THE REPUBLIC OF TURKEY
BAHÇEŞEHİR UNIVERSITY

ROLE OF ERG TRANSCRIPTION FACTOR ON EPITHELIAL MESENCHYMAL TRANSITION IN H358 LUNG CANCER CELL LINE

Master's Thesis

AHMED ASSAKER

ISTANBUL, 2016

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Name of the thesis: Role of ERG transcription factor on epithelial mesenchymal transition in H358 lung cancer cell line.
Name/Last Name of the Student: Ahmed Assaker
Date of the Defense of Thesis: 25/04/2016

The thesis has been approved by the Graduate School of Natural and Applied Sciences.

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ACKNOWLEDGEMENTS

At this level, I would like to express my ultimate gratitude to the work done in the Molecular Biology and Genetics Department at Bahçeşehir University. I would like thank my family for the unconditional support and understanding they have shown during this time. Above all, I need to thank my supervisor Assoc. Prof. Gülay Bulut for putting me back on the scientific track and adjusting me to be able to fulfill the requirements of this career path. I thank her for the motivation, guidance and support throughout these whole two years. And for teaching me as no one ever had done before.

I would like to show my gratitude to Abdülmecit Gökçe, who taught and helped me a lot in the laboratory. I cannot forget my laboratory colleagues İdil Temel and Elif Gelmez who made our laboratory such a cooperative and peaceful environment to work at.

I want to express my gratitude to the Turkish Scientific and Technological Research Council (TÜBİTAK) for the financial support and the scholarship (Project No. 114S428, PI: Gülay Bulut).

Finally, I thank everyone who supported me settling in this multi-cultured country, which I deeply love and I will always remember their contributions.

Istanbul, 2016 Ahmed ASSAKER

ABSTRACT

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Bioengineering

Thesis Supervisor: Assoc. Prof. Dr. Gülay BULUT

April 2016, 88 pages

Lung cancer is the most deadly cancer type worldwide and non-small cell lung cancer (NSCLC) contributes to approximately 85% of these cases. Epithelial mesenchymal transition (EMT) is an essential biological event for the metastatic abilities of cancer cells and it has been linked with poor prognosis in cancer and drug resistance during chemotherapy. In several studies attempting to understand the mechanism related to EMT, it was reported that a transcription factor called ETS related gene (ERG) was found to promote EMT in prostate cancer and in other cancer types. Based on these data, in this study it was hypothesized that ERG may also promote EMT in lung cancer. In order to test this hypothesis, ERG gene expression was established in H358 NSCLC cell line which has low endogenous ERG expression. Following confirmation of ERG upregulation in H358 cells, mRNA expressions of EMT marker genes including E-cadherin, Vimentin, ZEB1, Snail and Slug were analyzed. Upregulation in the mRNA expression levels of the mesenchymal marker, Vimentin and the transcription factors, Snail and ZEB1 were observed. These findings support that ERG may be an important target for the development of new therapies for lung cancer in the future.

Keywords: Non-Small Cell Lung Cancer (NSCLC), H358 Cells, Epithelial Mesenchymal Transition (EMT), ETS-Related Gene (ERG), RT-PCR, Gene Expression

ÖZET

ERG TRANSKRİPSİYON FAKTÖRÜNÜN H358 AKCİĞER KANSERİ HÜCRE HATTINDA EPİTELYAL VE MEZENKİMAL GEÇİŞ ÜZERİNDEKİ ETKİSİ

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Biyomühendislik

Tez Danışmanı: Doç. Dr. Gülay BULUT

Nisan 2016, 88 sayfa

Akciğer kanseri, dünyada kanser tipleri arasında en ölümcül kanser tiplerinden birisidir ve küçük hücreli dışı akciğer kanseri (NSCLC), tüm akciğer kanserlerinin yaklaşık %85'ini oluşturur. Epitel ve mezenkimal geçiş (EMT), kanser hücrelerinin metastatik yetenekleri için gerekli olan temel biyolojik bir olaydır ve kemoterapi sırasında, kanser ve ilaç direncinde zayıf prognoz ile bağlantısı kurulmuştur. EMT ile ilişkili mekanizmanın anlaşılması için yapılan çok sayıda araştırmada, ETS ilişkili gen (ERG) olarak adlandırılan bir transkripsiyon faktörünün prostat ve diğer kanser türlerinde EMT'yi teşvik ettiği bulunmuştur. Bu verilere dayanarak, ERG'ün akciğer kanserinde de EMT'yi teşvik edebileceği hipotezi bu çalışmada öne sürüldü. Bu hipotezi test etmek için, az endojen ERG ifadesine sahip olan H358 hücre hattında, ERG gen ifadesi belirlendi. H358 hücrelerinde ERG artışı doğrulandıktan sonra, E-Kadherin, Vimentin, ZEB1, Snail ve Slug'ı içeren EMT markör genlerinin mRNA ekspresyonları analiz edildi. mRNA ekspresyon seviyesinde, mezenkimal markörü Vimentinde ve transkripsiyon faktörleri Snail ve ZEB1'de artış olduğu gözlendi. Bu bulgular, ERG'ün gelecekte akciğer kanseri için yeni tedavilerin geliştirilmesinde önemli bir hedef olabileceğini desteklemektedir.

Anahtar Kelimeler: Küçük Hücreli Dışı Akciğer Kanseri (NSCLC), H358 Hücreleri, Epitelyal Ve Mezenkimal Geçiş (EMT), ETS-Ilişkili Gen (ERG), RT-PCR, Gen Ekspresyonu

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ABBREVIATIONS

ALK	: Anaplastic Lymphoma Kinase
AML	: Acute Myeloid Leukemia
ampR	: Ampicillin resistance gene
ATCC	: American Tissue and Cell Collection
$CaCl_2$	: Calcium chloride
cDNA	: Complementary DNA
dH_2O	: Distilled water
DNA	: Deoxyribonucleic Acid
E-box	: Enhancer Box
E-cadherin	: Epithelial Cadherin
ECM	: Extracellular matrix
EGFR	: Epidermal Growth Factor Receptor
EMT	: Epithelial Mesenchymal Transition
ERG	: ETS-Related Gene
ETS	: E-26 Transforming Sequence
FDA	: Food and Drugs Administration (USA)
LB	: Luria Broth
N-cadherin	: Neuronal Cadherin
NSCLC	: Non-Small Cell Lung Cancer

OD	: Optical density
pERG	: ERG Plasmid
pEV	: Empty Vector Plasmid
qRT-PCR	: Quantitative Real-Time Polymerase Chain Reaction
RNA	: Ribonucleic Acid
RPM	: Revolutions per minute
RT-PCR	: Reverse Transcriptase-Polymerase Chain Reaction
SCLC	: Small Cell Lung Cancer
SOC	: Super Optimal Broth with Catabolite Repression
TMPRSS2	: Transmembrane Protease Specific Serine 2 Gene
ZEB	: Zinc-finger E-box Binding transcription factors

1. INTRODUCTION

Cancer is ranked as one of the top causes of death worldwide with 14.1 million new cancer cases and 8.2 million cancer deaths per year, as estimated in 2012 (Ferlay *et al*. 2013). These numerical data are expected to increase up to 22 million new cancer cases and 13 million cancer deaths per year within the next 20 years. For these reasons, cancer is one of the most important topics in research (Stewart *et al*. 2014). Among different types of cancers, lung cancer has been the most common in the world for decades, contributing to around 13 percent (1.8 million) new cancer cases per year, as estimated in 2012. The 5-year survival rate is very poor with approximately 15 percent (Jemal *et al*. 2010). And non-small cell lung cancer (NSCLC) contributes to more than 80 percent of these lung cancer cases.

Surgery is considered as the best option for the treatment of NSCLC. However, within the late diagnosis of NSCLC until advanced stages, only 25 percent of the patients are considered eligible for the surgical therapy (van Zandwijk 2001). This raises the importance and need for further research in cancer. The other alternative treatment strategy, chemotherapy fails to offer improvement for the treatment of NSCLC patients, as they acquire drug resistance even after combination therapy. Unfortunately, the treatment fails mainly due to the high metastatic behavior of the cancer disease (Xiao and He 2010).

The ability of NSCLC to resist drugs and acquire a high metastatic potential contribute to the poor prognosis of the patients. This is mainly due to the ability of NSCLC cells to undergo epithelial mesenchymal transition (EMT) (Xiao and He 2010). The trans-differentiation of epithelial cells into motile mesenchymal cells is called as “epithelial mesenchymal transition”. This is a process known to be important for development, wound healing and cancer progression (Lamouille, Xu and Derynck, 2014). ERG (ETS-related gene) is an oncoprotein overexpressed in numerous human cancers (Sashida *et al*. 2010). ERG overexpression is found to promote EMT in different cancer types and especially in prostate cancer EMT takes place through the ZEB1 axe (Leshem *et al*. 2011). A notable study has shown that, ERG is relatively overexpressed in NSCLC samples (Xi *et al*. 2008).

Therefore, this study aims to identify the importance of ERG transcription factor in mediating EMT in lung cancers, which can significantly contribute to the future studies that aims to identify a therapeutic target to treat NSCLC. For this purpose, ERG gene expression was established in H358 NSCLC cell line with low endogenous ERG to analyse the role of ERG expression on EMT markers. The specific EMT markers used for analysis were e-cadherin, Vimentin, ZEB1, Snail and Slug. E-Cadherin and is a well-defined epithelial marker. Vimentin is a well-defined mesenchymal marker (De Craene and Berx 2013). ZEB1 is a transcription factor known to activate invasion and metastasis (Spaderna *et al*. 2008). It sustains proliferative signaling (Liu *et al*. 2008), resists cell death (Takeyama *et al*. 2010) and enables replicative immortality (Ohashi *et al*. 2010). Snail and Slug are both activators of invasion, metastasis and angiogenesis (Peinado *et al*. (2004) and Shih *et al*. (2005)). They resist cell death (Vega *et al*. 2004) and enable replicative immortality as well (Emadi Baygi *et al*. (2010) and Liu *et al*. (2010)).

In this study, we hypothesized that ERG may promote EMT in lung cancer. In order to confirm this hypothesis, we established ERG expression in a NSCLC cell line, H358 with low endogenous ERG expression. We then evaluated the effect of ERG on EMT marker gene expression using RT-PCR. According to our data, we observed that ERG may regulate EMT marker gene expression and this can suggest ERG protein as a critical therapeutic target for treatment of NSCLC patients in the future.

“Literature review” section summarizes the previous studies performed in this field and provides detailed information related to lung cancer and its treatment strategies. Detailed information regarding techniques and experimental approach used to test the hypothesis is provided in the “materials and methods” and results are given in the related chapters. Discussion and conclusion sections contain the interpretation of the data produced on this study.

2. LITERATURE REVIEW

2.1 CANCER

Cancer is a fatal multifactorial genetic disease. Ninety percent of cancers are tumors that rise from epithelial cells (Alison 2001). Tumors are forms of abnormal cell growth that can be classified into two groups according to their behavior; benign and malignant. Benign tumors are localized and they are not critical to human health unless they start to apply pressure on vital organs. They are also referred to as pre-cancerous. Malignant tumors, acquire the ability to metastasize by spreading and invading other sites and they are one of the leading causes of death worldwide (Alison (2001) and Ferlay *et al.* (2013)). For a tumor to be called cancer, six different essential biological events must be acquired. These events include cells ability to sustain proliferative signaling, the ability to avoid growth suppressors, an acquired ability to counter cell death, enabling an endless replication, the induction of angiogenesis, and the activation of invasion and metastasis (Hanahan and Weinberg 2011). Invasion and metastasis have been the focus of many researches over the past decade as a critical tool for the treatment of cancer.

2.1.1 Sustaining Proliferative Signaling and Avoiding Growth Suppressors

The most essential characteristic of cancer cells is their ability to sustain their proliferation signals. Proliferation of normal cells is tightly controlled through the production and/or the release of growth signals to insure homeostasis between cell division and cell death. This control mechanism ensures maintenance of a steady cell number. These cellular growth signals are further controlled from one cell to its neighboring cells and their bioavailability in the extracellular matrix is controlled by complex network of proteases and sulphatases. However, cancer cells acquire the ability of controlling their own proliferation by themselves (Hanahan and Weinberg 2011). This control is achieved by one of the following ways or in combinations of them. For example, cancer cells can produce their own growth signaling ligand and response to it, they can control neighboring cells by stimulating them

to produce growth factors to which they will response to and proliferate (Cheng *et al*. (2008) and Bhowmick *et al*. (2004)). These cells can also increase the number of growth factor receptor proteins present on their cell membranes to enhance their sensitivity to growth signals and even induce a structural alteration causing receptors to sustain a ligand-independent stimulation. In many tumors, it has been observed that due to somatic mutations, continuous activation of growth factor receptors result in proliferative signaling circuits. Another mechanism to achieve continuous proliferating signals is the inhibition of the negative feedback loops that are responsible for the attenuation of proliferating signals. This inhibition is believed to be responsible for the acquired ability to resist some targeted chemotherapeutic agents (Frauwirth and Thompson 2002).

Cancer cells do not increase their numbers by only sustaining the proliferative signals, but they also evade through growth suppressors which is another critical cancer hallmark. Many growth suppressors are reported to be inactivated in several cancer types. Contact inhibition, for example is a mechanism that suppresses cell proliferation through cell-cell contact and involves growth suppressors in normal cells. This mechanism was found to be inhibited in many cancer types enabling cancerous cells to replicate regardless of their density (Puliafito *et al*. 2012).

2.1.2 Resisting Cell Death

Cell number is maintained at constant in normal tissues through cell death programs to balance hyperproliferation. Two major cell death programs are apoptosis and autophagy. Apoptosis is a tightly controlled cell death program that is activated in response to variety of cellular stress conditions such as DNA damage. DNA damage is a condition that cancer cell usually experience during tumor formation. Therefore apoptosis is considered as a natural barrier to cancer. Apoptosis is controlled by members of Bcl-2 family. Within this family Bcl-x_L, Bcl-w and Bcl-2 have inhibitory roles while Bax and Bak trigger apoptosis (Adams and Cory 2007). In addition to apoptosis inhibitors or triggering proteins, stress sensors are needed by apoptosis to be activated. Example of stress sensors is the tumor suppressor protein, TP53 that senses DNA damage. Cancer cells escape the apoptotic

program by different strategies including upregulation of apoptosis inhibiting proteins or downregulation of apoptosis inducing proteins. An alternative strategy, which is most common in many tumors, includes a mutation that leads to loss of TP53 function, so that the cell can no longer sense DNA damage (Vogelstein *et al*. 2000).

Autophagy is another controlled cell death program that breaks down cellular compartments using autophagosomes that surround cellular organelles and degrade them by fusion to lysosomes. It was found that mice with mutated Beclin-1, an autophagy inducer, are more susceptible to cancer than normal mice. This reveals that autophagy is another natural barrier to cancer (White and DiPaola 2009).

2.1.3 Unlimited Proliferation

Normal cells have a limited proliferative capacity, as they can undergo a controlled number of replicative cycles, unlike cancer cells that have an unlimited replicative capacity. This normal replicative capacity is governed by two biological events, senescence and crisis. Senescence is an irreversible cellular state when cells are viable, but cannot replicate. Crisis is a state that involves the activation of cell death programs (Hanahan and Weinberg 2011). These events are reported to be related with telomere shortening (Blasco 2005).

Telomeres are repeats of six nucleotides at the chromosomal terminal regions. They protect chromosomes by preventing chromosomal end-to-end fusion that results in DNA damage. Therefore the length of the telomeres is proportional to the number of replicative cycles a cell can have. Telomerase is a DNA polymerase that adds telomere repeats to the terminal parts of chromosomes after replication. Telomerase is found to be expressed in a very low amount in normal cells. However, it is expressed in much higher amounts in cancer cells. The presence of telomerase was found to be associated with cellular resistance to senescence and crisis while its absence is associated with one of these two cellular events (Shay and Wright 2000). The protein subunit of telomerase enhances cellular proliferation abilities in addition to providing resistance to apoptosis. It is also important in DNA repair mechanisms (Cong and Shay 2008).

2.1.4 Induction of Angiogenesis

Cancer cells are very active cells that need higher levels of nutrition and oxygen supply to sustain their activity. In addition, they need to excrete their waste products in a much higher fashion than normal cells. This raises the need for new capillaries and blood vessels to neutralize the increased supply requirements. The process of forming new blood-vessels is known as angiogenesis (Baeriswyl and Christofori 2009).

Angiogenesis is a natural process that occurs during embryogenesis as well as in adults. Examples of events that require angiogenesis for adults include wound healing and female menstrual cycle, when angiogenesis is temporarily activated and that is called angiogenic switch. However, in cancer cells that angiogenic switch is almost always activated leading to continuous formation of new blood-vessels (Hanahan and Folkman 1996).The angiogenic switch is controlled by pro-angiogenic and anti-angiogenic factors which release signaling proteins that bind to cellular receptors either to induce or inhibit angiogenesis. A well-known angiogenesis inducer is vascular endothelial growth factor-A (VEGF-A), while thrombospondin-1 (TSP-1) is known as an angiogenesis inhibitor (Baeriswyl and Christofori 2009).

VEGF-A gene encodes proteins involved in embryogenesis, development and different events in adults. Because of its critical role, VEGF signaling is controlled in three levels and its action it through (VEGF-1-3) receptors. Its gene expression level was found to be upregulated in tumors (Ferrara 2009). Proteins encoded by VEGF were found to be present in an inert form in the extracellular matrix. These proteins can be activated by matrix metalloproteinases (MMP), proteins that can degrade the extracellular matrix. MMPs can be upregulated by the ZEB1 transcription factor (Kessenbrock *et al*. 2010).

2.1.5 Invasion and Metastasis

Before 2000, the mechanisms through which invasion and metastasis occurred were unclear. It was known that, cancer rises first from epithelial cells, which acquire higher carcinogenic traits to become malignant, referred to as local invasion and distant metastasis.

Years later, the process by which cells gain the invasive and metastatic phenotypes were better understood and the cascade of these biological events is now published (Fidler (2003).

Invasion and metastasis start with local invasion, followed by escaping of the cell to the nearest blood-vessel or lymph-vessel in a process called intravasation. Cancer cell then circulates in the system until it escapes out of the vessel in a process called extravasation. Cells then start to form small nodules called micrometastases until they grow into a tumor through colonization (Talmadge and Fidler 2010). During this time, cells gain changes in their morphology and in their attachment to both the surrounding cells and the ECM. One of the most highlighted changes is the loss of E-cadherin, which is the main adhesion protein that connects epithelial cells to each other.

Overexpression of e-cadherin is defined as an inhibitor of invasion and metastasis, while its downregulation is associated with invasion and metastasis. Therefore it is considered as a key suppressor to invasion and metastasis and a key target for many studies (Berx and van Roy 2009). In addition to e-cadherin, other cell-to-cell or cell-to-ECM adhesion molecules are found to be downregulated in some highly advanced cancer types. On the other hand, adhesion molecules found in migrating cell types are found to be overexpressed in invasive tumors (Cavallaro and Christofori 2004).

That downregulation of epithelial adhesion molecules and the overexpression of adhesion molecules associated with migrating cells is similar to the cascade of events of a developmental regulatory program, known as the epithelial mesenchymal transition (EMT). EMT is a program that is known to be regulatory for invasion and metastasis (Hanahan and Weinberg 2011).

2.1.6 Cancer Treatment

Different strategies have been applied for the treatment of cancer. Surgical option remains the best choice, but it is applicable only at early stage cancers. Radiotherapy and combinations of anticancer drugs come as alternatives to surgery. Majority of anticancer

drugs target DNA synthesis. This means that these drugs will also target all proliferating cells, resulting in serious complication and life-threatening side effects. These serious complications related to cancer treatment lead to the new wave of cancer drugs and the new strategies for cancer studies (Alison 2001).

2.2 LUNG CANCER

Lung cancer, a carcinoma that raises form the epithelial cells of the respiratory system, is the most common cancer type in the world with addition of 13 percent of new cancer cases every year (Jemal *et al*. 2010). There are several different subtypes of lung cancer. For example, non-small cell lung cancer (NSCLC) contributes to approximately 85 percent of the cases. Small cell lung cancer (SCLC), contributes to approximately 10 percent of the cases and the remaining 5 percent belongs to some other histological variants (Jemal *et al*. 2009).

Smoking comes at the first place as the most related and well-documented risk factor leading to lung cancer. A comparison between long-term smokers and non-smokers shows that smokers have 10 to 30 folds higher probability of being lung cancer patients (Samet 1991). The relative risk of having lung cancer was found to increase with both, the number of cigarettes smoked per day, and the life long duration of smoking by days. Cutting the smoking habit was also found to decrease the risk of having lung cancer when former smokers were compared with ongoing smokers. Passive smoking was also related to a relative increase in the risk of having lung cancer. However the increase was much lower than that found for smokers (Pallis 2012).

2.2.1 Lung Cancer Treatment

Over the course of the last 50 years, lung cancer treatment strategies did not change much. These strategies remained as surgery, radiotherapy, chemotherapy or combination of these. Over the period of time, surgery has proven to be the best treatment strategy. Besides, radiotherapy also developed with more focused targeting and chemotherapy improved with less side effects and better post treatment quality of life. However, the 1- and 5-year

survival rate did not change much (Spiro and Silvestri 2005). Concluding that, NSCLC is a disease with a good prognosis if diagnosed at an early stage, when surgery is most effective. Unfortunately, 5-year survival rate remains low when diagnosed at late stages (Ettinger *et al*. (2010) and Klastersky and Paesmans (2001)), raising the need for new strategies to treat patients with metastatic lung cancer, like targeted therapy which targets a specific signaling pathway related to the growth or suppression of a tumor (Minguet *et al*. 2015).

Lung cancer staging was one of the huge advances in the treatment of this disease achieved over a course of 100 years since hinted by Parrot (1876), until the establishment of the current "Tumor-Node-Metastasis" (TNM) system (Spiro and Silvestri 2005). The known cancer staging system which uses four stages of cancer with the first three stages divided into A and B sub-stages, is now further sub-divided according to T, referring to the original tumor size, N, referring to the nearby nodes involved in the tumor, M, referring to the presence or absence of distant metastasis (Peters *et al*. 2012). Currently, accurate lung cancer staging is very critical for the choice of the treatment strategy and disease prognosis as they completely differ from one stage to another.

2.2.1.1 Early-stage lung cancer

Surgery remains the best treatment strategy of choice for early-stage (stages I and II) and a selected stage IIIA lung cancer patients, but patients must be medically fit for an operation and this unfortunately can be as low as only 25 percent of the cases (van Zandwijk 2001). A common reason for this value is the old age of patients diagnosed with lung cancer (Pallis 2012).

The radiotherapy option is an alternative choice for these patients. Chemotherapy for early-stage patients was found to result in 5.4 percent increase in 5-year survival rate (Pignon *et al.* 2008). A survival rate that varied according to the disease stage, with stage II and IIIA patients benefited the most from chemotherapy, but stage IA patients were found to have worse conditions after treatment.

2.2.1.2 Locally advanced lung cancer

Approximately 33 percent of patients diagnosed with NSCLC are at stages IIIA and IIIB (Pallis 2012). Chemotherapy is the standard treatment strategy for these patients who cannot undergo surgery. The treatment strategy includes combining radiotherapy to chemotherapy, while the surgical option was found to be associated with higher toxicity (Robinson 2007).

2.2.1.3 Metastatic lung cancer

Chemotherapy is the treatment strategy of choice for stage VI patients with metastatic lung cancer. However, a study has shown that chemotherapy offers an overall survival rate of only 9 percent for 12 months for these patients (Group 2008). It was also reported that two-drug treatment is more advantageous than single drug treatment. However three-drug treatment does not provide any advantage over two-drug treatment (Delbaldo *et al*. 2004).

2.2.2 Chemotherapy for Lung Cancer

Chemotherapy drugs are being used in combinations for the treatment of lung cancer and in different lines of treatment based on how patients response to the treatment (Peters *et al*. 2012). Platinum-based chemotherapy drugs are being used as first line treatment for lung cancer, as they contain platinum that is able to bind to DNA and causes cross linkage that triggers apoptosis. Cisplatin and carboplatin are examples of platinum-based chemotherapy drugs that are used in combination with taxanes (Ardizzoni *et al*. 2007). Taxanes are drugs that inhibit the microtubules function to arrest cells in G_2 and M phases, resulting in cell death. Paclitaxel and docetaxel are drugs to represent the taxanes group (Gradishar 2012). However this combination of chemotherapy drugs is associated with many side effects (Scarpace 2015).

Alternative combinations of chemotherapy drugs administered with platinum based drugs as first line treatment varied according to the lung cancer type. Pemetrexed (a folate antimetabolite drug) and cisplatin combination was found to offer higher survival rate for

non-squamous lung cancer patients, compared to a combination of cisplatin and gemcitabine, a nucleoside analog that was more beneficial for squamous cell lung cancer patients (Scagliotti *et al*. 2009). However, patients using cisplatin and pemetrexed combination suffered from severe nausea much more than the patients using cisplatin and gemcitabine. The patients who used cisplatin and gemcitabine suffered from higher rates of anemia and thrombocytopenia. Etoposide, a topoisomerase inhibitor and vincristine and vinorelbine, which are tubulin inhibitors are other chemotherapeutic agents used in combination with platinum-based drugs as first line treatment for lung cancer.

Maintenance treatment is a medical term used to describe the use of a single drug that was used as a first line treatment or the introduction of a new drug after finishing the platinum-based combination of drugs used as first line treatment. Maintenance drugs are also given according to the cell type, for example pemetrexed was found to improve the progression-free survival (PFS) of lung cancer patients with non-squamous cells. It was largely noted that maintenance treatment is offering improvement on PFS but not on overall survival (OS) (Peters *et al*. 2012).

Patients who still show cancer progression after first line treatment are offered second line treatment drugs. Combination of chemotherapeutic drugs failed to show an improvement when used as second line drugs compared to single drug therapy (Di Maio *et al*. 2009). Patients with NSCLC using erlotinib chemotherapy as second line or third line treatment have shown an improvement in their OS regardless to cell histology. Any patient with EGFR mutations should be offered targeted therapy for EGFR as second line treatment if not used as first line treatment (Rosell *et al*. 2012).

2.2.3 Targeted Therapy for Lung Cancer

Targeted therapy is a medical term used to describe the use of systemic effective drugs that work by blocking a specific protein associated with a mutated pathway leading to tumor growth. The first group of drugs to be put into clinical practice under this category was epidermal growth factor receptor (EGFR) inhibitors. EGFR controls cell proliferation and apoptosis signaling and is over expressed in many tumors. Anaplastic lymphoma kinase

(ALK) inhibitors that were identified in 2007, and vascular endothelial growth factor (VEGF) inhibitors can be found under this category (Minguet *et al*. 2015).

Gefitinib was the first drug to be approved for the treatment of NSCLC under the category of targeted drugs as an EGFR inhibitor. It works by binding to the tyrosine kinase domain of EGFR to inhibit its activation by ATP binding, resulting in the inhibition of its proliferative signaling pathway and the suppression of cancer growth (Sanford and Scott 2009). Erlotinib is another EGFR inhibitor that works in the same way as gefitinib and got the FDA approval for the treatment of NSCLC in 2004. Afatinib represents the second generation of EGFR inhibitors as it binds to it irreversibly and received its US Food and Drugs Administration (FDA) approval in 2013 to be used for first line treatment of NSCLC patients with EGFR mutations (Minguet *et al*. 2015).

Crizotinib is the first ALK inhibitor to be approved by FDA for the second line treatment of NSCLC in 2011 before being approved for the first line treatment in 2013. A main drawback of crizotinib was cancer resistance to the treatment which led to the introduction of alectinib to overcome that resistance (Sakamoto *et al*. 2011).

Both EGFR inhibitor and ALK inhibitors have shown very promising results and many drugs from these two families gained the FDA approval. For EGFR inhibitors, Gefitinib, Erlotinib, Afatinib and Rociletinib and for ALK inhibition Crizotinib, Ceritinib and Alectinib gained the FDA approval. Unfortunately, many NSCLC patients do not carry EGFR or ALK mutations. Thus they cannot benefit from EGFR or ALK inhibitors that target the related mutant proteins. For this reason, new drugs targeting other pathways are needed. For example, targeting angiogenesis, which is a process essential for cancer progression, is another alternative approach for cancer treatment.

Bevacizumab a monoclonal antibody that inhibits angiogenesis by binding to VEGF-A (Gordon *et al*. 2001) was the first drug to be approved by FDA in 2006 for the treatment of NSCLC. This was followed by the discovery of many drugs such as Vandetani, Sorafenib and Sunitinib (Minguet *et al*. 2015). Another drug nintedanib was the most promising drug among them as it targets many angiogenesis pathways (Dhillon *et al*. 2015).

However, the main drawback for these drugs was the acquired resistance against them (Engelman and Janne 2008). The degree to which cells have undergone epithelial to mesenchymal transition (EMT) was observed to be associated with the EGFR inhibitor sensitivity (Thomson *et al*. 2005).

2.3 EPITHELIAL TO MESENCHYMAL TRANSITION

Epithelial mesenchymal transition (EMT) is an evolutionary conserved program, essential for embryogenesis, gastrulation, heart development and wound healing (Xiao and He 2010). The ability of epithelial cells to acquire mesenchymal characteristics and to downregulate its epithelial characteristics were observed by Elizabeth Hay (1995, pp. 8-20).

Three types of EMT are documented. Type 1 is related to embryonic development when EMT is specific and well-defined (Chaffer *et al*. 2007). Type 2 is associated with wound healing, when EMT is mediated by cytokines and pro-inflammatory factors released by immune cells to stimulate cells to undergo EMT (Zeisberg *et al*. 2007) and Kim *et al*. (2006)). Type 3 is the oncogenic EMT type, when epithelial cells gain the essential mesenchymal phenotype traits needed for metastasis (Thiery 2002).

For epithelial cells to show mesenchymal characteristics a common program with specific hallmarks needs to be followed. The six key events of EMT are the breakdown of the epithelial cell-cell junctions, losing the apical-basal polarity and gaining a front-rear polarity, reorganization of the cytoskeletal architecture, downregulation of epithelial gene markers and overexpression of mesenchymal markers, increased cell protrusions and motility and the ability to degrade extracellular matrix (ECM) proteins to enable invasive behavior in the cancerous type (Lamouille *et al*. 2014). These hallmarks are controlled at the gene level by the down regulation of genes that define the epithelial characteristics, (Huang *et al*. (2012) and Peinado *et al*. (2007)). One of the hallmarks is the downregulation of E-cadherin, which is essential for tight junctions (Huang *et al*. 2012).

2.3.1 Transcription Factors Controlling EMT

Many transcription factors are known to be EMT regulators. Among these transcription factors, ZEB1 and ZEB2, Snail and Slug are the most well characterized ones as EMT regulators (Samatov *et al*. 2013). They are overexpressed early in EMT, thus believed to have a central role. Together they coordinate to repress a well-defined epithelial marker, E-cadherin, and induce a well-defined mesenchymal marker, Vimentin (De Craene and Berx 2013).

Zinc-finger E-box binding transcription factors (ZEB1/2) are proteins that can bind to the promoter sequence of epithelial markers such as E-cadherin to repress its expression (Vandewalle *et al*. 2009) and induce the expression of mesenchymal markers such as Vimentin (Bindels *et al*. 2006). ZEB1 and ZEB2 are essential for embryogenesis and are reported to be overexpressed in cancer samples (De Craene and Berx 2013).

Snail1, known as Snail, and Snail2, known as Slug, are members of the Snail family of transcription factors. They regulate their target genes by binding to the E-box of their regulatory region (Sánchez-Tilló *et al*. 2012). Just like ZEB1 and ZEB2, Snail1 and Snail2 repress the expression of epithelial markers, such as E-cadherin, and induce the expression of mesenchymal markers, such as Vimentin (Peinado *et al*. 2004). Normally, Snail1 and Snail2 are absent in epithelial cells, and their presence is documented in invasive tumors (Sánchez-Tilló *et al*. 2012).

2.3.2 EMT and Cancer Progression

The connection between EMT and cancer has been documented in many cancer types (Hugo *et al*. (2007) and Lee *et al*. (2006)). Poor differentiation of tumors compared to low-grade breast tumor has been linked with an overexpression of EMT inducing genes (Teschendorff *et al*. 2007). Prostate cancer progression has been strongly linked with the downregulation of E-cadherin, as an epithelial marker, and the upregulation of N-cadherin, as a mesenchymal marker (Gravdal *et al*. 2007). Many studies have linked E-cadherin downregulation with the poor prognosis of lung cancer (Xiao and He 2010).

ZEB1 was found to be involved in tumor invasiveness by repressing P- and R-cadherins, cell polarity proteins, components of tight junctions (occludin, claudin) and components of gap junctions (connexins). These activities are key steps for cancer progression to invasive carcinoma by the breakdown of the basement membrane that separates the epithelial compartments from its surrounding stroma. This ZEB1 overexpression was observed in many cancer types including NSCLC (Sánchez-Tilló *et al.* 2012).

Snail and Slug were found to repress epithelial markers such as components of desmosomes (desmoplakin), components of tight junctions (occludin, claudin), cytokeratins and mucin-1. At the same time, Snail and Slug activate the expression of mesenchymal and invasive genes such as Vimentin, fibronectin MMP1, MMP2 that promote cell migration. The expression of Snail is not present in normal epithelial cells while it is over expressed in cancer cells especially at the invasive front. Snail was also was observed to be over expressed in NSCLC (Sánchez-Tilló *et al.* 2012). Binding of Snail to VEGFR was also found to be important for the vascular development in angiogenesis, which is an essential process for cancer progression. Also EMT markers such as Slug and ZEB2 were reported to be associated with the induction of angiogenesis (Gill *et al*. 2012).

ZEB1, Snail and Slug seem to be responsible for many cancer hallmarks including resisting cell death and inducing replicative immortality. The hallmarks they are responsible for are grouped in Table 2.1 (Sánchez-Tilló *et al.* 2012).

Table 2.1: Association of EMT markers with cancer

Cancer hallmark	**ZEB1**	**Snail**	**Slug**
Activating invasion and metastasis	+	+	+
Inducing angiogenesis	-	+	+
Sustaining proliferative signaling	+	NA	NA
Resisting cell death	+	+	+
Enabling replicative immortality	+	+	+

(+) Represents promoting the hallmark. (-) Represents suppressing the hallmark. (NA) means that data is not available.

2.3.3 EMT and Drug Resistance

Recently, studies have shown that EMT is essential for cancer cells to gain resistance against conventional drugs, such as, gemcitabine-resistant pancreatic cancer cells (Wang *et al*. 2009) and paclitaxel-resistant ovarian carcinoma cells (Kajiyama *et al*. 2007).

The understanding of the biomolecular events of cancer progression lead to the discovery of a target protein and the development of targeted drugs, such as EGFR inhibitors (Xiao and He 2010). EGFR inhibitors proved to be very effective for the treatment of NSCLC cases carrying a mutant EGFR gene (Lynch *et al*. (2004) and Sordella *et al*. (2004)). However, most of the patients acquire resistance to the treatment (Engelman and Janne 2008). It was also observed that the degree of sensitivity of NSCLC to EGFR inhibitors is associated with the degree to which they have undergone EMT (Thomson *et al*. 2005).

Sensitivity to EGFR inhibitors was high in E-cadherin expressing NSCLC lines. However, Vimentin expressing NSCLC lines demonstrated insensitivity to EGFR inhibitors such as gefitinib and erlotinib (Rho *et al*. (2009) and Yao *et al*. (2010)), concluding that the degree to which cells undergo EMT determines their sensitivity to EGFR inhibitors in NSCLC (Thomson *et al*. (2005) and Yauch *et al*. (2005)).

More studies have related EMT markers to cancer drug resistance ability. Since the new strategies in cancer treatment involve the targeting of specific oncogenes, however, EMT enables the cancer cells to overcome this oncogenic dependency and change their morphology from the epithelial targetable type to mesenchymal type where treatment fails (Sánchez-Tilló *et al.* 2012).

EMT markers were observed to be responsible for resistance against specific drugs. ZEB1 was found to be responsible for the resistance of breast carcinoma cell lines to doxorubicin (Tryndyak *et al*. 2010), head and neck squamous carcinoma cell lines to erlotinib (Haddad *et al*. 2009), non-small lung carcinoma cell lines to gefitinib (Witta *et al*. 2006) and pancreatic carcinoma cell lines to gemcitabine, 5-fluorouracil and cisplatin (Arumugam *et al*. 2009).

Snail was found to be responsible for the resistance of breast cancer cell line to 5-fluorouracil (Zhang *et al*. 2012), lung carcinoma cell lines to cisplatin (Hsu *et al*. 2010) and ovarian adenocarcinoma cell lines and primary tumors to cisplatin (Haslehurst *et al*. 2012).

Slug was found to be responsible for the resistance of malignant mesothelioma to doxorubicin, paclitaxel and vincristine (Catalano *et al.* 2004), non-small cell lung carcinoma cell lines and primary lung adenocarcinoma to gefitinib (Chang *et al*. 2011) and ovarian adenocarcinoma cell lines and primary tumors to cisplatin (Haslehurst *et al*. 2012).

2.4 ETS-RELATED GENE (ERG)

ETS-related gene (ERG) is a member of E-26 transforming sequence (ETS) transcription factors family. It has a critical role in cell proliferation, differentiation, migration and invasion (Hollenhorst *et al*. 2011).

ETS is a family of transcription factors, expressed in the endothelial lineage cells for vascular formation during embryogenesis and in the endothelial cells of adults for angiogenesis (Sato 2001). It is documented to be involved in the cellular growth and differentiation and in organ formation. This family of transcription factors is subdivided into ETS-1, ERG, ELG, PEA3, ELK, ELF, TEL and PU-1 members. ERG is characterized by a helix–turn–helix DNA binding domain which recognizes a highly conserved DNA binding sequence of purine rich nucleotides. This gene contains 17 exons and 16 introns, which results in 9 isoforms due to alternative splicing. Isoform 1 was found to have the nuclear localization sequence (NLS); while isoform 8 have two nuclear export sequences (NES) (Hoesel *et al.* 2016).

Physiologically ERG overexpression is involved in angiogenesis and overall cell maintenance. Pathologically ERG overexpression was observed in different cancer types, including acute myeloid leukemia, Ewing's sarcoma, peripheral primitive neuro-ectodermal tumors and prostate cancer (Sashida *et al*. 2010).

2.4.1 Physiological Role of ERG

ERG is located on chromosome 21. The gene product is a transcription factor associated with many genes inducing hematopoiesis. ERG is found to be overexpressed in mesodermal tissues such as endothelial cells, where it probably induces endothelial differentiation. In addition to its role in endothelial differentiation, the expression patterns of ERG highlight its role in angiogenesis, hematopoiesis and platelet development.

The role of ERG in hematopoiesis was confirmed by homozygous ERG mutant mice models, which died before birth due to defects in blood cells formation. Heterogynous ERG mutated mice showed a decrease in hematopoietic cells (Sato 2001).

2.4.2 Pathological Role of ERG

The discovery of ERG overexpression and its fusion with the prostate-specific gene transmembrane protease serine 2 gene (TMPRSS2) was a huge achievement as it occurs in 80 percent of prostate cancer samples (Tomlins *et al*. 2005). Later, its relation with other types of cancer was reported. For example, ERG overexpression was found in T-cell lymphoma mice models. In acute myeloid leukemia (AML), upregulation of ERG expression contributed for a worse outcome compared to normally expressed ERG cases (Marcucci *et al*. 2005).

ERG was also associated with Ewing's sarcoma, as it is related to a chromosomal translocation that involves ETS family of transcription factors. The most common translocation pattern was found with FLI-1. However, ERG was the fusion pattern in 10 percent of Ewing's sarcoma cases. Surprisingly, this fusion was able to induce leukemia when expressed in mice models (Miettinen *et al*. 2011).

The association of ERG and acute myeloid leukemia (AML) was confirmed after several studies, which revealed that ERG expression level is associated with poor prognosis in AML. More interestingly, ERG is overexpressed in almost all AML cases (Martens 2011).

The most interesting finding was that ERG silencing with sh-RNAs resulted in the growth inhibition of AML human cell lines.

After several studies, it was concluded that the aggressive forms of prostate cancers are related to ERG overexpression (Attard et al. (2008) and Demichelis *et al*. (2007)). Other studies suggested that ERG overexpression contributes to tumorigeneses by promoting proliferation, invasion and cancer initiation along with progression (Carver *et al*. (2009), Tomlins *et al*. (2008), Saramaki *et al*. (2008) and Wang *et al*. (2008)). Recently, a study has shown that ERG exerts its action by inducing epithelial to mesenchymal transition (Gupta *et al*. 2010).

It was reported that ERG promotes EMT through ZEB1 (Leshem *et al*. 2011). This defines ERG as a target to inhibit cancer cell invasion especially in prostate cancer (Rahim *et al*. 2011). An interesting study has shown that, ERG is relatively overexpressed in NSCLC samples (Xi *et al*. 2008), which may be related with EMT.

Recently, ERG over expression and its influence on clinical outcomes has been the focus of many studies (Rahim and Üren 2013), as lots of investigations have related ERG expression level with the cancer stage and the clinical outcome (Attard *et al*. (2008), Demichelis *et al*. (2007), Nam *et al*. (2007) and Perner *et al*. (2006)).

2.4.3 ERG Targeting

Analysis identified 36000 target regions for ERG gene product (Martens 2011). Therefore targeting ERG or its related pathways may offer additional promising targets for the treatment of many different cancer types (Rahim and Üren 2013). On the other hand, targeting transcription factors may be difficult because they lack enzymatic activity and the necessity of big number of binding proteins needed for their activity. Surprisingly, a small molecule that inhibits EWS-FLI1 fusion protein in Ewing's sarcoma and ERG in prostate cancer models (Rahim *et al*. 2011) was reported. This small molecule caused a reduction in the invasive abilities of prostate cancer cells.

3. MATERIALS AND METHODS

Details of the materials and methods performed in the study are available in this section.

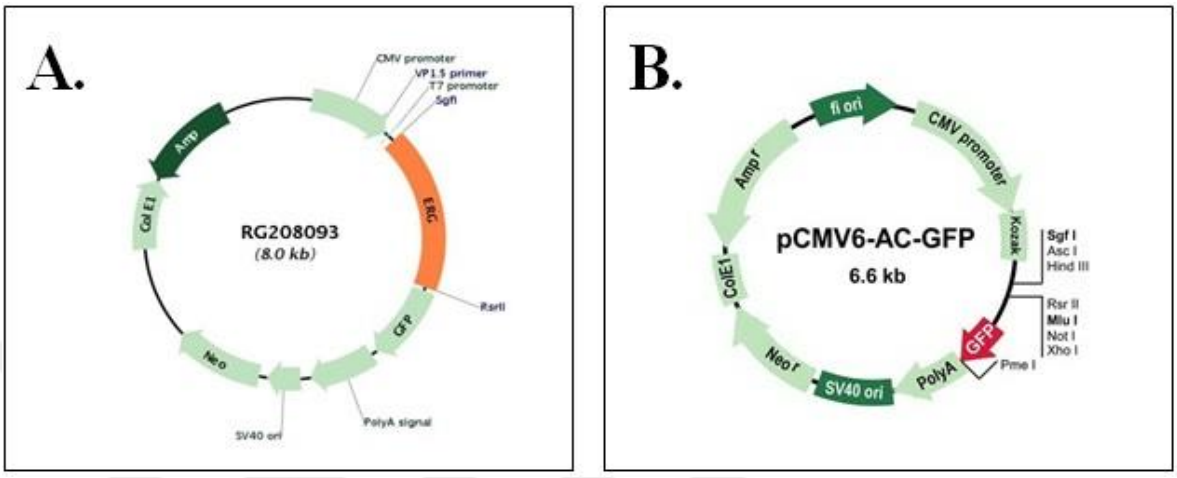
3.1 DESIGN OF THE ERG PLASMID

The expression plasmid containing the open reading frame of wild type human ERG transcription factor (GenBank, NM_182918) and the empty plasmid with the same backbone structure were obtained from Origene, USA (RG208093 and PS100010, respectively). Information regarding the plasmids is given on Table 3.1 and the plasmid maps are shown in Figure 3.1.

Table 3.1: List of the plasmids

Plasmid	**Vector**	**Gene reference**	**Insert name**	**Insert (bases)**	**Insert (amino acids)**
pEV	pCMV6-AC-GFP	-	Empty vector (no insert)	-	-
pERG	pCMV6-AC-GFP	NM_182918	Human ERG, variant 1	1440	479

Figure 3.1: Plasmid maps. ERG plasmid (pERG) (A) and the empty plasmid (pEV) (B)



3.1.1 *E. Coli* Top10 Transformation with ERG Plasmid

ERG plasmid (pERG) was transferred into competent *E.coli* TOP10 cells through heat shock transformation procedure. In order to enhance transformation efficiency, DNA concentration in the transformation mixture was kept less than 5% of total reaction volume. And also, different DNA concentrations were tested (1ng/µL, 100pg/µL and 10pg/µL) in order to have optimal number of colonies and isolated single colonies on the plate.

Bacterial transformation procedure:

E.coli TOP10 competent cells were kept on ice for 5-10 minutes to thaw.

1. Ampicillin (Amp, 100µg/ml) containing LB-Agar plates were stored at 4°C. They were brought to room temperature before each transformation reaction.

2. Plasmid DNA (in three different concentrations: 1ng/µL, 100pg/µL and 10pg/µL in a total volume of 4 µL) was added onto 100µl of competent *E. coli* Top10 in an Eppendorf tube. The transformation mixture was mixed gently.

3. The mixture was incubated on ice for 30 minutes.

4. A brief heat shock was performed by incubating the tubes in water bath (Stuart, USA) set to 42ºC for 30 seconds. The tubes were then transferred onto ice for 2 minutes immediately.

5. 250µL of SOC (Super optimal broth with catabolite repression, Gibco, USA) was added to the transformation mixture. The tubes were incubated in a shaking water bath set to 37ºC for 1 hour.

6. Transformed cells were plated on Ampicillin containing LB-Agar plates for selection.

7. Plates were incubated overnight (16 hours) at 37ºC in a bacterial incubator (VWR, USA).

3.1.2 Transformation Control

The presence of the plasmid and its size were confirmed after each transformation. For this confirmation, bacterial culture was started from a single colony, followed by plasmid DNA isolation by using PureLink® HiPure Plasmid Midiprep Kit (Invitrogen, USA). The presence of the individual plasmid was then confirmed by agarose gel electrophoresis.

3.1.3 Plasmid DNA Isolation and Purification

1. 10 mL Equilibration Buffer was added to the HiPure Midi Column and was allowed to flow by gravity.

2. An overnight grown culture was harvested by centrifugation at 4000 rpm for 10 minutes.

3. 4 mL Resuspension Buffer with RNase A was added to the cell pellet. The pellet was vortexed for proper mixing.

4. 4 mL of Lysis Buffer was added and mixed gently by inverting the tube. The tube was then incubated at room temperature for 5 minutes.

5. 4 mL of Precipitation Buffer was added and mixed by inverting the tube several times until a completely homogenous solution formed. Tubes were centrifuged at 4000 rpm for 30 minutes.

6. Supernatant was loaded onto the equilibrated column and allowed to drain by gravity. Column was washed twice with 10 mL Wash Buffer. Flow-through was discarded.

7. A sterile 15-mL centrifuge tube was placed under the column and 5 mL Elution Buffer was added to the column to elute the DNA.

8. 3.5 mL isopropanol was added to the eluted solution and the tube was mixed. The sample was then centrifuged at 4000 rpm for 30 minutes at 4°C. Supernatant was carefully removed and discarded.

9. Pellet was resuspended in 3 mL of 70% ethanol. The tube was again centrifuged at 4000 rpm for 15 minutes at 4°C and the supernatant was carefully discarded. The pellet was allowed to air-dry for 10 minutes.

10. DNA pellet was then resuspended in 200 µL TE Buffer.

11. The plasmid DNA concentration and purity were determined as described in Section 3.1.4.

12. DNA was stored at -20°C for long-term use.

3.1.4 Plasmid DNA Concentration and Purity Determination

Plasmid DNA concentration and purity were measured using MultiScan GO spectrometer (Thermo Scientific, USA) according to the formulas below:

DNA concentration (µg/mL) = OD_{260} × dilution ratio × 50 µg/mL

DNA purity = OD_{260}/ OD_{280}

3.1.5 Agarose Gel Electrophoresis

1. 100 mL of 1% agarose was prepared and poured into the agarose gel electrophoresis system (Bio-Rad, USA).

2. 5µL DNA marker (100ng/µL, Intron Biotech, Korea) was loaded.

3. 20µL each DNA sample was loaded to the wells.

4. Agarose gel was allowed to run in 1XTAE buffer for 1 hour at 80V on a power supply (Wealtec, USA).

5. Gel was then visualized using ChemiDoc MP imaging system (Bio-Rad, USA).

3.1.6 Stock Preparation of Plasmid Containing Bacteria

Followed by confirmation of the size of the individual plasmid, a glycerol stock of the bacteria hosting the plasmid was prepared for long-term storage.

Bacterial stock preparation procedure:

1. An individual single colony on the LB-Amp plate was transferred to 10mL LB-Amp solution with a sterile loop.

2. The culture was incubated in a shaking water bath at 37°C overnight (16 hours).

3. 2mL of the overnight culture was used for plasmid DNA isolation.

4. After confirmation of the presence of the plasmid DNA, 50µL LB-Glycerol (30% Glycerol, 70% LB) solution was mixed with 750µL transformed bacterial culture and transferred into cryovials.

5. Cryovials were stored at -80°C for long-term storage.

3.1.7 High Volume Bacteria Culture

High volume culture (50-100mL) was prepared from a single bacterial colony to provide the required DNA amount for mammalian cell transfection.

Bacterial culture preparation procedure:

1. Bacterial cell from the -80°C stock was inoculated into the 10mL LB-Amp medium using a sterile loop.

2. The culture was incubated overnight at 37°C.

3. Next day, the culture was transferred to 100mL LB-Amp medium, so that the starting bacterial density at OD_{600nm} is 0.1. The culture was incubated overnight at 37°C.

4. Following 16 hours of incubation, the culture was transferred to 50mL centrifuge tube and centrifuged at room temperature at 4000rpm for 20 minutes. The supernatant was then discarded.

5. Pellets were stored at -80°C for long-term storage.

3.2 MAMMALIAN CELL CULTURE

Non-small cell lung cancer (NSCLC) cell line, H358 was purchased from the American Tissue and Cell Collection (ATCC, USA). Overview information about this cell line with low ERG expression is given in in Table 3.2.

Table 3.2: Overview for H358 cell line

Catalog No	Organism	Tissue	Disease	Morphology	Properties	Medium
CRL5807	Human	Lung	Carcinoma	Epithelial	Adherent	RPMI-1640[1]

[1] Catalog no: 302001 (ATCC, USA).

H358 cells were cultured in its specific culture medium (Table 3.2) with 10% fetal bovine serum (FBS, Multicell, Brazil) and incubated at 37°C with 5% CO_2 in cell culture CO_2 incubator (NUVE, Turkey). The cells were monitored under phase contrast microscope (Nikon, USA) everyday and culture medium was changed in intervals as suggested by ATCC. Adherent cells were sub-cultured according to the protocol given below. All cell culture steps were performed in Biosafety Cabinet Type II (NUVE, Turkey) and the equipment and reagents used during these steps were sterile.

3.2.1 Cellular subculture

1. Culture medium on the cells was discarded. Cells were washed with phosphate buffered saline solution (PBS, Multicell, Brazil) to remove any traces of serum that may serve as a trypsin inhibitor.

2. Trypsin-EDTA solution (0.5% EDTA, Multicell, Brazil) was added onto the cells. Cells were incubated at 37°C with 5% CO_2 for 5 minutes.

3. Cellular detachment from the plastic surface was monitored under the microscope. As the cells became in suspension form, total growth medium was added to deactivate the trypsin solution.

4. The cells were split to new flasks in 1/3-1/6 dilution ratios.

3.2.2 Cryopreservation

For long-term storage of cells, -80°C glycerol stocks were prepared according to the following protocol.

Cells Cryopreservation:

1. Cell suspension was obtained as in 3.2.1, step 3.

2. Cells were centrifuged (Eppendorf, USA) at room temperature at 1000rpm for 5 minutes.

3. Supernatant was discarded.

4. Pellet was resuspended in cryopreservation medium (serum containing 10% DMSO) at a concentration of 1×10^6 cells/mL.

5. The cells were stored at -80°C for long-term storage.

3.2.3 Thawing

1. Frozen cells from -80°C stock were quickly placed in a 37°C water bath for thawing (maximum 2 minutes).

2. Cells were transferred to 15-mL Falcon tubes containing 9 mL medium.

3. Tubes were centrifuged at room temperature at 1000rpm for 5 minutes. Supernatant was discarded.

4. Total medium was added onto the cells and fresh culture started in a new flask.

3.3 TRANSIENT OVER EXPRESSION OF ERG IN H358 CELL LINE

The efficiency of transfection depends on cell confluency at the time of transfection and the ratio of the transfection agent (µL) to the plasmid DNA (ng) amount. Therefore, prior to the transfection of H358 cell line with pERG or pEV, the ideal cell number and transfection agent to DNA ratio optimizations were performed. ERG mRNA expression was analyzed at 24, 48 and 72 hours after transfection.

3.3.1 H358 Cell Number Optimization

FreeStyle Max (Invitrogen, USA) transfection agent was used to transfect H358 cells with ERG. H358 cells were plated to 6-well plate according to the protocol below at 300.000, 600.000 and 900.000 cells/well and confluency was monitored under the microscope after 24 hours.

Cell number optimization:

1. Cells were trypsinized and brought into suspension.

2. 20µL of the cell suspension was loaded to a hemocytometer.

3. Cell counting was performed twice under 10X magnification power and the average of cell numbers was taken.

4. Cell density and total cell number values were calculated with the following formulas.

Cell Density (Cells/ml) = Number of Cells × Dilution Factor × 10^4

Total Cell Number = Cell Density × Total Volume

5. 2ml of different cell densities were added to a 3.5cm^2 petri dishes.

6. Cell density was monitored under microscope after 24 hours.

3.3.2 Transfection Agent to Plasmid DNA Ratio Optimization

The ratio of the transfection agent (µL) to DNA (ng) amount is another important factor that affects the efficiency of transfection. Different transfection agent (µL) to plasmid DNA (µg) ratios (2:1 and 4:1) were used to transfect H358 cells with pERG or pEV plasmids. The protocol regarding these experiments is given below.

Transfection agent to plasmid DNA ratio optimization:

1. Cells were plated in culture dishes.

2. Cells were incubated for 24 hours at 37°C with 5% CO_2.

3. The next day, growth medium was changed.

4. The FreeStyle Max and plasmid DNA were diluted in OptiPRO SFM (Gibco, USA) with 2:1 and 4:1 ratios (transfection agent (µl) : DNA (µg)) and mixed slowly.

5. The mixture was allowed to stand at room temperature for 15-20 minutes and then was added dropwise on top of the cells.

6. Cells were incubated for 48 hours at 37°C with 5% CO_2. Cell viability was monitored under the microscope.

3.3.3 Determination of pEV and pERG Transfection Efficiency

The plasmids (pEV and pERG) used for this study carries the reporter gene, green fluorescent protein (GFP). Transfection efficiency of cells was analyzed by monitoring the expression of GFP under fluorescent microscope (Leica, USA) at 24, 48 and 72 hours after transfection. The ratio of the total number of cells to the green fluorescent cells in a single microscope visual area was determined. The ratio obtained was regarded as an indicator of transfection efficiency.

Transfection efficiency (%) = number of green glowing cells / total number of cells × 100

3.4 ANALYSIS OF H358 ERG EXPRESSION AT mRNA LEVEL

H358 cells were transiently transfected with ERG transcription factor and its expression level was analyzed with the procedure below.

1. H358 cells transfected by pEV or pERG were incubated for 24, 48 or 74 hours at 37°C with 5% CO_2.

2. Cell viability was monitored under the microscope at regular intervals.

3. At the end of the incubation time, H358 cells were collected, centrifuged and their pellet was used for RNA isolation.

3.4.1 RNA Isolation Protocol

Total RNA isolation from the pERG or pEV transfected H358 cells was performed using the PureLink RNA Isolation Kit (Invitrogen, USA). The protocol for this procedure is given below.

1. H358 cells were trypsinized and harvested by centrifugation at 4000rpm for 5 minutes at 4°C.

2. 0.3mL of lysis buffer solution was added to the pellet.

3. Tubes were vortexed at high speed until the cell pellet is completely dispersed.

4. A homogenous solution was obtained using a Homogenizer at its top speed for 45 seconds.

5. 0.3mL ethanol (70%) was added to the cell homogenate before vortexing.

6. The mixture was loaded on top of a column at room temperature with 12000g for 30 seconds. RNA was allowed to bind to the column.

7. 700µL of wash buffer was loaded to the column. The column was centrifuged with 12000g for 30 seconds. The flow-through was discarded.

8. 500µL of wash buffer II was loaded to the column. The column was centrifuged at room temperature at 12000g for 30 seconds.

9. The column was centrifuged once more at room temperature at 12000g for 2 minutes to dry.

10. The column was then placed in an RNase free microcentrifuge tube and 100µL of elution buffer was loaded on top of it before being incubated at room temperature for 3 minutes.

11. The total RNA was eluted by centrifugation at 14000g for 1 minute.

3.4.2 Determination of Total RNA Concentration and Purity

Total RNA concentration and purity were measured using MultiScan GO spectrometer (Thermo Scientific, USA) according to the following formulas respectively.

RNA concentration (µg/mL) = OD_{260} × Dilution Ratio × 40 µg/mL

RNA purity = OD_{260}/ OD_{280}

3.4.3 cDNA Synthesis

The cDNA synthesis reaction was performed according to the contents given on Table 3.3 with 100ng template RNA and the program available in Table 3.4.

Table 3.3: cDNA reaction mix

Content	**Volume (µL)**
qScript cDNA supermix	4
Template RNA, 100 ng	*
DEPC-treated dH_20, to complete the volume to 20 µL	**
Total volume	**20**

*depends on the concentration of RNA
**depends on the amount of RNA template used in the reaction

Table 3.4: cDNA synthesis program

Step	**Conditions**
RT enzyme activation	25°C temperature for 5 minutes
cDNA synthesis	42°C temperature for 30 minutes
RT enzyme inactivation	85°C temperature for 5 minutes
Holding	4°C holding temperature

3.4.4 Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR)

The cDNA synthesis was followed by reverse transcriptase-polymerase chain reaction (RT-PCR). The RT-PCR reaction contents, primer sequences and cycle program are given in Tables 3.5, 3.6, 3.7, respectively.

Table 3.5: The RT-PCR mix

Content	**Volume (µL)**
2X PCR Master Mix	10
Forward Primer, 10 µM	2
Reverse Primer, 10 µM	2
cDNA template (1/10 dilution)	2
DEPC-treated dH_20, to complete the volume to 20 µl	4
Total Volume	**20**

Table 3.6: List of RT-PCR primers

Primer Information		**Product size (bp)**
Homo sapiens ERG (NM_182918)		
Forward	5’ ACCGTTGGGATGAACTACGGCA 3’	284
Reverse	5’ TGGAGATGTGAGAGAAGGATGTCG 3’	
Homo sapiens GAPDH (NM_002046.5)		
Forward	5’ GTGAAGGTCGGAGTCAACG 3’	247
Reverse	5’ TGATTTTGGAGGGATCTCGC 3’	

Table 3.7: RT-PCR cycle program

Step	**Conditions**
Initial denaturation	95°C for 5 minutes
40 Cycles of the Following Three Steps	
Denaturation	95°C for 15 seconds
Annealing	56°C for 20 seconds
Extension	72°C for 20 seconds
Final Extension	40°C for 2 minutes

The PCR products were separated according to their band sizes on 1.5% agarose gel electrophoresis. The samples were run along with 5µL marker (100ng/µL, Thermo, USA). The expression level of each gene was determined using the concentration of the reference band on the marker and by densitometric analysis on the ChemiDoc MP imaging system (Bio-Rad, USA).

3.4.5 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

In addition to RT-PCR, quantitative RT-PCR (qRT-PCR), which is another method with more sensitivity, was performed to analyze the ERG mRNA expression levels in H358 cells. The results obtained through both procedures were compared. In qRT-PCR, Superscript III Platinum One Step SYBR RT-PCR kit (Invitrogen, USA) was used, and GAPDH gene expression was chosen for normalization. For specificity of the primers, melting curve analysis was performed. Experiments were completed in triplicates. CFX Manager software was used to analyze the data (Bio-Rad, USA).

qRT-PCR procedure:

The contents of the reaction mix are given on Table 3.8

Table 3.8: qRT-PCR reaction mix

Content	**Volume (µl)**
SuperScript® III RT/Platinum® Taq Enzyme Mixture	0.5
2X SYBR® Green Reaction Mixture	12.5
Forward Primer, 10 µM	0.5
Reverse Primer, 10 µM	0.5
Template RNA, 100 ng	*
DEPC-treated dH_20, to complete the volume to 25 µl	**
Total	**25**

*depends on the concentration of RNA

**depends on the amount of RNA template used in the reaction

The cycle program present on Table 3.9 was followed for gene expression analysis. This cycle program was determined based on the melting curve program of the CFX96 machine (Bio-Rad, USA).

Table 3.9: qRT-PCR cycle program

Step	**Conditions**
cDNA Synthesis	50°C for 4 minutes
Initial denaturation	95°C for 5 minutes
40 cycles for the following two steps	
Denaturation	95°C for 15 seconds
Extension	56°C for 30 seconds
Fluorescent signal measurement after every cycle	
Final Extension	40°C for 1 minute
Melting Curve Analysis	
Gradual Increase of Temperature I	Between 40°C- 60°C, at 0.05°C increments
Gradual Increase of Temperature II	Between 60°C- 95°C, at 0.5°C increments

The GeneBank information and primer sequences of the genes (ERG, GAPDH, E-cadherin, Vimentin, Snail, Slug and ZEB1) and the size of their amplification products are given on Table 3.10. GAPDH was used as an internal control as in RT-PCR.

Table 3.10: Primer sequences of EMT marker genes

Primer Information		**Product size**
Homo sapiens ERG (NM_182918)		
Forward	5’ ACCGTTGGGATGAACTACGGCA 3’	284
Reverse	5’ TGGAGATGTGAGAGAAGGATGTCG 3’	
Homo sapiens GAPDH (NM_002046.5)		
Forward	5’ GTGAAGGTCGGAGTCAACG 3’	247
Reverse	5’ TGATTTTGGAGGGATCTCGC 3’	
Homo sapiens E-Cadherin (NM_004360.3)		
Forward	5’ TACACCATCCTCAGCCAAGA 3’	248
Reverse	5’ ACCTGACCCTTGTACGTGGT 3’	
Homo sapiens Vimentin (NM_003380.3)		
Forward	5’ AGGTGGACCAGCTAACCAAC 3’	249
Reverse	5’ GGATTTCCTCTTCGTGGAGT 3’	
Homo sapiens Snail (NM_005985.3)		
Forward	5’ TGCAGGACTCTAATCCAGAGTTT 3’	218
Reverse	5’ CTCATCTGACAGGGAGGTCA 3’	
Homo sapiens Slug (NM_003068.4)		
Forward	5’ TGGTTGCTTCAAGGACACAT 3’	211
Reverse	5’ GTGCTACACAGCAGCCAGAT 3’	
Homo sapiens ZEB1 (NM_001128128.2)		
Forward	5’ TGCTCTGCAAAGAAATTGGGA 3’	221
Reverse	5’ GGAAATCTTGCGTCCACAGG 3’	

3.5 ANALYSIS OF EMT MARKER GENE EXPRESSION AT mRNA LEVEL USING RT-PCR

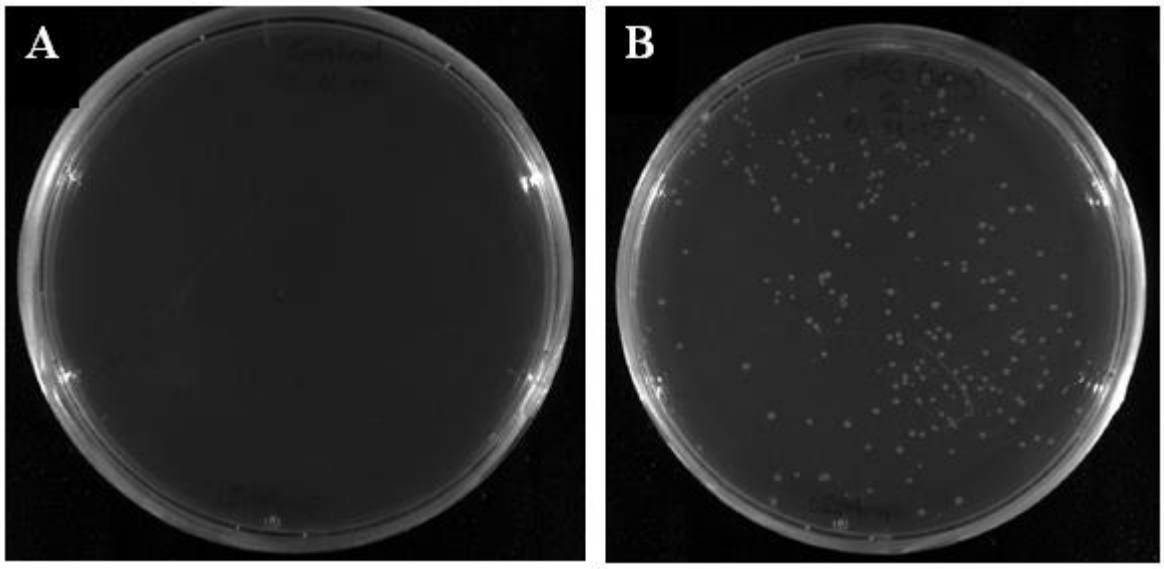
The mRNA expression analysis of the EMT marker genes in H358 cells transfected with the ERG gene was performed using the RT-PCR procedure. Protocols between 3.4.1-3.4.4 were followed in this part of the study.

4. RESULTS

4.1 *E.COLI* TOP10 TRASNFORMATION WITH ERG PLASMID

The vectors used in this study carry the ampicillin resistance (ampR) gene for selection of host bacteria containing the plasmid. Thus, the plasmid transformation using the $CaCl_2$ heat shock procedure was completed by selection of the *E.coli* TOP10 bacteria carrying the pEV or the pERG plasmids on LB-Agar plates with ampicillin (100ng/mL). *E.coli* colonies transformed with the pERG plasmid on LB-Amp plate and the negative control plate are shown in Figure 4.1.

Figure 4.1: *E.coli* TOP10 colonies on LB-Agar plates after pERG transformation



A: negative control (instead of plasmid DNA, dH_2O added during transformation), B: pERG. Colonies were obtained after 16 hour incubation at 37°C following transformation.

4.2 CONFIRMATION OF TRANSFORMATION

Presence of the plasmid in the transformed *E.coli* TOP10 bacteria was confirmed by comparing plasmid DNA band size pattern with the predicted one on agarose gel electrophoresis. The concentration values of the isolated plasmid DNA samples were

approximately 500-1000ng/μL and their purity values were between 1.8-1.9. Contamination was not observed on the pERG and pEV samples. Briefly, purity and concentration values were sufficient for the future transfection experiments.

4.3 MAMMALIAN CELL CULTURE STUDIES

H358 lung carcinoma cell line was grown in RPMI 1640 medium with 10% FBS as recommended by ATCC.

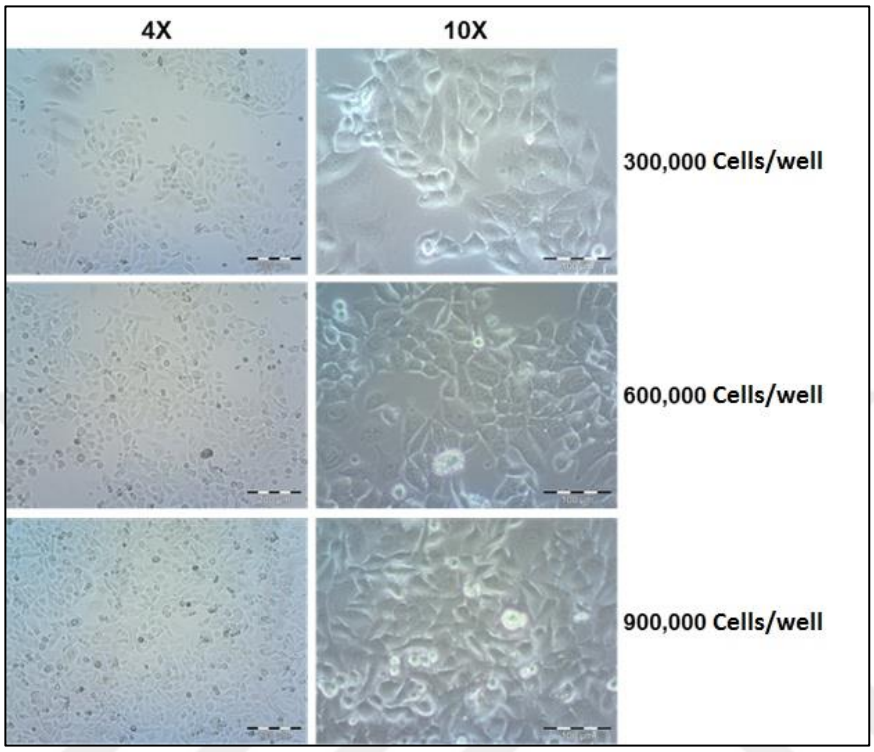
4.4 TRANSIENT ERG TRANSFECTION IN H358 NSCLC CELLS

Prior to the transfection of H358 cells with ERG, cell number and transfection agent (μL) to DNA (μg) ratio optimization studies were performed.

4.4.1 H358 Cell Number Optimization

FreeStyle Max transfection agent was used for the transfection of H358 cells with the pERG and pEV plasmids. According to the transfection protocol, cell confluency at the time of transfection is recommended as 70%. For this reason, in the first step, H358 cells were plated at different densities in order to obtain 70% confluency. It was observed that plating 400,000 cells/well in a 6-well plate one day before transfection maintains the 70% confluency at the time of transfection (Figure 4.2).

Figure 4.2: H358 cell number optimization

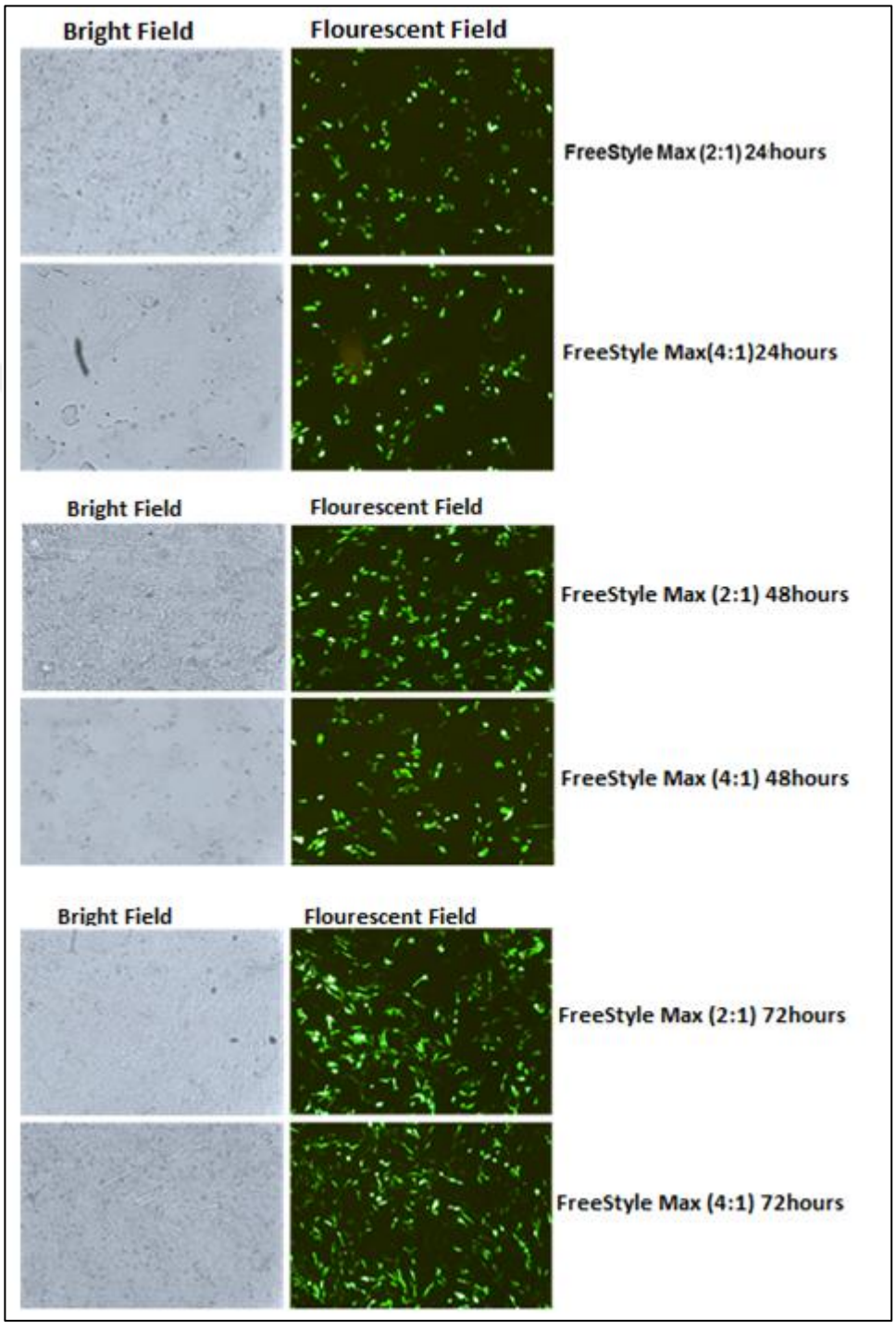


H358 cells were plated in 6-well plates as 300.000, 600,000 and 900,000 cells/well. One day after seeding, cell confluencies were examined under microscope with 4X and 10X magnifications.

4.4.2 Transfection Agent to Plasmid DNA Ratio Optimization

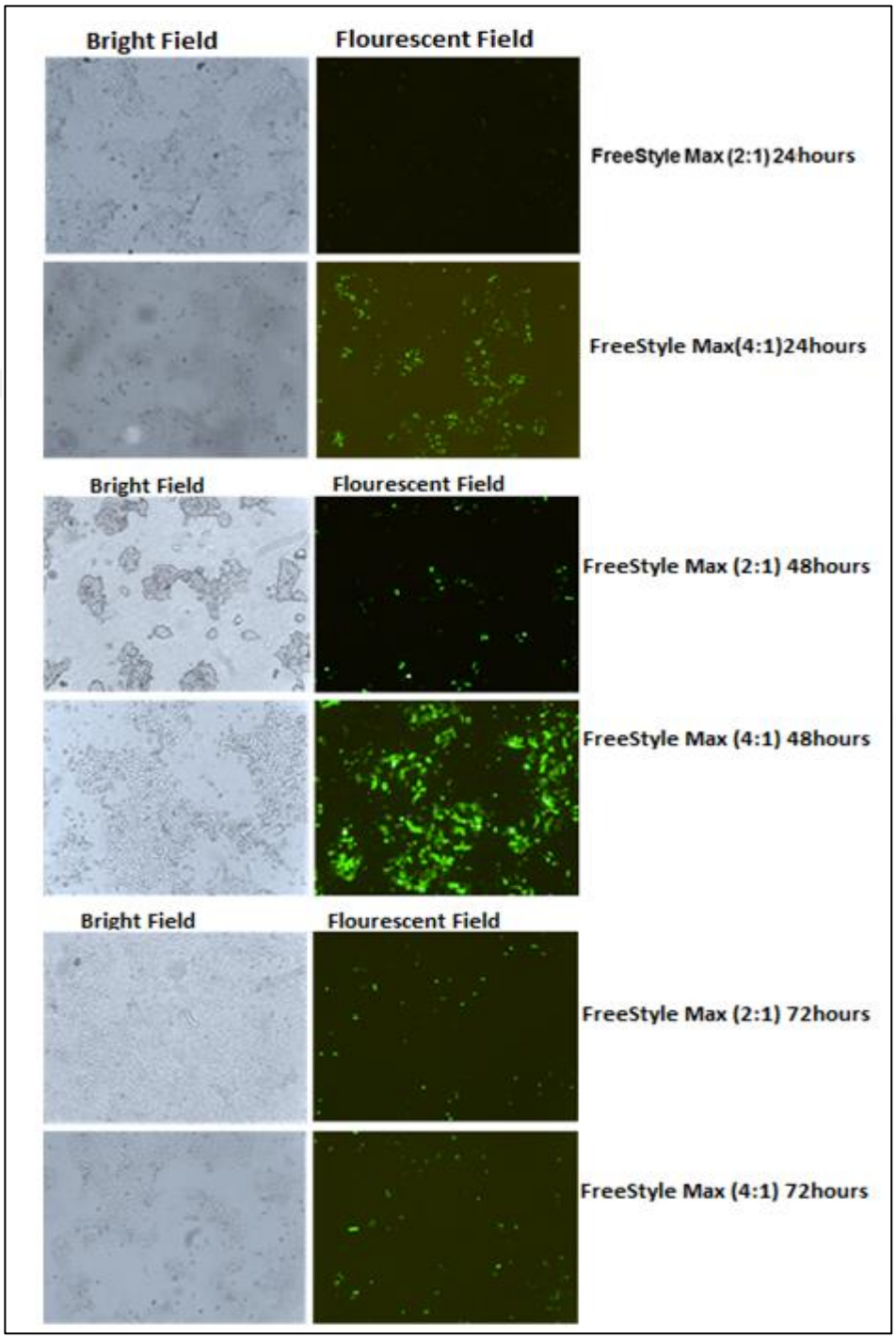
Lipid-based transfection method was performed for ERG gene transfer to H358 cells. For this purpose, optimizations with 2:1 and 4:1 (transfection reagent (µL): DNA (µg)) ratios were carried out on FreeStyle Max transfection reagent. Furthermore, in order to determine the time interval of the ERG gene expression, incubations at 24, 48 and 72 hours after the transfection with pEV and pERG plasmids were performed. The transfection efficiency was determined through observation of the fluorescent signal of GFP (Figures 4.3 and 4.4).

Figure 4.3: Transfection optimization of pEV transfected H358 cells



pEV plasmid was transfected with 2:1 and 4:1 ratios (transfection reagent: plasmid DNA), respectively, using FreeStyle Max reagent for transfection efficiency analysis. The transfection efficiency was observed at all 24, 48 and 72 hours intervals.

Figure 4.4: Transfection optimization of pERG transfected H358 cells



pERG plasmid was transfected with 2:1 and 4:1 ratios (transfection reagent: plasmid DNA), respectively, using FreeStyle Max reagent for transfection efficiency analysis. The transfection efficiency was observed at 24, 48 and 72 hours intervals.

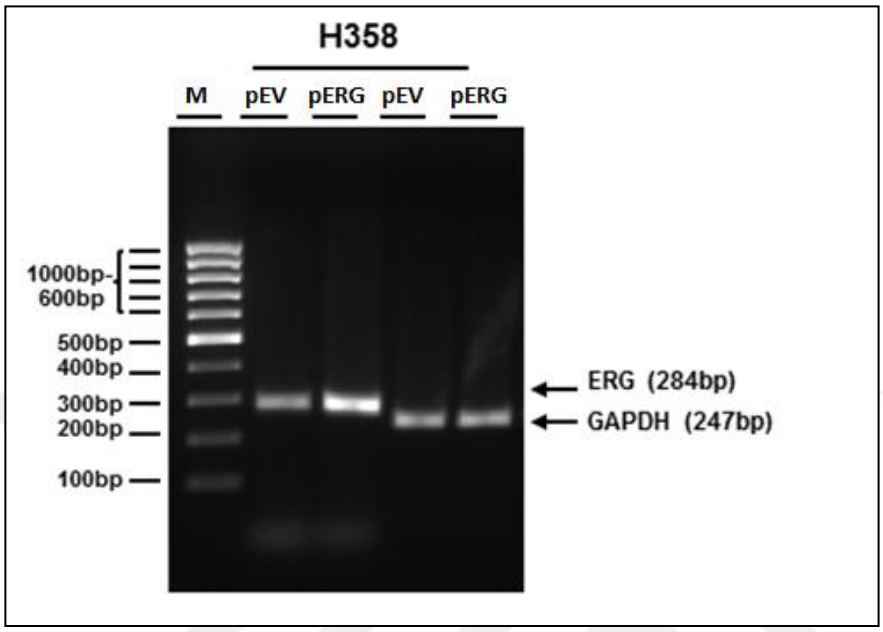
Positive pEV and pERG transfection was observed on FreeStyle Max transfection agent with both two ratios, 2:1 and 4:1 (Figures 4.3 and 4.4). For the pEV, the highest transfection efficiency was observed after 72 hours with the 4:1 ratio. For the pERG, highest transfection efficiency was observed after 48 hours with the 4:1 ratio. Based on these findings, the ratio to transfect H358 cells with pERG using FreeStyle Max was chosen as 4:1 and gene expression level were analyzed at all three time course settings (24, 48 and 72 hours after transfection).

4.4.3 Transient Gene Expression Analysis of ERG in H358 cells using RT-PCR

Following transfection of H358 cells with the ERG plasmid, ERG mRNA expression levels were determined at 24, 48 and 72 hours by the RT-PCR. Experiments were performed in triplicates. mRNA expression levels were also determined by qRT-PCR in triplicates due to the high sensitivity of this technique. During the first stage of ERG mRNA analysis, H358 cells were harvested at 24, 48 and 72 hours after transfection and the total RNA was isolated. Total RNA concentration values were around 100-250ng/µL and their purity levels were around 2.1-2.2.

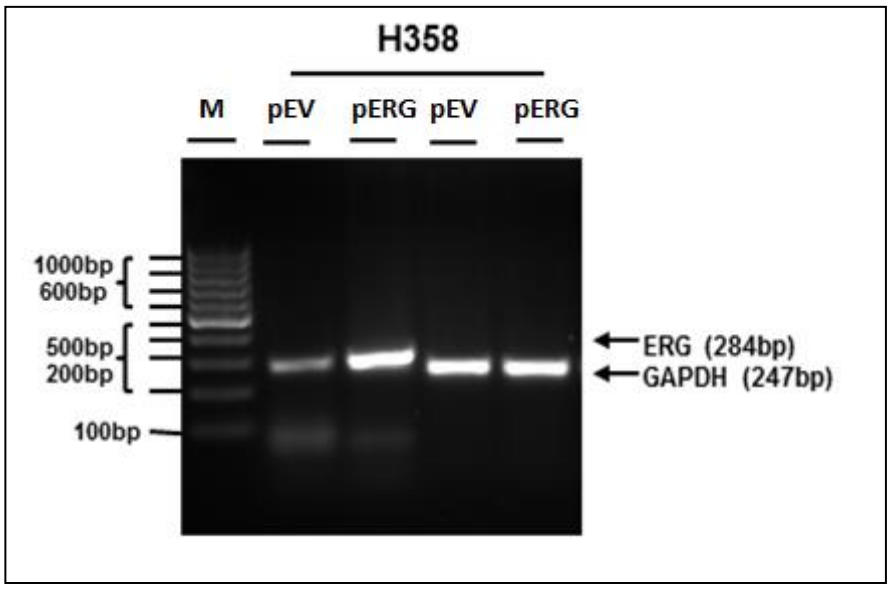
The mRNA molecules obtained by total RNA isolation were reverse-transcribed to its cDNA, which was amplified by RT-PCR to detect the ERG mRNA level. GAPDH house-keeping gene was used as a control gene. The resulting amplicons were analyzed by agarose gel electrophoresis. Figures 4.5-4.7, show ERG mRNA levels at 24, 48 and 72 hours after transfection of H358 cells with the ERG plasmid. Figure 4.8 shows ERG mRNA levels at 24, 48 and 72 hours after transfecting H358 cells with ERG by folds increase (pERG/pEV). The results show 2.1 folds increase after 24 hours, 2 folds increase after 48 hours and 5.1 folds increase after 72 hours.

Figure 4.5: ERG expression of H358 cells after ERG transfection (24 hour)



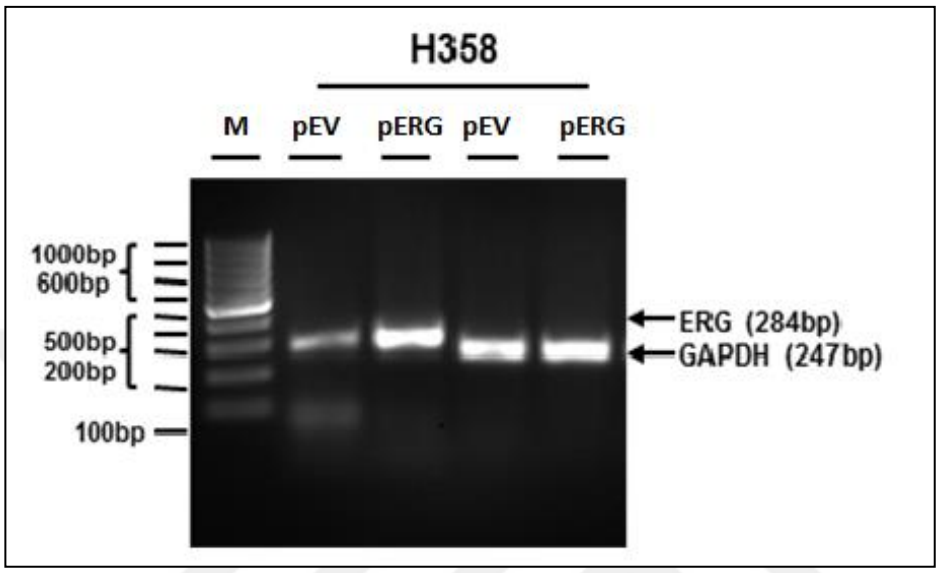
H358 cells were transfected with pEV and pERG (FreeStyle Max, 4:1 ratio). ERG expression was determined by RT-PCR method 24 hour after transfection. GAPDH gene was used as control.

Figure 4.6: ERG expression of H358 cells after ERG transfection (48 hour)



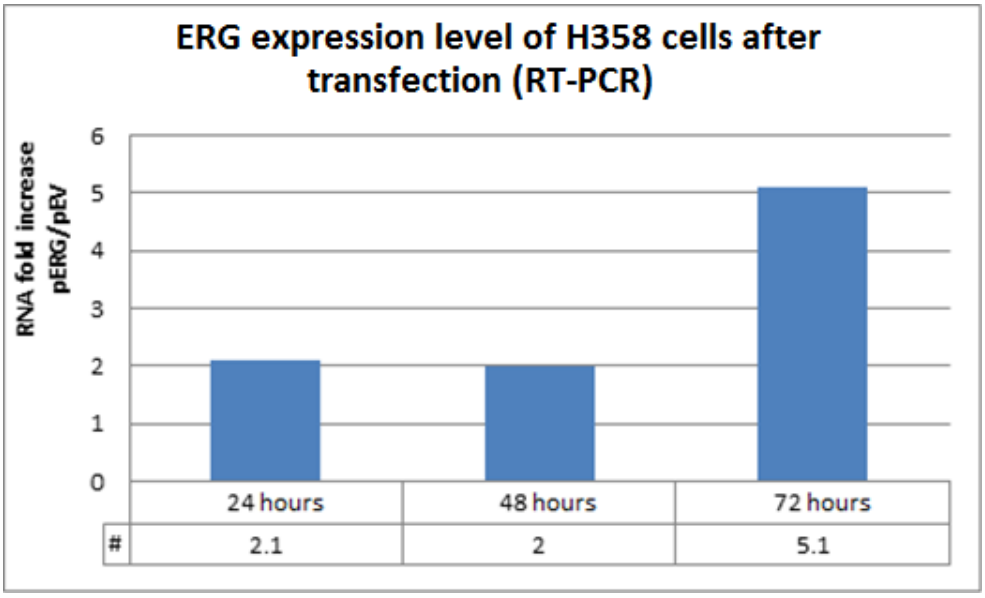
H358 cells were transfected with pEV and pERG (FreeStyle Max, 4:1 ratio). ERG expression was determined by RT-PCR method 48 hour after transfection. GAPDH gene was used as control.

Figure 4.7: ERG expression of H358 cells after ERG transfection (72 hour)



H358 cells were transfected with pEV and pERG (FreeStyle Max, 4:1 ratio). ERD expression was determined by RT-PCR method 72 hour after transfection. GAPDH gene was used as control.

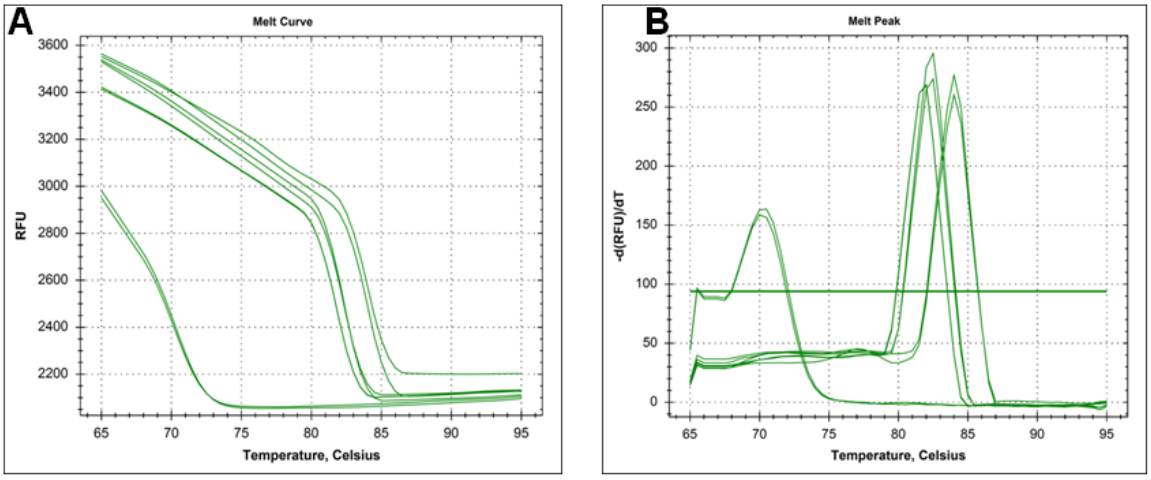
Figure 4.8: ERG mRNA level in H358 cells after ERG gene transfection



4.4.4 Transient Gene Expression Analysis of ERG in H358 cells using qRT-PCR

H358 cells have low endogenous ERG expression. In addition to RT-PCR, ERG mRNA expression levels after pERG transfection were evaluated by qRT-PCR. In a similar fashion, following transfection with pEV or pERG plasmids, total RNA was isolated at 48 and 72 hours and qRT-PCR was performed. The expression levels of ERG and GAPDH mRNAs were determined. The GAPDH expression level, which remained constant, was used for normalization. In addition, a melting curve analysis was performed to test the reliability of the data. Melting curve analysis showed that the primers used for the amplification of GAPDH and ERG are having a high level of specificity and did not produce any non-specific products (Figure 4.9A). The melting peak analysis produced a single peak melting curve, which shows that single products of ERG and GAPDH genes were obtained (Figure 4.9B).

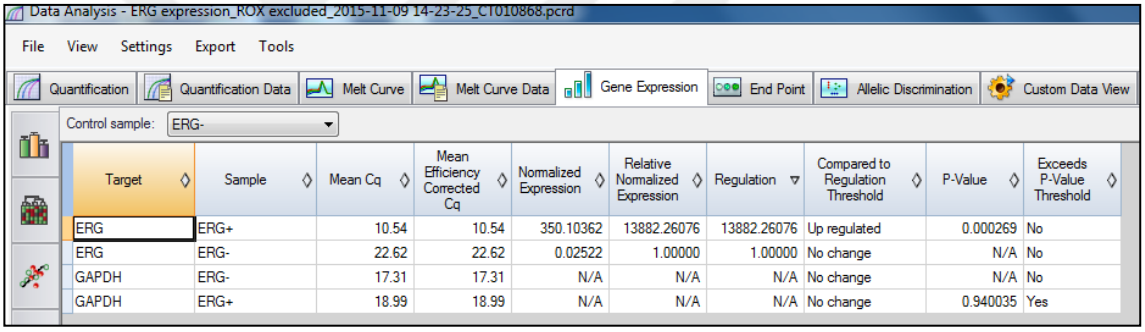
Figure 4.9: Melting curve (A) and melting peak (B) results of ERG expression analysis for H358 cells



Related analysis refers to the specificity of the primers used. Especially with non-specific binding in melting peak analysis, multiple peaks will be observed per sample. Non-specific binding was not observed in the relevant data.

The raw data obtained by qRT-PCR was normalized by the expression levels of GAPDH to compare ERG expression levels at 48 hour after transfection. H358 cells, transfected with pERG were compared with the same cells transfected with pEV, and approximately 14,000 folds increase (13,882 folds) were observed (Figure 4.10). Whereas GAPDH mRNA expression levels did not change. This demonstrates the consistency of the data. Additionally, when the gene expression regulation value was compared to threshold regulation value, an increase in ERG expression (up-regulated, $p<0.001$) and no change in GAPDH expression (no change, $p>0.05$) was observed statistically.

Figure 4.10: Raw data from qRT-PCR for H358 cells (48 hour)

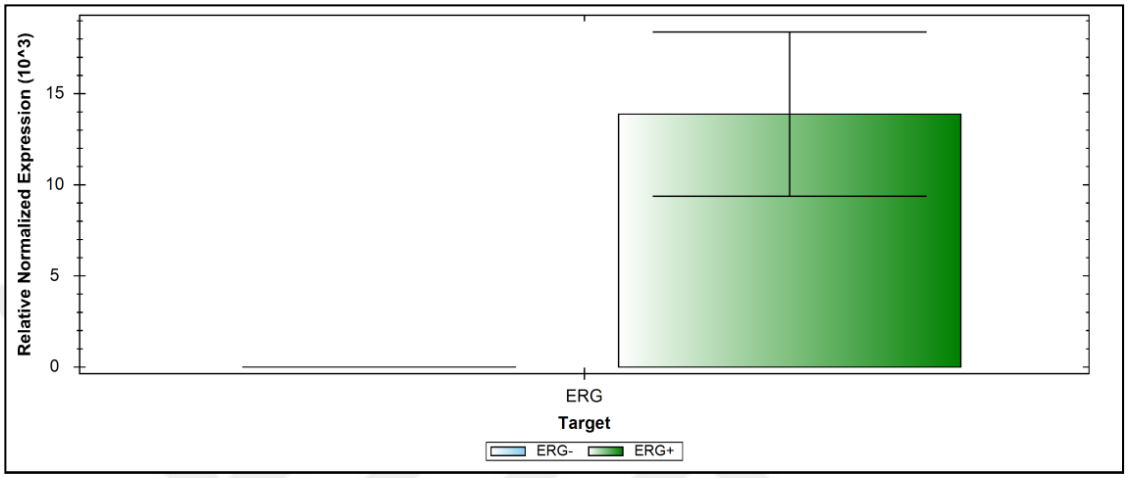


Target	Sample	Mean Cq	Mean Efficiency Corrected Cq	Normalized Expression	Relative Normalized Expression	Regulation	Compared to Regulation Threshold	P-Value	Exceeds P-Value Threshold
ERG	ERG+	10.54	10.54	350.10362	13882.26076	13882.26076	Up regulated	0.000269	No
ERG	ERG-	22.62	22.62	0.02522	1.00000	1.00000	No change	N/A	No
GAPDH	ERG-	17.31	17.31	N/A	N/A	N/A	No change	N/A	No
GAPDH	ERG+	18.99	18.99	N/A	N/A	N/A	No change	0.940035	Yes

Data include; threshold cycles for samples (Cq), normalized expression level (normalized expression), relative normalized expression, regulation, change compared to regulation threshold and statistical probability value (p-value). These data showed that ERG expression levels were increased approximately 14,000 times in the pERG transfected cells while no change was observed in GAPDH (normalization gene).

The relative normalized ERG expression level bar graph that was created as a result of 48 hour qRT-PCR also shows in a similar way that ERG expression was in the basal level in pEV transfected cells while ERG expression level was increased significantly in pERG transfected ones (Figure 4.11).

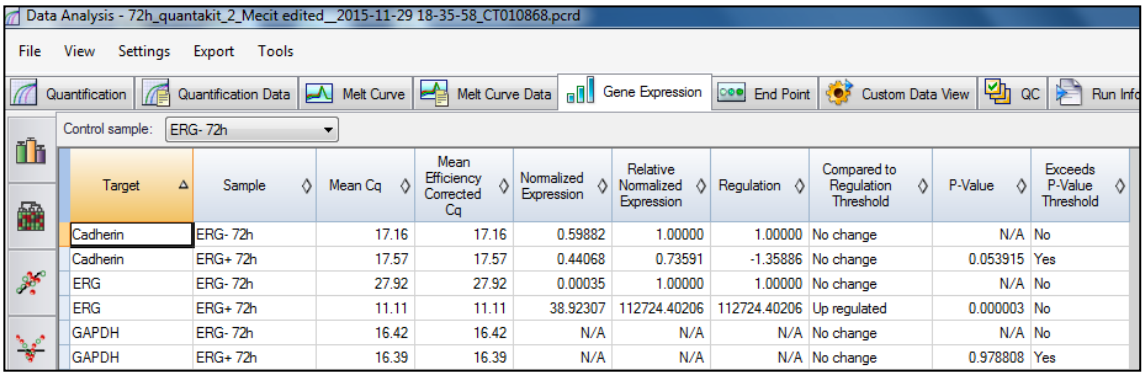
Figure 4.11: Relative normalized expression level bar graph for qRT-PCR analysis of H358 cells (48 hour)



ERG (-) represents pEV transfected sample and ERG (+) represents pERG transfected sample in the graph. Data show that the mRNA expression level (after the normalization with GAPDH expression level) of ERG transcription factor was increased approximately 14,000 times after the pERG plasmid transfection ($p<0,001$).

Raw data of 72 hour qRT-PCR analysis show that the ERG expression increased in 113,000 folds in pERG transfected H358 cells compared to pEV transfected same cell line when ERG expression was normalized to GAPDH expression (Figure 4.12). The comparison of GAPDH expression levels in both pEV and pERG-transfected cells did not show any difference. This demonstrates the consistency of the data. Additionally, when the gene expression regulation value was compared to threshold regulation value, an increase in ERG expression (up-regulated, $p<0.001$) and no change in GAPDH expression (no change, $p>0.05$) can be seen statistically.

Figure 4.12: Raw data from qRT-PCR for H358 cells (72 hour)

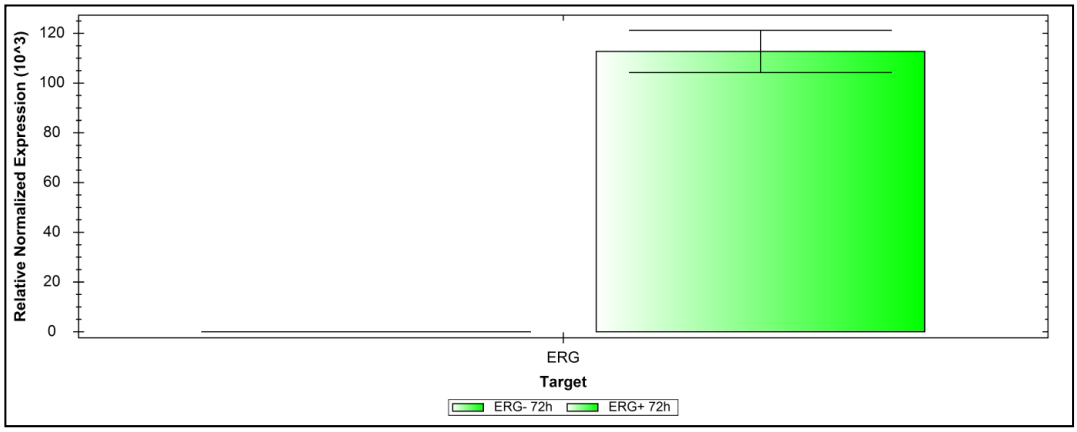


Target	Sample	Mean Cq	Mean Efficiency Corrected Cq	Normalized Expression	Relative Normalized Expression	Regulation	Compared to Regulation Threshold	P-Value	Exceeds P-Value Threshold
Cadherin	ERG- 72h	17.16	17.16	0.59882	1.00000	1.00000	No change	N/A	No
Cadherin	ERG+ 72h	17.57	17.57	0.44068	0.73591	-1.35886	No change	0.053915	Yes
ERG	ERG- 72h	27.92	27.92	0.00035	1.00000	1.00000	No change	N/A	No
ERG	ERG+ 72h	11.11	11.11	38.92307	112724.40206	112724.40206	Up regulated	0.000003	No
GAPDH	ERG- 72h	16.42	16.42	N/A	N/A	N/A	No change	N/A	No
GAPDH	ERG+ 72h	16.39	16.39	N/A	N/A	N/A	No change	0.978808	Yes

Data include; threshold cycles for samples (Cq), normalized expression level (normalized expression), relative normalized expression, regulation, change compared to regulation threshold and statistical probability value (p-value). These data showed that 72 hour ERG expression levels were increased approximately 113,000 times in the pERG transfected cells while no change was observed in GAPDH (normalization gene).

The relative normalized ERG expression level bar graph that was created as a result of 72 hour qRT-PCR also shows similarly that the ERG expression was in the basal level in pEV transfected cells while ERG expression level was increased significantly in pERG transfected ones (Figure 4.13).

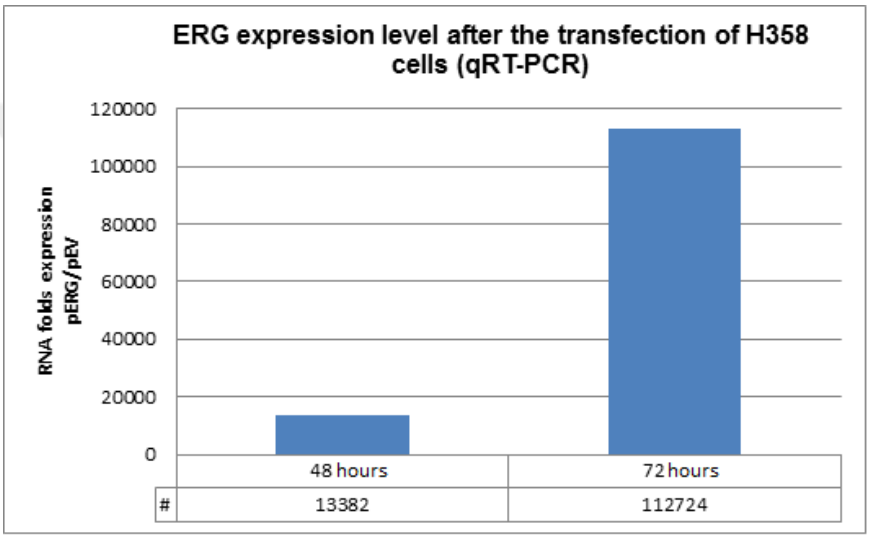
Figure 4.13: Relative normalized expression level bar graph for qRT-PCR analysis of H358 cells (72 hour)



ERG(-) represents pEV transfected sample and ERG(+) represents pERG transfected sample in the graph. Data showed that the mRNA expression level (after the normalization with GAPDH expression level) of ERG transcription factor was increased approximately 113,000 times after the pERG plasmid transfection ($p<0,001$).

The fold increase in ERG mRNA levels (pERG/pEV) for 48 and 72 hour groups determined by qRT-PCR are given in Figure 4.14. According to these results, 13,382 fold increase and 112,724 fold increase were obtained in the ERG mRNA levels of H358 cells at 48 and 72 hour after transfection, respectively.

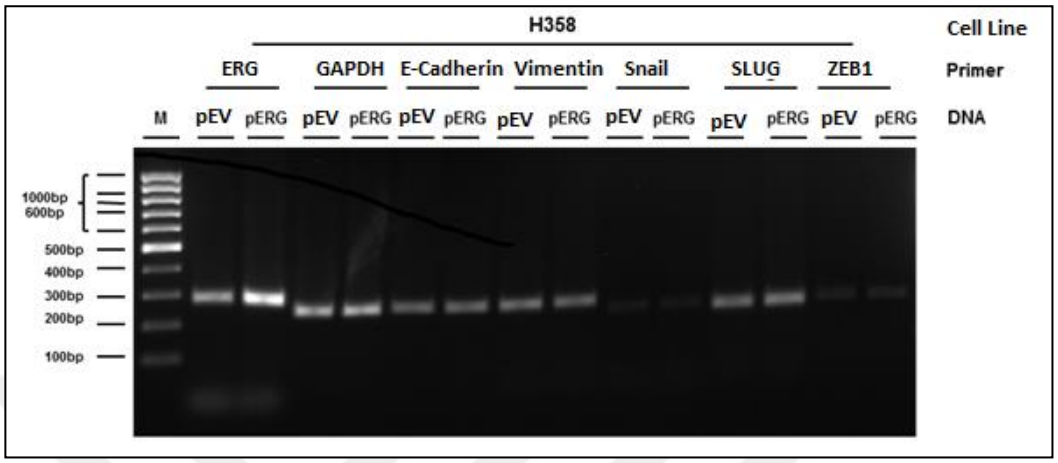
Figure 4.14: ERG mRNA level for H358 cells after ERG gene transfection (48 and 72 hours, qRT-PCR)



4.5 EMT MARKER GENE EXPRESSION ANALYSIS IN ERG TRANSFECTED H358 CELLS USING RT-PCR

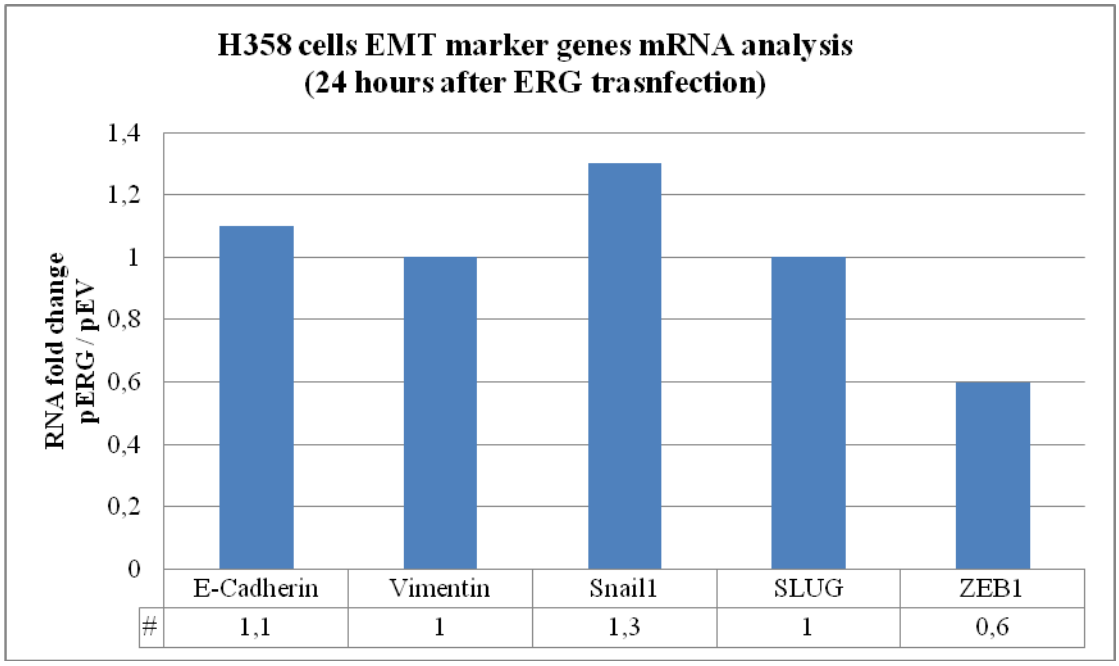
Following confirmation that ERG expression was successful in H358 cells, variations in mRNA expression levels of EMT markers were analyzed using RT-PCR in the next step. For this reason, after transfection of pEV and pERG into H358 cells, samples from 24, 48, 72 hours samples were taken respectively and RT-PCR was set up with equal amounts of total RNA. Corresponding RT-PCR results were separated in agarose gels (Figure 4.15; 24 hour, Figure 4.17; 48 hour, Figure 4.19; 72 hour). Densitometric analysis was performed based on the reference band of the marker. The averages of these numerical values were obtained and pERG expression levels of transfection rates were compared to pEV expression levels of transfection. This correlation was used for the calculation of how many folds the corresponding gene's expression had varied (Figure 4.16; 24 hour, Figure 4.18; 48 hour, Figure 4.20; 72 hour).

Figure 4.15: mRNA expression of EMT genes after ERG transfection (24 hour)



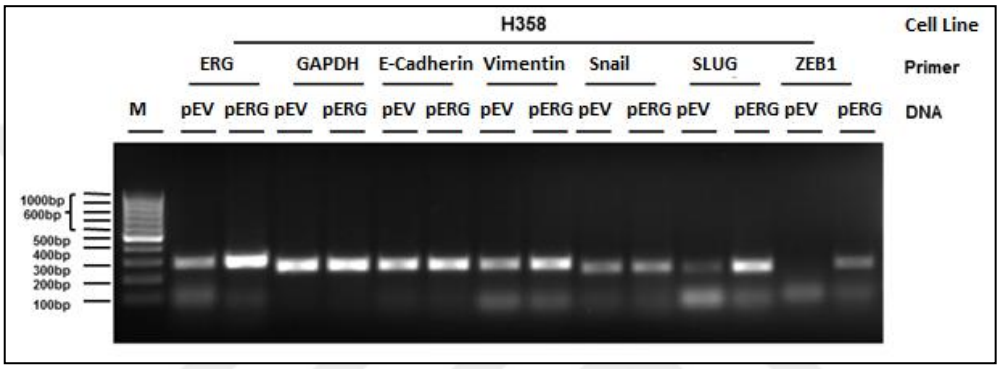
The bands for ERG and EMT genes on a 1.5% agarose gel for H358 cell line after 24 hour following transfection was observed. Brightness of the bands is proportional to the amount of PCR product in the corresponding wells.

Figure 4.16: mRNA expression of EMT genes after ERG gene transfection (fold increase, 24 hour)



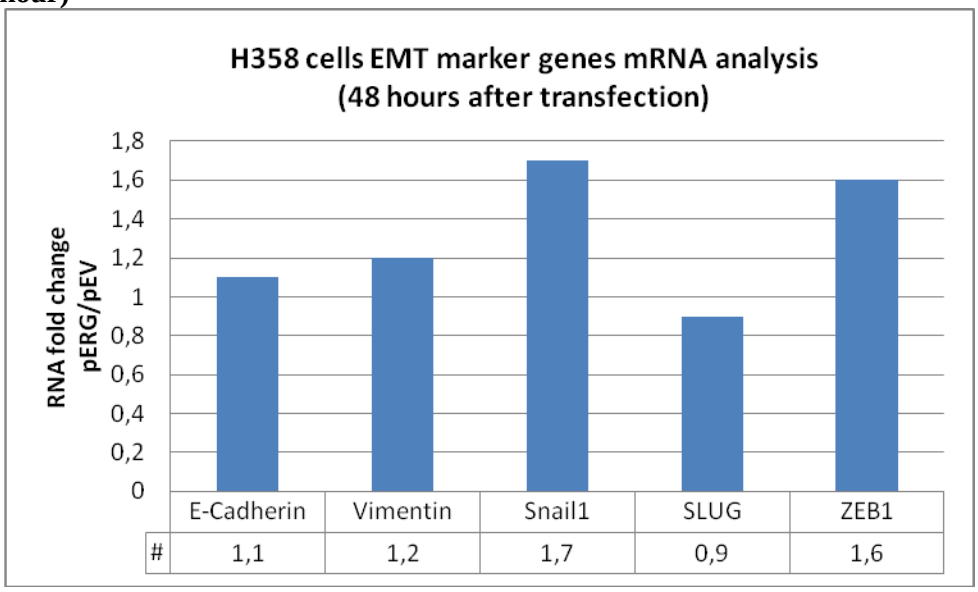
After the comparisons of the averages of 24 hour samples, it was observed that E-cadherin amount increased slightly, Vimentin and Slug expression level did not change and ZEB1 expression level decreased. The only gene with increased expression level in 24 hour samples was Snail (Figure 4.16).

Figure 4.17: mRNA expression of EMT genes after ERG transfection (48 hour)



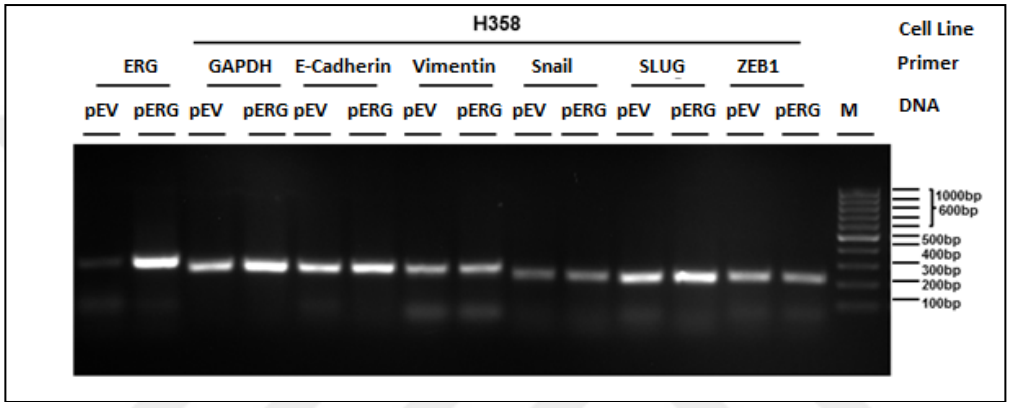
The bands for ERG and EMT genes on a 1.5% agarose gel for H358 cell line after 48 hour following transfection was observed. Brightness of the bands is proportional to the amount of PCR product in the corresponding wells.

Figure 4.18: mRNA expression of EMT genes after ERG gene transfection (fold increase, 48 hour)



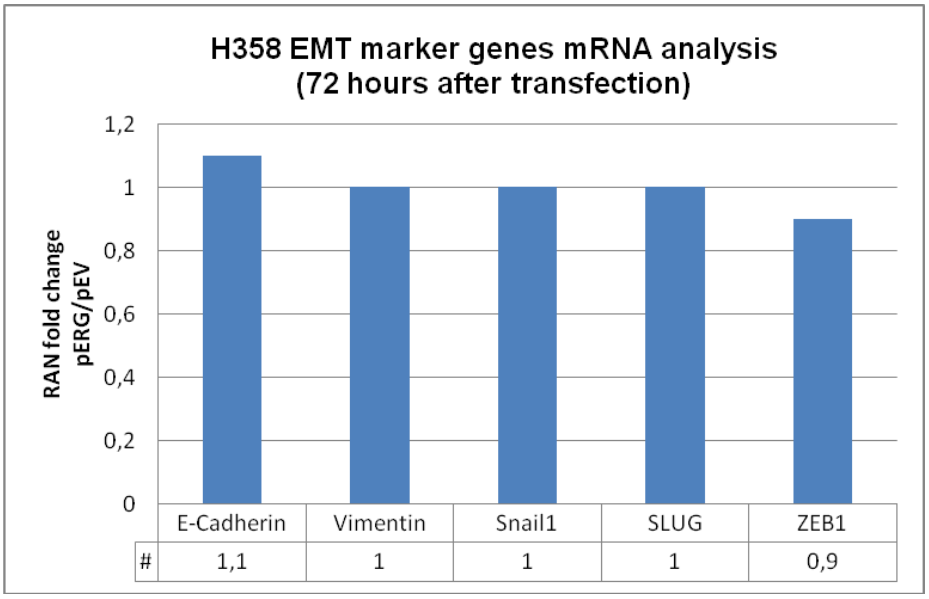
After the comparison of the averages of 48 hour samples, it was observed that E-cadherin amount increased slightly as similar to 24 hour experiment, Vimentin and Snail and ZEB1 gene expression levels increased and Slug expression level decreased slightly (Figure 4.18).

Figure 4.19: mRNA expression of EMT genes after ERG transfection (72 hour)



The bands for ERG and EMT genes on a 1.5% agarose gel for H358 cell line after 72 hour following transfection was observed. Brightness of the bands is proportional to the amount of PCR product in the corresponding wells.

Figure 4.20: mRNA expression of EMT genes after ERG gene transfection (fold increase, 72 hour)



After the comparison of the averages of 72 hour samples, it was observed that E-cadherin amount increased slightly, Vimentin and Snail and Slug gene expression levels did not changed and ZEB1 expression level decreased slightly (Figure 4.20).

According to these results, 48 hours after ERG transfection, a significant increase in the mRNA levels of EMT marker genes, Snail and ZEB1 were observed. This is consistent with the literature.

5. DISCUSSION

In this study, it is hypothesized that ERG transcription factor may promote epithelial mesenchymal transition (EMT) in lung cancer. In order to confirm this hypothesis, ERG expression was established in a non-small cell lung cancer (NSCLC) cell line, H358 with low endogenous ERG expression. The effect of ERG on expression of EMT marker genes was evaluated using RT-PCR. The data obtained from this study suggests that ERG may regulate expression of EMT marker genes and this transcription factor can be a critical therapeutic target for treatment of NSCLC patients in the future.

Lung cancer is the most common cancer type in the world, which contributes to 13 percent of the new cancer cases every year, as estimated in 2012. Non-small cell lung cancer contributes to more than 80 percent of the lung cancer cases. This disease has a very poor 5-year survival rate, which is approximately 15 percent of all lung cancer cases (Jemal *et al*. 2010).

Surgery is considered as the best option for the treatment of NSCLC. However, only 25 percent of the patients are considered eligible for the surgical therapy. This is attributed to the old age of the patients and/or the late diagnosis of the disease (van Zandwijk 2001). Despite improvements in chemotherapy, this alternative treatment strategy fails to offer significant advances in the survival rate of patients diagnosed with lung cancer. This is due to the high metastatic behavior of NSCLC, which enables cancer cells to gain resistance against the chemotherapeutic agents. The high metastatic behavior is mainly due to the ability of NSCLC cells to undergo EMT (Xiao and He 2010). This observation raises the need for a specific therapeutic target that regulates EMT.

ETS-related gene (ERG) is a member of E-26 transforming sequence (ETS) transcription factors family. The members of this family have critical roles in cell proliferation, differentiation, migration and invasion (Hollenhorst *et al*. 2011). ERG is characterized by a helix–turn–helix DNA binding domain which recognizes a highly conserved DNA binding sequence of purine rich nucleotides (Hoesel *et al.* 2016). Physiologically ERG

overexpression is involved in angiogenesis and overall cell maintenance. Pathologically ERG overexpression was observed in different cancer types such as, prostate cancer (Tomlins *et al*. 2005), acute myeloid leukemia (Marcucci *et al*. 2005) and Ewing's sarcoma (Sashida *et al*. 2010). ERG overexpression was observed in 80 percent of prostate cancer samples (Tomlins *et al*. 2005) and ERG fusion pattern was observed in 10 percent of Ewing's sarcoma cases. This fusion was able to induce leukemia when expressed in mice models (Miettinen *et al*. 2011). ERG was reported to exert its pathological effects by inducing epithelial to mesenchymal transition (Gupta *et al*. 2010). It was also reported that ERG promotes EMT through ZEB1 (Leshem *et al*. 2011). These observations define ERG as a target to inhibit cancer cell invasion, especially in prostate cancer (Rahim *et al*. 2011). In addition, many studies have related ERG expression level with cancer stage and clinical outcome (Attard *et al*. (2008), Demichelis *et al*. (2007), Nam *et al*. (2007) and Perner *et al*. (2006)). Although overexpression of ERG in NSCLC samples were reported previously (Xi *et al*. 2008), any direct correlation between ERG and EMT in NSCLC has not been shown yet.

This study hypothesizes that ERG may promote EMT in lung cancer in a similar way it does in other cancer types. In order to confirm this hypothesis, ERG gene expression was established in a NSCLC cell line, H358 with low endogenous ERG expression. The effect of ERG on mRNA expression of EMT marker genes including e-cadherin, vimentin, snail, slug and ZEB1 was then evaluated using RT-PCR. The data obtained from this study suggest that ERG may regulate expression of EMT marker genes and that it can be a critical therapeutic target for the treatment of NSCLC patients.

The presence of EMT was confirmed with downregulation of e-cadherin (Gupta *et al.* 2010), upregulation of vimentin (Lamouille *et al*. 2014) and upregulation of the transcription factors snail, slug and ZEB1 (Pena *et al*. (2006), Leshem *et al.* (2011)). E-cadherin is a well-documented epithelial marker that is essential for cell-cell adhesion through the formation of adhesive junctions (Lamouille *et al*. 2014). Vimentin is a mesenchymal marker that induces changes in cell shape, motility, and adhesion (Mendez *et al*. 2010). The transcription factor ZEB1 was reported to repress E-cadherin expression

(Vandewalle *et al*. 2009) and induce expression of vimentin (Bindels *et al*. 2006). ZEB1 was found to be involved in tumor invasion and its overexpression was observed in many cancer types including NSCLC (Sánchez-Tilló *et al.* 2012). In addition, ZEB1 was found to be responsible for the resistance of breast carcinoma cell lines to doxorubicin (Tryndyak *et al*. 2010), head and neck squamous carcinoma cell lines to erlotinib (Haddad *et al*. 2009), non-small lung carcinoma cell lines to gefitinib (Witta *et al*. 2006) and pancreatic carcinoma cell lines to gemcitabine, 5-fluorouracil and cisplatin (Arumugam *et al*. 2009). The transcription factors Snail and Slug, similar to ZEB1 were found to repress E-cadherin expression and induce expression of vimentin (Peinado *et al*. 2004). Snail was found to be responsible for the resistance of breast cancer cell line to 5-fluorouracil (Zhang *et al*. 2012), lung carcinoma cell lines to cisplatin (Hsu *et al*. 2010) and ovarian adenocarcinoma cell lines and primary tumors to cisplatin (Haslehurst *et al*. 2012). Slug was found to be responsible for the resistance of malignant mesothelioma to doxorubicin, paclitaxel and vincristine (Catalano *et al.* 2004), non-small cell lung carcinoma cell lines and primary lung adenocarcinoma to gefitinib (Chang *et al*. 2011) and ovarian adenocarcinoma cell lines and primary tumors to cisplatin (Haslehurst *et al*. 2012).

In this study, H358 cell line was used as a model for NSCLC. This adherent cell line with epithelial morphology is derived from metastatic alveoli of a male lung cancer patient and it shows relatively low endogenous ERG expression.

ERG overexpression in H358 cells at 24, 48 and 72 hours after transfection with the ERG plasmid was determined by RT-PCR and at 48 and 72 hours after transfection with qRT-PCR. In both procedures, overexpression of ERG gene was observed. In RT-PCR data, overexpression of ERG was observed 2.1 fold at 24 hours, 2 fold at 48 hours and 5.1 fold at 72 hours after transfection. The level of increase in ERG overexpression was shown much higher in qRT-PCR data due to the sensitivity of the procedure (Bleve *et al*. 2003). In qRT-PCR data, 13,382 fold increase at 48 hours, and 112,724 fold increase at 72 hours after transfection of H358 cells with the ERG plasmid were observed. Melting curve and melting peak analysis ensured the specificity of the primers because non-specific binding was not monitored during the qRT-PCR analysis.

Following confirmation of ERG overexpression in H358 cells, EMT marker gene expression levels were analyzed to test the role of ERG on EMT in H358 cells. According to the analysis of mRNA expression level of EMT marker genes at 24 hours after ERG transfection, e-cadherin mRNA level slightly increased, vimentin and slug levels did not change, ZEB1 mRNA level was decreased. The only EMT marker that showed an increase in its mRNA expression level was snail. This data at 24 hours cannot predict EMT since most of the EMT marker genes did not show any significant changes in their expression levels. This can be attributed to the early analysis time frame after ERG transfection, which may not have allowed enough time for the EMT marker genes to be expressed.

According to the analysis of mRNA expression level of EMT marker genes at 48 hours after ERG transfection, e-cadherin mRNA expression level slightly increased, vimentin, snail and ZEB1 mRNA levels showed an increase in their expression, while slug showed a slight decrease. These results support the hypothesis, as the expression level of the mesenchymal marker vimentin increased. Two transcription factors, considered as mesenchymal markers, Snail and ZEB1 also showed a significant increase in their mRNA expression levels. These results obtained at 48 hours after ERG transfection of H358 cells indicate that ERG overexpression induces EMT. This is also parallel to the data reported by others in prostate cancer (Leshem *et al.* (2011) and (Pellecchia *et al*. 2012)). According to the previous reports, e-cadherin expression level would be expected to show a decrease as well (Gupta *et al.* 2010). This slight decrease observed in e-cadherin mRNA expression level can be attributed to the presence of non-transfected cells.

According to the analysis of mRNA expression level of EMT marker genes at 72 hours after ERG transfection, e-cadherin mRNA expression levels slightly increased, vimentin, snail and slug mRNA levels did not show any significant change in their expression, while ZEB1 showed a slight decrease. These results do not predict EMT since most of the marker genes did not show any significant changes in their expression levels. These results were expected because the transfection efficiency of H358 cells with the ERG plasmid at 72 hours was shown to be very low. An alternative to overcome this problem of variation in transfection efficiency can be constructing a H358 cell line with stable ERG expression. In

this way, presence of non-transfected cells during expression analysis can be eliminated. The application of qRT-PCR to analyze expression of EMT marker genes is another tool to support these findings since qRT-PCR is more sensitive than RT-PCR. These results can also be confirmed by expression analysis of EMT marker genes in the protein level using western blot assay. Silencing ERG gene in NSCLC cell lines with high endogenous ERG expression and analyzing the effect of this procedure on EMT marker gene expression can also be another way to test the role of ERG in EMT in lung cancer.

Another alternative to support the hypothesis in this study is to analyze expression of additional EMT markers, such as TWIST1/2. These markers are reported to be downregulators for expression of epithelial genes and activators for expression of mesenchymal genes (Lamouille *et al*. 2014). FOXC2 transcription factor can also be used as it is reported to be overexpressed in cancer cells which show mesenchymal phenotype. TCF3/4 and KLF8 transcription factors were also found to directly repress the expression of e-cadherin, so they are used as EMT markers (Thiery *et al*. 2009). Goosecoid is another transcription factor, used to predict ERG induced EMT in prostate cancer (Leshem *et al.* 2011).

A small molecule, YK-4-279 was reported to inhibit ERG dependent prostate cancer motility and invasion (Rahim *et al*.2011). The results presented in this study indicate that ERG expression can induce EMT in a similar way as in other cancer types. Therefore, the administration of YK-4-279 on NSCLC cell lines with endogenous ERG expression can be critical to show the role of ERG on EMT in lung cancer. In this perspective, ERG gene silencing and treatment of NSCLC cells having endogenous ERG expression with YK-4-279 is expected to produce similar results. Another interesting approach can be administration of YK-4-279 in combination with chemotherapeutic drugs to which cancer cells gain resistance during cancer therapy. For example, cisplatin loses its effect on lung carcinoma cell lines due to snail overexpression (Hsu *et al*. 2010), gemcitabine loses its effect on pancreatic carcinoma cells due to ZEB1 overexpression (Arumugam *et al*. 2009), paclitaxel loses its effect on the malignant mesothelioma cells due to slug overexpression (Catalano *et al.* 2004) and gefitinib loses its effect on non-small cell lung cancer cell lines

and primary lung adenocarcinoma cells due to slug overexpression (Chang *et al*. 2011). Because ERG was reported to mediate the expression of these transcription factors, it is strongly possible that a combination therapy including YK-4-279 and anyone of these chemotherapeutic agents may open new therapeutic options for many cancer patients in the future.

The results of this study highlight ERG as a new therapeutic target for the treatment of lung cancer, which is one of the main causes of cancer deaths worldwide. More comparative studies are required to clarify the potential of ERG as a therapeutic target and its role on EMT in NSCLC.

6. CONCLUSION

The results obtained from this study confirm that ERG overexpression in lung cancer promotes EMT in a similar way to other cancer types. Our data suggests that ERG can be a potential target for lung cancer treatment in conjugation with the existing chemotherapeutics.

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APPENDIX

BUFFERS AND RECIPES

A list of the ingredients of every buffer used for the experiments is as follows.

50X TAE Stock Buffer

For 1 L solution; 242 g tris free base (Sigma, Germany), 57.1 mL glacial acetic acid (Sigma, Germany) and 100 mL 0.5M EDTA (Sigma, Germany) were added to 750 mL deionized water. Volume was completed to 1 L with deionized water.

Agarose Gel

For 1% agarose gel; 1 g agarose (Sigma, Germany) were added to 75 mL 1X TAE buffer. Volume was completed to 100 mL with 1X TAE buffer. The mixture was heated by microwave for 2 minutes. After cooling to approximately 60°C 2 µL ethidium bromide (Sigma, Germany) were added.

LB Liquid Medium

For 1 L medium; 10 g tryptone (Sigma, Germany), 10 g NaCl (Sigma, Germany), and 5 g yeast extract (Sigma, Germany) were dissolved in 800 mL deionized water. Volume was completed to 1 L with deionized water. The mixture was then autoclaved. Ampicillin was added to the autoclaved medium after cooling to 55°C so the final concentration will be 100µg/mL. For 30% glycerol containing LB medium; glycerol (Sigma, Germany) was added to the autoclaved LB medium so the final glycerol concentration will be 30%.

LB Agar Medium

For 1 L medium; 10 g tryptone (Sigma, Germany), 10 g NaCl (Sigma, Germany), 20 g Agar (Sigma, Germany) and 5 g yeast extract (Sigma, Germany) were dissolved in 800 mL

deionized water. Volume was completed to 1 L with deionized water. The mixture was then autoclaved. Ampicillin was added to the autoclaved medium after cooling to 55°C so the final concentration will be 100µg/mL.

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