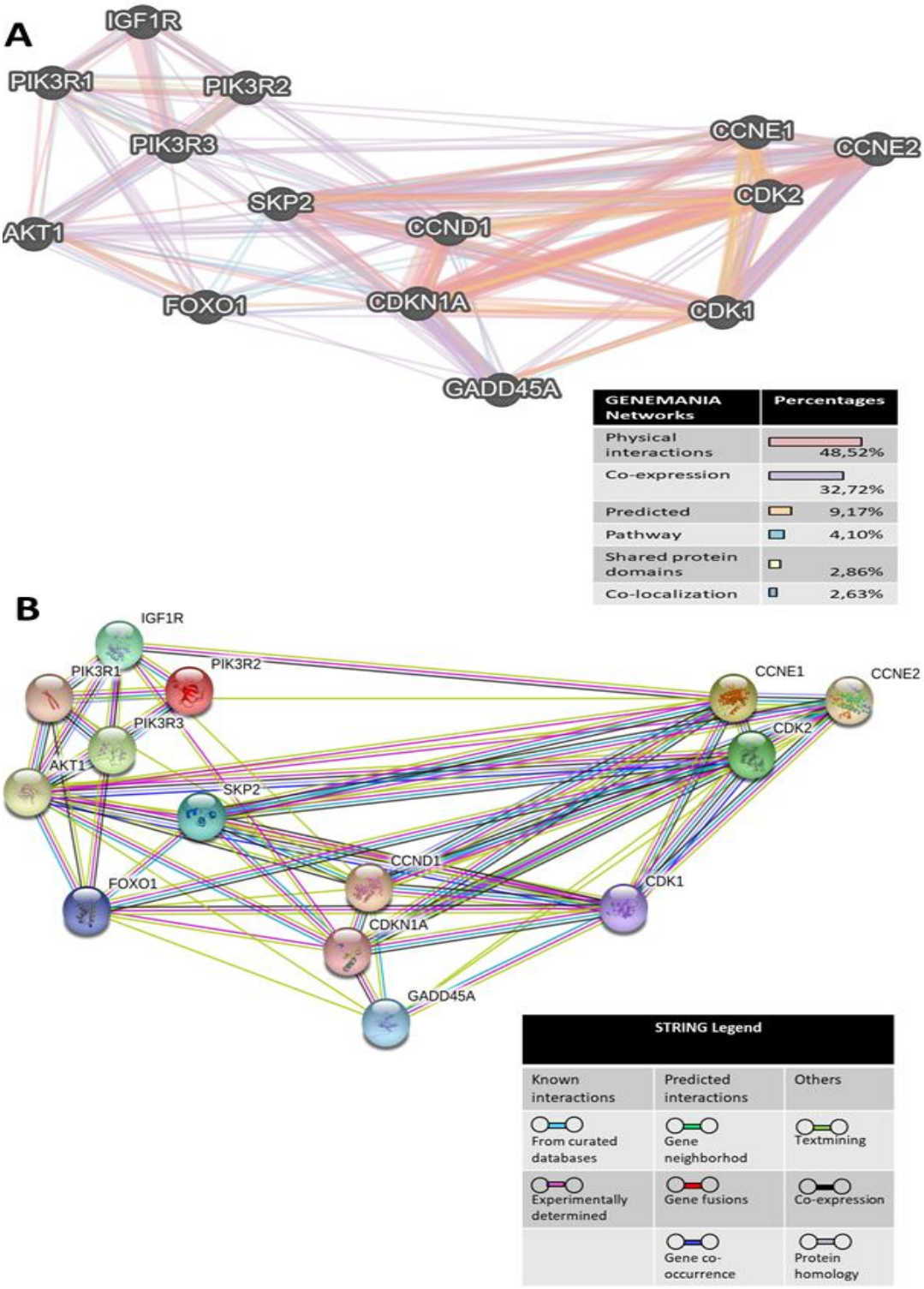

Figure 3.4 Gene interaction networks of MCF-7. A. GeneMANIA and B. STRING

In MDA-MB-231 cells, SAC mechanism related genes; BUB1, BUB1B, CCNB1, CCNB2, CDC20, CDC23, CDK1, MAD2L1, PTTG1, and TTK were used for the network analyses. Again, as in MCF-7, network results were consistent with literature and further, GeneMANIA and STRING network results were confirming each other. Gene interaction networks for MDA-MB-231 are shown in Figure 3.5.

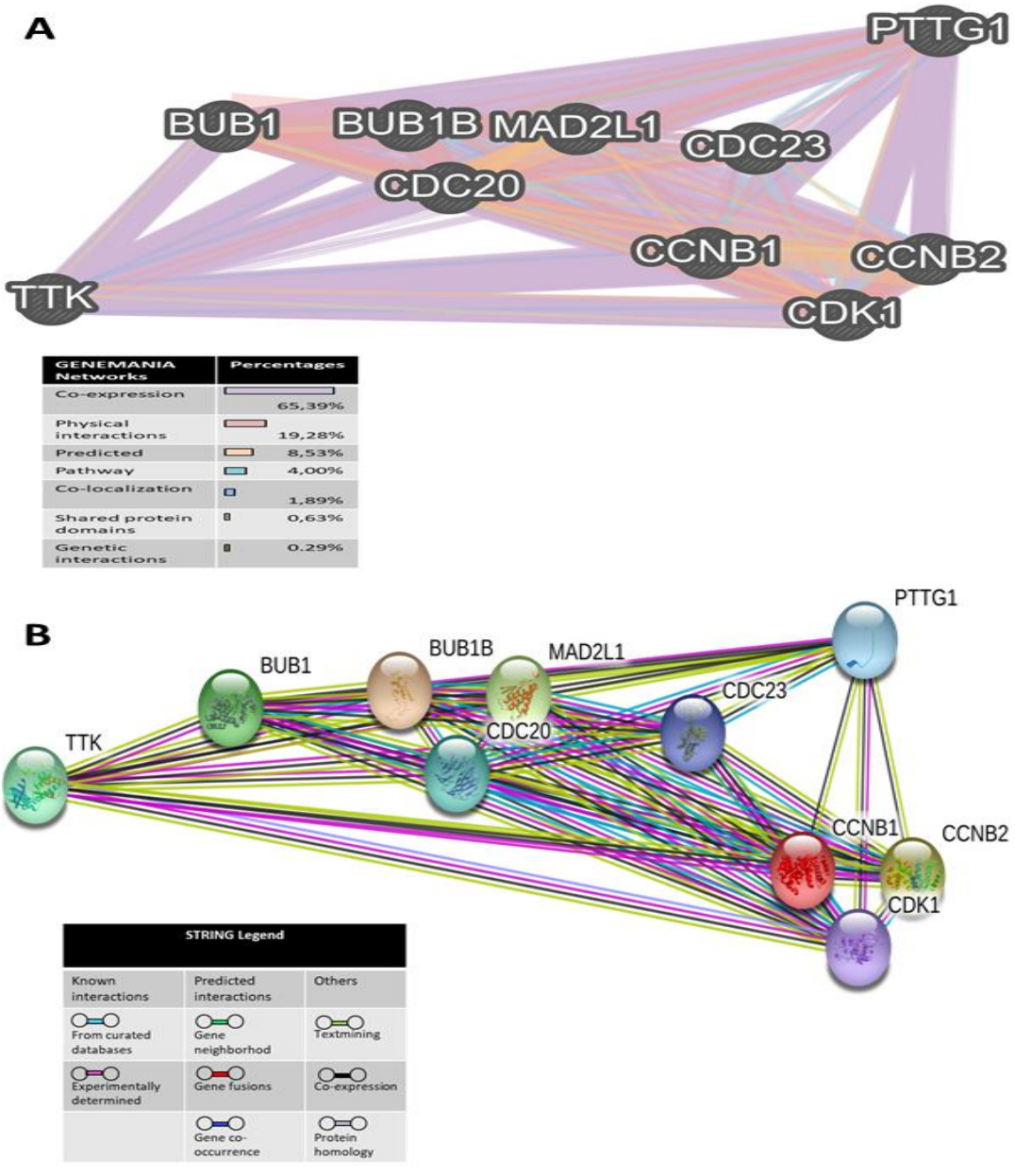

Figure 3.5 Gene interaction networks of MDA-MB-231. A. GeneMANIA and B. STRING

3.1.5 Enrichment Maps of Differentially Regulated MCF-7 and MDA-MB-231 Genes

In order to visualize the relationship between gene sets of regulated pathways and biological processes, enrichment map was built. Genes belonging to KEGG regulated pathways which are given in Table 3.4 and genes belonging to GO Biological Process terms were used for the analysis. In this representation, the nodes indicating a regulated pathway or a biological process and edges referring to an overlap between two nodes.

Enriched clusters of MCF-7 cells were ‘cell cycle regulation’ cluster with 103 nodes, ‘cellular response to stimulus’ cluster with 189 nodes, ‘Wnt signaling’ cluster with 3 nodes. These clusters were obtained by setting node adjusted p value to ≤0.001 and represented in Figure 3.6A. Nodes having FOXO1 gene showed the importance of FOXO1 regulation in ‘cellular response to stimulus’ cluster (Figure 3.6A1) and nodes having FOXO1 together with their neighbors showed how the effect of FOXO1 enlarged through ‘cell cycle regulation’ cluster (Figure 3.6A2). In addition, in order to increase the significance, node adjusted p value was adjusted to ≤1x10-15 and a subnetwork was obtained (Figure 3.6B and Appendix Table 4.2). With this adjustment, cell cycle regulation became the main cluster with 26 nodes and cellular response to stimulus was the other cluster with 8 nodes. FOXO1 regulated nodes (Figure 3.6B1) and their neighbors (Figure 3.6B2) showed strikingly how FOXO1 regulation is important for cell cycle regulation.

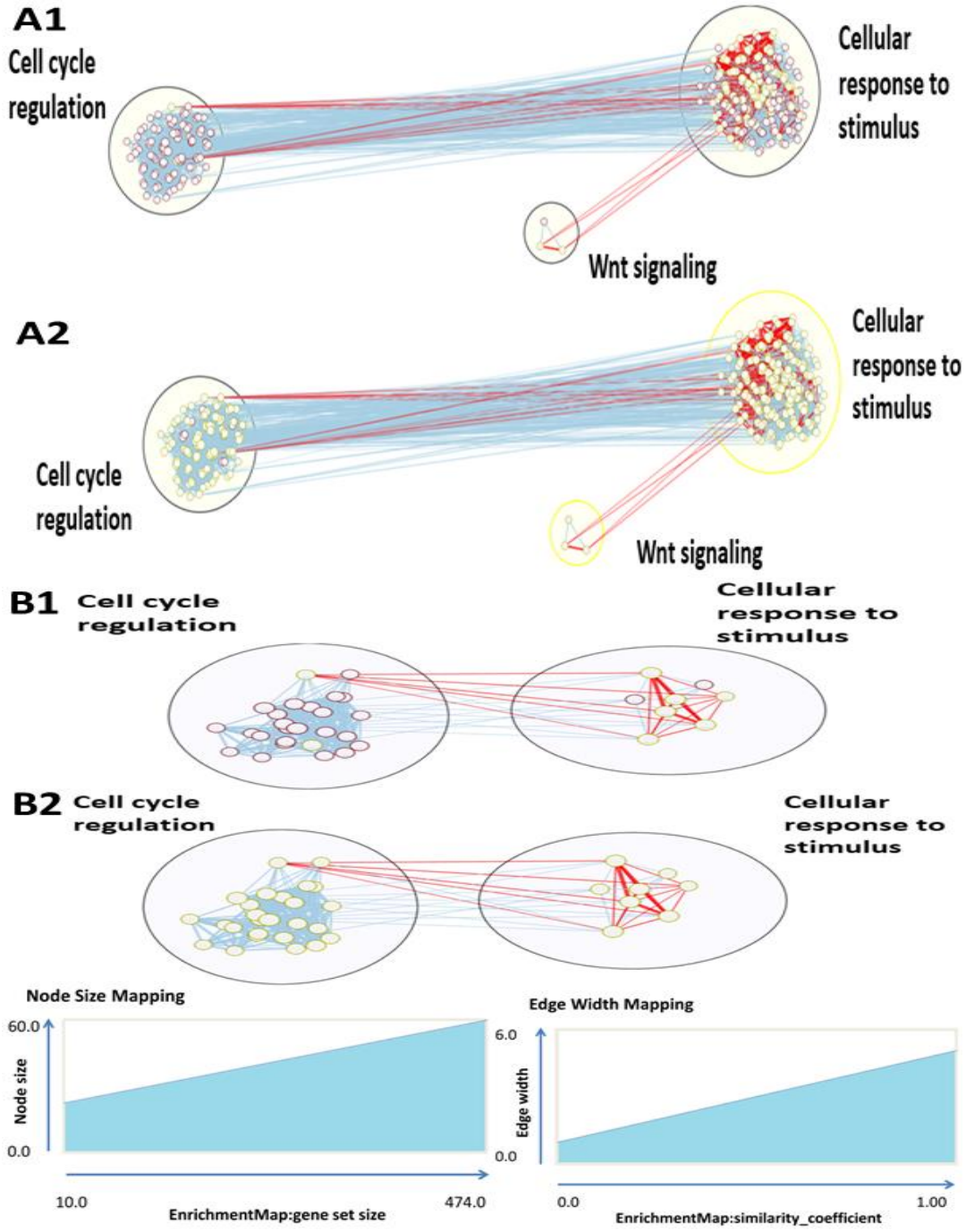

Figure 3.6 Enrichment maps of differentially regulated MCF-7 genes

FOXO1 related pathways and biological processes (A1 and B1) and the mentioned nodes together with their neighbors (A2 and B2) were represented by setting adjusted p value to ≤0.001 (A1 and A2) and ≤1x10^{-15}(B1 and B2). Yellow sided

nodes represent FOXO1 related pathways and biological processes among all enriched clusters which are shown as red sided nodes. Red edges represent interactions between FOXO1 related pathways, and biological processes among all interactions are shown as blue edges.

The only enriched cluster of MDA-MB-231 cells was 'cell cycle regulation' cluster with 129 nodes (adjusted p value was set to ≤0.001). This result was further proof of Emodin's effect on cell cycle regulation (Figure 3.7A). Figure 3.7B showed the most significant nodes by setting adjusted p value to ≤1x10-15. Among these nodes, mitotic cell cycle process was the most significant and this node included SAC mechanism related BUB1, BUB1B, CCNB1, CCNB2, CDC20, CDC23, CDK1, MAD2L1, PTTG1, and TTK genes which constituted selected gene cluster for MDA-MB-231 cells.

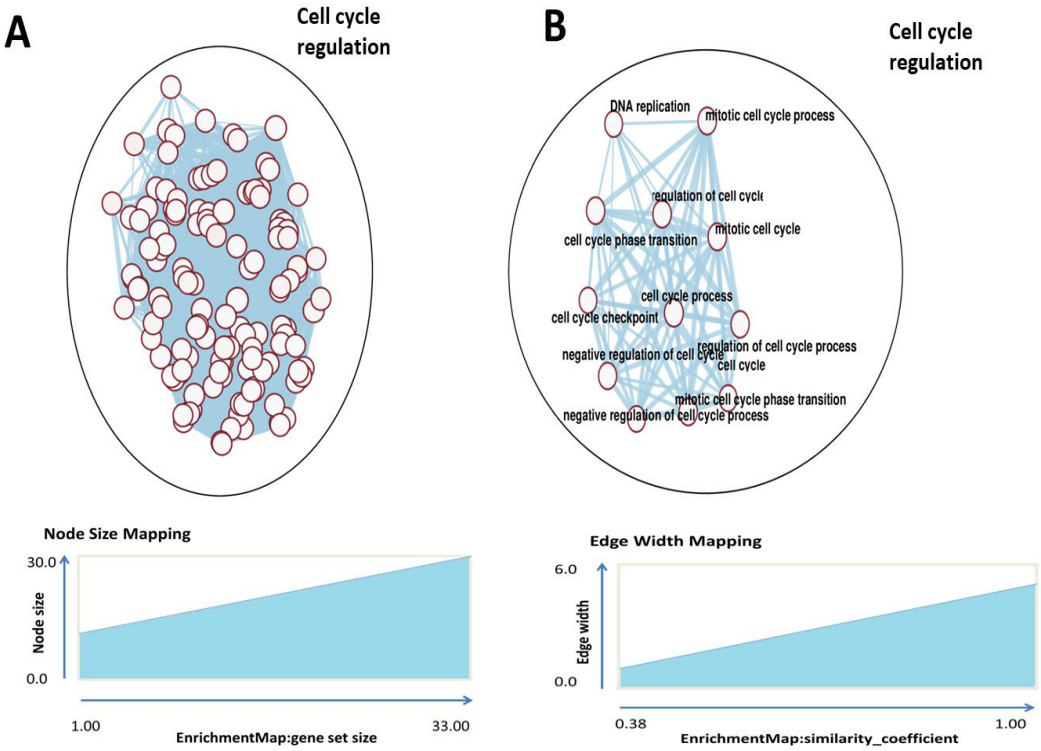

Figure 3.7 Enrichment maps of differentially regulated MDA-MB-231 genes by adjusting p value to ≤0.001 (A) and to ≤1x10^{-15}

Furthermore, in order to visualize the overlaps between two cell lines, enriched networks Figure 3.6A1 and Figure 3.7A were merged and their intersection points were taken. Cell cycle regulation was the only common cluster. When the adjusted p value was set to value $\leq 1x10^{-22}$, the cluster included further pathways namely cell cycle, cell cycle DNA replication, cell cycle G1/S phase transition, cell cycle phase transition, cell cycle process, cellular response to DNA damage stimulus, cellular senescence, DNA-dependent DNA replication, DNA replication, G1/S transition of mitotic cell cycle, mitotic cell cycle, mitotic cell cycle process, mitotic cell cycle phase transition, nuclear DNA replication, and regulation of cell cycle. All these results indicated that, Emodin showed its mechanism of action through the regulation of cell cycle related processes in both cell lines regardless of ER status.

3.2 Identification of Differentially Expressed MCF-7 and MDA-MB-231 miRNAs upon Emodin Treatment and Determination of miRNA-Gene Targets from Databases

3.2.1 Differentially Expressed miRNAs of MCF-7 and MDA-MB-231 Cell Lines

Following raw data normalization, differentially expressed miRNAs observed after Emodin treatment were identified through univariate two sample t-test with a significance level of 0.05 and a fold change value of 2. Among differentially expressed miRNAs in MCF-7, 28 miRNAs were found to be upregulated and 10 miRNAs as downregulated, whereas among differentially expressed miRNAs in MDA-MB-231, 25 miRNAs were found to be upregulated and 26 miRNAs as downregulated. All differentially expressed miRNAs in both cell lines are given in Figure 3.8.

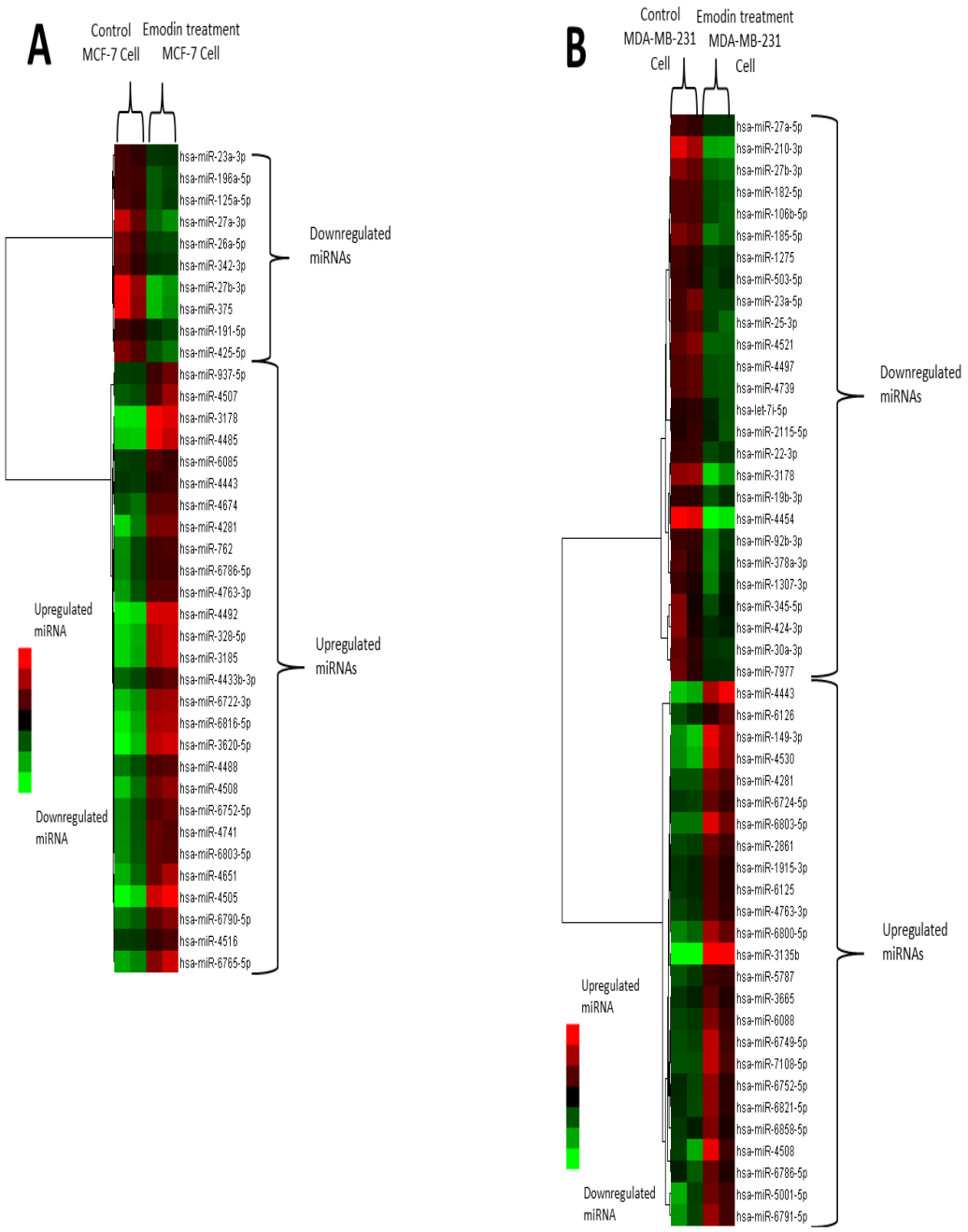

Figure 3.8 Hierarchical clustering of cell lines and the differentially expressed miRNAs obtained with Emodin treatment

Differentially expressed miRNAs successfully clustered in control and Emodin treated MCF-7 cells (A) and MDA-MB-231 (B) cells into two groups as downregulated and upregulated miRNAs. 'Red' color for upregulated and 'green'

color for downregulated differentially expressed miRNAs, while miRNAs with no significant differences in expression are colored as black.

3.2.2 miRNA-Gene Targets from miRNA Databases

For each miRNA, miRNA-gene targets were determined as previously explained in the first part of Figure 2.3. In order to have a robust and a reliable analysis, gene targets of predicted target databases, TargetScan and miRDB were intersected and gene targets of validated target databases, miRTarBase and DIANA-TarBase v8 were added.

For 28 upregulated MCF-7 miRNAs, there were totally 9979 gene targets from databases which were obtained by intersecting predicted target databases, TargetScan and miRDB which resulted 6879 genes and adding validated target databases, miRTarBase and DIANA-TarBase v8 which resulted 3100 genes as a result. Predicted and validated target databases could give the same target, therefore 9979 gene targets contained also the duplicated ones. When the total upregulated miRNA-gene targets were removed from duplicated ones, the resulting number became 5966 for upregulated MCF-7 miRNA-gene targets.

For 10 downregulated MCF-7 miRNAs, there were totally 26697 gene targets obtained from databases. The intersection of predicted target databases, TargetScan and miRDB was 2585 genes and validated target databases, miRTarBase and DIANA-TarBase v8 gave 24112 genes as gene targets of downregulated genes. Again, when duplicated gene targets were removed, the resulting number became 11597 for downregulated MCF-7 miRNA-gene targets.

Similarly, for 25 upregulated MDA-MB-231 miRNAs, a total of 11752 gene targets were obtained from databases. The intersection of predicted target databases, TargetScan and miRDB was 7831 genes and validated target databases, miRTarBase and DIANA-TarBase v8 gave 3921 genes as gene targets of upregulated MDA-MB-231 genes. When duplicated gene targets were removed,

the resulting number became 6900 for upregulated MDA-MB-231 miRNA-gene targets.

For 26 downregulated MDA-MB-231 miRNAs, a total of 38701 gene targets were obtained from databases. The intersection of predicted target databases, TargetScan and miRDB was 6616 genes and validated target databases, miRTarBase and DIANA-TarBase v8 gave 32085 genes as gene targets of downregulated MDA-MB-231 genes. When duplicated gene targets were removed, the resulting number of 12588 was obtained for downregulated MDA-MB-231 miRNA-gene targets.

3.3 Integration of Differentially Expressed MCF-7 and MDA-MB-231 Genes and Differentially Expressed MCF-7 and MDA-MB-231 miRNAs upon Emodin Treatment and Their Analysis

3.3.1 miRNA-Regulated Genes and miRNA-Gene Pairs

For each miRNA, miRNA-regulated genes were determined as explained previously in the second part of Figure 2.3. After finding the miRNA-gene targets from databases, miRNA-regulated genes were further determined by intersecting upregulated miRNA-gene targets with downregulated genes and downregulated miRNA-gene targets with upregulated genes.

For MCF-7, 1300 were identified as differentially expressed genes with Emodin treatment and within this, 514 genes were upregulated and 786 genes were downregulated. For upregulated miRNAs, miRNA-regulated genes were determined by intersecting 5966 upregulated miRNA-gene targets with 786 downregulated genes and the resulting intersect was composed of a total of ‘295 genes’. After running intersection analysis, all upregulated miRNAs were found to have differentially regulated targets. Therefore, as a result, in MCF-7 cells, 28 upregulated miRNAs were found to regulate 295 genes, which were downregulated, upon Emodin treatment. For downregulated miRNAs, miRNA-

regulated genes were determined by intersecting 11597 downregulated miRNA-gene targets with 514 upregulated genes and the resulting intersect was composed of ‘385 genes’. Like the upregulated miRNAs, all downregulated miRNAs were found to have differentially regulated targets after intersection analysis. Therefore, according to our results, in MCF-7 cells, 10 downregulated miRNAs regulated 385 genes, which were upregulated upon Emodin treatment. As a result, a total of ‘680 MCF-7 genes’ were regulated by miRNAs upon Emodin treatment and this constitutes 52% of all the differentially regulated MCF-7 genes.

Similar analysis and calculations were carried out for MDA-MB-231 cells, and as a result 283 differentially expressed genes were found after Emodin treatment, and within them, 51 genes were identified as upregulated and 232 genes were downregulated. For upregulated miRNAs, miRNA-regulated genes were determined by intersecting 6900 upregulated miRNA-gene targets with 232 downregulated genes and the resulting intersect was composed of ‘69 genes’. After intersection analysis, 22 upregulated miRNAs were found to have differentially regulated targets, and the remaining 3 upregulated miRNAs did not have any differentially regulated target at all. Therefore, in MDA-MB-231, a total of 22 upregulated miRNAs regulating 69 genes through downregulation with Emodin treatment. For downregulated miRNAs, miRNA-regulated genes were determined by intersecting 12588 downregulated miRNA-gene targets with 51 upregulated genes and the resulting intersect was composed of ‘44 genes’. After intersection analysis, only 25 downregulated miRNAs were found to have differentially regulated targets, the remaining 1 downregulated miRNA did not have any differentially regulated targets. Therefore, in MDA-MB-231 cells, 25 downregulated miRNAs regulated 44 upregulated genes after Emodin treatment. As a result, ‘113 MDA-MB-231 genes’ were regulated by miRNAs and this makes up 40% of all differentially regulated MDA-MB-231 genes.

In summary, 52% of Emodin responsive MCF-7 genes and 40% of Emodin responsive MDA-MB-231 genes were identified as candidate miRNA targets in miRNA-gene regulatory pairs. Thus, in both ER+ and ER- cells, miRNAs function

as important regulators in Emodin action mechanism, which seems to be more prominent in ER+ MCF-7 cells. In both cell lines, miRNAs regulate their target genes mostly through upregulation by increasing their expressions.

In MCF-7 cells, there were 28 upregulated miRNAs which targeted 295 downregulated genes and these generated 478 miRNA-gene pairs. In addition, there were 10 downregulated miRNAs which targeted 385 upregulated genes and these generated 1021 miRNA-gene pairs. These results and their genomic locations are given in Table 3.6 and miRNA-target gene pairs of MCF-7 are shown in Figure 3.9.

Table 3.6 Differentially expressed miRNAs upon Emodin treatment in MCF-7 cells

#	UP regulated 28 MCF-7 miRNAs	Genomic locations	Probe sets	Fold changes	FDR corrected pvalues	miRNA-gene pairs
1	hsa-miR-4505	14q24.3	MIMAT0019041_st	26,32	1,32E-01	68
2	hsa-miR-3178	16p13.3	MIMAT0015055_st	25,64	1,32E-01	3
3	hsa-miR-4492	11q23.3	MIMAT0019027_st	20,41	1,20E-01	13
4	hsa-miR-3620-5p	1q42.13	MIMAT0022967_st	19,61	1,55E-01	4
5	hsa-miR-4485	11p15.4	MIMAT0019019_st	18,18	1,32E-01	2
6	hsa-miR-328-5p	16q22.1	MIMAT0026486_st	13,69	1,32E-01	8
7	hsa-miR-3185	17q21.32	MIMAT0015065_st	13,33	1,32E-01	51
8	hsa-miR-6816-5p	22q11.21	MIMAT0027532_st	12,66	1,32E-01	7
9	hsa-miR-6722-3p	9q34.3	MIMAT0025854_st	9,09	1,32E-01	22
10	hsa-miR-6765-5p	14q32.33	MIMAT0027430_st	9,09	1,62E-01	19
11	hsa-miR-4281	5q35.2	MIMAT0016907_st	7,14	1,96E-01	9
12	hsa-miR-4508	15q11.2	MIMAT0019045_st	7,14	1,96E-01	5
13	hsa-miR-4651	7q11.23	MIMAT0019715_st	6,66	2,14E-01	19
14	hsa-miR-6790-5p	19p13.3	MIMAT0027480_st	4,55	1,35E-01	16
15	hsa-miR-6803-5p	19q13.42	MIMAT0027506_st	4,16	1,73E-01	15
16	hsa-miR-4741	18q11.2	MIMAT0019871_st	4,16	1,77E-01	20
17	hsa-miR-6752-5p	11q13.2	MIMAT0027404_st	4	1,96E-01	9
18	hsa-miR-4763-3p	22q13.31	MIMAT0019913_st	4	2,17E-01	36
19	hsa-miR-4507	14q32.33	MIMAT0019044_st	4	2,29E-01	8
20	hsa-miR-4674	9q34.3	MIMAT0019756_st	3,57	1,32E-01	17
21	hsa-miR-4488	11q12.2	MIMAT0019022_st	3,44	1,55E-01	4
22	hsa-miR-762	16p11.2	MIMAT0010313_st	3,33	2,29E-01	13
23	hsa-miR-4433b-3p	2p14	MIMAT0030414_st	3,23	1,32E-01	13
24	hsa-miR-6786-5p	17q25.3	MIMAT0027472_st	3,23	2,29E-01	2
25	hsa-miR-937-5p	8q24.3	MIMAT0022938_st	2,7	1,97E-01	23
26	hsa-miR-6085	15q22.2	MIMAT0023710_st	2,44	1,32E-01	9
27	hsa-miR-4516	16p13.3	MIMAT0019053_st	2,33	1,32E-01	11
28	hsa-miR-4443	3p21.31	MIMAT0018961_st	2,22	1,32E-01	52
						Totally 478 pairs
#	**DOWN regulated 10 MCF-7 miRNAs**	**Genomic locations**	**Probe sets**	**Fold changes**	**FDR corrected pvalues**	**miRNA-gene pairs**
1	hsa-miR-27b-3p	9q22.32	MIMAT0000419_st	16,07	2,45E-01	113
2	hsa-miR-375	2q35	MIMAT0000728_st	11,48	1,97E-01	69
3	hsa-miR-27a-3p	19p13.12	MIMAT0000084_st	6,31	2,29E-01	185
4	hsa-miR-425-5p	3p21.31	MIMAT0003393_st	4	1,77E-01	52
5	hsa-miR-26a-5p	3p22.2	MIMAT0000082_st	3,05	1,97E-01	136
6	hsa-miR-196a-5p	17q21.32	MIMAT0000226_st	2,7	1,32E-01	67
7	hsa-miR-125a-5p	19q13.41	MIMAT0000443_st	2,63	1,61E-01	64
8	hsa-miR-342-3p	14q32.2	MIMAT0000753_st	2,41	2,42E-01	46
9	hsa-miR-191-5p	3p21.31	MIMAT0000440_st	2,18	1,96E-01	206
10	hsa-miR-23a-3p	19p13.12	MIMAT0000078_st	2,14	1,32E-01	83
						Totally 1021 pairs

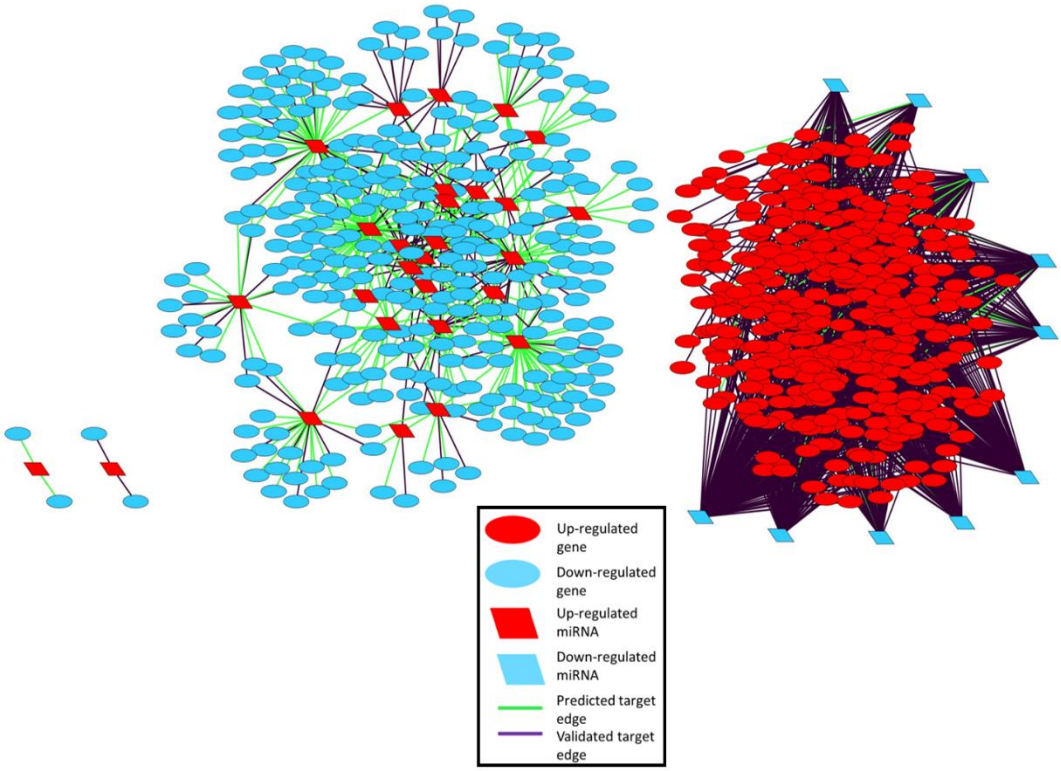

Figure 3.9 miRNA-gene pairs of Emodin treated MCF-7 cells

hsa-miR-4505 having 26.32-fold change was identified as the most upregulated miRNA in MCF-7 cells and also it has the largest number of miRNA-gene pairs with a total of 68 target genes. Among the downregulated miRNAs, hsa-miR-27b-3p having 16.07-fold change was the most downregulated miRNA, and with 206 target genes, hsa-miR-191-5p has the largest number of miRNA-gene pairs in these cells.

In MDA-MB-231 cells, 22 upregulated miRNAs were observed which targeted 69 downregulated genes and these interactions generated 87 miRNA-gene pairs. In addition, there were 25 downregulated miRNAs targeting 44 upregulated genes, generating 174 miRNA-gene pairs as given in Table 3.7. Genomic locations of differentially expressed miRNAs in MDA-MB-231 are also listed in Table 3.7. Chromosome 19 attracted attention due to its high number of differentially expressed miRNAs.

Table 3.7 Differentially expressed miRNAs upon Emodin treatment in MDA-MB-231 cells

#	UP regulated 25 MDA-MB-231 miRNAs	Genomic locations	Probe sets	Fold changes	FDR corrected pvalues	miRNA-gene pairs
1	hsa-miR-3135b	6p21.32	MIMAT0018985_st	243,9	1,15E-04	14
2	hsa-miR-4443	3p21.31	MIMAT0018961_st	14,49	2,31E-03	12
3	hsa-miR-149-3p	2q37.3	MIMAT0004609_st	11,63	5,97E-03	7
4	hsa-miR-4530	19q13.2	MIMAT0019069_st	10	4,37E-03	6
5	hsa-miR-6803-5p	19q13.42	MIMAT0027506_st	7,14	1,01E-02	1
6	hsa-miR-4508	15q11.2	MIMAT0019045_st	6,25	5,49E-02	1
7	hsa-miR-6800-5p	19q13.33	MIMAT0027500_st	5,55	5,97E-03	2
8	hsa-miR-6791-5p	19p13.3	MIMAT0027482_st	4,35	2,11E-02	2
9	hsa-miR-7108-5p	19p13.3	MIMAT0028113_st	4,35	2,16E-02	4
10	hsa-miR-5001-5p	2q37.1	MIMAT0021021_st	4	2,56E-02	3
11	hsa-miR-6749-5p	11q13.1	MIMAT0027398_st	3,85	3,01E-02	0
12	hsa-miR-4281	5q35.2	MIMAT0016907_st	3,57	3,28E-03	1
13	hsa-miR-6088	19q13.32	MIMAT0023713_st	2,85	1,75E-02	1
14	hsa-miR-6821-5p	22q13.33	MIMAT0027542_st	2,77	3,82E-02	1
15	hsa-miR-2861	9q34.11	MIMAT0013802_st	2,7	6,36E-03	2
16	hsa-miR-6752-5p	11q13.2	MIMAT0027404_st	2,7	4,82E-02	0
17	hsa-miR-6724-5p	21p11.2	MIMAT0025856_st	2,56	7,92E-03	2
18	hsa-miR-5787	3p21.31	MIMAT0023252_st	2,44	3,28E-03	1
19	hsa-miR-6126	16p13.3	MIMAT0024599_st	2,44	1,97E-02	4
20	hsa-miR-6786-5p	17q25.3	MIMAT0027472_st	2,38	4,34E-02	0
21	hsa-miR-4763-3p	22q13.31	MIMAT0019913_st	2,33	8,74E-03	8
22	hsa-miR-6858-5p	Xq28	MIMAT0027616_st	2,22	6,77E-02	2
23	hsa-miR-6125	12q14.1	MIMAT0024598_st	2,08	1,17E-02	1
24	hsa-miR-1915-3p	10p12.31	MIMAT0007892_st	2,04	1,24E-02	6
25	hsa-miR-3665	13q22.3	MIMAT0018087_st	2	2,39E-02	6
						Totally 87 pairs

Table 3.7 cont.

#	DOWN regulated 26 MDA-MB-231 miRNAs	Genomic locations	Probe sets	Fold changes	FDR corrected pvalues	miRNA-gene pairs
1	hsa-miR-4454	4q32.2	MIMAT0018976_st	56,38	9,74E-03	1
2	hsa-miR-210-3p	11p15.5	MIMAT0000267_st	11,83	2,31E-03	12
3	hsa-miR-3178	16p13.3	MIMAT0015055_st	9,32	2,85E-03	0
4	hsa-miR-27b-3p	9q22.32	MIMAT0000419_st	4,54	2,31E-03	9
5	hsa-miR-185-5p	22q11.21	MIMAT0000455_st	4,39	2,31E-03	6
6	hsa-miR-4521	17p13.1	MIMAT0019058_st	4,23	2,31E-03	1
7	hsa-miR-4497	12q24.11	MIMAT0019032_st	3,16	8,21E-04	1
8	hsa-miR-4739	17q25.3	MIMAT0019868_st	3,1	1,22E-03	1
9	hsa-miR-182-5p	7q32.2	MIMAT0000259_st	3,09	8,21E-04	16
10	hsa-miR-106b-5p	7q22.1	MIMAT0000680_st	3,06	2,31E-03	13
11	hsa-miR-25-3p	7q22.1	MIMAT0000081_st	3,05	6,15E-03	14
12	hsa-miR-23a-5p	19p13.12	MIMAT0004496_st	2,98	7,81E-03	1
13	hsa-miR-378a-3p	5q32	MIMAT0000732_st	2,81	3,46E-02	7
14	hsa-miR-92b-3p	1q22	MIMAT0003218_st	2,8	1,92E-02	8
15	hsa-miR-30a-3p	6q13	MIMAT0000088_st	2,38	1,02E-02	11
16	hsa-miR-1307-3p	10q24.33	MIMAT0005951_st	2,31	6,06E-02	2
17	hsa-miR-345-5p	14q32.2	MIMAT0000772_st	2,3	9,83E-02	4
18	hsa-miR-7977	3q26.32	MIMAT0031180_st	2,27	2,32E-02	2
19	hsa-miR-424-3p	Xq26.3	MIMAT0004749_st	2,23	7,60E-02	2
20	hsa-miR-22-3p	17p13.3	MIMAT0000077_st	2,22	2,31E-03	6
21	hsa-miR-27a-5p	19p13.12	MIMAT0004501_st	2,16	2,31E-03	17
22	hsa-miR-1275	6p21.31	MIMAT0005929_st	2,15	2,31E-03	1
23	hsa-miR-19b-3p	13q31.3	MIMAT0000074_st	2,12	1,12E-02	12
24	hsa-miR-503-5p	Xq26.3	MIMAT0002874_st	2,03	6,15E-03	10
25	hsa-miR-2115-5p	3p21.31	MIMAT0011158_st	2,03	2,39E-02	2
26	hsa-let-7i-5p	12q14.1	MIMAT0000415_st	2,02	1,26E-02	15
						Totally 174 pairs

miRNA-target gene pairs of MDA-MB-231 cells are shown in Figure 3.10.

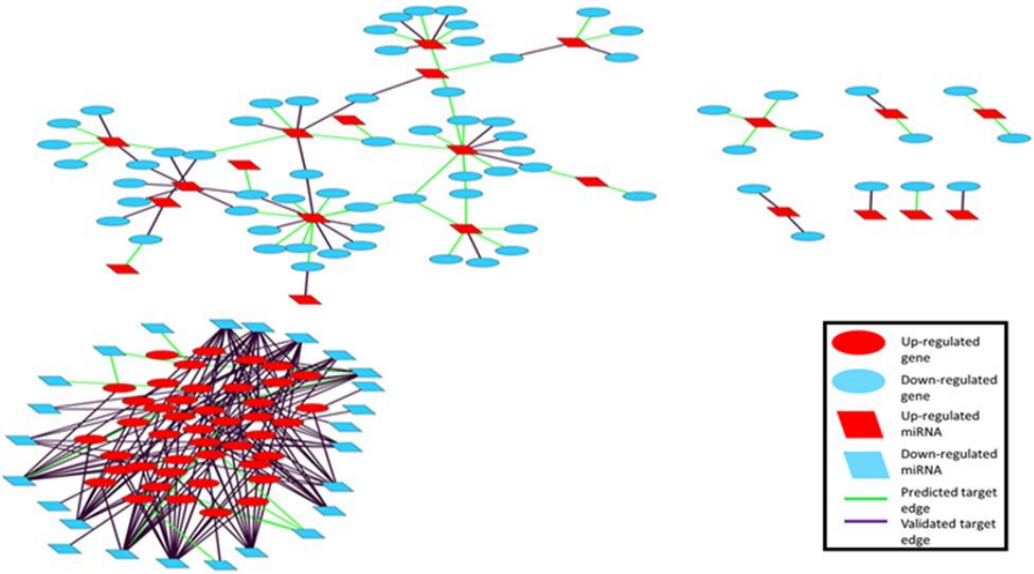

Figure 3.10 miRNA-gene pairs of Emodin treated MDA-MB-231 cells

In MDA-MB-231 cells, hsa-miR-3135b having 243.9-fold change was found to be the most upregulated miRNA and also it has the largest number of miRNA-gene pairs with a total of 14 target genes. hsa-miR-4454 having 56.38-fold change was the most downregulated miRNA in these cells, but it shows only 1 integrated target gene. The largest number of miRNA-gene pairs among the downregulated miRNAs belong to hsa-miR-27a-5p with a total of 17 target genes.

Furthermore, among 9 common miRNAs regulated in both cell lines, only hsa-miR-27b-3p, hsa-miR-4443, and hsa-miR-4763-3p had integrated miRNA target genes in both cell lines, a total of 13 target genes as represented in Figure 3.11.

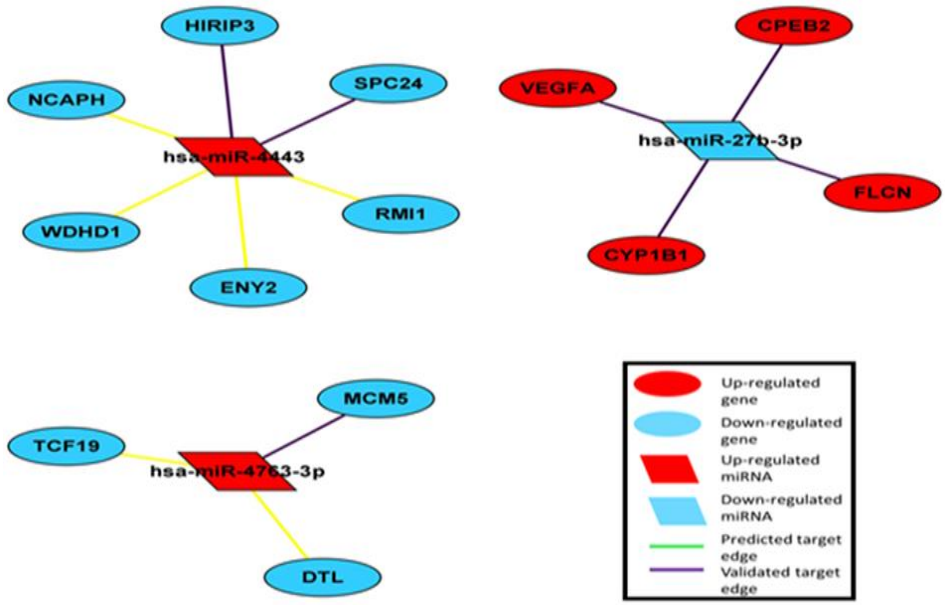

Figure 3.11 miRNA-gene pairs of common miRNA regulated genes and common miRNAs present both in Emodin treated MCF-7 and MDA-MB-231 cells

3.3.2 Functional Enrichment Analyses of miRNA-Regulated MCF-7 and MDA-MB-231 Genes

3.3.2.1 Gene Ontology Enrichment Analysis of miRNA-Regulated Genes

In order to understand the role of miRNAs on molecular action mechanism of Emodin, GO functional annotation was performed. The GO functional annotation composed of three categories, namely, biological process, cellular component, and molecular function. The top 5 enriched GO items are given in Table 3.8 for miRNA regulated MCF-7 genes, MDA-MB-231 genes and the intersection genes in both cells.

Table 3.8 Gene ontology enrichment analysis of miRNA-regulated genes

GO Enrichment Analysis	GO biological process			GO cellular component			GO molecular function		
Cell line	Term name	Gene#	Adjusted pvalue	Term name	Gene#	Adjusted pvalue	Term name	Gene#	Adjusted pvalue
miRNA-regulated MCF-7 genes	response to endogenous stimulus	138	7.4xE-19	intracellular membrane-bounded organelle	496	9.4xE-19	protein binding	582	6.2xE-12
	cellular response to stress	157	9.5xE-18	nucleus	368	1.9xE-16	enzyme binding	126	6.9xE-08
	response to organic substance	216	1.8xE-17	intracellular anatomical structure	586	4.6xE-16	DNA-binding transcription activator activity, RNA polymerase II-specific	41	9.9xE-06
	cellular response to endogenous stimulus	119	2.2xE-16	membrane-bounded organelle	534	6.3xE-16	DNA-binding transcription activator activity	41	1.3xE-05
	cellular response to organic substance	183	1.1xE-15	organelle lumen	292	8.3xE-16	binding	621	3.0xE-04
miRNA-regulated MDA-MB-231 genes	cell cycle	51	5.5xE-19	intracellular non-membrane-bounded organelle	63	1.43xE-08	microtubule binding	12	3.0xE-05
	mitotic cell cycle	38	4.9xE-17	non-membrane-bounded organelle	63	1.5xE-08	tubulin binding	12	9.9xE-04
	cell cycle process	43	7.5xE-17	nuclear lumen	56	3.9xE-08	ATPase activity	12	1.4xE-02
	mitotic cell cycle process	33	2.2xE-14	nucleus	76	4.7xE-08	ATP binding	22	2.3xE-02
	chromosome segregation	20	1.3xE-11	spindle	16	1.8xE-07	microtubule motor activity	5	4.1xE-02
Common miRNA regulated genes of common miRNAs in both cells	DNA metabolic process	7	4.6×E-04	chromosome	6	3.8×E-02	-	-	-
	cellular response to stress	8	4.1×E-03	-	-	-	-	-	-
	cellular macromolecule biosynthetic process	7	1.9×E-02	-	-	-	-	-	-
	DNA replication	4	3.0×E-02	-	-	-	-	-	-
	cell cycle	7	3.5×E-02	-	-	-	-	-	-
Intersection genes of miRNA-regulated MCF-7 and MDA-MB-231 genes	cellular response to stress	17	1.4xE-05	nucleoplasm	19	4.2xE-03	-	-	-
	DNA metabolic process	11	7.6xE-04	MCM complex	2	3.6xE-02	-	-	-
	cellular macromolecule metabolic process	25	2.9xE-03	autolysosome	2	3.6xE-02	-	-	-
	chromosome segregation	7	4.6xE-03	nucleus	29	3.7xE-02	-	-	-
	cell cycle	13	1.2xE-02	chromosome	11	4.0xE-02	-	-	

According to the results, miRNA-regulated Emodin responsive genes in MCF-7 were mainly concentrated on terms relating ‘response to stimulus’ indicating a

cellular response created by Emodin and for MDA-MB-231 cells on ‘cell cycle related processes’. miRNA-regulated Emodin responsive genes of common miRNAs were enriched most significantly in ‘DNA metabolic process’, and ‘chromosome’ cellular component.

In GO analysis, the intersection of miRNA-regulated Emodin responsive genes of both MCF-7 and MDA-MB-231 cells were also used. The intersection results identified 42 genes which were most significantly enriched in ‘cellular response to stress’ and overall, the ‘nucleoplasm’ was found to be the most significant cellular component in both cells.

In addition to this analysis, for MDA-MB-231, GO analysis was performed for gene targets of miRNAs which were located on chromosome 19. This genomic location was attracted attention due to its high number of differentially expressed miRNAs. According to the results, cell cycle was the most significant biological process, indicating the importance of chromosome 19 for Emodin’s mechanism of action through miRNA regulation.

3.3.2.2 KEGG Pathway Enrichment Analysis of miRNA-Regulated Emodin Responsive Genes

The breast cancer related enriched pathways of miRNA-regulated Emodin responsive genes in both MCF-7 and MDA-MB-231 cells are given in Table 3.9 and all the other results are given in Appendix Table 4.3. ‘Cellular senescence’ and ‘DNA replication’ were found to be the most significantly regulated pathways for MCF-7 and MDA-MB-231 cell lines respectively. Interestingly, the intersected genes were not enriched to any pathway related to both ER+ and ER- breast cancers.

Table 3.9 KEGG pathway enrichment analysis of miRNA-regulated genes with Emodin treatment

Cell line	KEGG pathway name	Gene#	Adjusted pvalue	Up regulated miRNA#	Down regulated gene target#	miRNA-gene pairs#	Down regulated miRNA#	Up regulated gene target#	miRNA-gene pairs#
miRNA-regulated MCF-7 genes	Cellular senescence	23	6.4xE-6	15	13	21	10	10	31
	Cell cycle	19	5.1xE-5	12	13	18	9	6	22
	p53 signaling pathway	14	1.1xE-4	5	4	6	10	10	45
	MAPK signaling pathway	30	2.5xE-4	12	12	20	10	18	54
	mTOR signaling pathway	20	3.7xE-4	13	6	17	10	14	49
	FoxO signaling pathway	17	2.0xE-3	13	9	17	9	8	27
	Apoptosis	17	3.3 xE-3	3	3	3	10	14	41
	Pathways in cancer	40	8.4xE-3	19	19	36	10	21	78
	Transcriptional misregulation in cancer	19	2.7xE-2	15	5	17	10	14	42
	Breast cancer	16	2.9xE-2	11	7	15	9	9	33
miRNA-regulated MDA-MB-231 genes	DNA replication	4	9xE-3	4	4	5	-	-	-

Later, MCF-7 miRNAs were examined regarding the number of pathways, miRNA-gene pairs, and the unduplicated gene targets, and the results are shown in Table 3.10. Among upregulated miRNAs, hsa-miR-4505 was regulated in 9 pathways. Furthermore, having 29 miRNA-gene pairs and 9 unduplicated gene targets as the most, it was the most effective upregulated miRNA for MCF-7 pathways. Among downregulated miRNAs, hsa-miR-191-5p was regulated in all 10 pathways. In addition to this, having 87 miRNA-gene pairs and 39 unduplicated gene targets as the most, it was the most effective downregulated miRNA for MCF-7 pathways. Therefore, it can be concluded that among all differentially regulated miRNAs, hsa-miR-191-5p was found to be the most effective miRNA for the regulation of MCF-7 cellular pathways. miRNAs that regulate the majority of

the pathways could have particular importance for understanding the action mechanism of Emodin.

Table 3.10 MCF-7 pathways related with each differentially regulated miRNAs

#	Up regulated MCF-7 miRNAs	Pathway numbers	MCF-7 pathways	miRNA-gene pair numbers in pathways	Unduplicated gene target numbers in pathways
1	hsa-miR-4505	9	B, CC, CS, F, MK, MT, P, PC, T	29	9
2	hsa-miR-3178	1	CC	1	1
3	hsa-miR-4492	6	CC, CS, F, MT, P, PC	7	3
4	hsa-miR-3620-5p	1	MT	1	1
6	hsa-miR-328-5p	4	B, MT, PC, T	4	2
7	hsa-miR-3185	7	B, CS, F, MK, MT, PC, T	17	6
8	hsa-miR-6816-5p	6	A, B, CS, F, MT, PC	10	3
9	hsa-miR-6722-3p	5	B, MK, MT, PC, T	8	4
10	hsa-miR-6765-5p	5	CC, CS, F, MK, PC	6	2
11	hsa-miR-4281	4	MK, MT, PC, T	4	3
12	hsa-miR-4508	6	B, F, MK, MT, PC, T	6	1
13	hsa-miR-4651	7	B, CC, CS, F, P, PC, T	7	2
14	hsa-miR-6790-5p	1	T	1	1
15	hsa-miR-6803-5p	7	B, CS, F, MK, MT, PC, T	10	4
16	hsa-miR-4741	4	CC, MT, P, T	4	3
17	hsa-miR-6752-5p	1	T	1	1
18	hsa-miR-4763-3p	4	CC, CS, PC, T	5	4
19	hsa-miR-4507	4	CC, F, MK, P	4	3
20	hsa-miR-4674	4	CS, F, MK, PC	4	2
21	hsa-miR-4488	2	PC, T	2	1
22	hsa-miR-762	6	CC, CS, F, MT, P, PC	7	3
23	hsa-miR-4433b-3p	4	B, CC, CS, PC	4	1
25	hsa-miR-937-5p	2	A, CS	2	1
26	hsa-miR-6085	6	B, CC, CS, MT, PC, T	6	4
27	hsa-miR-4516	3	CS, F, MK	3	1
28	hsa-miR-4443	9	A, B, CC, CS, F, MK, MT, PC, T	17	6
#	Down regulated MCF-7 miRNAs	Pathway numbers	MCF-7 pathways	miRNA-gene pair numbers in pathways	Unduplicated gene target numbers in pathways
1	hsa-miR-27b-3p	10	A, B, CC, CS, F, MK, MT, P, PC, T	46	22
2	hsa-miR-375	10	A, B, CC, CS, F, MK, MT, P, PC, T	35	11
3	hsa-miR-27a-3p	10	A, B, CC, CS, F, MK, MT, P, PC, T	60	27
4	hsa-miR-425-5p	7	A, CS, MK, MT, P, PC, T	14	8
5	hsa-miR-26a-5p	10	A, B, CC, CS, F, MK, MT, P, PC, T	49	24
6	hsa-miR-196a-5p	10	A, B, CC, CS, F, MK, MT, P, PC, T	41	15
7	hsa-miR-125a-5p	10	A, B, CC, CS, F, MK, MT, P, PC, T	34	17
8	hsa-miR-342-3p	10	A, B, CC, CS, F, MK, MT, P, PC, T	20	10
9	hsa-miR-191-5p	10	A, B, CC, CS, F, MK, MT, P, PC, T	87	39
10	hsa-miR-23a-3p	10	A, B, CC, CS, F, MK, MT, P, PC, T	36	20

Furthermore, the genes in the enriched pathways were also analyzed regarding the number of miRNA-gene pairs which they were part of. Among targets of upregulated miRNAs, IGF1R was the most targeted gene with 24 miRNA-gene pairs. Also, IGF1R was one of the targets of hsa-miR-4505 which was the most upregulated miRNA and had the largest number of miRNA-gene pairs. Among targets of downregulated miRNAs, CDKN1A was the most targeted gene with 49 miRNA-gene pairs and CDKN1A was one of the targets of hsa-miR-191-5p which had the largest number of miRNA-gene pairs. On top of all, all these genes were among the FOXO1-related MCF-7 gene cluster. So, these results drew further attention.

In MDA-MB-231 cells, DNA replication was the only pathway enriched for miRNA-regulated genes and was composed of 4 downregulated genes namely, MCM5, PRIM1, RFC3, and RNASEH2C which were targeted by 4 upregulated miRNAs (hsa-miR-3665, hsa-miR-4443, hsa-miR-4763-3p, and hsa-miR-6126).

3.3.3 Integration of miRNAs with FOXO1-Related Emodin Responsive MCF-7 Gene Cluster

Differentially expressed genes in MCF-7 cells after Emodin treatment were analyzed by examining the pathways through enrichment analysis in combination with literature studies. As a result of these analyses, a gene cluster composed of FOXO1-related genes was obtained and used for the construction of a gene interaction network as given in Figure 3.4.

The gene interaction network was reconstructed by using GeneMANIA 3.5.2 with the addition of the differentially expressed miRNAs which were found to be involved in gene interactions obtained from miRNA-target gene databases (Figure 3.12A). miRNA-gene pairs of this network are represented in Figure 3.12B to show the shared miRNAs more precisely. Layouts of the gene nodes were adjusted

according to the regulations of FOXO1 related Emodin responsive genes as described previously.

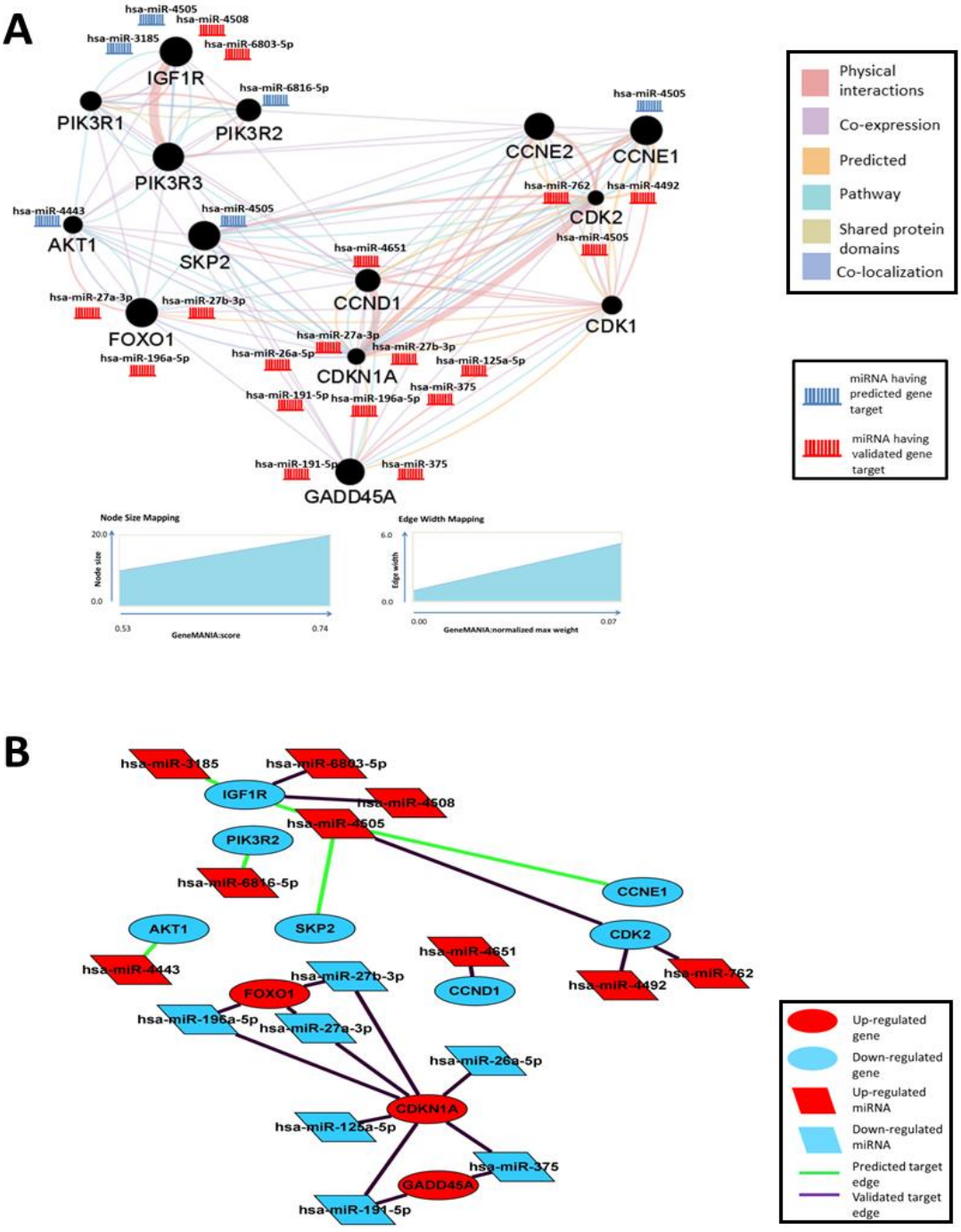

Figure 3.12 Gene and miRNA interaction network of FOXO1-related Emodin responsive-MCF-7 genes

(A) Gene interaction network analysis by using GeneMANIA with related miRNAs. (B) miRNA-gene pairs.

As a result, it turned out that, 10 of the 14 genes in the network were regulated by miRNAs. Among upregulated genes, CDKN1A was found to be the target of a maximum of 7 miRNAs. Moreover, it was also observed as among the target genes of hsa-miR-27b-3p, the most downregulated miRNA. Among the downregulated genes, IGF1R was the target of a maximum of 4 miRNAs and it was also among the target genes of hsa-miR-4505 which was the most upregulated miRNA. Finally, hsa-miR-4505 was the miRNA with the largest number of target genes in this network. In addition to the genes used in the network, GADD45B and GADD45G which were among MCF-7 gene cluster were also found to be regulated by hsa-miR-191-5p and hsa-miR-27a-3p miRNAs respectively.

According to the genomic locations of miRNAs (Table 3.6), hsa-miR-196a-5p, which regulates CDKN1A, FOXO1 genes, and hsa-miR-3185, which regulates IGF1R gene, are located on chromosome 17. Furthermore, hsa-miR-27a-3p, which regulates CDKN1A, FOXO1 genes, and hsa-miR-6803-5p, which regulates IGF1R gene, are located on chromosome 19. In addition to these, hsa-miR-26a-5p, which regulates CDKN1A gene, hsa-miR-191-5p, which regulates CDKN1A, GADD45A genes, and hsa-miR-4443, which regulates AKT1 gene are located on chromosome 3. This shows that chromosome 3, chromosome 17 and chromosome 19 were important for Emodin's action mechanism on FOXO pathway through miRNA regulation.

All the regulated pathways, miRNAs and miRNA-gene pair numbers of each individual gene in the gene cluster are given in Table 3.11. The overall high number of regulated pathways and miRNA-gene pairs further showed the importance of miRNA regulation in FOXO1-related Emodin responsive gene interaction network in MCF-7 cells. Furthermore, miRNA-gene pairs consisting of downregulated miRNAs and their upregulated target genes were all obtained from validated target databases which increased their reliability.

Table 3.11 miRNA targets of FOXO1-related MCF-7 gene cluster

miRNA-regulated genes		Pathways	miRNA names	miRNA-gene pairs #
Upregulated miRNA target genes	AKT1	A, B, CS, F, MK, MT, PC	hsa-miR-4443	7
	CCND1	B, CC, CS, F, P,PC	hsa-miR-4651	6
	CCNE1	CC, CS, P, PC	hsa-miR-4505	4
	CDK2	CC, CS, F, P, PC	hsa-miR-762, hsa-miR-4492, hsa-miR-4505	15
	IGF1R	B, F, MK, MT, PC, T	hsa-miR-3185, hsa-miR-4505, hsa-miR-4508, hsa-miR-6803-5p	24
	PIK3R2	A, B, CS, F, MT, PC	hsa-miR-6816-5p	6
	SKP2	CC, F, MT, PC	hsa-miR-4505	4
Downregulated miRNA target genes	CDKN1A	B, CC, CS, F, P, PC, T	hsa-miR-26a-5p, hsa-miR-27a-3p, hsa-miR-27b-3p, hsa-miR-125a-5p, hsa-miR-191-5p, hsa-miR-196a-5p, hsa-miR-375	49
	FOXO1	CS, F, PC, T	hsa-miR-27a-3p, hsa-miR-27b-3p, hsa-miR-196a-5p	12

A, Apoptosis; B, Breast cancer; CC, Cell cycle; CS, Cellular senescence; F, FOXO signaling pathway; MK, MAPK signaling pathway; MT, mTOR signaling pathway; P, p53 signaling pathway; PC, Pathways in cancer; T, Transcriptional misregulation in cancer

3.3.4 Enrichment Maps of miRNA-Regulated Emodin Responsive Genes

Enrichment maps were constructed for both ER+ and ER- cell lines by setting the adjusted p value ≤ 0.001. In this representation, nodes indicated a regulated pathway or a biological process and edges referred to an overlap between two nodes.

In Figure 3.13, a subnetwork was represented for MCF-7 by setting node adjusted p value $\leq 1 \times 10^{-8}$ in order to visualize the most significant nodes. The main cluster of

MCF-7 was composed of nodes related with “cellular response to stimulus” and “regulation of cellular processes”. The most significant nodes are shown in Figure 3.13A and the most significant one is “response to endogenous stimulus”. “Regulation of cellular metabolic process”, “cellular response to stimulus”, “apoptotic process”, “regulation of signal transduction”, “cell cycle” were among the other biological processes in which miRNA-regulated MCF-7 genes were clustered. Furthermore, as shown in Figure 3.13B, all miRNA-regulated biological processes in MCF-7 were found to be related to FOXO1 and this again reveals the importance of FOXO1 in both gene expressions and miRNA regulation in Emodin treated cells.

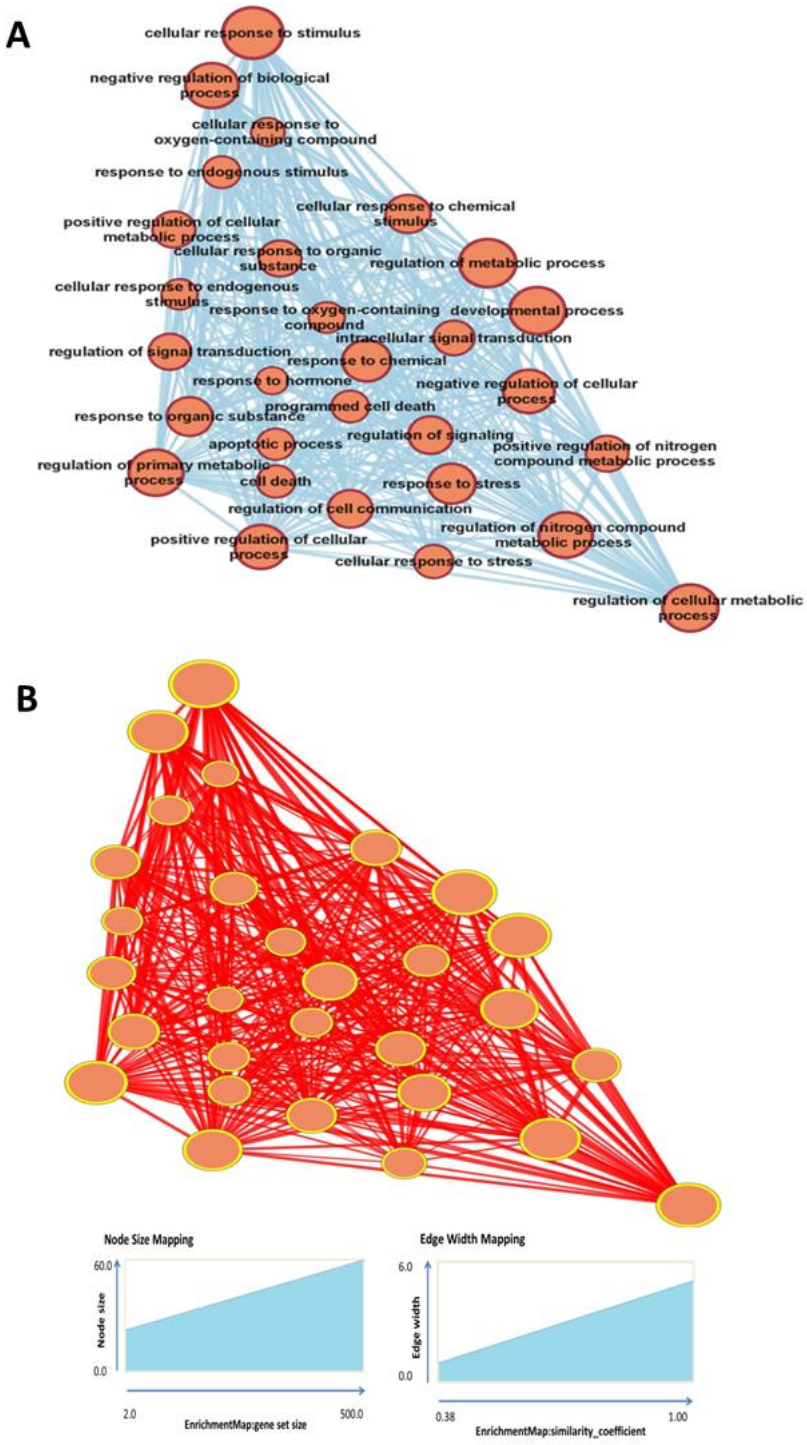

Figure 3.13 Enrichment maps of miRNA-regulated MCF-7 genes

(A) Enrichment map of miRNA-regulated MCF-7 genes (adjusted p value≤10^{-8}).

(B) FOXO1 related regulated pathways and biological processes (nodes with

yellow border line) and their interactions (red edges) of miRNA-regulated MCF-7 genes (adjusted p value≤10^{-8}).

In addition, in order to further show the importance of each miRNA target in the MCF-7 gene cluster for the regulation in enrichment map results, the number of nodes having each individual gene and the number of edges between these nodes showing the overlaps are given for each gene in Table 3.12. As seen from the Table, among the total of 171 nodes and 8047 edges, the gene with the highest node number was found as AKT1 having 152 nodes and the gene with the highest edge number was FOXO1 having 6971 edges. These results confirmed the importance of these genes in the miRNA regulation.

Table 3.12 Node and edge numbers of miRNA targets of FOXO1-related MCF-7 gene cluster in MCF-7 enrichment map

Selected gene at adjusted p ≤$1x10^{-3}$	Nodes with selected gene	Edges with selected gene
ALL	171	8047
AKT1	152	6882
CCND1	117	4952
CCNE1	103	4129
CDK2	108	4510
CDKN1A	122	5092
FOXO1	147	6971
GADD45A	113	4614
IGF1R	86	2862
PIK3R2	111	4433
SKP2	56	1251

According to enrichment map results of MDA-MB-231 as given in Figure 3.14, ‘cell cycle processes’ was observed as the only cluster.

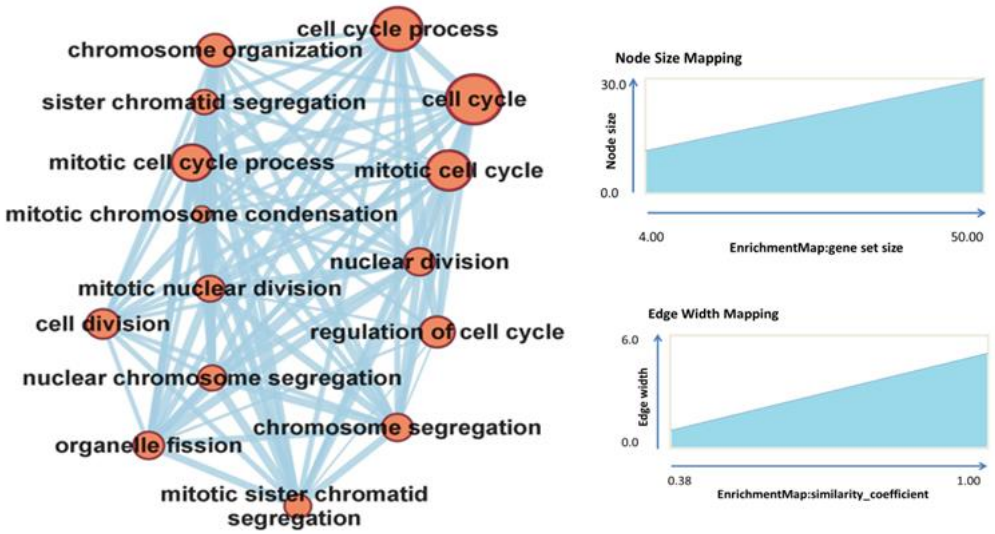

Figure 3.14 Enrichment map of miRNA-regulated MDA-MB-231 genes (adjusted p value≤0.001)

Among the 15 nodes of the cluster, cell cycle was the most significant one observed and it had the largest number of genes. Moreover, the other nodes obtained were also significantly related with cell cycle processes, and from the most significant to least; listed as ‘mitotic cell cycle’, ‘cell cycle process’, ‘mitotic cell cycle process’, ‘chromosome segregation’, ‘mitotic nuclear division’, ‘sister chromatid segregation’, ‘mitotic sister chromatid segregation’, ‘nuclear division’, ‘nuclear chromosome segregation’, ‘organelle fission’, ‘cell division’, ‘chromosome organization’, ‘mitotic chromosome condensation’, ‘regulation of cell cycle’. These nodes though, through different pathways further showed the importance of miRNA regulation also in ER- MDA-MB-231 cells with Emodin.

CHAPTER 4

DISCUSSION AND CONCLUSION

4.1 Discussion

Breast cancer, one of the most frequently diagnosed cancers, has high mortality rates worldwide and unfortunately this rate is expected to increase (Sung et al., 2021). As Estrogen plays important roles in breast cancer initiation and progression, estrogen receptors are important drug targets which have been widely used for treatment. However, resistance to anti-estrogen therapies is important obstacle to overcome. Furthermore, improving alternative therapy strategies for breast cancer cells that do not possess estrogen receptor is also important (Feng et al., 2018; Massarweh and Schiff, 2006). In addition, it is critical to develop drugs with less side effects to decrease death rates from breast cancer. Phytoestrogens showing anti-carcinogenic effects have been investigated for breast cancer treatments with fewer side effects (Tanwar et al., 2021). Emodin is an important phytoestrogen acting as a tyrosine kinase inhibitor which has been studied for its anti-carcinogenic properties in various different cancer cases (Wei et al., 2013) and also in breast cancer (Huang et al., 2013).

miRNAs are important regulators of gene expressions in a variety of biological processes. Their dysregulation can be an indicator of a disorder and returning back to their normal levels can be a treatment approach (Fu et al., 2019). They are widely used in breast cancer studies regarding diagnosis (Adhami et al., 2018) or treatment (Grimaldi et al., 2021). In addition, since one miRNA can regulate more than one gene and one gene can be regulated with multiple miRNAs (Xu et al., 2020), it is important to study gene and miRNA expression profiles conjunctly and interrelatedly. Network systems biology approaches were used for this purpose (Guzzi et al., 2015; Licursi et al., 2019). Integrated analyses of mRNA and miRNA

expression profiles are also being used in breast cancer studies (Evangelista et al., 2021). However, there is no information in literature about the interaction of miRNAs with the gene expressions showing the similarities and the differences of Emodin effect in breast cancer cells with different receptor status.

In our study, anti-carcinogenic effects of phytoestrogen Emodin on (ER+) MCF-7 and (ER-) MDA-MB-231 breast cancer cell lines are examined at both transcriptional and post-transcriptional level by identifying their microarray-based gene and miRNA expression profiles through integrated analysis. Our aim is to identify putative therapeutic targets in each cell line upon Emodin treatment.

Previous microarray-based gene expression data by Sakallı (2010) were reanalyzed and used for integration analyses. Revealed mRNA expression profiles showed that regardless of ER status, Emodin showed anti-carcinogenic properties and anti-proliferative effects in both cell lines. Also, in both cell lines these effects were predominantly through decreasing gene expressions. In addition to this, regarding the fold changes, the number of pathways and the number of genes regulated, Emodin affect was more explicit in MCF-7 cell. Because Emodin is a phytoestrogen and has structural similarities to estrogen, binding Emodin to ER increases the effect of Emodin in ER+ cell line. It was shown that, this proliferation inhibition was through genomic and nongenomic pathways (Huang et al., 2013; Sui et al., 2014). In addition to this, ER is not the only route for Emodin to show its anti-carcinogenic actions, according to the study of Song et al. (2014); Emodin inhibited EMT and metastasis in TNBC and inhibited tumor growth. Our analyses results are in accordance with the previous study results. In MCF-7, MDA-MB-231 and their intersect genes; the most significantly regulated pathway was ‘cell cycle’. Analysis results showed that Emodin suppressed cell cycle related mechanisms in both cell lines.

According to microarray-based miRNA expression data analysis, more than half of the differentially expressed MCF-7 genes and almost half of the differentially expressed MDA-MB-231 genes were regulated by miRNAs upon Emodin

treatment. This shows the importance of miRNAs for the action mechanism of Emodin on MCF-7 and MDA-MB-231 breast cancer cells which is more observed in MCF-7.

In both cell lines, GO and KEGG enrichment results of differentially regulated genes and miRNA target genes were compatible with each other. This showed that differentially expressed genes and therefore their enriched biological processes and pathways are mostly regulated by differentially expressed miRNAs. miRNAs were revealed to be important regulators for Emodin's anticarcinogenic actions in both cell lines which is an important outcome of the study.

In MCF-7, prominent gene cluster was found out by interpreting pathway enrichment analysis results in combination with literature studies especially related with MCF-7 and Emodin. Examination of the genes in Table 3.3 showed that the cluster was composed of FOXO1 pathway related genes and FOXO1 was suggested to be one of the key regulatory genes of Emodin's mechanism of action in MCF-7. CDKN1A, FOXO1, GADD45A, GADD45B, GADD45G genes were upregulated and AKT1, CCND1, CCNE1, CCNE2, CDK1, CDK2, IGF1R, PIK3R1, PIK3R2, PIK3R3, SKP2 genes were downregulated upon Emodin treatment in MCF-7 gene cluster. These expressions were in the same line with literature and according to literature these differential expressions might contribute to Emodin's anti-carcinogenic activity. Furthermore, as seen in Figure 3.6, in addition to FOXO1 related pathways and biological processes, their neighbours were also visualized. Majority of the cell cycle regulation cluster were observed as highlighted meaning FOXO1 effect upon Emodin treatment enlarged indirectly to cell cycle related pathways. This further points out FOXO1 as a key regulatory gene.

Forkhead Box O (FOXO) transcription factors have important regulatory roles in energy metabolism (Gross et al., 2009), cell cycle progression, stress response (Obsil and Obsilova, 2008), apoptosis and cancer (Fu and Tindall, 2008). Having functions as tumour suppressors, their suppressions can be an indicator of cancer

initiation or progression. FOXO1 is one of the members of FOXO transcription factor family. In a variety of cancer cases as glioblastoma (Yan and Wu, 2018), prostate (Dong et al., 2006), endometrial (Goto et al., 2008) and ovarian (Liu et al., 2015), FOXO1 was suppressed. According to the study of Guttilla and White (2009), overexpression of FOXO1 inhibited MCF-7 cell proliferation.

Previously it was shown that the phosphoinositide 3-kinase (PI3K) pathway induction inhibited FOXO mediated growth arrest and apoptosis in MCF-7 cells. According to the study, PI3K knockdown with small interfering RNA (siRNA) decreased cell viability and induced apoptosis regardless of ER status. In addition, phosphorylated AKT1 and CCND1 protein levels were decreased. Moreover, PI3K suppression activated FOXO proteins and induced cell cycle arrest in MCF-7. In addition, FOXO1 inhibition suppressed apoptosis. Decreased level of CCND1 was proposed to be a downstream effect of FOXO induction (Reagan-Shaw and Ahmad, 2006).

It was shown that Emodin suppressed MCF-7 cell proliferation through decreasing protein levels of ERα and ER regulated CCND1 (Huang et al., 2013). In a similar manner, according to the study of Sui et al. (2014); in MCF-7, protein expressions of AKT, BCL-2 and CCND1 were decreased and cell cycle was suppressed at G0/G1 phase and in addition to these apoptosis was induced. Furthermore it was stated that BCL-2 and CCND1 expressions were suppressed through ERα genomic pathway inhibition and AKT expression was suppressed through ERα nongenomic pathway inhibition. In another study, Emodin treatment, decreased AKT1 protein levels in MCF-7 as well as suppressed tumor growth of MCF-7 injected mice (Li et al., 2021).

In addition, it was reported that other than the growth factors ERα also binds to phosphorylated FOXO1 and induces its export from the nucleus (Guttilla and White, 2009). Therefore, by binding to ER and inactivating it, Emodin could further promote FOXO1 activity. According to the study of Campbell et al. (2001), it was shown that AKT was required for the phosphorylation and activation of

ERα. Again the study of Sun et al (2001); showed that AKT2 promoted ERα transcriptional activity via phosphorylating it. Furthermore, ERα activated PI3K/AKT2 pathway by binding to PI3K through a ligand independent route. Therefore, Emodin's inhibitory effects to both AKT, ERα and consequently to PI3K pathway are collectively important regarding Emodin's anti-carcinogenic activities via ERα regulation.

CDKN1A is an important regulator of cell cycle progression at G1 and its overexpression induced MCF-7 growth arrest and apoptosis (Sheikh et al., 1995). According to the study of Carroll et al. (2000), a pure estrogen antagonist arrested MCF-7 cells in quiescence, G0 state. Its mechanism of action showed that upon ER suppression, CyclinE-CDK2 activity was inhibited arresting the cells in G0 state. It was also declared that this inhibition was dependent on CDKN1A. Furthermore, mentioned actions were dependent on decreased CCND1 expression. Another study showed that a phytochemical treatment to MCF-7 induced CDKN1A and GADD45A expressions while decreasing the expressions of CDK1, CCNB1, CCNB2 proteins and consequently cell cycle arrested at G2/M transition. In the study it was also proposed that CCNB1/CCNB2-CDK1 dissociation might be due to the induction of CDKN1A and GADD45A expressions (Katagi et al., 2016). It was also previously shown that both CDKN1A and GADD45A genes are among the targets of FOXO (Farhan et al., 2017). Therefore, in our study, considering the recently mentioned literatures, upon Emodin treatment, CCND1 expression was decreased inducing CDKN1A expression, resulting in CyclinE-CDK2 suppression. In addition, CDK1 expression was decreased either through increased expressions of CDK1 inhibitors CDKN1A and GADD45A or through direct Emodin effect. Furthermore, because CDKN1A and GADD45A genes are the targets of FOXO, FOXO1 induction upon Emodin could contribute to the increase of their gene expressions and subsequent actions.

In addition to Emodin's mentioned effects, it has the ability to act as a tyrosine kinase inhibitor (Jayasuriya et al., 1992). Previously it was shown that Emodin suppressed the growth of tyrosine kinase HER-2/neu-overexpressing breast cancer

cells (Zhang et al., 1999). In our study, upon Emodin treatment, IGF1R gene expression was decreased. IGF1R is a tyrosine kinase receptor and its inhibition is being used in combination with conventional drugs for the treatment of breast cancer (Hartog et al., 2012). Zhang's study (2005) also indicated that, estrogen indirectly induced breast cancer proliferation using non-genomic pathway through inducing IGF1R and therefore activating PI3K/AKT pathway. Although our analysis results are at gene expression level, a decrease in IGF1R gene expression with Emodin, subsequently will decrease IGF1R protein expression. In addition, Emodin could directly bind and suppress tyrosine kinase receptor IGF1R. This suggests that the non-genomic route for Emodin's anti-carcinogenic actions started from the suppression of tyrosine kinase receptor IGF1R.

Using the genes of the predetermined MCF-7 gene cluster, gene interaction networks were constructed for gene and protein level and the layouts were adjusted according to the literature studies. Network results and literature studies were in compatible with each other. Considering all these findings, we propose that, after Emodin binding to ER and also to IGF1R, as a subsequent effect, PI3K cannot be phosphorylated and thus cannot activate its downstream molecule AKT1. Hence, SKP2 which has roles in the turn-over of proteins cannot be induced by AKT1 and FOXO1 cannot be degraded and stays in its active form in the nucleus (Huang et al., 2007; Wang et al., 2012). Therefore, through induction of FOXO1 and its downstream targets, Emodin inhibits proliferation of MCF-7 cells and induces apoptosis as supported with above mentioned studies. Emodin inhibited BCL2 expression in MCF-7 cells and this suppression is an indicator of apoptosis. As well as other researcher's studies (Sui et al., 2014), this finding was also confirmed with our previous study (Sakallı, 2010). CCND1 suppression could result from inactivation of PI3K or activation of FOXO1 or ER suppression or both in combination. Upon CCND1 inhibition, CDKN1A is induced which inhibits CyclinE-CDK2 activity; as a result cell cycle is arrested. Further, CDK1 expression is decreased through CDK1 inhibitors CDKN1A and GADD45A genes or through

direct Emodin effect. The induction of FOXO1 targets, CDKN1A and GADD45A genes by FOXO1 also contributes to the anti-proliferative effects of Emodin.

As well as Emodin's physical binding to receptors and initiated downstream processes, Emodin could induce this network through its direct effect to each of the gene products or direct effects to regulatory molecules like transcription factors or to miRNAs for post-transcriptional regulation or each of the above mechanisms could work together for the activation of FOXO1 related gene cluster and induce anti-proliferative effects in MCF-7 cells.

According to the miRNA-microarray analysis results of MCF-7, hsa-miR-4505 was the most upregulated miRNA with the most miRNA-gene pairs and this is declared for the first time with this study. hsa-miR-27b-3p was the most downregulated miRNA. It was previously found out that miR-27b was an oncogenic miRNA in MCF-7 cells and in xenograft nude mice which are fed with a standard diet, its suppression decreased the tumor growth (Hannafon et al., 2019). In addition, hsa-miR-191-5p had the largest number of miRNA-gene pairs among downregulated miRNAs. miR-191 has shown to regulate important cellular processes like proliferation, apoptosis, migration in many cancer cases including breast cancer (Nagpal and Kulshreshtha, 2014). Furthermore it was shown that miR-191/425 cluster induced breast cancer progression, invasion and metastasis through repressing DICER1 and therefore inhibiting global miRNA processing (Zhang et al., 2018). Moreover, being an estrogen-responsive miRNA, in MCF-7 cell, miR-191 induced proliferation and migration through estrogen induced-ER dependent suppression of its target gene (Nagpal et al., 2013) and suppressed apoptosis (Sharma et al., 2017).

According to our study, FOXO1 related-MCF-7 gene cluster is predominantly regulated by miRNAs. Ten of the 14 genes of the network were regulated by miRNAs. Among upregulated genes, CDKN1A being the target of 7 miRNAs was prominent and it was one of the target genes of the most downregulated miRNA, hsa-miR-27b-3p. Among downregulated genes, IGF1R being the target of 4

miRNAs was another prominent gene of the network and it was among target genes of the most upregulated miRNA, hsa-miR-4505.

FOXO1 which was proposed to be the key regulatory gene in the network was regulated by miR-27a-3p, hsa-miR-27b-3p and hsa-miR-196a-5p miRNAs. CDKN1A was also co-target of these miRNAs. Li et al. (2013b) stated that miR-27a expression was higher in breast cancer with metastasis and induction of miR-27a and its cluster resulted in cell migration, invasion, and hepatic metastasis. In addition, regarding a study with breast cancer patients, this miRNA was proposed to be prognostic marker for cancer progression and patient survival (Tang et al., 2012). According to the study of Guttilla and White (2009), multiple miRNAs including miR-27a were highly expressed in MCF-7, while FOXO1 expression was found to be very low. These miRNAs targeted FOXO1 and repressed FOXO1 expression. FOXO1 overexpression suppressed cell proliferation and promoted cell death. It was concluded that the linkage between those miRNAs and FOXO1 could be used for therapeutic and/or prognostic studies. As stated above, miR-27b was found out as an oncogenic miRNA in MCF-7 cells (Hannafon et al., 2019). Regarding miR-196a, Milevskiy et al. (2019) stated that this miRNA was regulated by ERα and is a promising biomarker for ER+ breast cancer progression to becoming therapy resistant breast cancer. In addition, miR-196a induced tumor growth and angiogenesis in MCF-7 cell (Jiang et al., 2018). All these studies also showed the importance of the regulations of co-miRNAs of CDKN1A and FOXO1 genes for Emodin's anticarcinogenic effects in MCF-7 cells.

CDKN1A also have co-miRNAs with GADD45A which are hsa-miR-191-5p and hsa-miR-375. Since regulations of these two genes lead to cell cycle inhibition (Katagi et al., 2016) and they are targets of FOXO1 (Farhan et al., 2017), their co-regulations by miRNAs are important and further contribute to FOXO1's downstream effects. According to the study of Sharma et al. (2017), miR-191-5p overexpression inhibited apoptosis in MCF-7 cells. Furthermore, CDKN1A protein expression was decreased upon hsa-miR-191-5p induction and its protein levels were increased upon hsa-miR-191-5p suppression (Sharma et al., 2017). As hsa-

miR-191-5p, hsa-miR-375 was also proposed to be an oncogenic miRNA, having roles in breast cancer progression. Its inhibition suppressed cell growth and induced apoptosis in breast cancer cells (Tang et al., 2020).

Moreover, in the second enrichment map in which miRNA-regulated genes were used, FOXO1 related pathways and biological processes were visualized in every miRNA regulated MCF-7 node of subnetwork (Figure 3.13B). This indicates the importance of FOXO1 regulation in miRNA regulated pathways and biological processes. Furthermore, high node and edge numbers of miRNA-targets of FOXO1 related MCF-7 gene cluster (Table 3.10) showed the importance of these genes for the overall miRNA regulation.

On the other hand in MDA-MB-231 cells, both GO and KEGG pathway enrichment analyses showed that, the cell cycle was the most significantly regulated biological process and also had the highest number of differentially regulated genes. Those genes were all suppressed after Emodin treatment. Detailed examination of cell cycle pathway showed that, the spindle assembly checkpoint (SAC) mechanism which is a self-control mechanism in mitosis was prominently regulated in MDA-MB-231. This was also supported with GO enrichment analysis results. Condensed chromosome, spindle, centromeric region, microtubule cytoskeleton, nucleus were among the enriched cellular components. Microtubule binding, microtubule motor activity, DNA helicase activity were among the enriched molecular functions. BUB1, BUB1B, CCNB1, CCNB2, CDC20, CDC23 (APC/C component), CDK1, MAD2L1, PTTG1, TTK gene expressions that have roles in SAC mechanism were suppressed upon Emodin treatment in MDA-MB-231 and a gene interaction network was constructed with these genes according to the literature studies. In addition, enrichment results were visualized through enrichment map (Figure 3.7). Cell cycle regulation was the only enriched cluster and the most significant node was revealed as ‘mitotic cell cycle process’ in which SAC mechanism related genes were included. These results further showed the importance of the regulation of SAC mechanism in MDA-MB-231 cells upon Emodin treatment.

SAC is a self-monitoring system for appropriate alignment of all chromosomes at metaphase prior to anaphase. It is activated upon unattached or mis-attached sister chromatids to the mitotic spindle leading to mitotic arrest eventually. In breast cancer, SAC proteins are overexpressed and their suppression is a potential target for treatment (Marques et al., 2015).

In prometaphase, unattached or mis-attached kinetochores cause the formation of mitotic check point complex (MCC) composed of SAC proteins which are BUB1B, BUB3, MAD2L1, CDC20. TTK is needed for recruitment of check point proteins BUB1, BUB3, BUB1B and activate check point signalling. Upon this signal, MCC formation occurs. Subsequent to MCC formation, APC/C is inhibited and as a result securin and CyclinB could not be degraded which result in mitotic arrest. After all sister chromatids attach to the mitotic spindle and align at the metaphase plate, MCC disassembles leading CDC20 free. CDC20 now is able to activate APC/C. Activated APC/C degrades securin (PTTG1) and CyclinB. Upon securin degradation which is the inhibitor of separase; separase is released and cleaves cohesin protein complex which holds sister chromatids together. Following these, chromosomes are separated. After CyclinB1 degradation which leads to inactivation of CDK1, the cell can exit mitosis (Marques et al., 2015; Bolanos-Garcia and Blundell, 2011; Ji et al., 2017).

SAC is a potential target for breast cancer treatment. In order to suppress mitosis by targeting SAC, there are two approaches. First is by targeting core SAC components (like TTK, BUB1, BUB1B, etc.) which are responsible for checkpoint signal and whose suppressions cause mitosis inhibition. Upon their suppressions tumour cells are forced to exit mitosis prematurely, resulting in massive chromosome mis-segregation leading to cell death. The other approach is inhibiting mitotic exit by targeting SAC components like CENPE which are involved in kinetochore microtubule attachment not in SAC signal generation. Inhibiting mitotic exit prolongs the time cells spend in mitosis and eventually tumour cells die through apoptosis. In addition to these, premature CyclinB degradation is a signal for premature mitotic exit, resulting in aneuploid cells (Marques et al., 2015).

Therefore, we suggest that, upon Emodin treatment, in MDA-MB-231 cells, core SAC components BUB1, BUB1B, CDC20, CDC23, MAD2L1, PTTG1, TTK were suppressed, furthermore CyclinB (CCNB1, CCNB2) genes were degraded prematurely and CDK1 was inactivated subsequently; and all of these caused premature mitotic exit and massive aneuploidy leading to cell death.

Among these TTK and BUB1 are among top ten down regulated genes. TTK is the spindle assembly checkpoint kinase promoting correct kinetochore microtubule attachment and often overexpressed in TNBC. High expression of TTK was correlated with high tumour grade and worse survival particularly in TNBC (Al-Ejeh et al., 2014). TTK was suggested to be an attractive therapeutic target for TNBC (Maire et al., 2013). Furthermore, Maia et al. (2015) showed that, inhibition of TTK, inhibited tumour growth in MDA-MB-231 xenografts and enhanced treatment efficacy.

BUB1, being a mitotic checkpoint protein, is overexpressed in breast cancer and associated with proliferation, progression and poor prognosis (Han et al., 2015; Takagi et al., 2013). Its suppression reduced cancer stem cell potential of MDA-MB-231 cell line (Han et al., 2015).

BUB1B is a member of mitotic check point complex and important for proper mitosis process. Its elevated expression is associated with poor survival in early breast cancer patients regarding analysis lasted for 15 years (Maciejczyk et al., 2013). According to another study which used tumour samples of invasive ductal carcinoma patients, high BUB1B expression was positively associated with Ki-67 expression which indicates cell proliferation extent (Du et al., 2011). Furthermore, BUB1B is one of five genes of Molecular Grade Index prognostic assay which classifies patient's tumour grades (Rajput et al., 2011).

MAD2L1 is another component of mitotic check point complex. Its overexpression in breast cancer is associated with proliferation (Marques et al., 2015). Furthermore, there is a study stating that MAD2L1 overexpression delayed breast cancer but facilitated oncogene independent outgrowth (Rowald et al., 2016).

CDC23 protein is the regulated component of Anaphase Promoting Complex, APC, in MDA-MB-231 upon Emodin treatment. Achari et al. (2014) stated in their study that, miR-34c, which was expressed lower in breast cancer and associated with poor prognosis, suppressed proliferation in MDA-MB-231 and this suppression was thought to be associated with CDC23 inhibition which caused G2/M arrest (Achari et al., 2014).

CDC20 is the activator protein of APC/C during mitosis. In a study, its inhibition induced mitotic arrest in MDA-MB-231 (Bah et al., 2014). In another study performed by Karra et al. for 20 years, it was found that high CDC20 and securin expression was associated with aneuploidy and was common in short breast cancer survival and TNBC (Karra et al., 2014).

Furthermore, CENPE, CENPF, CENPA and several other centromere proteins responsible for chromosome segregation (CENPN, CENPW, CENPU, and CENPL) are down regulated upon Emodin treatment. CENPE is an important centromere protein being part of spindle assemble check point. CENPE inhibition suppressed proliferation of TNBC and CENPE was proposed to be therapeutic target for patients with TNBC (Kung et al., 2014). CENPF is needed for recruitment of checkpoint proteins and its expression was found to be associated with poor prognosis and chromosomal instability (O'Brien et al., 2007). CENPA is one of the genes of MammaPrint® and one of five genes of Molecular Grade Index prognostic assays. CENPA overexpression was associated with genomic instability. Its expression was found to be related with shorter disease free survival in breast cancer patients (Rajput et al., 2011). In MDA-MB-231, CENPA was among top 10 down regulated genes upon Emodin treatment.

All these literature studies give the evidence for the importance of SAC mechanism suppression for cell growth inhibition in MDA-MB-231 cells after Emodin treatment.

Our integrative analysis of miRNA-mRNA microarray results showed that, MDA-MB-231 cells which are ER negative, though through different pathways, were also

to a great extent regulated by miRNAs. Functional enrichment analysis of differentially expressed genes and miRNA-regulated genes were compatible with each other. In both analyses, cell cycle was the predominant biological process. This was also visualized through enrichment map. While in the first enrichment map, ‘cell cycle regulation’ was the only enriched cluster; in the second enrichment map, the only enriched cluster was ‘cell cycle processes’. Therefore, we show that, miRNA regulation is the major part of the cell cycle suppression effect of Emodin in MDA-MB-231 cell line.

The most upregulated miRNA with 244-fold increase was observed as hsa-miR-3135b. Although its high expression was also associated with apocrine cell tumour morphology in TNBC (Koleckova et al., 2021), its high fold change should be further studied to examine the relation with Emodin’s anticarcinogenic effect.

The most downregulated miRNA with 56 fold decrease was hsa-miR-4454. This miRNA targeted only one gene, HSPA1A, expression of which has proposed to be related with anoikis resistance in MDA-MB-231 (Zhong et al., 2018). There are no more study in the literature regarding hsa-miR-4454 expression in MDA-MB-231 cells.

Among down regulated miRNAs, having the largest number of miRNA-gene pairs, hsa-miR-27a-5p drew attention. In MDA-MB-231, miR-27a overexpression increased proliferative and angiogenic properties (Mertens-Talcott et al., 2007) and regarding its roles in tumour growth miR-27a was proposed to be a promising biomarker for prediction of prognosis (Wu et al., 2018). Therefore, downregulation of hsa-miR-27a-5p expression could be a way of Emodin’s antiproliferative effect.

According to the enrichment analyses, DNA replication was the only pathway of miRNA-regulated MDA-MB-231 genes and suppressed upon Emodin treatment. Four genes of this pathway (MCM5, PRIM1, RFC3, and RNASEH2C) were regulated by 4 miRNAs (hsa-miR-4443, hsa-miR-6126, hsa-miR-4763-3p, and hsa-miR-3665). Roles in DNA replication and breast cancer relations of these genes were also reported in other studies. MCM5 had low prognostic value for breast

cancer patients (Lie et al., 2021). PRIM1 had roles in Estrogen induced breast cancer development (Lee et al., 2019). RFC3 had low prognostic value and induced metastasis in MDA-MB-231 (He et al., 2017). RNASEH2C was reported to have roles in breast cancer progression and indicate patients with high metastasis risk (Deasy et al., 2019). Regarding miRNAs, whereas hsa-miR-3665 has been studied for its tumour suppressor potential in endometrial carcinoma (Ni et al., 2020) and hsa-miR-6126 is a tumour suppressor miRNA in ovarian cancer cells potentials (Kanlikilicer et al., 2018); hsa-miR-4443 induced migration and invasion in MDA-MB-231 (Wang et al. 2020) and hsa-miR-4763-3p is stated to be potential biomarker for human adult fulminant myocarditis (Nie et al., 2020).

Differentially regulated common genes of MCF-7 and MDA-MB-231 cell lines were most significantly enriched in cell cycle related processes after Emodin treatment and genes belonging to cell cycle and proliferation related pathways were all downregulated. Therefore it was concluded that regardless of ER possession, Emodin regulated both cell lines through suppressing cell cycle and proliferation related pathways.

Furthermore, according to analysis of intersection of miRNA regulated genes of both cell lines and common miRNA regulated genes of common miRNAs, it was revealed that cellular response to stress and cell cycle related processes were regulated by miRNAs. Therefore, miRNAs are again revealed to be important regulators regarding common genes in both cell lines.

As shown in Figure 3.11, there were three miRNAs, which had integrated target genes in both cell lines. Downregulated hsa-miR-27b-3p, which targeted CPEB2, CYP1B1, FLCN, VEGFA genes, and upregulated hsa-miR-4443 and hsa-miR-4763-3p miRNAs which targeted ENY2, HIRIP3, NCAPH, RMI1, SPC24, WDHD1 and DTL, MCM5, TCF19 genes respectively, are important. There are studies in the literature regarding hsa-miR-27b-3p and hsa-miR-4443 miRNAs. hsa-miR-27b-3p was defined primarily as an oncogenic miRNA in MCF-7 (Hannafon et al., 2019) and induced tumor progression, cell invasion and

metastasis in MDA-MB-231 (Shen et al., 2020). Therefore, downregulation of hsa-miR-27b-3p upon Emodin treatment could be important for suppression of proliferation in both cell lines. However, according to studies, CYP1B1 and VEGFA genes have tumorigenic effects in breast cancer cells. CYP1B1 increased proliferation and metastasis in MCF-7 (Kwon et al., 2016) and VEGFA induced metastasis in MDA-MB-231 (Kim et al., 2017). In addition to these, CPEB2 and FLCN genes were reported to have breast cancer inhibitory effects. CPEB2 has tumour suppressor role in human mammary epithelial cells (Tordjman et al., 2019) and FLCN suppresses breast tumor growth (El-Houjeiri et al. 2021)

Although upregulation of hsa-miR-4443 expression correlated with drug resistance in both MCF-7 and MDA-MB-231 cell lines according to the study of (Chen et al., 2016) and induced migration and invasion in MDA-MB-231 (Wang et al. 2020), its revealed targets which were downregulated for both cell lines in our study, have anti-tumorigenic effects according to literature. ENY2 was described as a biomarker for lung metastasis in TNBC (Xie et al., 2019). NCAPH (Lu et al., 2020) and RMI1 (Zhang et al., 2023) were identified as a prognostic biomarker for breast cancer. SPC24 knockdown suppressed breast cancer progression and induced apoptosis through regulating PI3K/AKT signaling (Zhou et al., 2018). WDHD1 was stated to be a biomarker for poor breast cancer prognosis (Thangavelu et al., 2018) and found to be essential for PTEN inactive TNBC (Ertay et al., 2020). Regarding HIRIP3, having roles in chromatin assembly, Rafique et al. suggested in their study that, the genes of chromosome region in which HIRIP3 resides, upregulated with Estrogen and as a result, unfolding of chromatin structure regulated breast cancer in MCF-7 (Rafique et al., 2015). However downregulation of HIRIP3 upon Emodin in MCF-7 could have a different significance.

While there is no study regarding breast cancer, for human adult fulminant myocarditis hsa-miR-4763-3p was found to be a biomarker (Nie et al., 2020). According to our study, this miRNA targeted DTL, MCM5, TCF19 genes upon Emodin treatment and related literature studies showed the importance of repression of these genes in breast cancer. In a study in which breast cancer cell

also used, DTL induced cancer progression (Cui et al., 2019). As stated above, MCM5 had low prognostic value for breast cancer patients (Liu et al., 2021). TCF19 was proposed to be related with Estrogen and Progestogen related breast cancer tumorigenesis (Deng et al., 2022).

Finally, genomic locations of differentially expressed miRNAs were also examined for each cell line; chromosome 3, chromosome 17 and chromosome 19 were found to be important in MCF-7 cell line with respect to FOXO signaling pathway. In MDA-MB-231 cell line, chromosome 19 was the most related chromosome for cell cycle regulation.

This study is the first integrative analysis of gene and miRNA expressions of two breast cancer cells having either ER and not, upon treatment with phytoestrogen Emodin. By comparing the effects of Emodin in two cell lines, the different effects of Emodin to cells with different ER status were examined. Furthermore, with integrative approach, the effects of Emodin were defined at both transcriptional and post-transcriptional level. Potential key genes, miRNAs and miRNA-gene interactions were determined which presents potential therapeutic targets.

According to the results, Emodin showed anti-carcinogenic effects in both cell lines but these effects were more remarkably observed in ER positive MCF-7 cells rather than MDA-MB-231, which shows the importance of the interaction of Emodin and ER for Emodin's anticarcinogenic effects. Moreover, in both cell lines, miRNAs were shown to be important regulators for gene expressions after Emodin treatment. Therefore, in MCF-7 it is proposed that the suppression of proliferation is started with the downstream effects of the interaction of Emodin with ER and miRNAs regulate the processes post transcriptionally. Constructed pathways should be further investigated for the potential therapeutic targets.

Different types of breast cancers have different treatment approaches ranging from hormone, chemo, radio or targeted therapy, to surgery or their combinations. Therefore, it is important to compare the treatment mechanisms of breast cancers with different ER status. Moreover, since cancer has a dynamic, rapid evolving and

adaptive nature, searching alternative ways is important to overcome potential resistance to currently available drugs and therefore increase the treatment success. In addition, with studies using natural products, less toxic molecules are explored. FOXO1 pathway and SAC mechanism which are prominent pathways for Emodin's action mechanism in MCF-7 and MDA-MB-231 cells respectively will serve for mentioned alternative therapy options.

4.2 Conclusion

- Emodin affected both cell lines at gene and miRNA level, with more in ER+ MCF-7 cells, with mechanisms which suppress proliferation in both breast cancers.
- In MCF-7 cells, FOXO1-related gene cluster was regulated prominently.
- Ten genes of the FOXO1-related MCF-7 gene cluster composing of upregulated CDKN1A, FOXO1, GADD45A and downregulated AKT1, CCND1, CCNE1, CDK2, IGF1R, PIK3R2, SKP2 genes were regulated by miRNAs according to miRNA target gene databases.
- According to the integrated analyses results together with literature, upregulations of CDKN1A, FOXO1 and IGF1R genes and downregulations of hsa-miR-27a-3p, hsa-miR-27b-3p, hsa-miR-191-5p, hsa-miR-196a-5p, and hsa-miR-375 miRNAs of the FOXO1-related MCF-7 gene cluster drew attention for Emodin's mechanism of action. In addition, being the highest-ranking upregulated miRNA and with the largest number of miRNA-gene pairs hsa-miR-4505, which was related to breast cancer for the first time in this study, stood out.
- In MDA-MB-231, prominently, SAC mechanism related genes were suppressed which leads to cell cycle repression and miRNAs regulated cell cycle processes. With the largest number of miRNA-gene pairs, and being supported with literature, downregulated hsa-miR-27a-5p drew attention regarding Emodin's action mechanism in MDA-MB-231.

- In both cell lines, cell cycle related genes were regulated. Regarding miRNAs, cellular response to stress and cell cycle related processes were regulated. Downregulation of hsa-miR-27b-3p, which is an oncogenic miRNA for both cell lines according to the literature, attracted attention.

REFERENCES

Achari, C., Winslow, S., Ceder, Y., Larsson C. (2014). Expression of miR-34c induces G2/M cell cycle arrest in breast cancer cells. BMC Cancer, 14,538. doi: 10.1186/1471-2407-14-538

Adhami, M., Haghdoost, A.A., Sadeghi, B., Afshar, R.M. (2018) Candidate miRNAs in human breast cancer biomarkers: a systematic review. Breast Cancer, 25(2), 198–205. doi: 10.1007/s12282-017-0814-8

Agarwal, V., Bell, G.W., Nam, J., Bartel, D.P. (2015). Predicting effective microRNA target sites in mammalian mRNAs. ELife, 4, e05005. doi: 10.7554/eLife.05005

Al-Ejeh, F., Simpson, P.T., Sanus, J.M., Klein, K., Kalimutho, M., Shi, W., ... Khanna, K.K. (2014). Meta-analysis of the global gene expression profile of triple-negative breast cancer identifies genes for the prognostication and treatment of aggressive breast cancer. Oncogenesis, 3(4), e100. doi: 10.1038/oncsis.2014.14

Ambros, V. (2003). MicroRNA pathways in flies and worms: growth, death, fat, stress, and timing. Cell, 113(6), 673–676. doi: 10.1016/s0092-8674(03)00428-8

American Cancer Society. (2015). Cancer Facts & Figures. Atlanta, Georgia. https://www.cancer.org/research/cancer-facts-statistics/all-cancer-facts-figures/cancer-facts-figures-2015.html

Bah, N., Maillet,L., Ryan, J., Dubreil, S., Gautier, F., Letai, A., ... Barillé-Nion, S. (2014). Bcl-xL controls a switch between cell death modes during mitotic arrest. Cell Death Dis., 5(6), e1291. doi: 10.1038/cddis.2014.251

Bartel, D.P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. Cell, 116(2), 281–297. doi: 10.1016/s0092-8674(04)00045-5

Bhat, S.S., Prasad, S.K., Shivamallu, C., Prasad, K.S., Syed, A., Reddy, P., ... Amachawadi, R.G. (2021). Genistein: A Potent Anti-Breast Cancer Agent. Curr Issues Mol Biol, 43(3), 1502–1517. doi: 10.3390/cimb43030106

Bhattacharjee, M., Upadhyay, P., Sarker, S., Basu, A., Das, S., Ghosh, A., ... Adhikary, A. (2020). Combinatorial therapy of Thymoquinone and Emodin synergistically enhances apoptosis, attenuates cell migration and reduces stemness efficiently in breast cancer. Biochim. Biophys. Acta, 1864(11), 129695. doi: 10.1016/j.bbagen.2020.129695

Bolanos-Garcia, V.M., Blundell, T.L. (2011). BUB1 and BUBR1: multifaceted kinases of the cell cycle. Trends Biochem Sci, 36(3), 141–150. doi: 10.1016/j.tibs.2010.08.004.

Buruiană, A., Florian, Ș. I., Florian, A. I., Timiș, T. L., Mihu, C. M., Miclăuș, ... Șușman, S. (2020). The Roles of miRNA in Glioblastoma Tumor Cell Communication: Diplomatic and Aggressive Negotiations. International journal of molecular sciences, 21(6), 1950. doi: 10.3390/ijms21061950

Burstein, H.J. (2020). Systemic Therapy for Estrogen Receptor–Positive, HER2-Negative Breast Cancer. N Engl J Med, 383(26), 2557–2570. doi: 10.1056/NEJMra1307118

Cai, J., Niu, X., Chen, Y., Hu, Q., Shi, G., Wu, H., ... Yi, J. (2008). Emodin-induced generation of reactive oxygen species inhibits RhoA activation to sensitize gastric carcinoma cells to anoikis. Neoplasia, 10(1), 41–51. doi: 10.1593/neo.07754.

Campbell, R.A., Bhat-Nakshatri, P., Patel, N.M., Constantinidou, D., Ali, S., Nakshatri H. (2001). Phosphatidylinositol 3-kinase/AKT-mediated activation of estrogen receptor alpha: a new model for anti-estrogen resistance. The Journal of Biological Chemistry, 276(13), 9817–9824. doi: 10.1074/jbc.M010840200

Cansaran-Duman, D., Yangın, S., Çolak, B. (2021). The role of vulpinic acid as a natural compound in the regulation of breast cancer-associated miRNAs. Biol Res, 54(1), 37. doi: 10.1186/s40659-021-00360-4

Carroll, J.S., Prall, O.W.J., Musgrove, E.A., Sutherland, R.L. (2000). A Pure Estrogen Antagonist Inhibits Cyclin E-Cdk2 Activity in MCF-7 Breast Cancer Cells and Induces Accumulation of p130-E2F4 Complexes Characteristic of Quiescence. The Journal of Biological Chemistry, 275(49), 38221-38229. doi: 10.1074/jbc.M004424200

Ceylan, İ. (2018). Ht-29 insan kolorektal kanser hücre hattında emodin'in mir-25 ifadesine etkisi. Adnan Menderes Üniversitesi, Fen Bilimleri Enstitüsü, Yüksek Lisans Tezi. Aydın.

Chen, X., Zhong, S.L, Lu, P., Wang, D.D., Zhou, S.Y., Yang, S.J., ... Tang, J.H. (2016). miR-4443 Participates in the Malignancy of Breast Cancer. PLoS ONE, 11(8), e0160780. doi: 10.1371/journal.pone.0160780

Chen, Y., Wang, X. (2020). miRDB: an online database for prediction of functional microRNA targets. Nucleic Acids Research, 48(D1), D127–D131. doi: 10.1093/nar/gkz757

Chen, Y.Y., Chiang, S.Y., Lin, J.G., Ma, Y.S., Liao, C.L., Weng, S.W., ... Chung, J.G. (2010). Emodin, aloe-emodin and rhein inhibit migration and invasion in human tongue cancer SCC-4 cells through the inhibition of gene

expression of matrix metalloproteinase-9. Int J Oncol, 36(5), 1113–1120. doi: 10.3892/ijo_00000593

Cheng, H.Y., Papp, J.W., Varlamova, O., Dziema, H., Russell, B., Curfman, J.P., ... Obrietan, K. (2007). microRNA modulation of circadian-clock period and entrainment. Neuron, 54(5), 813–829. doi: 10.1016/j.neuron.2007.05.017

Condrat, C.E., Thompson, D.C., Barbu, M.G., Bugnar, O.L., Boboc, A., Cretoiu, D., ... Voinea, S.C. (2020). miRNAs as Biomarkers in Disease: Latest Findings Regarding Their Role in Diagnosis and Prognosis. Cells, 9(2), 276. doi: 10.3390/cells9020276

Connelly, C.M., Thomas, M., Deiters, A. (2012). High-throughput luciferase reporter assay for small-molecule inhibitors of microRNA function. Journal of biomolecular screening, 17(6), 822–828. doi: 10.1177/1087057112439606

Cui, H., Wang, Q., Lei, Z., Feng, M., Zhao, Z., Wang, Y., Wei, G. (2019). DTL promotes cancer progression by PDCD4 ubiquitin-dependent degradation. J Exp Clin Cancer Res., 38(1), 350. doi: 10.1186/s13046-019-1358-x.

Deasy, S.K., Uehara, R., Vodnala, S.K., Yang, H.H., Dass, R.A., Hu, Y., ... Hunter, K.W. (2019). Aicardi-Goutières syndrome gene Rnaseh2c is a metastasis susceptibility gene in breast cancer. PLoS Genet., 15(5), e1008020. doi: 10.1371/journal.pgen.1008020

Deng, Y., Huang, H., Shi, J., Jin, H. (2022). Identification of Candidate Genes in Breast Cancer Induced by Estrogen Plus Progestogens Using Bioinformatic Analysis. Int J Mol Sci., 23(19), 11892. doi: 10.3390/ijms231911892

Denkiewicz, M., Saha, I., Rakshit, S., Sarkar, J.P., Plewczynski, D. (2019). Identification of Breast Cancer Subtype Specific MicroRNAs Using Survival Analysis to Find Their Role in Transcriptomic Regulation. Front Genet., 10, 1047. doi: 10.3389/fgene.2019.01047

Di Leva, G., Croce, C. M. (2013). miRNA profiling of cancer. Current opinion in genetics & development, 23(1), 3–11. doi: 10.1016/j.gde.2013.01.004

Di Leva, G., Garofalo, M., Croce, C.M. (2014). MicroRNAs in Cancer. Annual Review of Pathology, 9, 287–314. doi: 10.1146/annurev-pathol-012513-104715

Dong, H., Lei, J., Ding, L., Wen, Y., Ju, H., Zhang, X. (2013). MicroRNA: Function, Detection, and Bioanalysis. Chemical Reviews, 113(8), 6207–6233. doi: 10.1021/cr300362f.

Du, J., Du, Q., Zhang, Y., Sajdik, C., Ruan, Y., Tian, X.X., Fang, W.G. (2011). Expression of cell-cycle regulatory proteins BUBR1, MAD2, Aurora A, cyclin A and cyclin E in invasive ductal breast carcinomas. Histol Histopathol., 26(6), 761–768. doi: 10.14670/HH-26.761

El-Houjeiri, L., Biondini, M., Paquette, M., Kuasne, H., Pacis, A., Park, M., ... Pause, A. (2021). Folliculin impairs breast tumor growth by repressing TFE3-dependent induction of the Warburg effect and angiogenesis. J Clin Invest., 131(22), e144871. doi: 10.1172/JCI144871

Enerly, E., Steinfeld I, Kleivi K, Leivonen S-K, Aure MR, Russnes HG, ... Børresen-Dale, A.L. (2011) miRNA-mRNA Integrated Analysis Reveals Roles for miRNAs in Primary Breast Tumors. PLoS ONE, 6(2): e16915. doi: 10.1371/journal.pone.0016915

Ertay A, Liu H, Liu D, Peng P, Hill C, Xiong H, ... Wang Y. (2021). WDHD1 is essential for the survival of PTEN-inactive triple-negative breast cancer. Cell Death Dis., 11(11), 1001. doi: 10.1038/s41419-020-03210-5

Evangelista, A.F., Oliveira, R.J., Silva, V.A.O., Vieira, R.A.D.C., Reis, R.M., Marques, M.M.C. (2021). Integrated analysis of mRNA and miRNA profiles revealed the role of miR-193 and miR-210 as potential regulatory biomarkers in different molecular subtypes of breast cancer. BMC Cancer, 21(1), 76. doi: 10.1186/s12885-020-07731-2

Farhan, M., Wang, H., Gaur, U., Little, P.J., Xu, J., Zheng, W. (2017). FOXO Signaling Pathways as Therapeutic Targets in Cancer. Int J Biol Sci, 13(7), 815–827. doi: 10.7150/ijbs.20052

Fazeli, S. (2016). Identification of Phenolic Compounds in Roots of Rheum Ribes L. M.Sc. Thesis, Middle East Technical University, Ankara.

Feng, Y., Spezia, M., Huang, S., Yuan, C., Zeng, Z., Zhang, L., ... Ren, G. (2018). Breast cancer development and progression: Risk factors, cancer stem cells, signaling pathways, genomics, and molecular pathogenesis. Genes & Diseases, 5(2), 77–106. doi: 10.1016/j.gendis.2018.05.001

Ferrero, G., Carpi, S., Polini, B., Pardini, B., Nieri, P., Impeduglia, A., ... Naccarati, A. (2021). Intake of Natural Compounds and Circulating microRNA Expression Levels: Their Relationship Investigated in Healthy Subjects With Different Dietary Habits. Front Pharmacol., 11, 619200. doi: 10.3389/fphar.2020.619200

Filipów, S., Łaczmański, Ł. (2019). Blood Circulating miRNAs as Cancer Biomarkers for Diagnosis and Surgical Treatment Response. Frontiers in genetics, 10, 169. doi: 10.3389/fgene.2019.00169

Finotti, A., Fabbri, E., Lampronti, I., Gasparello, J., Borgatti, M., Gambari, R. (2019). MicroRNAs and Long Non-coding RNAs in Genetic Diseases. Mol Diagn Ther., 23(2), 155–171. doi: 10.1007/s40291-018-0380-6

Friedman, R.C., Farh, K.K., Burge, C.B., Bartel, D.P. (2009). Most mammalian mRNAs are conserved targets of microRNAs. Genome Research, 19(1), 92–105. doi: 10.1101/gr.082701.108

Fu, Y., Chen, J., Huang, Z. (2019). Recent progress in microRNA-based delivery systems for the treatment of human disease. ExRNA, 1, 24. doi: 10.1186/s41544-019-0024-y

Genovesi, L.A., Carter, K.W., Gottardo, N.G., Giles, K.M., Dallas, P.B. (2011). Integrated analysis of miRNA and mRNA expression in childhood medulloblastoma compared with neural stem cells. PLoS One, 6(9), e23935. doi: 10.1371/journal.pone.0023935

Greco, S.J., Rameshwar, P. (2007). MicroRNAs regulate synthesis of the neurotransmitter substance P in human mesenchymal stem cell-derived neuronal cells. Proceedings of the National Academy of Sciences of the United States of America, 104 (39), 15484–15489. doi: 10.1073/pnas.0703037104

Grimaldi, A.M., Salvatore, M., Incoronato, M. (2021). miRNA-Based Therapeutics in Breast Cancer: A Systematic Review. Front Oncol., 11, 668464. doi: 10.3389/fonc.2021.668464

Gross, D.N., Wan, M., Birnbaum, M.J. (2009). The role of FOXO in the regulation of metabolism. Curr Diab Rep, 9(3), 208–214. https://doi.org/10.1007/s11892-009-0034-5

Guo J, Li W, Shi H, Xie X, Li L, Tang, H., ... Xie, X. (2013). Synergistic effects of curcumin with emodin against the proliferation and invasion of breast cancer cells through upregulation of miR-34a. Molecular and Cellular Biochemistry, 382 (1-2), 103–111, 2013. doi: 10.1007/s11010-013-1723-6

Guttilla, I.K., White, B.A. (2009). Coordinate regulation of FOXO1 by miR-27a, miR-96, and miR-182 in breast cancer cells. J Biol Chem, 284(35), 23204–23216. doi: 10.1074/jbc.M109.031427.

Guzzi, P.H., Di Martino, M.T., Tagliaferri, P., Tassone, P., Cannataro, M. (2015). Analysis of miRNA, mRNA, and TF interactions through network-based methods. EURASIP journal on bioinformatics & systems biology, 2015, 4. doi: 10.1186/s13637-015-0023-8

Hamam, R., Ali, A.M., Alsaleh, K.A., Kassem, M., Alfayez, M., Aldahmash, A., Alajez, N.M. (2016). microRNA expression profiling on individual breast cancer patients identifies novel panel of circulating microRNA for early detection. Sci Rep., 6, 25997. doi: 10.1038/srep25997

Han, J.Y., Han, Y.K., Park, G.Y., Kim, S.D., Lee, C.G. (2015). Bub1 is required for maintaining cancer stem cells in breast cancer cell lines. Sci Rep., 5, 15993. doi: 10.1038/srep15993

Hanahan, D., Weinberg, R.A. (2000). The Hallmarks of Cancer. Cell, 100(1), 57–70. doi: 10.1016/s0092-8674(00)81683-9

Hanna J, Hossain GS, Kocerha J. (2019). The Potential for microRNA Therapeutics and Clinical Research. Front Genet., 10, 478. doi: 10.3389/fgene.2019.00478

Hannafon, B.N., Cai, A., Calloway, C.L., Xu, Y.F., Zhang, R., Fung, K.M., Ding, W.Q. (2019). miR-23b and miR-27b are oncogenic microRNAs in breast cancer: evidence from a CRISPR/Cas9 deletion study. BMC Cancer, 19(1), 642. doi: 10.1186/s12885-019-5839-2

Harris, D.M., Besselink, E., Henning, S.M., Go, V.L., Heber, D. (2005). Phytoestrogens induce differential estrogen receptor alpha- or Beta-mediated responses in transfected breast cancer cells. Exp BioI Med., 230(8), 558–568. doi: 10.1177/153537020523000807

Hartog, H., Van Der Graaf, W.T., Boezen, H.M., Wesseling, J. (2012). Treatment of breast cancer cells by IGF1R tyrosine kinase inhibitor combined with conventional systemic drugs. Anticancer Res, 32(4), 1309–1318.

He, Z.Y., Wu, S.G., Peng, F., Zhang, Q., Luo, Y., Chen, M., Bao, Y. (2017). Up-Regulation of RFC3 Promotes Triple Negative Breast Cancer Metastasis and is Associated With Poor Prognosis Via EMT. Transl Oncol., 10(1), 1–9. doi: 10.1016/j.tranon.2016.10.004

Hill, M., Tran, N. (2018). MicroRNAs Regulating MicroRNAs in Cancer. Trends in cancer, 4(7), 465–468. doi: 10.1016/j.trecan.2018.05.002

Hsu, C.M., Yen, C.H., Wang, S.C., Liu, Y.C., Huang, C.T., Wang, MH, ... Hsiao, H.H. (2022) Emodin Ameliorates the Efficacy of Carfilzomib in Multiple Myeloma Cells via Apoptosis and Autophagy. Biomedicines, 10(7), 1638. doi: 10.3390/biomedicines10071638.

Hsu, S.D., Lin, F.M., Wu, W.Y., Liang, C., Huang, W.C., Chan, W.L., ... Huang, H.D. (2011). miRTarBase: a database curates experimentally validated microRNA-target interactions. Nucleic Acids Res., 39(Database issue), D163–D169. doi: 10.1093/nar/gkq1107

Huang, H., Tindall, D.J. (2007). Dynamic FoxO transcription factors. J Cell Sci, 120(15), 2479–2487. doi: 10.1242/jcs.001222

Huang, P.H., Huang, C.Y, Chen, M.C., Lee, Y.T., Yue, C.H., Wang, H.Y., Lin, H. (2013). Emodin and Aloe-Emodin Suppress Breast Cancer Cell Proliferation through ERα Inhibition. Evid Based Complement Alternat Med., 2013, 376123. doi: 10.1155/2013/376123

Huang, Q., Shen, H.M., Ong, C.N. (2004). Inhibitory effect of emodin on tumor invasion through suppression of activator protein-1 and nuclear factor-kappaB. Biochem. Pharmacol., 68, 361–371. doi: 10.1016/j.bcp.2004.03.032

Jayasuriya, H., Koonchanok, N.M., Geahlen, R.L., Mclaughlin, J.L., Chang, C.J. (1992). Emodin, a protein tyrosine kinase inhibitor from Polygonum cuspidatum. J Nat Prod, 55, 696–698. doi: 10.1021/np50083a026

Ji, Z., Gao, H., Jia, L., Li, B., Yu, H. (2017). A sequential multi-target Mps1 phosphorylation cascade promotes spindle checkpoint signaling. ELife, 6, e22513. doi: 10.7554/eLife.22513

Jiang, C.F., Shi, Z.M., Li, D.M., Qian, Y.C., Ren, Y., Bai, X.M., ... Jiang, B.H. (2018). Estrogen-induced miR-196a elevation promotes tumor growth and metastasis via targeting SPRED1 in breast cancer. Mol Cancer., 17(1), 83. doi: 10.1186/s12943-018-0830-0

Jørgensen, S., Paulsen, I.W., Hansen, J.W., Tholstrup, D., Hother, C., Sørensen, E., ... Pedersen, O.B. (2020). The value of circulating microRNAs for early diagnosis of B-cell lymphoma: A case-control study on historical samples. Sci Rep., 10(1), 9637. doi: 10.1038/s41598-020-66062-1

Kanlikilicer, P., Rashed, M.H., Bayraktar, R., Mitra, R., Ivan, C., Aslan, B., ... Lopez-Berestein, G. (2018). Ubiquitous Release of Exosomal Tumor Suppressor miR-6126 from Ovarian Cancer Cells. Cancer Res., 76(24), 7194–7207. doi: 10.1158/0008-5472.CAN-16-0714

Karagkouni, D., Paraskevopoulou, M.D., Chatzopoulos, S., Vlachos, I.S., Tastsoglou, S., Kanellos, I., ... Hatzigeorgiou, A.G. (2018). DIANA-TarBase v8: a decade-long collection of experimentally supported miRNA-gene interactions. Nucleic Acids Res., 46(D1), D239–D245. doi: 10.1093/nar/gkx1141

Karra, H., Repo, H., Ahonen, I., Löyttyniemi, E., Pitkänen, R., Lintunen, M., ... Kronqvist, P. (2014). Cdc20 and securin overexpression predict short-term breast cancer survival. Br J Cancer, 110(12), 2905–2913. doi: 10.1038/bjc.2014.252

Katagi, A., Sui, L., Kamitori, K., Suzuki, T., Katayama, T., Hossain A, ... Tokuda, M. (2016). Inhibitory effect of isoamericanol A from Jatropha curcas seeds on the growth of MCF-7 human breast cancer cell line by G2/M cell cycle arrest. Heliyon, 2(1), e00055. doi: 10.1016/j.heliyon.2015.e00055

Kim, M., Jang, K., Miller, P., Picon-Ruiz, M., Yeasky, T.M., El-Ashry, D., Slingerland, J.M. (2017). VEGFA links self-renewal and metastasis by inducing Sox2 to repress miR-452, driving Slug. Oncogene, 36(36), 5199–5211. doi: 10.1038/onc.2017.4

Kim, Y.G., Park, Y.H., Yang, E.Y., Park, W.S., Park, K.S. (2019). Inhibition of tamoxifen's therapeutic effects by emodin in estrogen receptor-positive breast cancer cell lines. Ann Surg Treat Res. 97(5), 230–238. doi: 10.4174/astr.2019.97.5.230

Ko, J.H., Sethi, G., Um, J.Y., Shanmugam, M.K., Arfuso, F., Kumar, A.P., ... Ahn, KS. (2017). The Role of Resveratrol in Cancer Therapy. Int J Mol Sci., 18(12), 2589, doi: 10.3390/ijms18122589

Koleckova, M., Ehrmann, J., Bouchal, J., Janikova, M., Brisudova, A., Srovnal, J., ... Kolar, Z. (2021). Epithelial to mesenchymal transition and microRNA expression are associated with spindle and apocrine cell morphology in triple-negative breast cancer. Sci Rep., 11(1), 5145. doi: 10.1038/s41598-021-84350-2

Kozomara, A., Birgaoanu, M., Griffiths-Jones, S. (2019). miRBase: from microRNA sequences to function. Nucleic Acids Res., 47(D1), D155–D162. doi: 10.1093/nar/gky1141

Kung, P.P., Martinez, R., Zhu, Z., Zager, M., Blasina, A., Rymer, I., ... Murray, B.W. (2014). Chemogenetic evaluation of the mitotic kinesin CENP-E reveals a critical role in triple-negative breast cancer. Mol Cancer Ther., 13(8), 2104–2115. doi: 10.1158/1535-7163.MCT-14-0083-T

Kurozumi, S., Yamaguchi, Y., Kurosumi, M., Ohira, M., Matsumoto, H., Horiguchi, J. (2017). Recent trends in microRNA research into breast cancer with particular focus on the associations between microRNAs and intrinsic subtypes. J Hum Genet., 62(1), 15–24. doi: 10.1038/jhg.2016.89

Kwon, Y.J., Baek, H.S., Ye, D.J., Shin, S., Kim, D., Chun, Y.J. (2016). CYP1B1 Enhances Cell Proliferation and Metastasis through Induction of EMT and Activation of Wnt/β-Catenin Signaling via Sp1 Upregulation. PLoS One, 11(3), e0151598. doi: 10.1371/journal.pone.0151598

Lagendijk, M., Sadaatmand, S., Koppert, L.B., Tilanus-Linthorst, M.M.A., de Weerd, V., Ramírez-Moreno, R., ... Martens, J.W.M. (2018). MicroRNA expression in pre-treatment plasma of patients with benign breast diseases and breast cancer. Oncotarget, 9(36), 24335–24346. doi: 10.18632/oncotarget.25262

Latini, A., Borgiani, P., Novelli, G., Ciccacci, C. (2019). miRNAs in drug response variability: potential utility as biomarkers for personalized medicine. Pharmacogenomics, 20(14), 1049–1059. doi: 10.2217/pgs-2019-0089

Lee, R.C., Feinbaum, R.L., Ambros, V. (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell, 75(5), 843–854. doi: 10.1016/0092-8674(93)90529-y.

Lee, W.H., Chen, L.C., Lee, C.J., Huang, C.C., Ho, Y.S., Yang, P.S., ... Tu, S.H. (2019). DNA primase polypeptide 1 (PRIM1) involves in estrogen-induced breast cancer formation through activation of the G2/M cell cycle checkpoint. Int J Cancer, 144(3), 615–630. doi: 10.1002/ijc.31788.

Lee, Y.S., Dutta, A. (2009). MicroRNAs in cancer. Annual Review of Pathology, 4, 199-227. doi: 10.1146/annurev.pathol.4.110807.092222.

Li, B., Zhao, X., Zhang, L., Cheng, W. (2021). Emodin Interferes With AKT1-Mediated DNA Damage and Decreases Resistance of Breast Cancer Cells to Doxorubicin. Frontiers in Oncology, 10, 588533. doi: 10.3389/fonc.2020.588533

Li, N., Wang, C., Zhang, P., You, S. (2018). Emodin inhibits pancreatic cancer EMT and invasion by up-regulating microRNA-1271. Mol Med Rep, 18(3), 3366–3374. doi: 10.3892/mmr.2018.9304

Li, W.Y., Chan, R.Y., Yu, P.H., Chan, S.W. (2013a). Emodin induces cytotoxic effect in human breast carcinoma MCF-7 cell through modulating the expression of apoptosis-related genes. Pharm Biol., 51(9), 1175–1181. doi: 10.3109/13880209.2013.782322

Li, X., Liu, X., Xu, W., Zhou, P., Gao, P., Jiang, S., ... Zhu, T. (2013b). c-MYC-regulated miR-23a/24-2/27a cluster promotes mammary carcinoma cell invasion and hepatic metastasis by targeting Sprouty2. J Biol Chem., 288(25), 18121–18133. doi: 10.1074/jbc.M113.478560

Liang, F., Yang, M., Tong, N., Fang, J., Pan, Y., Li, J., Zhang, X. (2018). Identification of six key miRNAs associated with breast cancer through screening large-scale microarray data. Oncology letters, 16(4), 4159–4168. doi: 10.3892/ol.2018.9175

Licursi, V., Conte, F., Fiscon, G., Paci, P. (2019). MIENTURNET: an interactive web tool for microRNA-target enrichment and network-based analysis. BMC Bioinformatics, 20(1), 545. doi: 10.1186/s12859-019-3105-x

Lin, Q., Ma, L., Liu, Z., Yang, Z., Wang, J., Liu, J., Jiang, G. (2017). Targeting microRNAs: a new action mechanism of natural compounds. Oncotarget, 8(9), 15961–15970. doi: 10.18632/oncotarget.14392

Lin, S.Y., Lai, W.W., Ho, C.C., Yu, F.S., Chen, G.W., Yang, J.S., ... Chung, J.G. (2009). Emodin induces apoptosis of human tongue squamous cancer SCC-4 cells through reactive oxygen species and mitochondria-dependent pathways. Anticancer Res., 29(1), 327–335.

Lin, S.Z., Xu, J.B., Ji, X., Chen, H., Xu, H.T., Hu, P., ... Tong ,H.F. (2015). Emodin inhibits angiogenesis in pancreatic cancer by regulating the transforming growth factor-β/drosophila mothers against decapentaplegic pathway and angiogenesis-associated microRNAs. Mol Med Rep., 12(4), 5865–5871. doi: 10.3892/mmr.2015.4158

Liu, C.G., Calin, G.A., Volinia, S., Croce, C.M. (2008). MicroRNA expression profiling using microarrays. Nat Protoc., 3(4), 563–578. doi: 10.1038/nprot.2008.14

Liu H. (2012). MicroRNAs in breast cancer initiation and progression. Cellular and molecular life sciences, 69(21), 3587–3599. doi: 10.1007/s00018-012-1128-9

Liu, Q., Hodge, J., Wang, J., Wang, Y., Wang, L., Singh, U., ... Fan, D. (2020) Emodin reduces Breast Cancer Lung Metastasis by suppressing Macrophage-induced Breast Cancer Cell Epithelial-mesenchymal transition and Cancer Stem Cell formation. Theranostics, 10(18), 8365–8381. doi: 10.7150/thno.45395

Liu, X., Liu, Y., Wang, Q., Song, S., Feng, L., Shi, C. (2021). The Alterations and Potential Roles of MCMs in Breast Cancer. J Oncol., 2021, 7928937. doi: 10.1155/2021/7928937

Lodish, H.F., Zhou, B., Liu, G., Chen, C.Z. (2008). Micromanagement of the immune system by microRNAs. Nat Rev Immunol, 8(2), 120–130. doi: 10.1038/nri2252

Loh, H.Y., Norman, B.P., Lai, K.S., Rahman, N.M.A.N.A., Alitheen, N.B.M., Osman, M.A. (2019). The Regulatory Role of MicroRNAs in Breast Cancer. International Journal of Molecular Sciences, 20(19), 4940. doi: 10.3390/ijms20194940

Long, H., Chen, H., Yan, J., Cheng, H. (2022). Emodin exerts antitumor effects in ovarian cancer cell lines by preventing the development of cancer stem cells via epithelial mesenchymal transition. Oncol Lett., 23(3), 95. doi: 10.3892/ol.2022.13215

Long, H., Wang, X., Chen, Y., Wang, L., Zhao, M., Lu, Q. (2018). Dysregulation of microRNAs in autoimmune diseases: Pathogenesis, biomarkers and potential therapeutic targets. Cancer Lett., 428, 90–103. doi: 10.1016/j.canlet.2018.04.016

Lu, H., Shi, C., Wang, S., Yang, C., Wan, X., Luo, Y., ... Li L. (2020). Identification of NCAPH as a biomarker for prognosis of breast cancer. Mol Biol Rep., 47(10), 7831–7842. doi: 10.1007/s11033-020-05859-9

Ma, J., Lu, H., Wang, S., Chen, B., Liu, Z., Ke, X., ... Fu, J. (2015). The anthraquinone derivative Emodin inhibits angiogenesis and metastasis through downregulating Runx2 activity in breast cancer. Int J Oncol., 46(4), 1619–1628. doi: 10.3892/ijo.2015.2888

Ma, Y., Chen, M., Wu, Z., Zhao, H., Yu, H., Yu, J., Zhang, J. (2013). Emodin can induce K562 cells to erythroid differentiation and improve the expression of globin genes. Molecular and Cellular Biochemistry, 382(1-2), 127–136. doi: 10.1007/s11010-013-1726-3

MacFarlane, L.A., Murphy, P.R. (2010). MicroRNA: Biogenesis, Function and Role in Cancer. Current Genomics, 11(7), 537–561. doi: 10.2174/138920210793175895

Maciejczyk, A., Szelachowska, J., Czapiga, B., Matkowski, R., Hałoń, A., Györffy, B., Surowiak, P. (2013). Elevated BUBR1 expression is associated with poor survival in early breast cancer patients: 15-year follow-up analysis. J Histochem Cytochem., 61(5), 330–339. doi: 10.1369/0022155413480148

Maia, A.R., de Man, J., Boon, U., Janssen, A., Song, J.Y., Omerzu, M., ... Medema, R.H. (2015). Inhibition of the spindle assembly checkpoint kinase TTK enhances the efficacy of docetaxel in a triple-negative breast cancer model. Ann Oncol., 26(10), 2180–2192. doi: 10.1093/annonc/mdv293

Maire, V., Baldeyron, C., Richardson, M., Tesson, B., Vincent-Salomon, A., Gravier, E., ... Dubois, T. (2013). TTK/hMPS1 is an attractive therapeutic target for triple-negative breast cancer. PLoS One., 8(5), e63712. doi: 10.1371/journal.pone.0063712

Marques, S., Fonseca, J., Silva, P.M., Bousbaa, H. (2015). Targeting the Spindle Assembly Checkpoint for Breast Cancer Treatment. Current Cancer Drug Targets, 15(4), 272–281. doi: 10.2174/1568009615666150302130010

Massarweh, S., Schiff, R. (2006). Resistance to endocrine therapy in breast cancer: exploiting estrogen receptor/growth factor signaling crosstalk. Endocrine-Related Cancer, 13(Suppl 1), S15–S24. doi: 10.1677/erc.1.01273

Merico, D., Isserlin, R., Stueker, O., Emili, A., Bader, G.D. (2010). Enrichment Map: A Network-Based Method for Gene-Set Enrichment Visualization and Interpretation. PLoS One, 5(11), e13984. doi: 10.1371/journal.pone.0013984

Mertens-Talcott, S.U., Chintharlapalli, S., Li, X., Safe, S. (2007). The oncogenic microRNA-27a targets genes that regulate specificity protein transcription factors and the G2-M checkpoint in MDA-MB-231 breast cancer cells. Cancer Res, 67(22), 11001–11011. doi: 10.1158/0008-5472.CAN-07-2416

Milevskiy, M.J.G., Gujral, U., Del Lama Marques, C., Stone, A., Northwood, K., Burke, L.J., ... Brown, M.A. (2019). MicroRNA-196a is regulated by ER and is a prognostic biomarker in ER+ breast cancer. Br J Cancer, 120(6), 621–632. doi: 10.1038/s41416-019-0395-8

Motameny, S., Wolters, S., Nürnberg, P., Schumacher, B. (2010). Next Generation Sequencing of miRNAs - Strategies, Resources and Methods. Genes (Basel), 1(1), 70–84. doi: 10.3390/genes1010070

Moore, B.T., Xiao, P. (2013). MiRNAs in bone diseases. Microrna. 2(1), 20–31. doi: 10.2174/2211536611302010004

Nagpal, N., Ahmad, H.M., Molparia, B., Kulshreshtha, R. (2013). MicroRNA-191, an estrogen-responsive microRNA, functions as an oncogenic regulator in human breast cancer. Carcinogenesis, 34(8), 1889–1899. doi: 10.1093/carcin/bgt107

Nagpal, N., Kulshreshtha, R. (2014). miR-191: an emerging player in disease biology. Front Genet, 5, 99. doi: 10.3389/fgene.2014.00099

National Cancer Institute. (2021). What Is Cancer?. https://www.cancer.gov/about-cancer/understanding/what-is-cancer. Last accessed 30.12.2021.

Ni, J., Liang, S., Shan, B., Tian, W., Wang, H., Ren, Y. (2020). Methylation associated silencing of miR 638 promotes endometrial carcinoma progression by targeting MEF2C. Int J Mol Med., 45(6), 1753–1770. doi: 10.3892/ijmm.2020.4540

Nie, X., He, M., Wang, J., Chen, P., Wang, F., Lai, J., ... Chen, C. (2020). Circulating miR-4763-3p Is a Novel Potential Biomarker Candidate for Human Adult Fulminant Myocarditis. Mol Ther Methods Clin Dev., 17, 1079–1087. doi: 10.1016/j.omtm.2020.05.005

Normann, L.S., Aure, M.R., Leivonen, S.K., Haugen, M.H., Hongisto, V., Kristensen, V.N., ... Sahlberg, K.K. (2021). MicroRNA in combination with HER2-targeting drugs reduces breast cancer cell viability in vitro. Sci Rep., 11(1), 10893. doi: 10.1038/s41598-021-90385-2

O'Brien, S.L., Fagan, A., Fox, E.J., Millikan, R.C., Culhane, A.C., Brennan, D.J., ... Gallagher, W.M. (2007). CENP-F expression is associated with poor prognosis and chromosomal instability in patients with primary breast cancer. Int J Cancer., 120(7), 1434–1443. doi: 10.1002/ijc.22413

O'Connell, R.M., Rao, D.S., Chaudhuri, A.A., Baltimore, D. (2010). Physiological and pathological roles for microRNAs in the immune system. Nature Reviews Immunology, 10(2), 111–122. doi: 10.1038/nri2708

Ouyang, T., Liu, Z., Han, Z., Ge, Q. (2019). MicroRNA Detection Specificity: Recent Advances and Future Perspective. Anal Chem., 91(5), 3179–3186. doi: 10.1021/acs.analchem.8b05909

Papadaki, C., Stoupis, G., Tsalikis, L., Monastirioti, A., Papadaki, M., Maliotis, N., ... Agelaki, S. (2019). Circulating miRNAs as a marker of metastatic disease and prognostic factor in metastatic breast cancer. Oncotarget, 10(9), 966–981. doi: 10.18632/oncotarget.26629

Pawlick, J.S., Zuzic, M., Pasquini, G., Swiersy, A., Busskamp, V. (2021). MiRNA Regulatory Functions in Photoreceptors. Front. Cell Dev. Biol., 8, 620249. doi: 10.3389/fcell.2020.620249

Perron, M.P., Provost, P. (2008). Protein interactions and complexes in human microRNA biogenesis and function. Front Biosci., 13, 2537–2547. doi: 10.2741/2865

Ponnusamy, L., Kothandan, G., Manoharan, R. (2020). Berberine and emodin abrogates breast cancer growth and facilitates apoptosis through inactivation of SIK3-induced mTOR and Akt signaling pathway. Biochim Biophys Acta Mol Basis Dis, 1866(11), 165897. doi: 10.1016/j.bbadis.2020.165897

Poy, M.N., Eliasson, L., Krutzfeldt, J., Kuwajima, S., Ma, X., Macdonald, P.E., ... Stoffel, M. (2004). A pancreatic islet-specific microRNA regulates insulin secretion. Nature, 432(7014), 226–230. doi: 10.1038/nature03076

Rafique, S., Thomas, J.S., Sproul, D., Bickmore, W.A. (2015). Estrogen-induced chromatin decondensation and nuclear re-organization linked to regional epigenetic regulation in breast cancer. Genome Biol., 16(1), 145. doi: 10.1186/s13059-015-0719-9

Rajput, A.B., Hu, N., Varma, S., Chen, C.H., Ding, K., Park, P.C., ... Feilotter, H.E. (2011). Immunohistochemical Assessment of Expression of Centromere Protein-A (CENPA) in Human Invasive Breast Cancer. Cancers (Basel)., 3(4), 4212–4227. doi: 10.3390/cancers3044212

Raudvere, U., Kolberg, L., Kuzmin, I., Arak, T., Adler, P., H. Peterson, J. Vilo. (2019). g:Profiler: a web server for functional enrichment analysis and conversions of gene lists (2019 update). Nucleic Acids Research, 47(W1), W191–W198. doi: 10.1093/nar/gkz369

Reagan-Shaw, S., Ahmad, N. (2006). RNA Interference–Mediated Depletion of Phosphoinositide 3-Kinase Activates Forkhead Box Class O Transcription Factors and Induces Cell Cycle Arrest and Apoptosis in Breast Carcinoma Cells. Cancer Res, 66(2), 1062–1069. doi: 10.1158/0008-5472.CAN-05-1018

Reimand J, Isserlin R, Voisin V, Kucera M, Tannus-Lopes C, Rostamianfar A, ... Bader GD. (2019). Pathway enrichment analysis and visualization of omics data using g:Profiler, GSEA, Cytoscape and EnrichmentMap. Nat Protoc., 14(2), 482–517. doi: 10.1038/s41596-018-0103-9

Ren, Z., Tong, H., Chen, L., Yao, Y., Huang S., Zhu, F., Liu, W. (2016). miR-211 and miR-429 are involved in emodin's anti-proliferative effects on lung cancer. International Journal of Clinical and Experimental Medicine, 9(2), 2085–2093.

Rowald, K., Mantovan, M., Passos, J., Buccitelli, C., Mardin, B.R., Korbel, J.O., ... Sotillo, R. (2016). Negative Selection and Chromosome Instability Induced by Mad2 Overexpression Delay Breast Cancer but Facilitate Oncogene-

Independent Outgrowth. Cell Rep., 15(12), 2679–2691. doi: 10.1016/j.celrep.2016.05.048

Russo J, Russo IH. (2006). The role of estrogen in the initiation of breast cancer. J Steroid Biochem Mol Biol. 102(1-5), 89–96. doi: 10.1016/j.jsbmb.2006.09.004

Saini, S., Arora, S., Majid, S., Shahryari, V., Chen, Y., Deng, G., … Dahiya, R. (2011). Curcumin modulates microRNA-203-mediated regulation of the Src-Akt axis in bladder cancer. Cancer Prev Res (Phila), 4(10), 1698–709. doi: 10.1158/1940-6207.CAPR-11-0267

Sakallı, E. (2010). Comparative Effects of Emodin on Biological Activities of MCF-7 and MDA-231 Cell Lines. M.Sc. Thesis, Middle East Technical University, Ankara.

Sakalli-Tecim, E., Uyar-Arpaci, P., Guray, N.T. (2022). Identification of Potential Therapeutic Genes and Pathways in Phytoestrogen Emodin Treated Breast Cancer Cell Lines via Network Biology Approaches. Nutrition and Cancer, 74(2), 592–604. doi: 10.1080/01635581.2021.1889622

Saldanha, A.J. (2004). Java Treeview—extensible visualization of microarray data. Bioinformatics, 20(17), 3246–3248. doi: 10.1093/bioinformatics/bth349

Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., ... Ideker, T. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res., 13(11), 2498–504. doi: 10.1101/gr.1239303

Sharma, S., Lu, H.C. (2018). microRNAs in Neurodegeneration: Current Findings and Potential Impacts. J Alzheimers Dis Parkinsonism, 8(1), 420. doi: 10.4172/2161-0460.1000420

Sharma, S., Nagpal, N., Ghosh, P.C., Kulshreshtha, R. (2017). P53-miR-191-SOX4 regulatory loop affects apoptosis in breast cancer. RNA, 23(8), 1237–1246. doi: 10.1261/rna.060657.117

Sheikh, M.S., Rochefort, H., Garcia, M. (1995). Overexpression of p21WAF1/CIP1 induces growth arrest, giant cell formation and apoptosis in human breast carcinoma cell lines. Oncogene, 11(9), 1899–1905.

Shen, S.J., Song, Y., Ren, X.Y., Xu, Y.L., Zhou, Y.D., Liang, Z.Y., Sun, Q. (2020). MicroRNA-27b-3p Promotes Tumor Progression and Metastasis by Inhibiting Peroxisome Proliferator-Activated Receptor Gamma in Triple-Negative Breast Cancer. Front Oncol., 10, 1371. doi: 10.3389/fonc.2020.01371

Shi, Z., Zhang, J., Qian, X., Han, L., Zhang, K., Chen, L., ... Kang, C. (2013). AC1MMYR2, an inhibitor of dicer-mediated biogenesis of Oncomir miR-21, reverses epithelial-mesenchymal transition and suppresses tumor growth

and progression. Cancer Res., 73(17), 5519–5531. doi: 10.1158/0008-5472.CAN-13-0280

Shrimali, D., Shanmugam, M.K., Kumar, A.P., Zhang, J., Tan, B.K., Ahn, K.S., Sethi, G. (2013). Targeted abrogation of diverse signal transduction cascades by emodin for the treatment of inflammatory disorders and cancer. Cancer Lett., 341(2), 139–149. doi: 10.1016/j.canlet.2013.08.023

Shriram, V., Kumar, V., Devarumath, R.M., Khare, T.S., Wani, S.H. (2016). MicroRNAs As Potential Targets for Abiotic Stress Tolerance in Plants. Front Plant Sci., 7:817. doi: 10.3389/fpls.2016.00817

Simon, R., Lam, A., Li, M.C., Ngan, M., Menenzes, S., Zhao, Y. (2007). Analysis of gene expression data using BRB-ArrayTools. Cancer Inform., 3, 11–17.

Singh, R., Mo, Y.Y. (2013). Role of microRNAs in breast cancer. Cancer Biol Ther., 14(3), 201–212. doi: 10.4161/cbt.23296

Song, X., Zhou, X., Qin, Y., Yang, J., Wang, Y., Sun, Z., ... Liu, S. (2018). Emodin inhibits epithelial-mesenchymal transition and metastasis of triple negative breast cancer via antagonism of CC-chemokine ligand 5 secreted from adipocytes. Int J Mol Med., 42(1), 579–588. doi: 10.3892/ijmm.2018.3638

Stahlhut Espinosa, C.E., Slack, F.J. (2006). The role of microRNAs in cancer. The Yale Journal of Biology and Medicine, 79(3-4), 131–140.

Sui, J.Q., Xie, K.P., Zou, W., Xie, M.J. (2014). Emodin Inhibits Breast Cancer Cell Proliferation through the ERα-MAPK/Akt-Cyclin D1/Bcl-2 Signaling Pathway. Asian Pac J Cancer Prev, 15(15), 6247–6251. doi: 10.7314/apjcp.2014.15.15.6247

Sun, M., Paciga, J.E., Feldman, R.I., Yuan, Z., Coppola, D., Lu, Y.Y., ... Cheng, J.Q. (2001). Phosphatidylinositol-3-OH Kinase (PI3K)/AKT2, activated in breast cancer, regulates and is induced by estrogen receptor alpha (ERalpha) via interaction between ERalpha and PI3K. Cancer Res., 61(16), 5985–5991.

Sun, Y., Wang, X., Zhou, Q., Lu, Y., Zhang, H., Chen, Q., ... Su S. (2015). Inhibitory effect of emodin on migration, invasion and metastasis of human breast cancer MDA-MB-231 cells in vitro and in vivo. Oncol Rep., 33(1), 338–346. doi: 10.3892/or.2014.3585

Sung, H., Ferlay, J., Siegel, R.L., Laversanne, M., Soerjomataram, I., Jemal, A., Bray, F. (2021). Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin., 71(3), 209–249. doi: 10.3322/caac.21660

Szklarczyk, D., Gable, A.L., Lyon, D., Junge, A., Wyder, S., Huerta-Cepas, J., ... Mering, C.V. (2019). STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide

experimental datasets. Nucleic Acids Res., 47(D1), D607–D613. doi: 10.1093/nar/gky1131

Takagi, K., Miki, Y., Shibahara, Y., Nakamura, Y., Ebata, A., Watanabe, M., ... Suzuki, T. (2013). BUB1 immunolocalization in breast carcinoma: its nuclear localization as a potent prognostic factor of the patients. Horm Cancer, 4(2), 92–102. doi: 10.1007/s12672-012-0130-x

Takahashi, K., Jia, H., Takahashi, S., Kato, H. (2021). Comprehensive miRNA and DNA Microarray Analyses Reveal the Response of Hepatic miR-203 and Its Target Gene to Protein Malnutrition in Rats. Genes, 13(1), 75. doi: 10.3390/genes13010075

Tamimi, R.M., Colditz, G.A., Hazra, A., Baer, H.J., Hankinson, S.E., Rosner, B., ... Collins, L.C. (2012). Traditional breast cancer risk factors in relation to molecular subtypes of breast cancer. Breast Cancer Res Treat., 131(1), 159–167. doi: 10.1007/s10549-011-1702-0

Tan, G.S., Chiu, C.H., Garchow, B.G., Metzler, D., Diamond, S.L., Kiriakidou, M. (2012). Small molecule inhibition of RISC loading. ACS Chem Biol., 7(2), 403–410. doi: 10.1021/cb200253h

Tan, W., Liu, B., Qu, S., Liang, G., Luo, W., Gong, C. 2018. MicroRNAs and cancer: Key paradigms in molecular therapy. Oncol Lett., 15(3), 2735–2742. doi: 10.3892/ol.2017.7638

Tang, W., Li, G.S., Li, J.D., Pan, W.Y., Shi, Q., Xiong, D.D., ... Rong, M.H. (2020). The role of upregulated miR-375 expression in breast cancer: An in vitro and in silico study. Pathol Res Pract., 216(1), 152754. doi: 10.1016/j.prp.2019.152754

Tang, W., Zhu, J., Su, S., Wu, W., Liu, Q., Su, F., Yu, F. (2012). MiR-27 as a prognostic marker for breast cancer progression and patient survival. PLoS One, 7(12), e51702. doi: 10.1371/journal.pone.0051702

Tanwar, A.K., Dhiman, N., Kumar, A., Jaitak, V. (2021). Engagement of phytoestrogens in breast cancer suppression: Structural classification and mechanistic approach. European Journal of Medicinal Chemistry, 213, 113037. doi: 10.1016/j.ejmech.2020.113037

Thangavelu, P.U., Reid, L.E., Lim, M., Molaei, M., McCart Reed, A.E., Saunus, J.M., Dray, E., Simpson, P.T., Lakhani, S.R., Duijf, P.H.G. 2018. PO-321 Causes and consequences of WDHD1 overexpression in breast cancer. ESMO Open, 3(Suppl 2), A1–A463.

Tribolet, L., Kerr, E., Cowled, C., Bean, A.G.D., Stewart, C.R., Dearnley, M., Farr, R.J. 2020. MicroRNA Biomarkers for Infectious Diseases: From Basic Research to Biosensing. Front Microbiol., 11, 1197. doi: 10.3389/fmicb.2020.01197

Tordjman, J., Majumder, M., Amiri, M., Hasan, A., Hess, D., Lala, P.K. (2019). Tumor suppressor role of cytoplasmic polyadenylation element binding protein 2 (CPEB2) in human mammary epithelial cells. BMC Cancer., 19(1), 561. doi: 10.1186/s12885-019-5771-5

Tosun, F., Akyüz-Kızılay, Ç. (2003). Anthraquinones and Flavonoids from Rheum Ribes. Journal of Faculty of Pharmacy Ankara, 32(1), 31–35.

Vafadar, A., Shabaninejad, Z., Movahedpour, A., Fallahi, F., Taghavipour, M., Ghasemi, Y., ... Mirzaei H. (2020). Quercetin and cancer: new insights into its therapeutic effects on ovarian cancer cells. Cell Biosci., 10, 32. doi: 10.1186/s13578-020-00397-0

Wang, G., Dai, F., Yu, K., Jia, Z., Zhang, A., Huang, Q., ... Pu, P. (2015). Resveratrol inhibits glioma cell growth via targeting oncogenic microRNAs and multiple signaling pathways. Int J Oncol., 46(4), 1739–1747. doi: 10.3892/ijo.2015.2863

Wang, J., Wang, J., Gu, Q., Yang, Y., Ma, Y., Zhu, J., Zhang, Q. (2020). [MiR-4443 promotes migration and invasion of breast cancer cells by inhibiting PEBP1 expression]. Nan Fang Yi Ke Da Xue Xue Bao, 40(12), 1712–1719. doi: 10.12122/j.issn.1673-4254.2020.12.03

Wang, Y., Keys, D.N., Au-Young, J.K., Chen, C. (2009). MicroRNAs in embryonic stem cells. Journal of Cellular Physiology, 218(2), 251–255. doi: 10.1002/jcp.21607

Wang, Y., Luo, Q., He, X., Wei, H., Wang, T., Shao, J., Jiang, X. (2018). Emodin Induces Apoptosis of Colon Cancer Cells via Induction of Autophagy in a ROS-Dependent Manner. Oncol Res., 26(6), 889–899. doi: 10.3727/096504017X15009419625178

Wang, Z., Fukushima, H., Inuzuka, H., Wan, L., Liu, P., Gao, D., ... F.H., Wei, W. (2012). Skp2 is a promising therapeutic target in breast cancer. Front Oncol., 1(57), 57. doi: 10.3389/fonc.2011.00057

Warde-Farley, D., Donaldson, S.L., Comes, O., Zuberi, K., Badrawi, R., Chao, P., ... Morris, Q. (2010). The GeneMANIA prediction server: biological network integration for gene prioritization and predicting gene function. Nucleic Acids Res., 38(Web Server issue), W214–220. doi: 10.1093/nar/gkq537

Wei, W.T., Lin, S.Z., Liu, D.L., Wang, Z.H. (2013). The distinct mechanisms of the antitumor activity of emodin in different types of cancer (Review). Oncology Reports, 30(6), 2555–2562. doi: 10.3892/or.2013.2741

Wightman, B., Ha, I., Ruvkun, G. (1993). Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. Elegans. Cell, 75(5), 855–862. doi: 10.1016/0092-8674(93)90530-4

Wu, J., Sun, Z., Sun, H., Li, Y. (2018). MicroRNA-27a promotes tumorigenesis via targeting AKT in triple negative breast cancer. Molecular Medicine Reports, 17(1), 562–570. doi: 10.3892/mmr.2017.7886

Wu, K.L., Tsai, Y.M., Lien, C.T., Kuo, P.L., Hung, A.J. (2019). The Roles of MicroRNA in Lung Cancer. Int J Mol Sci., 20(7), 1611. doi: 10.3390/ijms20071611

Xie, G., Yang, H., Ma, D., Sun, Y., Chen, H., Hu, X., ... Shao, Z.M. (2019). Integration of whole-genome sequencing and functional screening identifies a prognostic signature for lung metastasis in triple-negative breast cancer. Int J Cancer, 145(10), 2850–2860. doi: 10.1002/ijc.32329

Xu, L., Xiang, J., Shen, J., Zou, X., Zhai, S., Yin, Y., ... Sun, Q. (2013). Oncogenic MicroRNA-27a is a target for genistein in ovarian cancer cells. Anticancer Agents Med Chem., 13(7), 1126–1132. doi: 10.1002/ijc.32329

Xu, P., Vernooy, S.Y., Guo, M., Hay, B.A. (2003). The Drosophila microRNA Mir-14 suppresses cell death and is required for normal fat metabolism, Current Biology, 13(9), 790–795. doi: 10.1016/s0960-9822(03)00250-1

Xu, P., Wu, Q., Yu, J., Rao, Y., Kou, Z., Fang, G., ... Han, H. (2020). A Systematic Way to Infer the Regulation Relations of miRNAs on Target Genes and Critical miRNAs in Cancers. Front Genet., 11, 278. doi: 10.3389/fgene.2020.00278

Yang, X.R., Chang-Claude, J., Goode, E.L., Couch, F.J., Nevanlinna, H., Milne, R.L., ... Garcia-Closas, M. (2011). Associations of breast cancer risk factors with tumor subtypes: a pooled analysis from the Breast Cancer Association Consortium studies. J Natl Cancer Inst., 103(3), 250–263. doi: 10.1093/jnci/djq526

Yao, Y., Jiang, C., Wang, F., Yan, H., Long, D., Zhao, J., ... Zhang, Z. (2019) Integrative Analysis of miRNA and mRNA Expression Profiles Associated With Human Atrial Aging. Front. Physiol., 10, 1226. doi: 10.3389/fphys.2019.01226

Yu,. J.Q., Bao, W., Lei, J.C. (2012). Emodin regulates apoptotic pathway in human liver cancer cells. Phytother Res., 27(2), 251–257. doi: 10.1002/ptr.4703

Zhang, B., Tian, L., Xie, J., Chen, G., Wang, F. (2020). Targeting miRNAs by natural products: A new way for cancer therapy. Biomed Pharmacother.,130, 110546. doi: 10.1016/j.biopha.2020.110546

Zhang, L., Hao, C., Han, B., Zeng, G., Han, L., Cao, C., ... Zhang, Q. (2023). RMI2 is a novel prognostic and predictive biomarker for breast cancer. Cancer Med., 12(7), 8331–8350. doi: 10.1002/cam4.5533

Zhang, L., Lau, Y.K., Xia, W., Hortobagyi, G.N., Hung, M.C. (1999). Tyrosine kinase inhibitor emodin suppresses growth of HER-2/neu-overexpressing

breast cancer cells in athymic mice and sensitizes these cells to the inhibitory effect of paclitaxel. Clin Cancer Res, 5(2), 343–353.

Zhang, L., Lau, Y.K., Xi, L., Hong, R.L., Kim, D.S., Chen, C.F., ... Hung, M.C. (1998). Tyrosine kinase inhibitors, emodin and its derivative repress HER-2/neu-induced cellular transformation and metastasis-associated properties. Oncogene, 16(22), 2855–2863. doi: 10.1038/sj.onc.1201813

Zhang, N., Wang, J., Sheng, A., Huang, S., Tang, Y., Ma, S., Hong, G. (2021). Emodin inhibits the proliferation of MCF-7 human breast cancer cells through activation of Aaryl hydrocarbon receptor (AhR). Front. Pharmacol., 11, 622046. doi: 10.3389/fphar.2020.622046

Zhang, S., Li, X., Burghardt, R., Smith, R. 3rd, Safe, S.H. (2005). Role of estrogen receptor (ER) alpha in insulin-like growth factor (IGF)-I-induced responses in MCF-7 breast cancer cells. J Mol Endocrinol., 35(3), 433–447. doi: 10.1677/jme.1.01858

Zhang, X., Wu, M., Chong, Q.Y., Zhang, W., Qian, P., Yan, H., ... Zhu, T. (2018). Amplification of hsa-miR-191/425 locus promotes breast cancer proliferation and metastasis by targeting DICER1. Carcinogenesis, 39(12), 1506–1516. doi: 10.1093/carcin/bgy102

Zhong, Z., Pannu, V., Rosenow, M., Stark, A., Spetzler, D. (2018). KIAA0100 Modulates Cancer Cell Aggression Behavior of MDA-MB-231 through Microtubule and Heat Shock Proteins. Cancers (Basel), 10(6), 180. doi: 10.3390/cancers10060180

Zhou, J., Pei, Y., Chen, G., Cao, C., Liu, J., Ding, C., ... Niu, G. (2018). SPC24 Regulates breast cancer progression by PI3K/AKT signaling. Gene, 675, 272–277. doi: 10.1016/j.gene.2018.07.017

Zhou, S.S., Jin, J.P., Wang, J.Q., Zhang, Z.G., Freedman, J.H., Zheng, Y., Cai, L. (2018). miRNAS in cardiovascular diseases: potential biomarkers, therapeutic targets and challenges. Acta Pharmacol Sin., 39(7), 1073–1084. doi: 10.1038/aps.2018.30

Zhu, J., Xu, Y., Liu, S., Qiao, L., Sun, J., Zhao, Q. (2020). MicroRNAs Associated With Colon Cancer: New Potential Prognostic Markers and Targets for Therapy. Front. Bioeng. Biotechnol., 8:176. doi: 10.3389/fbioe.2020.00176

Zografos, E., Zagouri, F., Kalapanida, D., Zakopoulou, R., Kyriazoglou, A., Apostolidou, K., ... Dimopoulos, M.A. (2019). Prognostic role of microRNAs in breast cancer: A systematic review. Oncotarget, 10(67), 7156–7178. doi: 10.18632/oncotarget.27327

Zu, C., Qin, G., Yang, C., Liu, N., He, A., Zhang, M., Zheng, X. (2018). Low dose Emodin induces tumor senescence for boosting breast cancer chemotherapy

via silencing NRARP. Biochem Biophys Res Commun., 505(4), 973–978. doi: 10.1016/j.bbrc.2018.09.045

https://media.affymetrix.com/support/downloads/manuals/mirna_flashtag_manual.pdf,

last visited on February 2022.

APPENDICES

A. KEGG Pathway Results of Differentially Regulated Genes

Table 4.1 Up and down regulated genes of differentially regulated MCF-7, MDA-MB-231 and common pathways upon Emodin treatment

MCF-7				
Pathway name	**Gene#**	**Adjusted pvalue**	**UP regulated genes**	**DOWN regulated genes**
			CDK6	ANAPC7
			CDKN1A	BUB1B
			CUL1	CCND1
			GADD45A	CCNE1
			GADD45B	CCNE2
			GADD45G	CDC25A
				CDC45
				CDC6
				CDC7
				CDK1
				CDK2
				CDKN2C
Cell cycle	38	3,08E-12		CHEK1
				E2F2
				ESPL1
				GSK3B
				MCM2
				MCM3
				MCM4

			MCM5
			MCM6
			MCM7
			ORC1
			PCNA
			RBL1
			RBL2
			SKP1
			SKP2
			SMC1A
			TFDP1
			TFDP2
			TGFB2
DNA replication	17	2,06E-08	DNA2
			FEN1
			LIG1
			MCM2
			MCM3
			MCM4
			MCM5
			MCM6
			MCM7
			PCNA
			POLA1
			POLA2
			POLD1
			POLE2
			PRIM1
			RFC5
			RNASEH2A

p53 signaling pathway	22	9,88E-07	CASP3 CDK6 CDKN1A GADD45A GADD45B GADD45G PMAIP1 SERPINE1 SESN2 TNFRSF10B	BCL2 BID CCND1 CCNE1 CCNE2 CDK1 CDK2 CHEK1 GTSE1 RRM2 SESN3 TP53I3
Cellular senescence	33	8,37E-06	GADD45A CXCL8 SERPINE1 SQSTM1 CAPN2 CDKN1A FOXO1 GADD45G GADD45B CDK6	AKT1 CALM1 CCND1 CCNE1 CCNE2 CDC25A CDK1 CDK2 CHEK1 E2F2 ITPR1 ITPR2 MAPK13 MYBL2 NBN PIK3R1 PIK3R2 PIK3R3 PPP3CA RBL1 RBL2 TGFB2 ZFP36L2

Small cell lung cancer	22	1,18E-04	CASP3 CDK6 CDKN1A GADD45A GADD45B GADD45G LAMC1 XIAP	AKT1 BCL2 CCND1 CCNE1 CCNE2 CDK2 COL4A5 E2F2 FN1 LAMB1 PIK3R1 PIK3R2 PIK3R3 SKP2
Colorectal cancer	21	1,44E-04	CASP3 CDKN1A FOS GADD45A GADD45B GADD45G JUN PMAIP1 TGFA	AKT1 BCL2 BIRC5 CCND1 GSK3B LEF1 MSH2 MSH6 PIK3R1 PIK3R2 PIK3R3 TGFB2
Pancreatic cancer	18	1,07E-03	CDK6 CDKN1A GADD45A GADD45B GADD45G PLD1 TGFA VEGFA	AKT1 BRCA2 CCND1 E2F2 ERBB2 PIK3R1 PIK3R2 PIK3R3 RAD51 TGFB2

AGE-RAGE signaling pathway in diabetic complications	21	1,85E-03	CASP3 CXCL8 EGR1 F3 FOXO1 ICAM1 JUN PIM1 SERPINE1 VEGFA	AKT1 BCL2 CCND1 COL4A5 FN1 MAPK13 PIK3R1 PIK3R2 PIK3R3 PRKCD TGFB2
Apoptosis	25	3,40E-03	CAPN2 CASP3 CTSL DDIT3 ERN1 FOS GADD45A GADD45B GADD45G JUN MAP3K14 PMAIP1 TNFRSF10B XIAP	AKT1 BCL2 BID BIRC5 CASP2 ITPR1 ITPR2 PARP1 PIK3R1 PIK3R2 PIK3R3
FoxO signaling pathway	24	3,49E-03	CDKN1A FOXO1 GABARAPL1 GADD45A GADD45B GADD45G IRS2 PRKAA2 SGK1	AKT1 CAT CCND1 CDK2 C8ORF44-SGK3 IGF1R MAPK13 NLK PIK3R1 PIK3R2 PIK3R3 PLK4 RBL2 SKP2 TGFB2

Prostate cancer	20	3,89E-03	CDKN1A ETV5 FOXO1 TGFA	AKT1 AR BCL2 CCND1 CCNE1 CCNE2 CDK2 CREB3L4 E2F2 ERBB2 GSK3B IGF1R LEF1 PIK3R1 PIK3R2 PIK3R3
Human T-cell leukemia virus 1 infection	34	4,23E-03	ADCY7 CDKN1A EGR1 FOS FOSL1 ICAM1 IL15 JUN MAP3K14 MSX2 XIAP ZFP36	AKT1 ANAPC7 BUB1B CCND1 CCNE1 CCNE2 CDK2 CDKN2C CHEK1 CREB3L4 E2F2 ESPL1 HLA-DQB1 HLA-DRB1 NRP1 PIK3R1 PIK3R2 PIK3R3 PPP3CA RANBP1 TCF3 TGFB2

Platinum drug resistance	16	9,16E-03	CASP3 CDKN1A PMAIP1 XIAP	AKT1 BCL2 BID BIRC5 BRCA1 ERBB2 MSH2 MSH6 PIK3R1 PIK3R2 PIK3R3 TOP2A
Ferroptosis	11	1,61E-02	ACSL1 FTH1 GCLM HMOX1 MAP1LC3B PRNP SAT1 SAT2 SLC3A2 SLC7A11	PCBP2
Fanconi anemia pathway	13	1,80E-02	HES1	BLM BRCA1 BRCA2 BRIP1 FANCA FANCD2 FANCG FANCI FANCM RAD51 RMI1 RMI2

mTOR signaling pathway	25	2,22E-02	CLIP1	AKT1
			DDIT4	AKT1S1
			FLCN	EIF4E2
			FNIP1	FZD2
			FZD7	FZD3
			GRB10	GSK3B
			MAPKAP1	IGF1R
			PRKAA2	NPRL2
			RRAGC	PIK3R1
			RRAGD	PIK3R2
			SESN2	PIK3R3
			SGK1	SKP2
			SLC3A2	
Pathways in cancer	63	2,30E-02	ADCY7	AKT1
			CASP3	AR
			CDK6	BCL2
			CDKN1A	BID
			CUL1	BIRC5
			CXCL8	BRCA2
			EPAS1	CALM1
			FOS	CCND1
			FOXO1	CCNE1
			FZD7	CCNE2
			GADD45A	CDK2
			GADD45B	COL4A5
			GADD45G	E2F2
			HES1	ERBB2
			HMOX1	FGFR3
			IL15	FGFR4
			IL6R	FN1
			JUN	FZD2
			JUP	FZD3
			LAMC1	GSK3B
			PIM1	IGF1R
			PLD1	KITLG
			PMAIP1	LAMB1
			PPARD	LEF1
			TGFA	MECOM
			VEGFA	MSH2
			XIAP	MSH6

				PIK3R1 PIK3R2 PIK3R3 RAD51 RARA RPS6KA5 SKP1 SKP2 TGFB2
Longevity regulating pathway	17	2,96E-02	ADCY7 FOXO1 IRS2 PRKAA2 SESN2	ADIPOR2 AKT1 AKT1S1 CAT CREB3L4 EHMT2 EIF4E2 IGF1R PIK3R1 PIK3R2 PIK3R3 SESN3

MDA-MB-231

Pathway Name	Gene #	Adjusted p value	UP Regulated Genes	DOWN Regulated Genes
Cell cycle	20	6,70E-14		BUB1 BUB1B CCNA2 CCNB1 CCNB2 CCNE2 CDC20 CDC23 CDC25C CDC45

				CDC7 CDK1 CHEK1 MAD2L1 MCM5 PTTG1 RBL1 SKP2 TFDP1 TTK
Fanconi anemia pathway	11	2,18E-08		BLM BRCA1 BRCA2 BRIP1 FANCD2 FANCG FANCI FANCL RMI1 RMI2 USP1
Oocyte meiosis	13	2,93E-06	CPEB2	AURKA BUB1 CCNB1 CCNB2 CCNE2 CDC20 CDC23 CDC25C CDK1 FBXO5 MAD2L1 PTTG1
DNA replication	7	6,41E-05		MCM5 POLE2 PRIM1 RFC3 RFC5 RNASEH2A RNASEH2C

Progesterone-mediated oocyte maturation	10	1,23E-04	CPEB2	AURKA BUB1 CCNA2 CCNB1 CCNB2 CDC23 CDC25C CDK1 MAD2L1
p53 signaling pathway	7	7,30E-03		CCNB1 CCNB2 CCNE2 CDK1 CHEK1 GTSE1 RRM2
Cellular senescence	10	9,17E-03	CXCL8 SQSTM1	CCNA2 CCNB1 CCNB2 CCNE2 CDK1 CHEK1 FOXM1 RBL1
Homologous recombination	5	2,40E-02		BARD1 BLM BRCA1 BRCA2 BRIP1

Genes Regulated in Both Cell Lines

Pathway Name	Gene #	Adjusted p value	UP Regulated Genes		DOWN Regulated Genes	
			MCF-7	MDA-MB-231	MCF-7	MDA-MB-231
Fanconi anemia pathway	9	2,24E-08			BLM	BLM
					BRCA1	BRCA1
					BRCA2	BRCA2
					BRIP1	BRIP1
					FANCD2	FANCD2
					FANCG	FANCG
					FANCI	FANCI
					RMI1	RMI1
					RMI2	RMI2
Cell cycle	10	3,08E-06			BUB1B	BUB1B
					CCNE2	CCNE2
					CDC45	CDC45
					CDC7	CDC7
					CDK1	CDK1
					CHEK1	CHEK1
					MCM5	MCM5
					RBL1	RBL1
					SKP2	SKP2
					TFDP1	TFDP1
DNA replication	5	6,65E-04			MCM5	MCM5
					POLE2	POLE2
					PRIM1	PRIM1
					RFC5	RFC5
					RNASEH2A	RNASEH2A
p53 signaling pathway	5	1,98E-02			CCNE2	CCNE2
					CDK1	CDK1
					CHEK1	CHEK1
					GTSE1	GTSE1
					RRM2	RRM2
Homologous recombination	4	2,10E-02			BLM	BLM
					BRCA1	BRCA1
					BRCA2	BRCA2
					BRIP1	BRIP1

B. Enrichment Map Clusters of Differentially Regulated MCF-7 Genes

Table 4.2 Enrichment map clusters of MCF-7, adjusted p value ≤1xE-15

#	Cluster	Adjusted pvalue	Node name representing GO biological process terms or KEGG pathways	Node GO or KEGG ID	Genes size
1	Cell cycle regulation	3.25E-40	cell cycle	GO:0007049	82
2	Cell cycle regulation	1.01E-35	Cell cycle	KEGG:04110	38
3	Cell cycle regulation	1.65E-35	cell cycle process	GO:0022402	69
4	Cellular response to stimulus	4.67E-35	Pathways in cancer	KEGG:05200	61
5	Cell cycle regulation	2.02E-33	G1/S transition of mitotic cell cycle	GO:0000082	38
6	Cell cycle regulation	5.40E-33	regulation of cell cycle	GO:0051726	64
7	Cell cycle regulation	4.54E-32	cell cycle G1/S phase transition	GO:0044843	38
8	Cell cycle regulation	8.85E-32	mitotic cell cycle process	GO:1903047	55
9	Cell cycle regulation	1.24E-31	mitotic cell cycle	GO:0000278	59
10	Cell cycle regulation	7.34E-30	mitotic cell cycle phase transition	GO:0044772	46
11	Cell cycle regulation	1.72E-28	cell cycle phase transition	GO:0044770	46
12	Cell cycle regulation	3.96E-25	DNA replication	GO:0006260	32
13	Cell cycle regulation	8.30E-25	nuclear DNA replication	GO:0033260	20
14	Cell cycle regulation	9.90E-25	cellular response to DNA damage stimulus	GO:0006974	48
15	Cell cycle regulation	1.12E-24	Cellular senescence	KEGG:04218	32
16	Cell cycle regulation	2.02E-24	DNA-dependent DNA replication	GO:0006261	26
17	Cell cycle regulation	5.64E-24	cell cycle DNA replication	GO:0044786	20
18	Cell cycle regulation	2.54E-22	DNA replication initiation	GO:0006270	17
19	Cell cycle regulation	5.31E-22	negative regulation of cell cycle	GO:0045786	40
20	Cell cycle regulation	3.58E-20	regulation of mitotic cell	GO:0007346	40

			cycle		
21	Cell cycle regulation	6.83E-20	p53 signaling pathway	KEGG:04115	22
22	Cell cycle regulation	2.03E-19	regulation of cell cycle process	GO:0010564	41
23	Cell cycle regulation	2.17E-19	DNA metabolic process	GO:0006259	44
24	Cellular response to stimulus	4.22E-19	regulation of phosphate metabolic process	GO:0019220	59
25	Cellular response to stimulus	4.26E-19	regulation of phosphorylation	GO:0042325	56
26	Cellular response to stimulus	4.34E-19	regulation of phosphorus metabolic process	GO:0051174	59
27	Cell cycle regulation	5.39E-19	DNA replication	KEGG:03030	17
28	Cell cycle regulation	1.66E-17	regulation of cell cycle phase transition	GO:1901987	32
29	Cell cycle regulation	1.71E-17	regulation of mitotic cell cycle phase transition	GO:1901990	31
30	Cell cycle regulation	6.68E-17	negative regulation of cell cycle process	GO:0010948	28
31	Cellular response to stimulus	7.46E-17	Apoptosis	KEGG:04210	24
32	Cellular response to stimulus	2.22E-16	apoptotic signaling pathway	GO:0097190	33
33	Cellular response to stimulus	2.45E-16	FoxO signaling pathway	KEGG:04068	22
34	Cellular response to stimulus	8.21E-16	regulation of intracellular signal transduction	GO:1902531	55

C. KEGG Pathway Results of miRNA-Regulated MCF-7 Genes

Table 4.3 Up and down regulated genes of miRNA regulated MCF-7 pathways upon Emodin treatment

MCF-7				
Pathway name	**Gene#**	**Adjusted pvalue**	**UP regulated genes**	**DOWN regulated genes**
Cellular senescence	23	6.35E-06	CAPN2 CDK6 CDKN1A CXCL8 FOXO1 GADD45A GADD45B GADD45G SERPINE1 SQSTM1	AKT1 CALM1 CCND1 CCNE1 CDK2 E2F2 ITPR2 MAPK13 MYBL2 PIK3R2 PPP3CA TGFB2 ZFP36L2
Cell cycle	19	5.05E-05	CDK6 CUL1 CDKN1A GADD45A GADD45G GADD45B	CCND1 CCNE1 CDC6 CDK2 E2F2 ESPL1 MCM3 MCM5 MCM7 SKP2 TFDP1 TFDP2 TGFB2

p53 signaling pathway	14	1.07E-04	CASP3 CDK6 CDKN1A GADD45A GADD45B GADD45G PMAIP1 SERPINE1 SESN2 TNFRSF10B	CCND1 CCNE1 CDK2 SESN3
MAPK signaling pathway	30	2.55E-04	CASP3 CSF1 DDIT3 DUSP1 DUSP10 DUSP3 DUSP4 DUSP5 DUSP6 EPHA2 FOS GADD45A GADD45B GADD45G JUN MAP3K14 NR4A1 VEGFA	AKT1 ERBB2 FGFR3 FGFR4 IGF1R KITLG MAPK13 MECOM NLK PPP3CA RASGRF1 TGFB2
mTOR signaling pathway	20	3.68E-04	CLIP1 DDIT4 FLCN FNIP1 FZD7 GRB10 LPIN2 MAPKAP1 PRKAA2 RRAGC RRAGD SESN2 SGK1 SLC3A2	AKT1 AKT1S1 FZD2 IGF1R PIK3R2 SKP2

Small cell lung cancer	15	3.77E-04	CASP3 CDK6 CDKN1A GADD45A GADD45B GADD45G LAMC1 XIAP	AKT1 CCND1 CCNE1 CDK2 E2F2 PIK3R2 SKP2
Ferroptosis	10	3.99E-04	ACSL1 FTH1 GCLM HMOX1 MAP1LC3B PRNP SAT1 SAT2 SLC3A2 SLC7A11	
Pancreatic cancer	13	9.90E-04	CDK6 CDKN1A GADD45A GADD45B GADD45G VEGFA	AKT1 CCND1 E2F2 ERBB2 PIK3R2 RAD51 TGFB2
AGE-RAGE signaling pathway in diabetic complications	15	1.09E-03	CASP3 CXCL8 EGR1 F3 FOXO1 ICAM1 JUN PIM1 SERPINE1 VEGFA	AKT1 CCND1 MAPK13 PIK3R2 TGFB2

FoxO signaling pathway	17	2.03E-03	CDKN1A FOXO1 GABARAPL1 GADD45A GADD45B GADD45G PRKAA2 SGK1	AKT1 CCND1 CDK2 IGF1R MAPK13 NLK PIK3R2 SKP2 TGFB2
Apoptosis	17	3.34E-03	CAPN2 CASP3 CTSL DDIT3 ERN1 FOS GADD45A GADD45B GADD45G JUN MAP3K14 PMAIP1 TNFRSF10B XIAP	AKT1 ITPR2 PIK3R2
Human T-cell leukemia virus 1 infection	22	6.10E-03	CDKN1A EGR1 FOS FOSL1 ICAM1 JUN MAP3K14 MSX2 XIAP ZFP36	AKT1 CCND1 CCNE1 CDK2 E2F2 ESPL1 HLA-DQB1 NRP1 PIK3R2 PPP3CA TCF3 TGFB2

Pathways in cancer	40	8.42E-03	CASP3 CDK6 CDKN1A CUL1 CXCL8 EPAS1 FOS FOXO1 FZD7 GADD45A GADD45B GADD45G HES1 HMOX1 IL6R JUN LAMC1 PIM1 PMAIP1 VEGFA XIAP	AKT1 AR CALM1 CCND1 CCNE1 CDK2 E2F2 ERBB2 FGFR3 FGFR4 FZD2 IGF1R KITLG MECOM PIK3R2 RAD51 RARA SKP2 TGFB2
Fluid shear stress and atherosclerosis	16	1.42E-02	ARHGEF2 CTSL DUSP1 FOS HMOX1 ICAM1 JUN NCF2 PRKAA2 SQSTM1 VEGFA	AKT1 BMPR2 CALM1 MAPK13 PIK3R2

Osteoclast differentiation	15	1.56E-02	CSF1 FHL2 FOS FOSL1 FOSL2 FYN JUN MAP3K14 NCF2 SQSTM1	AKT1 MAPK13 PIK3R2 PPP3CA TGFB2
Colorectal cancer	12	1.73E-02	CASP3 CDKN1A FOS GADD45A GADD45B GADD45G JUN PMAIP1	AKT1 CCND1 PIK3R2 TGFB2
Glioma	11	2.06E-02	CDK6 CDKN1A GADD45A GADD45B GADD45G	AKT1 CALM1 CCND1 E2F2 IGF1R PIK3R2
Bladder cancer	8	2.09E-02	CDKN1A CXCL8 HBEGF VEGFA	CCND1 E2F2 ERBB2 FGFR3
Chronic myeloid leukemia	11	2.32E-02	CDK6 CDKN1A GADD45A GADD45B GADD45G	AKT1 CCND1 E2F2 MECOM PIK3R2 TGFB2

Transcriptional misregulation in cancer	19	2.67E-02	CDKN1A CEBPB CXCL8 DDIT3 DUSP6 ETV5 FOXO1 GADD45A GADD45B GADD45G JMJD1C KLF3 NR4A3 NUPR1	IGF1R MLLT3 PBX1 RARA TCF3
Breast cancer	16	2.97E-02	CDK6 CDKN1A FOS FZD7 GADD45A GADD45B GADD45G HES1 JUN	AKT1 CCND1 E2F2 ERBB2 FZD2 IGF1R PIK3R2
Shigellosis	22	4.05E-02	ARHGEF2 BCL10 CAPN2 CTTN CUL1 CXCL8 FOXO1 GABARAPL1 JUN MYL12A RRAGC RRAGD SQSTM1 WIPI1	AKT1 AKT1S1 CYTH2 ITPR2 MALT1 MAPK13 PIK3R2 PYCARD

Epstein-Barr virus infection	19	4.22E-02	CASP3	AKT1
			CDK6	CCND1
			CDKN1A	CCNE1
			GADD45A	CDK2
			GADD45B	E2F2
			GADD45G	HLA-DQB1
			HES1	MAPK13
			ICAM1	PIK3R2
			JUN	SKP2
			MAP3K14	

CURRICULUM VITAE

PERSONAL INFORMATION

Surname, Name: Sakallı Tecim, Elif

EDUCATION

Degree	Institution	Year of Graduation
MS	METU Biochemistry	2010
BS	METU Biology	2007
High School	Gölbaşı Anadolu High School, Ankara	2003

WORK EXPERIENCE

2013-Present	Republic of Türkiye Ministry of Agriculture and Forestry Agriculture and Forestry Expert
2013	Republic of Türkiye Ministry of Health Turkish Medicines and Medical Devices Agency Product Inspector Assistant
2010-2012	ATQ Biotechnology (Qiagen Türkiye) R&D Department Application Specialist

FOREIGN LANGUAGES

English C1, Spanish A2

PUBLICATIONS

1. Sakalli-Tecim, E., Gur-Dedeoglu, B. and Guray, N.T. “Systems Biology Based miRNA–mRNA Expression Pattern Analysis of Emodin in Breast Cancer Cell Lines”, (2023). Submitted

2. Sakalli-Tecim, E., Uyar-Arpaci, P. and Guray, N.T. “Identification of Potential Therapeutic Genes and Pathways in Phytoestrogen Emodin Treated Breast Cancer Cell Lines via Network Biology Approaches”, Nutrition and Cancer, 74 (2), 592-604 (2022), doi: 10.1080/01635581.2021.1889622.

3. Sakallı E. “Comparative Effects of Emodin on Biological Activities of MCF-7 and MDA-231 Cell Lines”. M.Sc. Thesis, Middle East Technical University, Ankara, 2010.