MOLECULAR CONSEQUENCES OF EMT SILENCING ON PANCREAS CANCER CELL LINES

by
Ezgi Kaşıkcı

Submitted to Graduate School of Natural and Applied Sciences
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in
Biotechnology

Yeditepe University
2018

MOLECULAR CONSEQUENCES OF EMT SILENCING ON PANCREAS CANCER CELL LINES

APPROVED BY:

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

Prof. Dr. Ahmet Arman .....

Prof. Dr. Dilek Telci .....

Assoc. Prof. Dr. Elif Damla Arısan .....

Assoc. Prof. Dr. Ömer Faruk Bayrak .....

DATE OF APPROVAL: ..../..../2018

Dedicated to Aysun Taneri….

ACKNOWLEDGEMENTS

First and foremost I want to thank my advisor Fikrettin Sahin, PhD. It has been an honor to work with him as his Ph.D. student. He has always been an excellent example for us. I want to thank him for the moral and financial support during my PhD.

I want to thank Ömer Faruk Bayrak, my honorary co-advisor. His guidance and criticism has helped me a lot during my PhD. I also want to thank Kursad Turksen, my honorary co-advisor. I am very lucky to have you as a mentor.

The members of the Molecular Diagnostic group have contributed immensely to my personal and professional time at Yeditepe University. I am grateful for having them as my coworkers and my friends for life. I want to thank; Safa Aydın, Huseyin &Ezgi Abdikzade, Taha Bartu Hayal, Burcin Asutay and Zeynep Busra Bolat for their moral and scientific supports.

My years in Yeditepe has introduce me someone that I can call a true friend. I am especially grateful to Esra Aydemir Coban for being the sister I have always longed for. We shared the best and the worst moments of life “together”.

Family is a gift that lasts forever and I am lucky to have 3 families now. The I want to thank my mother & father in law, Nur Kasikci and Serdar Kasikci for listening my crazy thoughts for hours and accepting me as their daughter. I want to thank my new grandmother, Kadriye Kasikci for her warm hugs. I want to thank my dad, Murat Boylu, for the love he has given me and for the sparkles in his eyes when I enthusiastically talk about my science stuff. I want to thank my grandmother, Cemile Taneri. You are and always will be in my heart and in my prayers.

“Spinning counter clockwise so each turn robs the planet of angular momentum, slowing its spin the tiniest bit, lengthening the night, pushing the dawn and giving me a little more time here, with you”. If I am Atlas, you are my P-body. We are e^2. I want to thank you, my lovely husband, Evren Kasikci. I love you more than mango-gelato coated with a thin layer of hot Belgium chocolate.

You know now it is your turn.. I want to thank you for like.. Everything. I think in a lot of ways unconditional love is a myth. My mom's the only reason I know it's a real thing.

“My Lord, increase me in knowledge.”

ABSTRACT

MOLECULAR CONSEQUENCES OF EMT SILENCING ON PANCREAS CANCER

Pancreatic cancer is known to spread rapidly and rarely detected in early stages. Not until quite advanced stages, no signs and symptoms are observed and in advanced stages it is nearly impossible to remove the tumor. This type of cancer is known to be aggressive and migrate to different sections of the body quickly. If metastasis of pancreatic cancer could be stopped it would be possible to manage the tumor without searching for additional tumor spread and if this treatment could be combined with drug resistance decrease it could be used as a good treatment option for pancreas cancer. Epithelial to Mesenchymal transition (EMT) is counted as one of the possible routes for initiating metastasis and drug resistance. Movement of epithelial cells are limited. Hence it must convert itself into a more motile cell type "mesenchymal" by using EMT pathways. In this study, important EMT transcription factors "Snail, Slug and Twist" were silenced by short hairpin technology on different pancreas cancer (adenocarcinoma) cell lines, Panc-1, MIA PaCa-2, BxPc-3, AsPc-1. It was shown that, this silencing causes the cell to turn back into epithelial stage; being less mobile and invasive, having decreased stem cell characteristics. After these favorable results from gene silencing, AsPc-1, metastatic pancreas cancer cell line, was subjected to chemotherapeutic agents and drug resistance was evaluated. EMT downregulated AsPc-1 cells have shown less resistance to chemotherapeutics compared to the control group. Combinational therapy (both gene therapy and drug induction) is a rising trend for cancer. During this study, together with gene therapy small molecule inhibitors of EMT were used to enhance the effect of gene downregulation. EMT downregulated AsPc-1 cells were treated with two EMT inhibitors, SD-208 and CX-4945, to understand the cumulative effect of gene therapy and small molecule inhibitors on migration and invasion. The results have shown that, it is possible to increase the effect of gene therapy by using chemical support but gene therapy is irreplaceable with small molecule inhibitors alone. Overall it was found that inhibition of EMT, either by gene therapy alone or with the enhancement by small molecules, is a good candidate for the treatment of pancreas cancer since it simultaneously minimize metastasis, stem cell properties and drug resistance.

ÖZET

EMT SUSTURULMASININ PANKREAS KANSERİ HÜCRELERİ ÜZERİNE MOLEKÜLER SONUÇLARI

Pankreas kanseri, hızlıca yayılması ve erken evrelerinde seyrek olarak fark edilmesi ile tanınır.. Gelişmiş evrelerine kadar hiçbir belirti ve semptom göstermemekle beraber, gelişmiş evrelerinde tümörün alınması neredeyse imkansızdır. Ayrıca bu kanser türü agresifliği ile tanınır. Eğer pankreas kanserinin metastazı durdurulursa, tümörün üstesinden gelmek mümkün olacaktır. Buna ilaveten, göç ile beraber ilaç dayanıklılığı da azalırsa bu metot pankreas kanseri için iyi bir tedavi opsiyonu haline gelecektir. Epitelden mezenkimale geçiş (EMG), metastazı başlatan ve ilaç dayanıklılığına neden olan yolaklardan bir tanesidir. Epitel hücrelerin hareketliliği sınırlıdır. Bu nedenle kendilerini daha hareketli bir hücre türü olan "mezenkimal" hücrelere "EMG" yolaklarını kullanarak dönüştürmeleri gerekmektedir. Bu çalışmada, önemli EMG transkripsyon faktörleri olan "Snail, Slug ve Twist" kısa hairpin teknolojisi ile çeşitli pankreas kanseri adenokarsinom hücre hatları, Panc-1, MIA PaCa-2 , BxPc-3 ve AsPc-1 hücrelerinde susturulmuştur. Bu susturma sonucu hücrelerin epitel özelliklerini geri kazanarak, daha az hareketli, daha az invazif ve daha az kanser kök hücre özellikleri olan hücrelere dönüştükleri bulunmuştur. Bu olumlu sonuçlar görüldükten sonra, metastatik özelliklere sahip olan AsPc-1 hücreleri seçilip, kemoterapik ajanlara maruz bırakılmış ve ilaç dayanıklılıkları test edilmiştir. EMT susturulan ASPC-1 hücrelerinin kontrol grubuna kıyasla kullanılan ilaçlara daha az dayanıklılık gösterdiği görülmüştür. Kombinasyon terapisi (gen terapisi ve küçük molekül inhibitörlerinin beraber kullanımı) kanser tedavisi için gözde bir opsiyon haline gelmektedir. Bu çalışma sırasında gen terapisi ile beraber EMG'nin küçük molekül inhibitörleri gen susturmasının etkisinin arttırılması için kullanılmıştır. EMG susturulmuş AsPc-1 hücreleri, EMT inhibitörü olan SD-208 ve CX-4945 ile muamele edilip, gen tedavi ve küçük molekül inhibitörlerinin kümülatif etkilerine bakılmıştır. Sonuçlar doğrultusunda küçük molekül inhibitörlerinin EMG'nin etkisini arttırdığı ancak gen tedavinin yerini dolduramayacağı bulunmuştur. Sonuç olarak EMG inhibisyonu tek başına veya küçük molekül inhibitörleri ile beraber, metastazı minimize etmesi, kanser kök hücre özelliğini azaltması ve ilaç duyarlılığını arttırması sebebiyle pankreas kanseri tedavisi için iyi bir adaydır.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS/ABBREVIATIONS

ABCG2	ATP-binding cassette sub-family G member 2
AD	Anno domini
Akt	Protein Kinase B
ALDH1	Aldehyde dehydrogenase
AMPK	Adenosine monophosphate-activated protein kinase
aSMA	Alpha-actin-2
ATP	Adenosine triphosphate
BC	Before Christ
BCR	Breakpoint cluster region protein
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
BRCA1/2	Breast cancer gene ½
c-MET	tyrosine-protein kinase Met or hepatocyte growth factor receptor
CA 19-9	Cancer antigen 19-9
CAT	Collective ameoboid transition
CCL2	CC-chemokine ligand 2
CCND1	Cyclin D1
CCNE1	Cyclin E1
CCR2	CC-chemokine receptor 2
CD	Cluster of differentiation
CGH	Comparative genomic hybridization
CSC	Cancer stem cell
CT	Computed tomography
DAPI	4',6-diamidino-2-phenylindole
dH2O	Distilled water
DMEM	Dulbecco’s Modified Eagle’s Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid

EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
EpCAM	Epithelial cell adhesion molecule
ESA	Paraoxonase 1, PON-1
EtBr	Ethidium bromide
EtOH	Ethanol
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GS	Goat Serum
GTP	Guanosine triphosphate
HGF	Hepatocyte growth factor
IGF	Insulin-like growth factor
INK4A	Cyclin-dependent kinase inhibitor 2A, multiple tumor suppressor 1
IMs	Inflammatory monocytes
JNK	c-Jun N-terminal kinase
KCL	Potassium chloride
KDR	Kinase insert domain receptor
KRAS	Kirsten rat sarcoma viral oncogene homolog
MAMs	Metastasis associated macrophages
MAPK	Mitogen-activated protein kinase
MAT	Mesenchymal to ameoboid transition
MET	Mesenchymal to epithelial transition
MIC	Minimum inhibition concentration
MiRNA	Micro-RNA
MMP2	matrix metallopeptidase 2
MMP9	matrix metallopeptidase 9
MTS	3-(4,5-di-methyl-thiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4 sulfo-phenyl)-2H-tetrazolium
MUC1	Mucin1
MYC	C-Myc
NaCl	Sodium chloride

NF-κB	Nuclear Factor kappa B
PAF	Paraformaldehyde
PAN-IN	Pancreatic Intraepithelial Neoplasia 1-A
PBS	Phosphate Buffered Saline
PC	Pancreas cancer
PCR	Polymerase chain raction
PDAC	Pancreatic ductal adenocarcinoma
PI	Propidium Iodide
PI3K	Phosphoinositide 3-kinase
PI3K-Akt-mTOR	Phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin
PSA	Penicillin/Streptomycin/Amphotericin
qPCR	Quantitative PCR
Ras	Rat sarcoma gene
RB	Retinoblastoma
Rho	rhodopsin
RNA	Ribonucleic acid
sh	Short hairpin
shSnail	Snail gene silenced with short hairpin
shSlug	Slug gene silenced with short hairpin
shTwist	Twist gene silenced with short hairpin
SMAD4	mothers against decapentaplegic homolog 4
SNAI	Snail1
SNAI	Snail2 or Slug
SP-1	Specificity protein 1
TAE	Tris base, acetic acid and EDTA
TdT	Terminal deoxynucleotidyl transferase
TE	Tris EDTA
TGF β3	Transforming Growth Factor β3
TGFβ	Transforming growth factor beta
TMZ	Temozolomide
TP53	Tumor protein p53
UV	Ultraviolet

VEGF	Vascular endothelial growth factor
Wnt	Wingless
Zeb-1	Zinc finger E-box binding homeobox 1
Zeb-2	Zinc finger E-box binding homeobox 2

1. INTRODUCTION

1.1. CANCER

Cancer is described as the abnormal expansion of cells with a potential to invade or migrate to the different parts of the body. Cancers cells can; synthesize growth signaling molecules and become insensitive to anti-growth signals, they can evade apoptosis and can enter replication continuously, and they can induce angiogenesis and activate metastasis in order to migrate distant tissues. These features are called the six hallmarks of cancer [1]. After a decade, it is stated that cancer cells can also create the inflammation that promotes tumor growth and reprogram their energy metabolism [2]. After cardiovascular diseases, cancer is the second leading cause of mortality worldwide.

Cancer is not a new disease. Signs of bone cancer was discovered in mummies in ancient Egypt and the oldest recorded case of breast cancer is again from ancient Egypt in 1500 B.C [3].

The word cancer is derived from the Greek word karkinos, meaning crab. It is used by Hippocrates (460–370 B.C) to describe the appearance of carcinoma tumors, which has the veins stretched on all sides looking like a crab's feet. It is translated to Latin "Cancer" by Celsus (25 BC – 50 AD) (also meaning crab). Galen (130-200 AD) used oncos (Greek word for swelling) to define tumors [3].

Today, cancer is still one of the most important field of interest and every year billions of dollars are spent for finding the cure or for understanding the underlining mechanism of cancer.

1.1.1. Cancer Initiation, Promotion and Progression

Every day, some of the cells in our organs come across different mutagens. We are exposed to mutagenic UV light, inhale carcinogenic gases, eat burnt food or exposed to chemicals. Minor damages are repaired by cell’s repair mechanisms and when there is a major damage

those cells are eliminated by our body's defense mechanisms. Despite all the effort, if a cell can escape from everything, this damage creates the initiation of cancer.

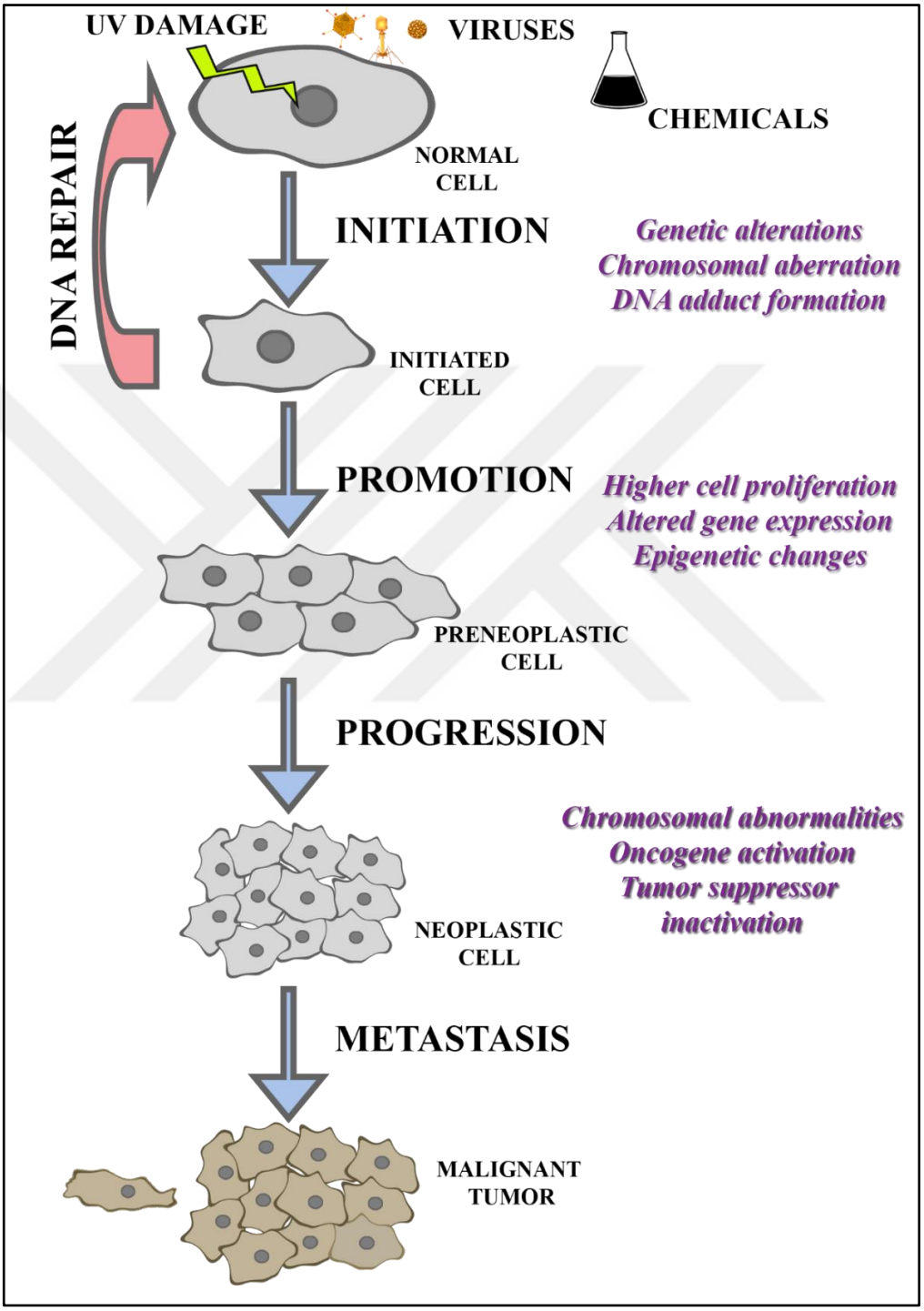


Figure 1.1 Schematic representation of cancer progression

With current researches it is now known that, most tumors start from a single mutated and transformed cell which is called cancer-initiating cells or cancer stem cell. This cell is known

to be able to divide for infinite numbers and create a tumor in body. Although there are theories that suggest, cytoplasmic exchange or cellular fusion between different types of cells can be effective for tumor formation, it is still questionable [4].

As shown in the Figure 1.1 cancer formation starts with cancer initiation step. In initiation step, genes responsible for biochemical signaling pathways such as proliferation, apoptosis evasion, apoptosis initiation and differentiation can be overexpressed, silenced or mutated and because of these alterations of the genes, transcription factor and protein amounts are changed [5]. Additionally cellular architecture, cellular morphology and structural properties of cells [6]. This step can be reversed by using single or combination of blocking agents. Currently many researchers work on developing blocking agents in order to stop cancer while it is in initiation step [7].

Second step of creating a tumor is called the promotion stage. This step can take time and considered a reversible process. This process involves creating a group of pre-neoplastic cells that actively proliferate. In this step it is possible to limit proliferation and growth rate by using chemo preventive agents and/or suppressive agents [7, 8].

Chemicals or mutagens can act as promoters. They don't attach directly to DNA or proteins, instead they generally attach to surface receptors that control intracellular signaling pathways. They can block contact inhibition signals or stop natural killer cells to recognize mutated cells. Reactive oxygen species can be classified as cancer promoters [9].

The third phase is called the progression phase. Progression is the step-by-step change of a benign tumor to a neoplasm then to malignancy. During this step, cells create chromosomal changes. It is known that most of the malignant tumors have altered number of chromosomes. Chromosomal abnormalities give rise to increased growth rate, invasiveness, metastasis and they change the characteristics and biochemical reactions of a cell. Additionally in this step cells activate oncogenes and deactivate tumor suppression genes [10].

1.1.2. Oncogenes and Tumor Suppressor Genes

Oncogenes and tumor suppressor genes are the two types of genes that act as a fate changer in cancer and tumor development.

1.1.2.1. Oncogenes

Without mutations, oncogenes are called proto-oncogenes. Proto-oncogenes create transcription factors and proteins that normally help cells to grow and control its important metabolic pathways. When so called a proto-oncogene, is mutated, it becomes a gene that creates proteins and transcription factors that are able to transform cells into cancer cells.

In most of the cases, a gain of function mutation caused; either by point mutations (changes the activity of the protein product), localized reduplication (overexpression of the protein product) or chromosomal translocation (change of promoter resulting an unfitting amount of gene expression) creates the harmful effects of an oncogene [11].

In normal conditions, proto-oncogenes can give rise to receptors such as; epidermal growth factor receptor (EGFR), kinase insert domain receptor (KDR), vascular endothelial growth factor receptor (VEGF). Proto-oncogenes can also encode intracellular proteins such as GTPase HRas (transforming protein p21) and Kras (V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) which normally act as downstream mediators of cell surface receptor pathways. Proteins used in cell cycle control such as cyclin D1 (CCND1) and cyclin E1 (CCNE1) are also proto-oncogenes [12].

Chromosomal translocations can also give rise to oncogenes. One example is Philadelphia chromosome found by Peter Nowell and David Hungerford. In this case BCR gene is fused with ABL1 gene creating BCR-ABL. When the fused gene is expressed, it encodes a protein having an increased protein tyrosine kinase activity [13].

1.1.2.2. Tumor Suppressor Genes:

Tumor suppressor genes normally protect cells from being a cancer cell. First identified tumor suppressor gene is retinoblastoma (Rb) gene. In normal condition, protein synthesized by retinoblastoma gene (pRB) controls excessive cell growth. It pauses cell cycle until the cell is completely ready. This gene is first identified in a rare childhood eye tumor. Extensive studies on this gene identified that when this it is lost or inactivated it causes many different cancer types [14].

The second identified tumor suppression gene is p53. It can activate DNA repair proteins, arrest growth by controlling cell cycle, initiate apoptosis, inhibit angiogenesis and control the expressions of microRNAs and transcription factors [15]. In 50% of cancer cases, p53 is found mutated.

Mutations in different tumor suppressor genes are found to cause different cancer types. For example BRCA1, BRCA2 is found mutated in breast cancers, mutated INK4 is found in melanomas, carcinomas and brain tumors, PTEN is found inactivated in brain tumors and carcinomas, APC is found mutated in colon/rectum carcinomas. There are many other tumor suppressor genes normally acts as guardian angels to cells but when silenced or have loss-of-function mutations they cause a cell to lose control [16].

1.1.3. Cancer Energy Metabolism

Due to the unlimited proliferation capacity, cancer cells need large amounts of energy and bio-macromolecules to supply newly synthesized DNA and cellular components. In order to fulfill these needs, cancer cells are found to alter their energy metabolism compared to normal healthy cells [17].

In 1956, Warburg et al. have suggested that, in order to survive in low oxygen levels and synthesize higher amounts of ATP, cancer cells improve the glycolytic activity and reduce oxidative phosphorylation. This phenomenon is later accepted as the Warburg Effect [18] and counted as one of the hallmarks of cancer [1].

Cancer cells has increased amount of glucose intake and this also results in generation of more intermediate glycolytic metabolites and ATP. In normal conditions, glycolysis is a less efficient mechanism for ATP generation but in the case of cancer, it is faster than oxidative phosphorylation and thus generating ATP faster to help the proliferation of cancer cells. Additionally fast glycolysis favor the increasing synthesis of lipids, nucleotides, NADPH and amino acids that are necessary for the growth of cancer cells [19].

Even though, primarily the main reason for cancer cells prefer glycolysis over oxidative phosphorylation was thought to survive in hypoxic conditions, recent studies revealed that preferring glycolysis also activates various pathways that helps cancer cells to survive. Activated pathways due to the glycolysis also support synthesis of metabolic requirements

for cellular proliferation. Anaerobic respiration is found to influence PI3K/AKT pathway. Activated PI3K by glycolysis can phosphorylate and activate AKT and mTOR which then triggers the activation of HIF1α, hypoxia induced factor 1 alpha. In this way, HIF1α, which normally ensures cell survival and proliferation in low oxygen, gets activated in even in high oxygen levels [20]. Apart from influencing HIF1α, the main target of PI3K is glucose transporters. Activated AKT stimulate the expression of glucose transporters and increase glucose influx to the cells [21].

Additionally, after the Warburg Effect is accepted, targeting glycolysis in cancer cells has become one of the main possible route in the combat with cancer [22-24].

To sum up, changes in the energy metabolism in cancer cells may contribute many different pathways that helps the cell to support the needs of fast proliferation, activates hypoxic survival pathways and promotes EMT thus metastasis.

1.1.4. Stem Cell Hypothesis and Properties of Cancer Stem Cell

Cancer stem cells are defined as minor segment of cells among cancer cells that have unlimited self-renewal potential and differentiation ability. These cells are also have the function of initiating the tumor and supplying drug resistant, highly metastatic and invulnerable cancer cells. Targeting cancer stem cells are important for cancer therapy since they generate the durable part of the tumor [25].

Cancer stem cells were first described by Dick et. al, in myeloid leukemia and afterwards cancer stem cells have studied broadly for every other cancer types [26]. This hypothesis suggests that cancer tumor is comprised of varied cell types. Chemotherapy can treat normal cancer cells without eliminating resistant cancer stem cells that have very active drug efflux pathways controlled by ABC-binding cassette family [27].

Occurrence of cancer stem cells have been studied in pancreas cancers extensively [28]. Pancreatic adenocarcinoma is a notorious cancer type due to its high metastatic ability. CD133+ and CXCR4 positive stem cells in pancreatic cancer tissue is found to be highly tumorigenic, drug resistant and metastatic. Additional studies defined pancreas cancer stem cells as high c-met expressed [29], CD44+, CD24+, ESA+ [28], EpCAM+ [30].

Still searching for a surface biomarker, Bailey et al has found a microtubule regulator family member DCLK1 has found positive in cells isolated from mouse PanIN model. These cells have shown an increased potential of tumorigenesis and drug resistance and can be categorized as a pancreas stem cell marker [31].

Normally identified as a stem cell marker, Nestin has found positive in 30 per cent of pancreatic adenocarcinoma (PDAC) tumors. In the study by Su et al. it is found that overexpression of Nestin increases cell motility and it is defined as a cancer stem cell marker [32]. Other studies has shown that, ALDH-1 (aldehyde dehydrogenase) and ABCG-2 cells can be counted as cancer stem cell markers in pancreatic adenocarcinoma [33-35].

1.1.4.1. Relationship between Cancer Stemness and EMT

Although still not completely identified, surface markers associated with cancer stem cells are linked to the pathways that control epithelial to mesenchymal transition. Additionally epithelial to mesenchymal transition is highly active in stem cells during developmental phase and it is highly possible for the same pathways to get activated in cancer stem cells due to EMT.

TGFβ, one of the main controller of EMT, has found to be linked to create tumor-initiating stem cells in breast and liver cancer [36, 37].

Another pathway responsible to control EMT is Wnt/β-catenin signaling pathway. Activators of Wnt signaling is found to cause high aggressiveness and increased stem cell features [38, 39]. A study by DiMeo et al has shown that Wnt signaling incubation reduced the self-renewal capacity of cancer cells by downregulating EMT transcription factor Slug and Twist [40] and another studies has shown that sustained β-catenin activation created tendency to increased tumorigenesis and stem cell proliferation. All of these studies suggest that inhibition of Wnt/β-catenin pathway can decrease EMT linked stem-cell like characteristic in cancer.

Sonic-Hedgehog signaling is an important regulator of EMT. Downregulation of Hedgehod signaling is found to inhibit CSCs and EMT due to lowering SNAIL transcription factor and increasing E-cadherin in pancreas cancer [41, 42].

1.2. PANCREAS

1.2.1. History

The pancreas, once referred as the “finger of the liver” in the “Talmud” written around 200BC and 200AD, has received the critical awareness of anatomists, physicians and surgeons later than many of the other organs. It was once thought to support and protect the stomach like a cushion until pancreatic ducts of humans are found in 1642. It took approximately 200 years for researchers to understand the digestive actions of pancreatic secretions. Digestion of starch, emulsification of fats and proteolytic activity of the pancreatic secretions are demonstrated in 1800’s. Kuhne, who introduced the term “enzyme”, has isolated trypsin in 1876 and later on discovery of pancreatic amylase, lipase and enterokinase make the researchers of the era interested in pancreas.

First histological study of pancreas was done in 1896 by Langerhans and first systematic description of a pancreatic disease was written in 1875 by Friedreich [43].

1.2.2. Anatomy

In adults, pancreas weighs between 70 to 110 grams and its length can be 12 to 20 cm. It’s a soft, extended and flattened gland. The head lies at the rear of the peritoneum of the posterior abdominal wall and has a lobular structure. It is covered with a thin connective tissue but not in a capsule. Its head is on the right side. The neck, body and tail of pancreas lies obliquely in the posterior abdomen and its tail extends as far as the gastric surface of the spleen [44]. Since it lies in abdominal cavity it is grouped as a retroperitoneal organ. Common bile duct’s distal end passes through pancreatic head and together with pancreatic duct, they enter duodenum.

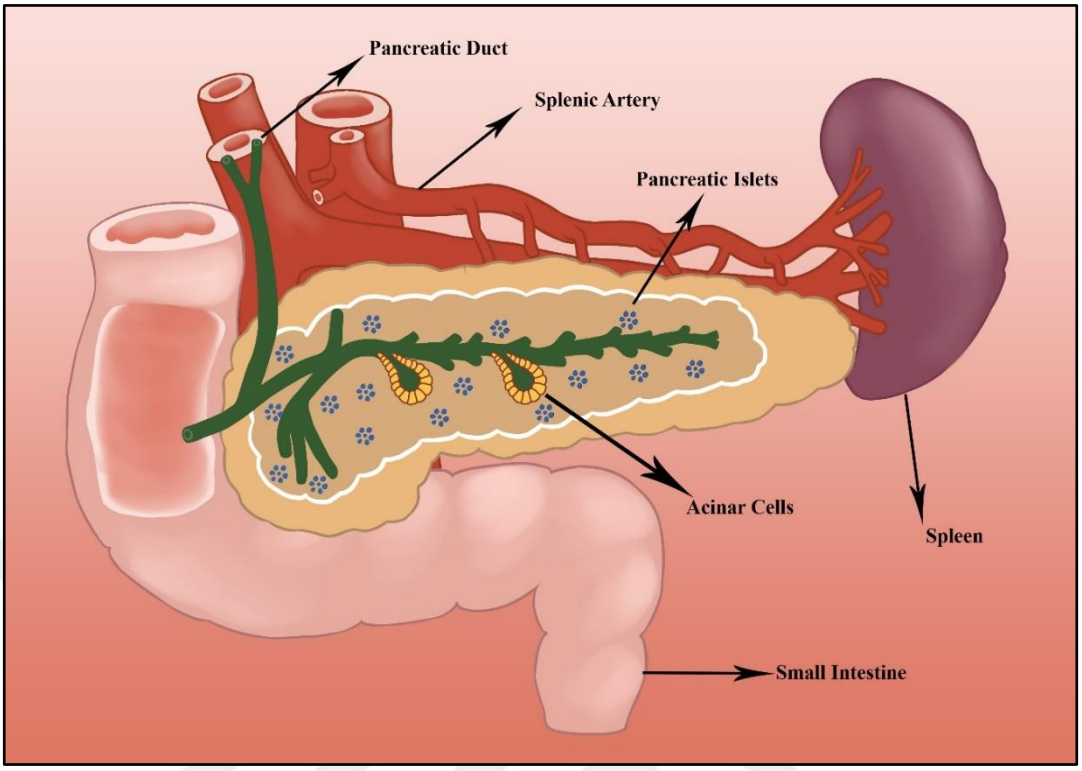


Figure 1.2 Schematic representation of pancreas anatomy, interior and surrounding organs

Its anatomical position generally protects pancreas from physical trauma. Still a severe blunt trauma to abdomen's upper parts by a car crush, such as a hit by the steering wheel, can cause an acute injury.

As shown in Figure 1.2, supply of blood to pancreas comes from two major arteries, the celiac and superior mesenteric arteries. Due to double blood supply, ischemia caused from vascular blockage is unusual. Pancreas is surrounded by both parasympathic and sympathic nervous systems. Nervous systems connected to pancreas controls both exocrine and endocrine structures [45].

1.2.3. Histology

Pancreas histologically looks like salivary glands, but less compact, and it is a compound and finely nodular gland. The lobules are visible on gross examination and they are connected by connective tissue which contains blood vessels, nerves, lymphatics and excretory ducts. It is a both exocrine (80 per cent) and endocrine (two per cent) organ. Islets of Langerhans, which are spherical clusters dispersed around the organ, create the endocrine

portion and the cells there are light stained. In contrast, exocrine portion is composed of dark-staining cells, tubular and spherical masses [46].

1.3. PANCREAS CANCER

Pancreas cancer is a sickness that malignant cells are found in the tissues of the pancreas. Pancreatic cancer often has poor prognosis and different from other types of cancer, even early diagnosis is not useful. Pancreatic cancer is known to spread fast and infrequently observed in early stages. It is reported to occur more in men than women. It also occurs more in blacks than woman and generally observed in urban areas rather than rural populations.

Until the quite advanced stages, signs and symptoms (which are normally seen in a simple flu like loss of appetite, losing weight, and depression) may not appear and since the complete removal is impossible, patient cannot survive.

Although initiation of pancreas cancer can have genetic factors, analytical studies shows that cigarette smoking can have an increasing effect on pancreas cancer. Also coffee consumption, chemicals exposed in working areas, diabetes, and pancreatitis can increase pancreas cancer risk and progression [47].

1.3.1. History

The earliest mention of pancreatic cancer was made by Giovanni Battista Morgagni (1670's), who is memorized as the histological father of modern-day anatomic pathology, after he has observed five cases of cancer in pancreas. During this period, pathologists have called a hardened mass within the pancreas as the pancreatic cancer, but it is also confused with the appearance of chronic pancreatitis.

Doctors in the 18^{th}-19^{th} centuries still had doubts about pancreas cancer. In 1830's cases of pancreas cancer started to be announced. First American writer on the subject is J. M. Da Costa. He has dismissed Morgagni's cancer reports but alternatively, he found records of 35 autopsies of patients who have diagnosed to have pancreatic cancer and he also added one case of his own. In at least one of his cases, it was possible to find microscopic diagnosis of adenocarcinoma.

At the later years of 19th century, Bard and Pit differentiated between duct and acinar cell cancers, noticed the likelihood of islet cell cancer. In 1898, Codivilla, an Italian surgeon, has performed the first partial pancreaticoduodenectomy [43].

In 1912, first removal of large parts of duodenum and pancreas together was made accomplished by the German surgeon Walther Kanusch. In 1918, an operation conducted on dogs has proved that it is possible for an animal to live after its whole duodenum is removed. In 1953, a series of operations made by Allen Oldfather Whipple in New York. And during these operations one of the patients' duodenum was totally removed and he lived two more years before dying of metastasis to the liver [48].

Development of blood transfusion and the discovery that vitamin K prevents bleeding with jaundice have improved operations and post-operative survival.

Remedy of pancreas cancer is still a predicament and only 10 per cent of sufferer with carcinoma are able to have the tumor removed. Death rate has declined from 21 per cent to zero per cent but survival rate for 5 years are still not changed [43].

1.3.2. Pancreas Cancer Types

There are two types of cells in pancreas; exocrine cells and endocrine cells. More than 95 per cent of pancreatic cancers are derived from exocrine cells and called exocrine tumors. Exocrine cells of pancreas secrete enzymes that help in digestion. Most commonly seen exocrine tumor is pancreatic ductal adenocarcinoma [48].

1.3.2.1. Exocrine Tumors

Pancreatic Adenocarcinoma is the most frequently seen pancreatic cancer type is pancreatic ductal adenocarcinomas. It is the forth-leading cause of cancer death in western world. Its prognosis is particularly poor and five-year survival rate is only four per cent.

This type of cancer occurs when cells within the digestive juice carrying ducts have mutations. Ductal adenocarcinomas can be found anywhere in the pancreas but most commonly they are found in the head of pancreas [49].

Adenocarcinoma proceeds with defined histopathological stages (PanIN, grade I-II-III) and disease progression occurs together with genetic modifications. Approximately all tumors have an oncogenic mutation in K-RAS gene. This mutation is considered as the initiator of pathogenesis step.

In advanced stages, loss of tumor suppression gene, INK4A, p53 mutations and loss of SMAD4 are observed.

It is important to note that, two pathways that control EMT, RAS and TGFβ signaling are disturbed in pancreatic cancer [50].

Other rare exocrine cancers are; acinar cell carcinoma, intraductal papillary mucinous neoplasm with invasive carcinoma, mucinous cystic neoplasm with invasive carcinoma, pancreablastoma, Serous cystadenocarcinoma, solid pseudopapillary neoplasm [49].

1.3.2.2. Neuroendocrine Tumors (NETs)

Endocrine cells (islets of Langerhans cells), produce hormones such as insulin, glucagon, somatostatin. When these cells grow abnormally, they produce neuroendocrine tumors. These type of tumors start occur in the hormone producing cells of the pancreas. NETs are observed in less than five per cent of all pancreatic tumors and they are considered as rare. These types of tumors are called as neuroendocrine tumors (NETs) or islet tumors. NETs can be related to genetic conditions and can be malignant or benign.

These types of tumors usually grow much more slowly than exocrine tumors. There are two types NETs; functioning or non-functioning.

Functioning tumors overproduce hormones. Excessive amounts of hormones cause clinical syndromes, depending on which hormone is being overproduced. Some types of functioning endocrine tumors are; gastrinoma, glucagonoma, insulinoma, somatostatinoma, VIPoma.

Non-functioning endocrine tumors are harder to detect and diagnose. Usually recognized while searching for another sickness. They are usually found in the head of the pancreas [51].

1.3.3. Metastatic Properties of Pancreatic Carcinoma

Symptoms of pancreatic cancer are similar to other generally occurring diseases. Also with respect to the position of the organ it is hard to take a biopsy sample and investigate the situation. Because of these aspects, when pancreatic cancer is recognized, most probably it has already spread to the blood stream and this spread may be the reason for observation of symptoms.

The spread of pancreatic cancer to other organs start with spreads to nearby lymph nodes. After lymph nodes, cancer spreads to liver or to peritoneal cavity. Another possible routes are spread to large intestine or lungs [52]. It is not common but also not impossible to observe metastatic secondary tumors around bones and brain [53, 54]

Pancreas cancer can also be a secondary cancer that have spread from other parts of body (kidney, skin, breast, lung etc.) but this is found in only about two per cent of pancreatic cancers.

1.3.4. Diagnosis

Clinical presentation and symptoms can vary with tumor location and disease stage. When tumor develops in the head of the pancreas, they usually cause obstructive jaundice and weight loss associated with diarrhea. Tumors of head and tail, usually cause abdominal pain and weight loss. Pain is frequent (up to 80-85 per cent) in pancreatic cancers [55].

In rare conditions, pancreatic tumors can cause duodenal obstruction or gastrointestinal bleeding. In 80 per cent of pancreatic cancer patients, it is possible to observe hyperglycemia or diabetes. Other possible symptoms are anorexia, asthenia, thrombosis, liver-function abnormalities [56].

Diagnosis type changes with the symptoms. For patients with obstructive jaundice or epigastric pain and weight loss, it is possible to use trans-abdominal ultrasound for initial imaging. However in this case, there is a possibility to miss tumors smaller than 3 cm. If ultrasound is negative but still there is a high clinical suspicion for pancreatic cancer, it is often suggested, to use a triple phase, helical multi detector row computed tomography scan with IV contrast material. If both ultrasound and CT are negative, it is possible to use

endoscopic ultrasonography. Also with endoscopic ultrasound biopsy it is possible to take tissue samples for histology [55].

After the biopsy sample is taken from the pancreas, it is possible to use markers in order to see the tumor formation. CA 19-9 is a monoclonal tumor marker, useful for detecting pancreatic cancer. It is not specific (73 per cent specificity) to only pancreatic cancer and normally present in the cells of biliary tract and other GI cancers. It is not recommended for screening but it can be used in differentiating chronic pancreatitis from pancreatic cancer, detecting metastasis [57].

After all it is so hard to recognize pancreatic cancer until it becomes too big to operate or start metastasizing.

1.3.5. Treatment

When pancreatic cancer is identified early, it is possible to try some therapeutic strategies. There are also some treatments that can help while controlling the disease during later stages in order to increase the survival times of patients.

1.3.5.1. Surgery

Surgery for pancreatic cancer is removing pancreas completely or partially. This removal depends on the place of the tumor and the size of the tumor. Only 20 per cent of pancreatic cancer patients are able to have surgery because generally patients are diagnosed when the disease has already spread [58].

When a patient is lucky enough to have surgery, the operation must be combined with radiation therapy or/and chemotherapy. There are different types of surgery;

Laparoscopy: In laparoscopy, surgeon opens several small holes in the abdomen and with micro cameras he finds the place of tumor and searches for metastasis to the other parts of abdomen [58].

Surgery: A surgeon can choose from different surgery methods explained below:

If cancer is not spread and it is found around the head of the pancreas, it is possible for the surgeon to do a Whipple procedure which includes the removal of the head of pancreas and part of small intestine, stomach, bile duct. Surgeon then can reconnect the digestive tract and biliary system. This method is chosen by experienced surgeons.

If it is located around the tail of the pancreas, it is possible for the surgeon to make a distal pancreactectomy. In this procedure, the surgeon removes the tail and body of the pancreas together with the spleen.

If the tumor is spread, the last possible surgery type is total pancreactectomy. In this procedure, surgeon removes pancreas completely together with a part of small intestine, a part of stomach, common bile duct, gallbladder, spleen and the lymph nodes nearby.

After all of these operation types, patients will need the take pancreatic enzymes (e.g. insulin, amylase, lipase, and protease) extrinsically [58].

1.3.5.2. Radiation Therapy

Radiation therapy is using high energy x-rays in order to disrupt cancer cells. Radiation oncologist can use radiation therapy in the situations below:

After the surgery, if there is a high chance of the cancer to turn back (e.g. after a large tumor removal), when it is possible to shrink the borderline of the tumor before surgery, when the tumor is unresectable, when the patient has severe pain because of the metastatic cancer [59].

1.3.5.3. Chemotherapy

Chemotherapy can be used at any stages of pancreatic cancer. It can be used before surgery together with radiation, in order to shrink the tumor (asneoadjuvant treatment). It can be used after surgery for killing remaining cancer cells (adjuvant treatment). It is also used when it is possible to remove the tumor with surgery. When it is given with radiation it is called chemo radiation therapy. In this case it will have severe side effects but it will work better [60].

Some chemotherapeutic drugs used for exocrine pancreatic cancer types are; gemcitabine, 5-fluorouracil, Irinotecan, oxaliplatin, albumin-bound paxlitaxel, capecitabine, cisplatin, paclitaxel and docetaxel. When the patient is healthy enough, drugs can be given in combinations.

There are some targeted therapy options for exocrine pancreatic cancers. Erlotinib targets EGF receptors on the surface of cancer cells. It is known that, EGF promotes cancer cell's growth. Erlotinib can be used in combination with other drugs.

For the treatment of neuroendocrine tumors of pancreas, most commonly used chemotherapy drugs are; doxorubicin (or liposomal doxorubicin), streotozocin, fluorouracil, dacarbazine, temozolomide, thalidomide and capecitabine [61].

There are also targeted therapy options for neuroendocrine tumors. Sunitinib is one of the chemicals used as targeted therapeutic. It inhibits multiple receptor tyrosine kinases which include; platelet-derived growth factor receptors and vascular endothelial growth receptors. By this way this chemical inhibits both angiogenesis and cell proliferation [62].

Other chemical used as a targeted therapeutic is Everolimus. It is an immunosuppressant which is used after organ transplant in order to prevent rejections. It is an inhibitor of mTOR protein which has roles in regulation of cell growth, proliferation, motility, transcription and protein synthesis [63].

Even if the tumor is removed or different therapy options are tried in the patients, it is nearly impossible to rescue the patient or increase the survival rate of the patient in 5 years.

Because of these reasons, pancreatic cancer is chosen as a target of the genetic therapy in this study.

1.4. METASTASIS

1.4.1. Definition of Metastasis

Metastasis is defined as the spread of cancer cells from the primary site and colonize on another part of the body. This complex procedure, is the cause of the majority of cancer mortalities [64]. Considering the inefficiency of the process, from a tumor mass consists of

approximately 10^9 cells only a million cells can penetrate through the circulatory system and most of them quickly die [65], metastasis can be defined as a highly dangerous series of biochemical events.

The process of metastasis occur when a tumor cell spread from the initial tumor, penetrate the basement membrane, travel in vascular system, locate into a new site and proliferate. It can only be completed when multiplex molecular networks work together to endorse; cancer-cell invasion in primary site or invasion of a single-cell, generation of a suitable microenvironment around the primary site, generation of blood vessels, intravasation, migration through lymph system, extravasation and metastatic outgrowth [66].

There are two suggested models of cancer progression; linear and parallel progression model. Linear progression model suggests that, metastasis takes place only after the initial tumor reaches a certain size. On the contrary, new studies, involving the tracking of tumor cells, propose that metastasis to distant organs can happen in early stages of cancer, defined as parallel progression model.

Progression type can differ with cancer type. For instance in cases of pancreas and breast cancer, metastasis is commonly assist the early staged tumor. Also cells that survive in circulation can arrive distant organs (micrometastasis) and remain undetected for several years since they are kept in balance between apoptosis and proliferation even the primary epithelial tumor disappears [67, 68].

In order to understand metastasis completely, steps of the total concept must be explained one by one.

These steps include; benign growth of the tumor, becoming invasive, intravasation (entering into the blood vessel), survival in the blood stream, attachment to the inner wall of the vein in final destination, extravasation (escaping from the blood vessel) and micro metastasis, colonization in the secondary organ.

1.4.2. Steps and Genetics of Metastasis

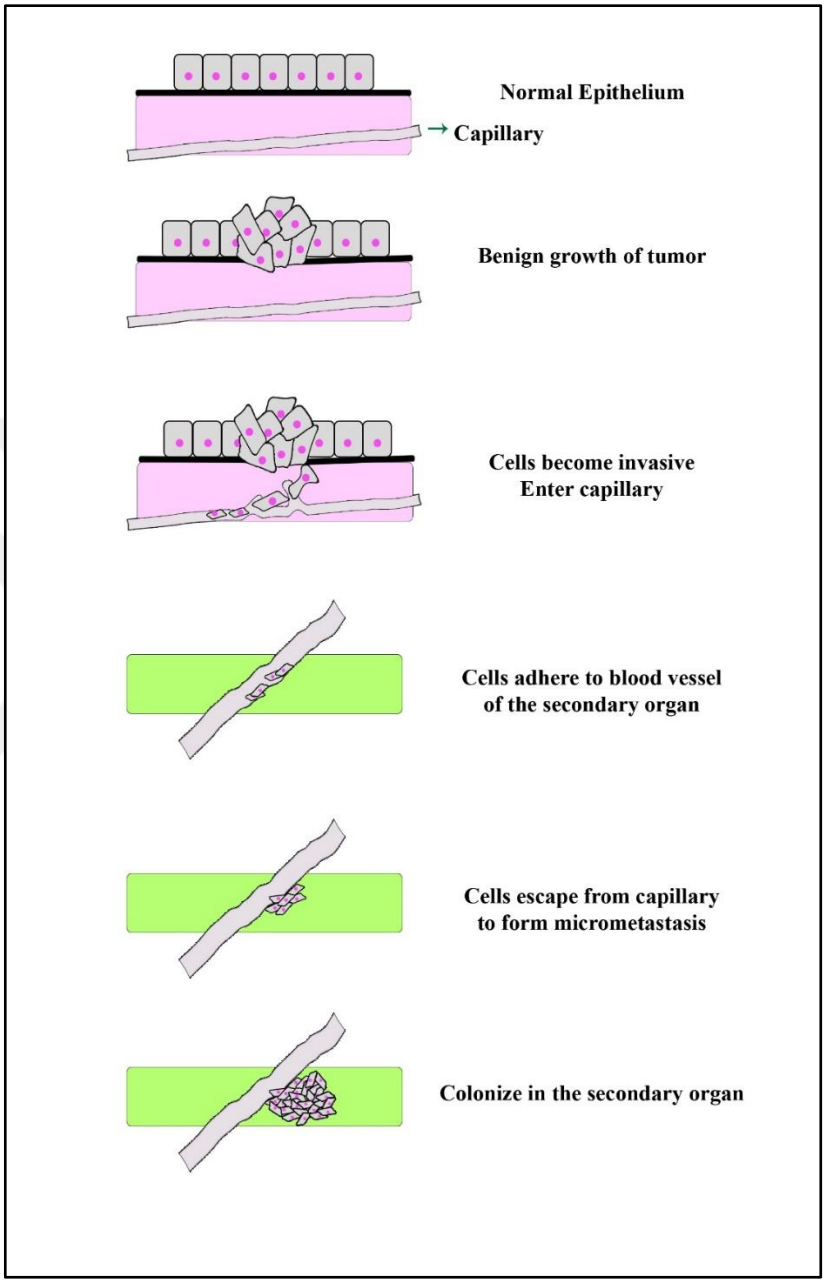


Figure 1.3 Schematic representation of metastatic progression

As illusturated in the Figure 1.3, a cancer cell must escape from the primary tumor site, enter into the capillary. If it can manage to survive the huge shear stress of the blood circulation, it needs to escape the capillary. After the escape it must adapt and colonize at the secondary tumor site.

1.4.2.1. Initial Steps of Metastasis, Local Invasion

When a cell is mutated enough to fulfill the hallmarks of cancer, the next natural step is losing cellular polarity and disconnecting from the basement membrane.

In order to detach from the basal membrane cancer cells change the condition of their surrounding niche by releasing matrix metalloproteinases such as (MMP-1, MMP-2, MMP-3 and MMP-13, MMP-28) and transmembrane proteases including TMPRSS4. These enzymes are used to breakdown the extracellular matrix proteins (laminin, collagen, fibronectin and other basement membrane glycoproteins) allowing cell separate from the initial tumor and migrate away [69, 70]. Lysis of ECM proteins frees the connection of cancer cell to the basal membrane. Destruction of ECM proteins separates the transmembrane proteins, including integrins and cadherins on the cellular membrane, from the basal membrane, allowing the cancer to move freely, enabling free space for angiogenesis and lymphogenesis [71]. Additionally the changes due to the activity of metalloproteinases and the lost connection between the cancer cell and basal membrane, activates and deactivates various pathways controlling proliferation, migration, cell cycle and tumorigenesis [72].

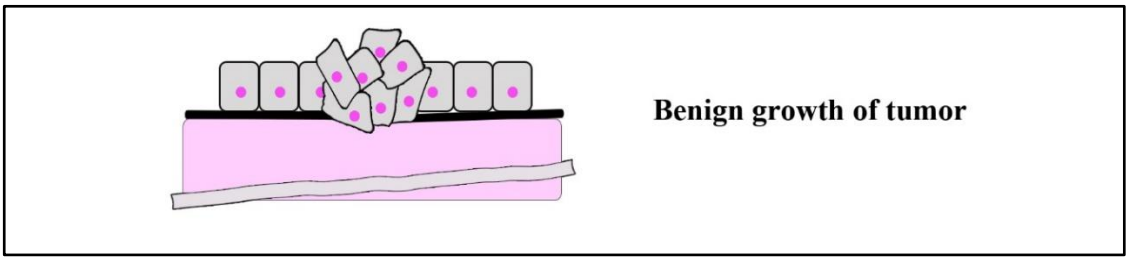


Figure 1.4 Schematic representation of benign growth of the primary tumor

The changes in microenvironment around the cell can trigger the cancer cell to search a way to prepare itself for invasion. The increase of growth factors such as transforming growth factor-β (TGF- β), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), epidermal growth factor (EGF) and insulin-like growth factor (IGF) can start EMT [73-77]. These factors can also trigger mesenchymal differentiation as well as controlling c-met signaling and tumor stroma interactions. Together with changes in ECM and MMP levels, cytokines and growth factors can trigger invasion.

When the conditions gets suitable, there are numerous strategies that a cell can try and interconvert due to the situation arises from its niche in order to start invasion. In the case of epithelial tissue organization, normal organ tissue assembly, certainly does not suit single cell invasion pathways. The most important key factor for keeping a cell in its usual place is E-cadherin. E-cadherin is a calcium-dependent transmembrane protein found in adherens junctions that mediates cell to cell connection [78, 79]. While playing essential roles during gastrulation, neurulation and organogenesis, e-cadherin also controls epithelial to mesenchymal transition (EMT), one of the most important pathways that control metastasis [80].

EMT is controlled by a group of transcription factors such as Snail, Slug, Twist, Zinc-Finger E-Box Binding Homeobox one and two. These transcription factors control the expression levels of markers coordinating mesenchymal and epithelial state of the cell [76, 81]. The process of EMT will be described in EMT section in detail.

Additionally cancer cell can choose the collective amoeboid transition (CAT) or mesenchymal to ameboid transition (MAT) instead of EMT. EMT is considered as an inflexible process, used to maintain mesenchymal characteristic by changing gene transcription. But in the case of MAT or its opposite AMT, changes within the cell happen fast and with controlled by instantaneous changes in cellular environment. [82].

CAT is a type of collective invasion and in this case individual cells gets disconnected from cellular clusters, by using amoeboid type of migration. During CAT, amoeboid cells decrease cellular attachment to ECM and pass through small gaps within ECM and MMP activation is not necessary [83]. This type of invasion is mainly chosen by melanoma [84].

MAT is the conversion of mesenchymal cells to amoeboid cells. It is observed in the cases of breast cancer [85], fibrosarcoma [86], and in melanoma [87, 88]. It is highly dependent on Rac and Rho/ROCK pathways and it requires additional help from proteases such as MMPs, serine proteases and cathepsins [89-91]. MAT can be controlled and initiated by the changes in tumor microenvironment or by regulatory kinases such as EphA2 Kinase [87]. Additionally mutations and inhibition of tumor suppressor proteins p53 and p27kip1 is found to promote MAT in fibroblasts and in melanoma [88, 92].

Even if the cells invade in groups or as individuals, the cells need to make morphological changes or changes in their environment in order to initiate metastasis.

1.4.2.2. Intravasation

By definition, intravasation is the entry of a foreign substance or a tumor cell into the bloodstream by passing through the wall of blood vessels. Intravasation is essential for a cancer cell that is committed to distant metastasis. Lymphatic metastasis is also an additional way out in order to reach circulation [93].

Intravasation through a blood vessel can either be active or passive. The choice of active/passive intravasation changes by the tumor type, blood vessel's conditions around the tumor and the conditions of the tumor microenvironment. Cells need to change themselves and their environment at first. Successful intravasation is controlled by various elements. This long list includes; tumor microenvironment [94], proteases [95], and proteinases (especially MMPs) [96], macrophages [97], signaling molecules (prominently TGF-β) [98, 99] and environmental conditions [100]. Each factor is crucial for its distinctive help to the metastatic tumor cell to; invade through the basal membrane, attach to the endothelial cell junctions and pass through them.

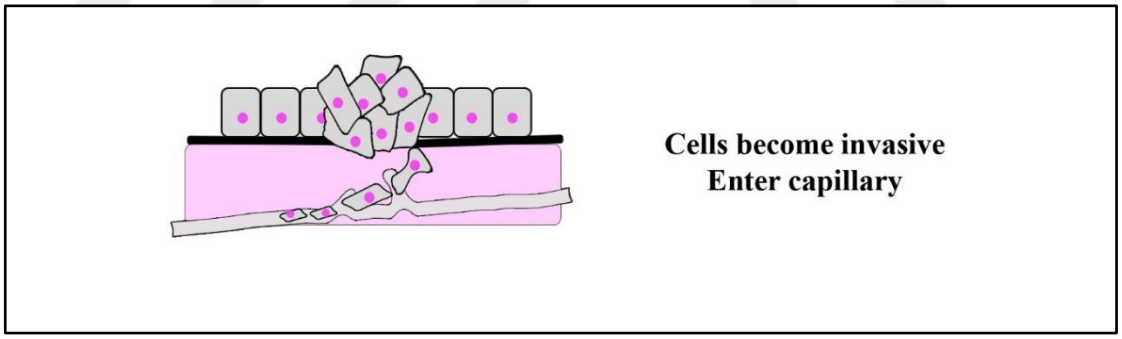


Figure 1.5 Schematic representation of intravasation of primary tumor cells and early metastasis

In order to materially penetrate through the blood vessels, degradation by proteases and proteinases is necessary. As mentioned in the previous section, metastatic cancer cells are known to increase their protease expression. Interestingly with the recent researches it is now known that majority of proteases and matrix metalloproteinases, cathepsins and serine proteases are originated from the surrounding stromal cells [101-104].

Additionally to enter into a blood vessel, the tumor cell must also be able to contact with the vessels. Tumor make it possible by angiogenesis. Angiogenesis, one of the hallmarks of cancer, is cell's method to create their own nutrition supply and an exit to the main circulation vessels [105]. Angiogenesis is controlled by secreted growth factors by the tumor. When a cell has decreased amount of oxygen, hypoxia induced factor 1-alpha (HIF1α) is expressed by the tumor cell. Increased HIF1α triggers many different pathways that secrete growth factors for vascular endothelial growth (VEGF) [106], basic fibroblast growth factor [107-109].

Overall angiogenesis makes it possible for the tumor cells to contact with blood vessels and additional help from MMPs, EMT or amoeboid transformation makes intravasation possible.

1.4.2.3. Survival in the Blood Stream

After a cell successfully enter into the blood vessel, in order to stay alive it must survive blood's shear stress, turbulence, attacks of immune system, lack of nutrition and pass through microvessels and small capillaries [110].

When a tumor cell enters into bloodstream, it needs to interact cells and components within the blood vessels. Some of these elements act against the cancer cell while the others may help and shield the cell through the hard conditions of stream [94].

The most important contribution to cancer cell survival is done by platelets. The interaction of cancer with platelets for example is first identified by Trousseau in nineteenth century [111]. Later researches on the topic identified that tumor cells are able to trigger platelets, forming clots and survive better in the blood vessels [112, 113].

Additionally with the studies among the cancer patients, it is now well known that, tumor growth is directly proportional with the elevated blood clotting, abnormalities in platelets and tendency to have thromboembolic anomalies [114].

During their normal lifespan, platelets are used in coagulation and hemostasis. They are tiny, anuclear cells synthesized by the bone marrow. They have short lifespan, only a week, and in a healthy individual it is possible to find 400 billion of them in a one liter of blood. They are used to stop the outflow after a tissue cut or injury in a blood vessel [115, 116].

In order to physically and chemically protect itself from the harsh environment and immune system, the tumor cell covers itself with platelets creating an embolus aggregate. Interaction with platelets are maintained by two types of mediators; membrane bound adhesion molecules (predominantly fibrin), coagulation factors and released chemicals. Cell surface receptors that are found in both platelets and tumor cells such as αIIbβ3 integrin receptors and released chemicals like 12(S)-HETE can be given as an example to both of the mediators given above [117-120].

With the attachment to platelets, platelets get activated start to help tumor cells to stay alive in the vessels, increase the tumor cell proliferation, help to arrest in the final destination also help extravasation and increase the tumor-ECM interaction [121].

1.4.2.4. Arrest of the Tumor Cell and Extravasation

In order to escape from the blood vessel, the circulating tumor cell must first arrest inside the micro vessel of the chosen organ. The cell must contact with and attach to the capillary endothelium. The first proof of the cancer cell arrest was demonstrated by Wood et al. [122]. Later Jones et al. used microcinematography methods and observed that, when rabbit V2 carcinoma model was injected to a rat model, a single cancer cell covered with six platelet aggregate can arrest in the final destination, just after two minutes [123]. Additional studies also proven the arrest theory [124].

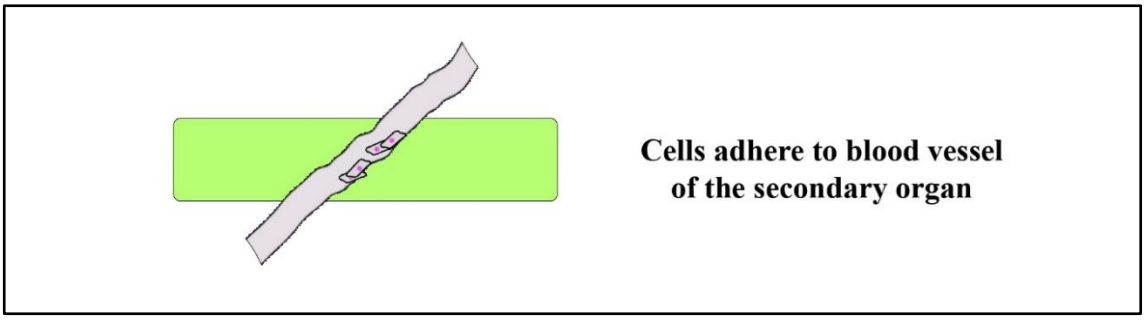


Figure 1.6 Schematic representation of the attachment onto the interior wall of the blood vessel

Platelets that has protected the tumor from the harsh bloodstream environment also help the cancer cell to arrest and contribute extravasion. They provide a survival signal which then

allows the increase of CC-chemokine ligand two (CCL2) activating CCR2 receptor containing inflammatory monocytes (IMs) to differentiated/transformed to metastasis associated macrophages (MAMs) [125, 126]. MAMs are known to promote extravasation, macrophage assisted metastasis, and help cancer cell to survive while in contact with endothelial cells [127].

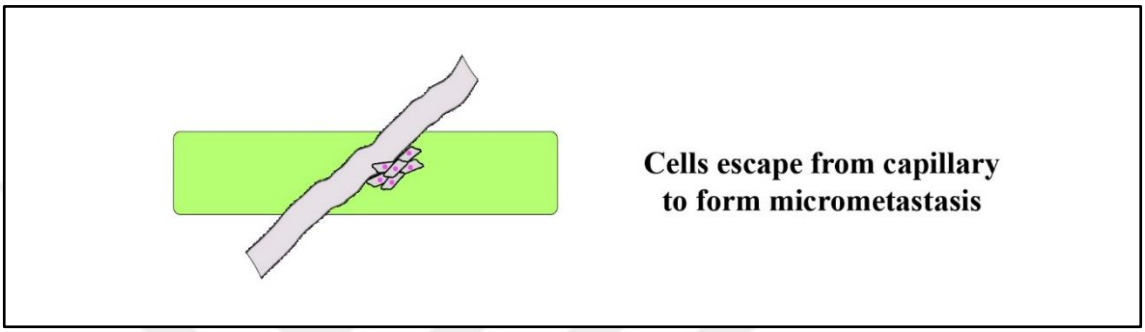


Figure 1.7 Schematic representation of extravasation and escaping of the cell through the secondary organ

Additionally, it is found that, platelets released factors such as S1P and IPA acting on cytoskeletal rearrangement, cell-cell communication and changes in actin structure in order to control endothelial permeability. These changes in the endothelial layer of the vessels could also control extravasation [128, 129].

Because of the limitations of imaging techniques on humans, animal models are frequently used to identify the dynamics of extravasation. A study by Leong et al. used the mouse model to observe extravasation. This study found that, cancer cells uses a special structure called invadopodium. They first pass through the endothelial layer by their invadopodium and then pull the rest of the cell to extravascular stroma [130].

VEGF is another factor that contributes extravasation. Studies done in animal models shows that cells lack of expressing VEGF or VEGF-mediated Src kinase activity, in normal conditions leads to a breakdown in endothelial barrier, showed reduced extravasation [131, 132].

1.4.2.5. Survival in the Secondary Site

The last step of metastasis is the survival in the secondary site. When cancer cell successfully get out of the blood vessel it needs to attach and adapt to the final organ's conditions. It is also important to state that, when a cancer cell metastasize (for example a pancreas cancer cell) to another organ (to liver), it is still a pancreas cell. If the cell cannot manage to adapt the environment it may get eliminated.

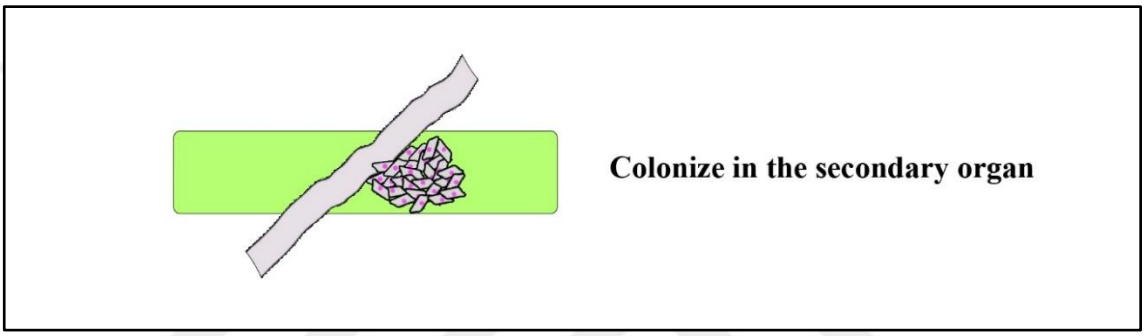


Figure 1.8 Schematic representation of the colonization in the secondary organ and adaptation

It is found that some cells prefer their secondary organs while most of the time the secondary site is determined by anatomical and mechanical consideration. Thus there are currently two hypotheses on a tumor cell's decision on secondary organ site.

The first hypothesis is "seed and soil". This hypothesis is first proposed by Paget in 1889, after investigating 735 cases of metastatic breast cancer autopsies. He suggested that metastasis depends on the interaction between the primary tumor and specific organ microenvironments [133]. For example a study done by Procter et al. has shown that, rat sarcoma tumor cells prefer colonization in lung even when the tumor is injected into the blood circulation from several different routes [134]. However Procter also stated that the results of the study suggests the result may verify the "seed and soil" hypothesis and also the "anatomical mechanics" hypothesis.

The "anatomy mechanics", proposed by Ewing in 1929, suggests that, secondary site is only determined because of the anatomy of the circulatory system and some other mechanical factors. Both of these theories above may be true and interchangeable with the tumor type.

1.5. EPITHELIAL TO MESENCHYMAL TRANSITION (EMT)

Metastasis requires movement, in contrast; the most malignant tumors originate from epithelial tissue (carcinomas). Researchers suggest that, these invading and metastasizing cells may also have mesenchymal characters. For this reason, it is suggested that the transition from being an epithelial cell to a mesenchymal cell occurs during cancer development [73].

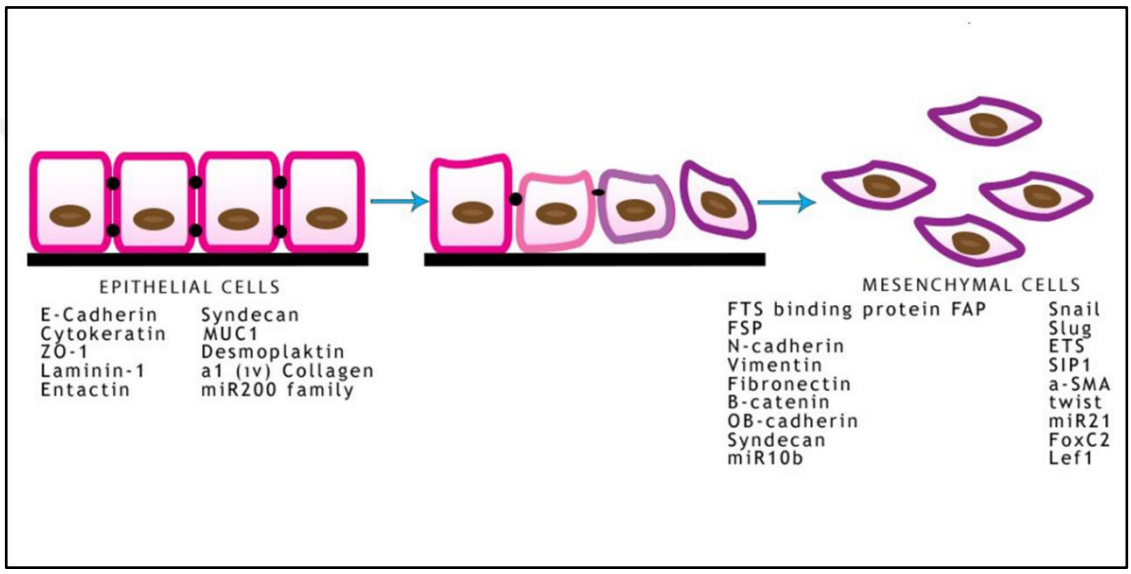


Figure 1.9 Schematic representation of changed morphology during EMT and major epithelial and mesenchymal hallmark genes

Epithelial cells can be differentiated from mesenchymal cells with respect to their shapes. They appear as columnar or polygonal and they are closely attached to each other with cell-cell adhesion molecules. Their migratory potential is limited. On the other hand mesenchymal cells are loosely attached to each other and prone to movement. They only have focal cell-cell contacts [135].

When the transition from epithelial to mesenchymal occurs; cells with epithelial characteristics, lose their shape and expression of epithelial markers (e-cadherin, cytokeratins, occludin and claudin) are deactivated or silenced while expression mesenchymal markers (N-cadherin, vimentin and fibronectin) get activated. ECM degrading enzymes are produced and specific microRNAs are synthesized [135].

1.5.1. Types of EMT

Epithelial to mesenchymal transition occurs in different occasions within a lifetime. It is important while embryonic development and essential for mesoderm formation. It is known to contribute wound healing and tissue regeneration and cause organ fibrosis. EMT is also known to get activated during tumor progression and one of the reasons of metastasis [136].

In all of these circumstances, activated transition mechanisms share many similar elements but they also have clear distinctions between each other[137]. EMT mechanisms can be classified in 3 groups:

1.5.2. Type One EMT

Type one EMT occurs during embryogenesis and organ development [138]. The trophoectoderm cells undergo EMT to attach placenta in order to maintain its need of nutrient and gas exchange [139]. EMT during this stage is important for pregnancy since failure of placental anchorage ends up with miscarriage. But this is not the only occurrence of EMT-one during embryonic development. In early stages of embryonic development, a structure called the “primitive streak”, is formed in within the epiblast layer [140].

In this step, epithelial cells in the middle of epiblast layer, previously expressing e-cadherin and exhibit apical-basal polarity, goes into EMT and gets extended inwards [141]. This occurs with the programmed changes within epithelial cells that includes; activation of cell migration proteins and differentiation. After the activation, epithelial cells that create primitive streak that form in embryonic mesoderm between epiblast and hypoblast and later on give rise to axial, paraxial, intermediate, and lateral plate mesodermal layers [142].

Type-one EMT is mainly controlled by Wnt signaling. It is found that, when an embryo’s Wnt3 is silenced, it cannot start gastrulation associated EMT [142]. TGF superfamily proteins (NodaI and Vg1) intervene the action of Wnts and their absence creates mesodermal defects. Wnts also cooperate with FGF receptors, snail, Eomes and mesps transcription factors during EMT [143, 144]. In gastrulation of invertebrates, Snail and Twist play an important role while in vertebrates, TGF-β induced activation of Snail-1 and Snail-2 found to be highly active [145, 146].

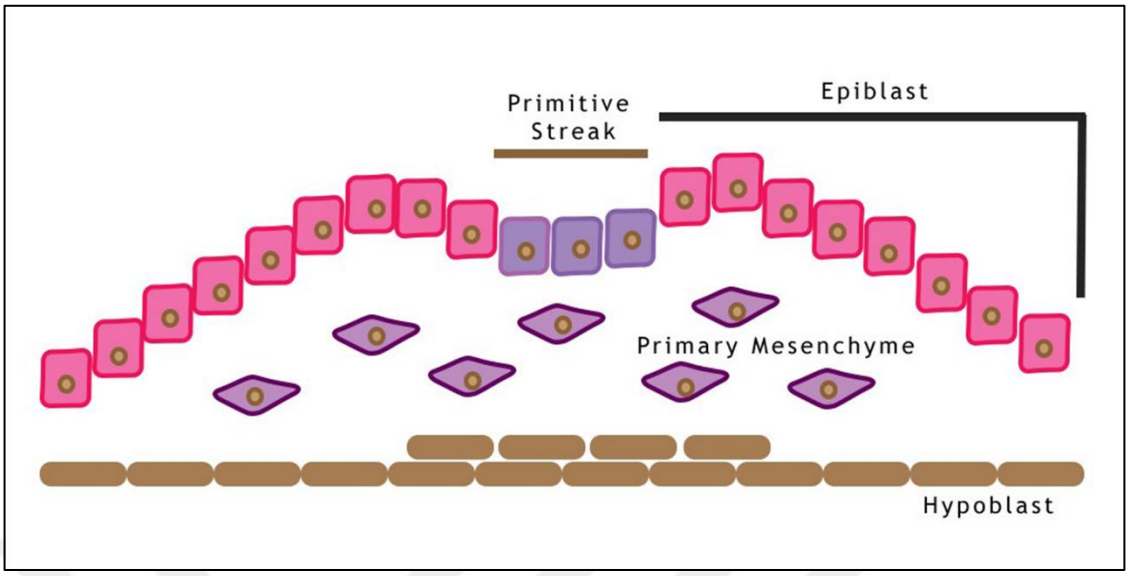


Figure 1.10 Schematic representation of Type-one EMT and the formation of primitive streak during embryonic development

While the embryonic development occurs, epithelial cells within neuroectoderm layer generate migratory neural crest cells. These cells undergo an EMT and as a result, they migrate from neural folds and distribute between other cell types [144]. Those dispersed cells undergo further differentiation, creating different cell types such as melanocytes, cells that provide pigment to the skin and neurons. For this step, activate EMT program is similar to the one associated with gastrulation and it is also mediated by Wnts, FGFs, BMPs, c-Myc signals in which migration is predominantly controlled with BMPs. Type one EMT is not associated with inflammation.

1.5.3. Type Two EMT

Different from type-one EMT, the type-two EMT is controlled with the signals associated with trauma and inflammation. It gets activated during tissue repair, such as wound healing and in normal conditions it is beneficial. However when the inflammation signal becomes persistent it can result in organ and tissue fibrosis, ends up with organ failure (such as kidney fibrosis) [147].

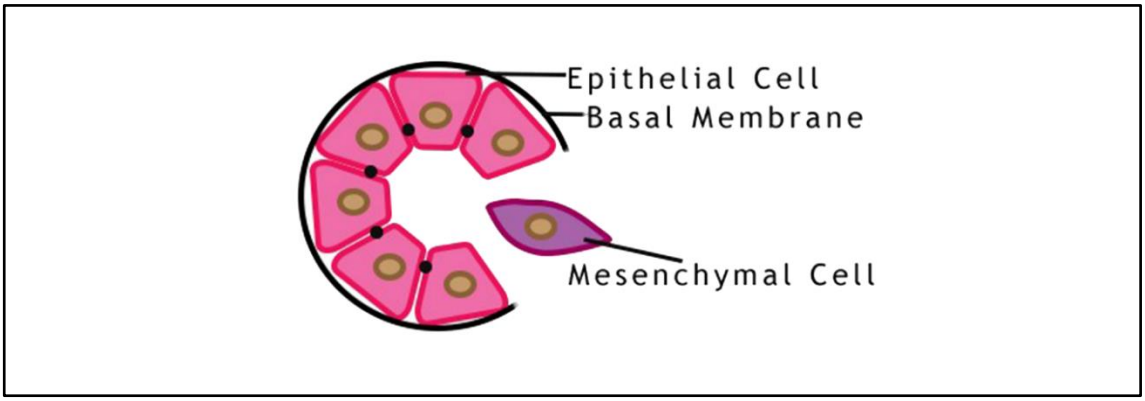


Figure 1.11 Schematic representation of type-two EMT, responsible for fibrosis

First phase of type-two EMT is characterized by the loss of cell-cell contact and apico-basal polarity (repression of tight junction and epithelial membrane junction proteins-such as E-cadherin and Zonula occludens). This leads to the loss of cell-cell connection and cells become destabilized. After the first phase, cells start to express both epithelial and mesenchymal markers. During this stage cytoskeletal proteins are rearranged and matrix metalloproteinases (especially MMP2 and MMP9) are upregulated in order to disrupt underlying basal membranes [148]. Type-two EMT is concluded with the formation of spindle-shaped fibroblast which expresses the mesenchymal markers such as αSMA and vimentin. Those cells have enhanced cell migration capacity and their epithelial markers (e.g. E-cadherin and ZO-1) are silenced [149].

1.5.4. Type Three EMT

Uncontrolled proliferation of epithelial cells and angiogenesis are hallmarks of the initiation of epithelial cancers [1]. Inhibition of metastasis and invasiveness are studied widely in order to understand the biochemical and genetic changes. As a result of the studies among epithelial cancer cells, epithelial to mesenchymal transition is proposed as a critical mechanism for attaining malignant phenotype [150].

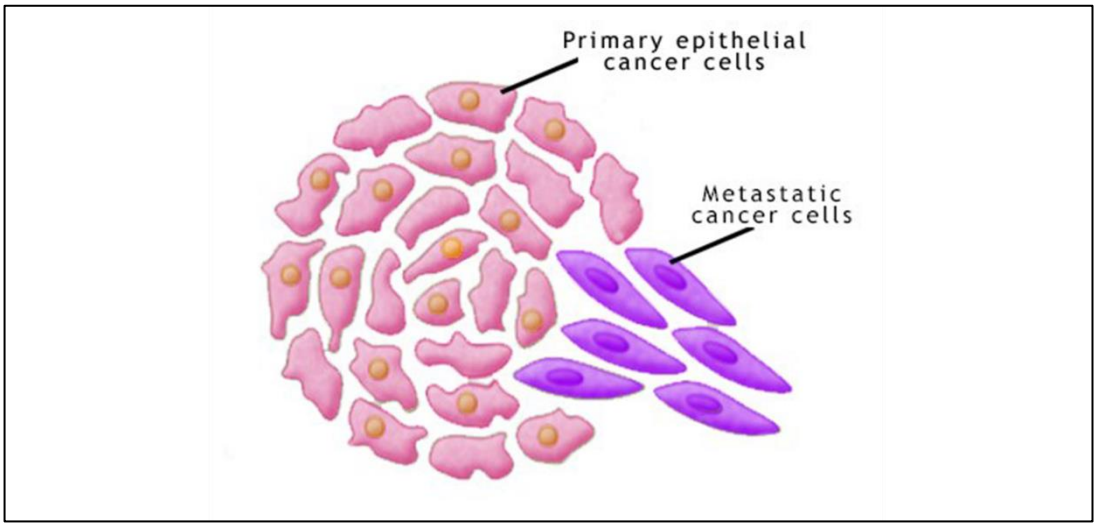


Figure 1.12 Schematic representation of type-three EMT, responsible for cancer metastasis

In epithelial based cancers (carcinomas), it is possible to observe mesenchymal phenotype and expression of genes which are normally considered as mesenchymal markers (e.g. α-SMA, FSP1, vimentin and desmin) [151]. The corresponding cells are found at the invasive front of primary tumors. It is also known that after the cancer metastasis is completed and cells start to establish secondary colonies far away from the initial site, they no longer exhibit mesenchymal characteristic. With respect to this information, it is suggested that, these cells gain their epithelial characteristics by the opposite mechanism, mesenchymal to epithelial transformation (MET) [152]. The complete signaling pathways of EMT and its effect during the metastasis of carcinomas are still unknown but prevention of metastasis by silencing EMT promises hope for further studies.

1.5.5. EMT Signaling Pathways

EMT is activated and deactivated with different signaling pathways during tumor progression. It can be triggered with extracellular signals, with both soluble and non-soluble factors. Soluble factors like, TGFβ family members, FGF, HGF, EGF family members predominantly trigger EMT while collagen and hyaluronic acid can be considered as important non-soluble EMT inducers [153].

Different receptors involved in EMT signaling. TGFβ is one of the most important receptors together with ECM receptors, tyrosine kinase receptors.

Inside the cell, the signal is processed with SMAD signaling and small GTPases (such as RAS). Also transcription regulators; snail, ZEB and bHLH families are up-regulated and/or activated. These regulators generally repress E-cadherin which is the main epithelial adhesion protein [154].

1.5.5.1. TGF-β and SMAD Signaling

Transforming growth factor beta is the most important EMT- inducing soluble factors in many tumor cells. It has 2 defined functions in tumor development. The first function that occurs in early tumor development inhibits tumor growth. TGFβ inhibits MYC and ID transcription factors and induces p15 and p21 cell cycle inhibitors. It can also induce apoptosis in many different cell types [155]. In later stages of tumor, when tumor cells become over resistant to cytostatic effects of TGFβ, it starts to act as tumor promoter. The main explanation of this is the role of TGFβ as an EMT inducer. In case of pancreatic carcinomas, when they are treated with TGFβ, they lose epithelial markers and gain mesenchymal markers [156].

With the binding of TGFβ to its receptor TβRII, type I receptor (TβRI or activin receptor-like kinase ALK) is trans activated. It is a serine/threonine kinase which phosphorylates SMAD2 and SMAD3. Phosphorylated SMAD2 and 3 forms heterodimers with SMAD4 and moved to nucleus. There the SMAD heterodimers can interact with DNA-binding transcription factors in order to activate and repress their transcription. TGFβ can also trigger EMT with SMAD-independent pathway which involves ERK/MAPK, PI3K, p38, JNK, RhoA and other signaling molecules [157] and it may require the activation of transcription factors SP-1 and NF-κB [158, 159].

1.5.5.2. Other Soluble Factors

It is observed that soluble factors different than TGFβ may trigger EMT. Some of them belongs to the TGFβ superfamily (BMPs) [160], some are growth factors that perform their actions by receptor tyrosine kinases (HGF, VEGF) [161] and the last group signals by using other different pathways (TNFα, Periostin) [162] . These factors can be synthesized by the tumor cell or surrounding cells.

1.5.5.3. Ras Signaling

GTP-binding protein RAS is responsible for cell differentiation, proliferation and survival. Additionally it has been identified as a perquisite of EMT. It is generally active in many tumors whether as a result of a mutation or because of stimulation by receptor tyrosine kinases. It can activate several downstream pathways and RAF-MAPK is found to be necessary for induction of EMT [163].

1.5.5.4. Transcription Repressors: Snail, ZEB and bHLH (basic helix-loop-helix) Families

In recent years, the effect of ZEB, Snail and bHLHs on EMT is extensively studied. Zeb1 and Zeb2 are zinc finger transcription factors and they are known to repress the transcription of e-cadherin directly or indirectly by repressing miRNAs which normally act as the repressors of EMT promoters; TGFβII and ZEB1 (feed-forward loop) [164].

Basic helix-loop-helix family members important for EMT are E47 and Twist. They have repressive effect on e-cadherin. Their effect is similar to Snail-superfamily zinc-finger transcriptional repressors; Snail (SNAI1) and Slug (SNAI2) which also represses e-cadherin gene [154].

1.5.5.5. Other Signaling Pathways

NF-κB signaling has been associated with tumor formation for a long time because of its anti-apoptotic effect. In the recent years, it is found that NF-κB is a central regulator of EMT. Also hedgehog signaling is found to repress e-cadherin and snail [165, 166].

Wnt signaling, most important regulator of EMT during development, is linked to EMT by the intracellular domain of the cadherins that can activate β-catenin/wnt signaling [167] Notch signaling is also found to be partially control EMT since their down-regulation leads to partial MET.

1.5.5.6. Cadherins

In order to conduct cell-cell adhesion, expression of cadherins (a large family of type 1 transmembrane proteins) have an important place and many EMT pathways eventually lead to a switch in the expression of these proteins [167]. During EMT, cells start to decrease their e-cadherin and increase expression of n-cadherin. In most of the carcinoma cases expression of e-cadherin is lost by signaling pathways or by silencing mutations. This loss itself can start progression of pancreatic tumors. Also re-expression of e-cadherin is known to trigger MET [168]. On the other hand, N-cadherin is considered as a promoter of invasion and metastasis [168].

1.5.6. Small Molecule Inhibitors of EMT

The main purpose of this thesis is inhibition of metastasis. The initial starting point is trying gene silencing at the first place in order to understand its effect. If gene silencing is found to be successful for inhibition of metastasis, instead of using viral gene silencing methods, small molecules that blocks EMT[169]. By this way it would be possible to use this therapy strategy in real pancreatic patients since viral gene therapy options are not ethical and forbidden in human trials. Additionally, these small molecules can be used together with gene therapy (in combination) to enhance the effect [170].

The main controller of EMT is TGF-β pathway. If TGF-β is blocked by pharmacological agents, it is also possible to block the downstream gene targets of TGF-β pathway. By recent studies, it was found that, via small molecules it is possible to block TGF-β receptor kinases [171]. These molecules change the gene expressions epigenically, meaning that making a chance in cellular phenotype without changing the genome [172].

In recent years there have been many different small molecule inhibitors of TGF-β receptor kinases including; A-8301 [173], GW-6604 [174], Ki26894 [175] and SD208 [176].

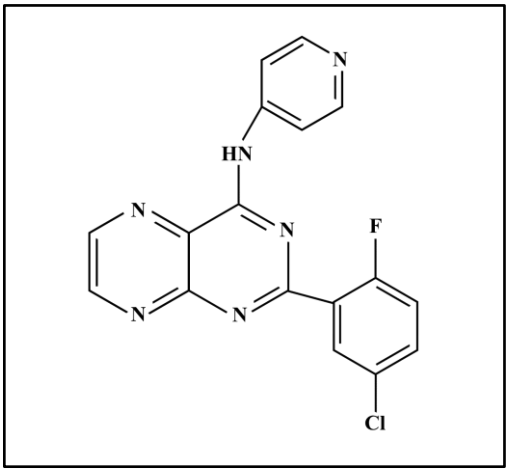


Figure 1.13 Chemical structure of SD-208

One of the chosen small molecule for this study is SD208. This chemical has been used in various researches and it is found that it decreases invasiveness, cancer progression, inflammation and decreases fibrosis [176-179]. Additionally it has been used for pancreatic cancer [180] and in other carcinomas in order to decrease metastatic activity. But there has never been a research that it was used in combination to genetic silencing approach.

Another approach for decreasing the tumorigenic and metastatic ability is targeting EMT's side pathways such as casein kinase pathway. This pathway changes various EMT controllers including Foxc2 and α-SMA. There are several commercially available CK2 inhibitors including, LRP6ICD [181] , 7,7′-Diazaindirubin [182], D4476 [183], LH846 [184], and CX-4945 (Silmitasertib) [185].

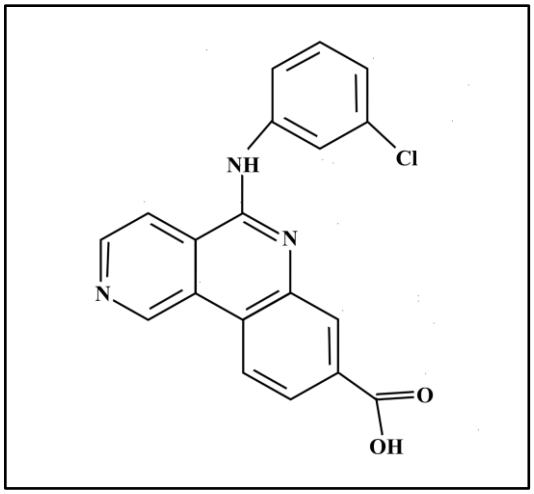


Figure 1.14 Chemical structure of CX-4945

The second chosen small molecule inhibitor for this study is CX-4945 (Silmatasertib). This small molecule has been used on tumor cells and endothelial cells and the inhibition of CK2 via CX-4945 has stopped cell cycle, showed anti-tumorigenic effects and inhibited angiogenesis [186]. Again this chemical has not been used for pancreatic carcinomas and not used in combination with gene therapy.

1.5.7. EMT and Pancreas Cancer Stem Cells

It has been found that beneath pancreatic cancer cells, there is a subpopulation of CD133+ cells which are highly tumorigenic and drug resistant. Also within this population, there are both CD133+ and CXCR4+ cells (pancreatic cancer stem cells) that creates the invasive front of the pancreatic tumors. These cells are considered as the reason for pancreas cancer metastasis. Targeting CSCs and EMT can be a good therapeutic option in order to inhibit metastasis [187].

1.5.8. Relationship between EMT, Pancreas Cancer and Metastasis

EMT principles have been discovered in cell culture models and tumor models. In pancreatic carcinoma, Javle *et al.* saw a correlation between EMT markers (high fibronectin, high vimentin and low e-cadherin) in 36 pancreatic carcinoma samples (surgically removed) [188].

It is found that, down regulation of e-cadherin can be used as a marker for predicting whether the pancreatic cancer will spread to other tissues[189] .

N-cadherin expression is not normally observed in healthy pancreas was detected in some pancreatic cancer patients while vimentin was increased in metastasis compared to the primary tumors.

Transcriptional repressor snail was found to be expressed in 36% to 78% of pancreatic cancer tissues [190]. Undifferentiated tumor cell lines (MIA PaCa-2 and Panc-1) were found to have higher snail expression compared to the differentiated ones (Capan-1, HPAF, AsPc-1). Slug expression on the other hand was found to get increased in pancreatic carcinoma but up to date there is no published correlation between slug expression and clinical data [191]

Twist expression is not observed in pancreatic carcinoma but it is also found that hypoxia increases twist expression and induce EMT [191] Zeb1 is not normally expressed in healthy pancreatic tissue and only weekly expressed in well differentiated pancreatic adeno-carcinoma. However it is found to be overexpressed in dedifferentiated pancreatic carcinoma [192].

Mutations in the TGFβ pathway is observed in pancreatic cancer. Inactivation of TGFβ is observed in 55% of cases. On the contrary overexpression of TGFβ is also correlated with decreased survival in pancreatic carcinoma [193, 194].

A connection between EMT and invasion/metastasis is investigated in many studies[195] [196] . For example EMT program is found to be active in highly metastatic pancreatic cells isolated from animals. Also e-cadherin and ZEB1 seems to be important for creating a link between EMT and invasion[197].

1.6. THE AIM OF THE STUDY

Purpose of this study is to evaluate the changes of gene expression, protein synthesis amounts, cellular characteristics of various pancreas cancer cell lines after treating with short hairpins in order to silence Snail, Slug and Twist genes, main transcription factors that regulate and orchestrate EMT, epithelial to mesenchymal transition. This study was done to understand that whether it is possible to observe a decrease in migration potential, invasiveness and cancer stem cell treats if EMT was silenced.

Additionally, the cells were treated with small molecule inhibitors for direct and indirect inhibitors of EMT pathways. This was done to evaluate if the gene therapy is replaceable with small molecule inhibitors of EMT or is it beneficial to use this two methods together.

2. MATERIALS AND METHODS

2.1. CELL CULTURE

For this study, five different pancreas cancer cell lines (Panc-1 (ATCC® CRL-1469™), MIA PaCa-2 (ATCC® CRL-1420™), BxPc-3 (ATCC® CRL-1687™), AsPc-1 (ATCC® CRL-1682™), healthy immortalized pancreas cell; hTert-HPNE (ATCC® CRL-4023™) and HEK-293T Cells (ATCC® CRL-1573™) were planned to be used. Properties of all cells and their specific cell culture conditions are listed in the table below.

Table 2.1 Cells used for the study and their culture medium specifications

Name of the Cell	**Cell Type**	**Disease**	**Cell Culture Condition**
Panc1	Pancreas/Duct Epithelial	Epitheloid Carcinoma	High glucose DMEM + 10%FBS+1%PSA
MIA PaCa-2	Pancreas Epithelial	Carcinoma	High glucose DMEM + 10%FBS+ 2.5%HS 1%PSA
BxPc-3	Pancreas Epithelial	Adenocarcinoma	RPMI medium+ 10%FBS+1%PSA
AsPc-1	Pancreas-Derived from metastatic site	Adenocarcinoma	RPMI medium+ 10%FBS+1%PSA

For cell culture; High Glucose DMEM (Gibco, 11965092), RPMI 1640 Medium (Gibco, 11875093), Fetal Bovine Serum (Gibco, 16000044), Antibiotic/Antimycotic (Gibco,

15240062), Horse Serum (Gibco, 16050130), , Puromycin dihydrochloride (Thermofisher, A1113803) were used as/in culture mediums. As consumables, Corning® cell culture flasks (25cm^2, CLS430372; 75cm^2, CLS430641; 175cm^2, CLS431306), Corning® multi-well cell culture plates (6-well plate, CLS3506; 12-well plate, CLS3512; 24-well plate, CLS3526; 48-well plate, CLS3548; 96-well plate; CLS3595), Corning® cell culture dishes (100mm x 20mm, CLS430293; 60mm x 15mm, CLS430166), Corning® sterile serological pipettes (5ml, CLS4487-200EA; 10ml, CLS4101-200EA; 25ml, CLS4251-200EA), Axygen Scientific pre-sterilized falcon tubes with conical bottom (15ml, SCT-15ML-R-S; 50ml, SCT15ML25S), Expel pipette tips were used.

After cells were purchased or thawed from a previous stock, they were cultured in their specialized cell culture media in a humidified chamber (In-Vitro-Cell Direct Heat Microbiological CO2 Incubators - Humidity Sensor Control, NU-5820) at 37 °C and 5 per cent CO_2, Culture medium changed twice a week and then confluency reaches 65-70 per cent; while excess cells were frozen/stored in liquid nitrogen, the deficient amount were left to grow for experiments.

2.2. GENE EXPRESSION ANALYSIS OF SNAIL SLUG AND TWIST

In order to find out the expression levels of Snail-1 (SNAI-1), Snail-2/Slug (SNAI-2), Twist-1 before genetic manipulation and to indicate levels of epithelial and mesenchymal markers if pancreas cancer cell lines, selected genes were analyzed with Real-time PCR.

Cells (Panc-1, MIA PaCa-2, BxPc-3, AsPc-1) were cultured. Approximately 1x10^6 cells are trypsinized and pelleted. RNAs were isolated by High Pure RNA Isolation Kit (#11828665001, Roche, USA) using the manufacturer's protocol. Briefly; previously pelleted cells (trypsinized, centrifuged, stored in -80) were re-suspended in 200 µl PBS. 400 µl Lysis/Binding Buffer was added and vortexed for 15 s. Mixture was transferred to High Pure Filter Tube and centrifuged 15 s at 8,000 × g. Flow through was discarded. For each sample a mixture of 90 µl DNase I Incubation Buffer and add 10 µl DNase I was pipetted onto glass fiber fleece in the upper reservoir of the filter tube. Samples were incubated at +15 to +25°C for 15 minutes. After incubation, 500 µl Wash Buffer I was added to the upper reservoir and centrifuged 15 s at 8,000 × g. Flow through was discarded. 500 µl Wash Buffer II was added to the upper reservoir and centrifuged 15 s at 8,000 × g. Flow through was

discarded. 200 µl Wash Buffer II was added to the upper reservoir and centrifuged for 2min at maximum speed. Flow through was discarded. Collection tube was discarded and the filter tube was inserted into a clean, sterile 1.5 ml micro centrifuge tube. 50 µl Elution buffer or DEPC treated water was added to the upper reservoir of the filter tube and incubated for two minutes. The tube assembly was centrifuged for one min at 8,000 × g. Isolated RNA was either stored or used for cDNA synthesis after measurement of concentration.

Purity of RNAs were measured by using NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, ND-2000). Isolated RNA purity (A269/280) must be approximately two.

Complementary DNAs from the isolated RNAs were reverse transcribed with QuantiTect Reverse Transcription Kit (Qiagen, USA, #205310). Briefly; for a total volume of 14µl, two µl gDNA wipeout solution, 1000ng template DNA and dH2O was mixture and incubated at 42 °C for three minutes. For a total volume of six µl, four µl Buffer 5x, one µl RT enzyme and one µl RT Primer mix was prepared and added to the first mixture after initial incubation. The new mixture was then incubated at 42 °C for 30 minutes then incubate at 95°C for five minutes.

For Taqman reactions, 2X TaqMan® Gene Expression Master Mix (Thermo Scientific, 4369016) was used and the plate was subjected to; 50°C, two mins 95°C, 10 mins 40 cycles of the following: 95°C, 15 sec 60°C, one min with plate reading at the end of each cycle. For reactions using SYBR Green, 2X SYBR green master mix (Applied Biosystems, 1409155) was used and the plate was subjected to 50°C, two mins 95°C, and 10 mins 40 cycles of the following: 95°C, 15 sec 58°C, one min with plate reading at the end of each cycle.

Primers for Realtime-PCR (both Taqman primers and sybr green primers) were given in the table below. Gene expression folds were normalized by housekeeping genes (β-Actin, GAPDH or 18S) by ΔΔCt method.

Table 2.2 Taqman catalog numbers and sequences for the primers used in this study

Primers	**Sequences/Catalog Number**
Snail	Hs00195591_m1
Slug	Hs00161904_m1
Twist	Hs01675818_s1
Beta Actin	Hs01060665_g1
GAPDH	Hs02758991_g1
18S	Hs03003631_g1
E-cadherin	Hs01023894_m1
N-cadherin	Hs00983056_m1
Vimentin	Hs00958111_m1
CD133	Hs01009259_m1
CD24	Hs02379687_s1
CD44	Hs01075864_m1
CXCR4	Hs00607978_s1
EPCAM	Hs00901885_m1
OCT3/4	Hs04260367_gH
NANOG	Hs02387400_g1
KLF4	Hs00358836_m1
cMYC	Hs00153408_m1
SOX2	Hs01053049_s1
c-MET	Hs01565584_m1
MMP1	Hs00899658_m1

MMP9	Hs00957562_m1
MUC1	Hs00159357_m1
ABCG2	Hs01053790_m1
Vimentin [198]	5’-GAG AAC TTT GCC GTT GAA GC-3’ 5’-GCT TCC TGT AGG GGC AAT C-3’
N-cadherin [199]	5’-ACA GTG GCC ACC TAC AAA GG-3’ 5’-CCG AGA TGG GGT TGA TAA TG-3’
Fibronectin [199]	5’-TCC CTC GGA ACA TCA GAA AC-3’ 5’-CAG TGG GAG ACC TCG AGC AG-3’
hMTA1 [200]	5’ GCT GTT ACA CCA CAC AGT CTT 3’ 5’ GGA CTC ATG TTA CTG CGG TTT 3’
hMTA2 [200]	5’ CCG ACG GCC TTA TGC TCC T 3’ 5’ CTG GGC CAC CAG ATC TTT GAC 3’
hIntegrin1β [201]	5’ GAA GGG TTG CCC TCC AGA 3’ 5’ GCT TGA GCT TCT CTG CTG TT 3’
TGFβ1 [202]	5’ ATG ACA TGA ACC GAC CCT TC 3’ 5’ ACT TCC AAC CCA GGT CCT TC 3’
TGFβ R1 [202]	5’ ACC TTC TGA TCC ATC CGT T 3’ 5’ CGC AAA GCT GTC AGC CTA G 3’
hSmad2 [203]	5’ CTG GCT CAG TCT GTC AAC CA 3’ 5’ CTG CCT CCG ATA TTC TGC TC 3’
hSmad3 [203]	5’ CCA GTG CTA CCT CCA GTG TT 3’ 5’ CTG GTG GTC GCT AGT TTC TC 3’
Beta Actin [204]	5’ TTC TAC AAT GAG CTG CGT GTG 3’ 5’ GGG GTG TTG AAG GTC TCA AA 3’

2.3. GENE THERAPY

2.3.1. Gene Silencing with Short Hairpin Technology

In order to understand the effects of silencing the EMT related genes on pancreas cancer cell lines, cells were transfected with lentiviral particles containing shRNAs (Snail, Santa Cruz, sc-38398-V; Slug, Santa Cruz, sc-38393-V; Twist, Santa Cruz, sc-38604-V, control shRNA Lentiviral Particles-A, Santa Cruz, sc-108080; GFP shRNA Lentiviral Particles, Santa Cruz, sc-45924-V).

In order to start transfection, cells were seeded to 6-well plates. After 24 hours of incubation, culture medium was removed and new culture medium containing eight µg of polybrene (filter sterilized while preparation) was given to cells. Cells were incubated with polybrene for 4 to 6 hours. After incubation, the lentiviral particles (20µl- 1x105 IFU) were added to the medium and cells were incubated with the medium for approximately 18 hours.

After incubation, medium was changed again to the normal culture medium and cells are cultured for an additional 24 hours before starting the puromycin (one-two µg/ml) selection. GFP observation indicates successful transfection.

After puromycin selection, cells were grown and pelleted for RNA isolation. Changes in gene expression was examined by Realtime-PCR.

In order to observe changes in protein levels after short hairpin silencing; ELISA assay (Bioassay Technology Laboratory; E3964H, Human Snail Homolog-1 (SNAI-1) ELISA Kit, E1877H, Human Snail Homolog-2 (SNAI-2) ELISA Kit, E3531H, Human Twist Related Protein (TWIST1) ELISA Kit and E0704H, Human GAPDH ELISA Kit- for normalization) was used. Briefly; cells were grown and approximately one million cells per ml of medium was trypsinized and frozen. Proteins from these cells were isolated by RIPA lysis and extraction buffer (Thermo Scientific; 89900) and Halt Protease Inhibitor Cocktail (Thermo Scientific; 78430) with ice incubation and vortexing cycles. Cell debris was removed by centrifugation at 14000 x g for 15 minutes. Protein concentrations were measured with Pierce BCA Protein Assay Kit (Thermo Scientific; 23225) as instructed in product's manual. Protein amounts were equalized with respect to BCA results and diluted with respect to assay's ranges. Elisa assay was also done with respect to product's manual. Briefly; standard

solutions were diluted as instructed. Same amounts of total protein from each cells were added to the wells of elisa kit. On proteins snail, slug, twist antibody and on both proteins and standards streptavidin-HRP was added. Mixture was incubated at 37°C for an hour. After incubation chromagen A and chromagen B solution was added and mixture was incubated at 37°C for 10 minutes. Stop solution was then added to mixture and absorbance was measured at 450 nm. Results were calculated with respect to standard curve made by values of standards. Results were normalized with respect to GAPDH elisa kit.

2.3.2. Changes in Gene Expression Analysis with Downregulation of Snail, Slug And Twist or with Combination Therapy

Changes in gene expression after gene therapy and after combination therapy was analyzed via qPCR method with primers given in table one. For Taqman reactions, 2X TaqMan® Gene Expression Master Mix (Thermo Scientific, 4369016) was used and the plate was subjected to; 50°C, 2 mins 95°C, 10 mins 40 cycles of the following: 95°C, 15 sec 60°C, one min with plate reading at the end of each cycle. For reactions using SYBR Green, 2X SYBR green master mix (Applied Biosystems, 1409155) was used and the plate was subjected to 50°C, 2 mins 95°C, and 10 mins 40 cycles of the following: 95°C, 15 sec 58°C, one min with plate reading at the end of each cycle. Gene expression folds were normalized by housekeeping genes (β-Actin, GAPDH or 18S) by ΔΔCt method.

2.4. PROLIFERATION RATE CHANGE

2.4.1. MTS Assay

After gene expressional change and changes in protein levels are confirmed with Realtime-PCR, ICC and Elisa, in order to understand how gene therapy effects cellular proliferation rate, cells were subjected to MTS assay. Briefly cells were trypsinized and counted. 1000, 2000, 4000, 8000, 10000 and 12000 cells were seeded on the wells of a 96-well plate. Additionally 2000 cells from each type of cells were seeded on different 96-well plates for absorbance measurement for three days.

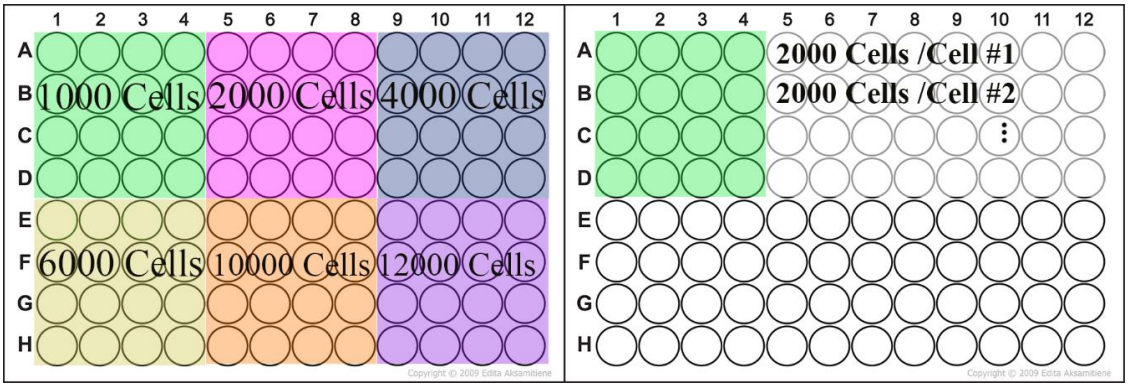


Figure 2.1 A sample assay plate for proliferation rate change

MTS assay is made to the cells seeded for proliferation curve after attachment and for three days cellular proliferation was analyzed with MTS assay. Absorbance was measured with Elisa Reader at 490 nm. MTS assay solution was prepared with using CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega)

2.5. SCRATCH ASSAY

In order to understand how gene therapy effects migration rate, scratch assay was used. Briefly, genetically manipulated cells together with their parental cells were trypsinized and counted. Sets of 25000, 50000, 75000 and 100000 cells were seeded on 12-well plates for optimization of confluency.

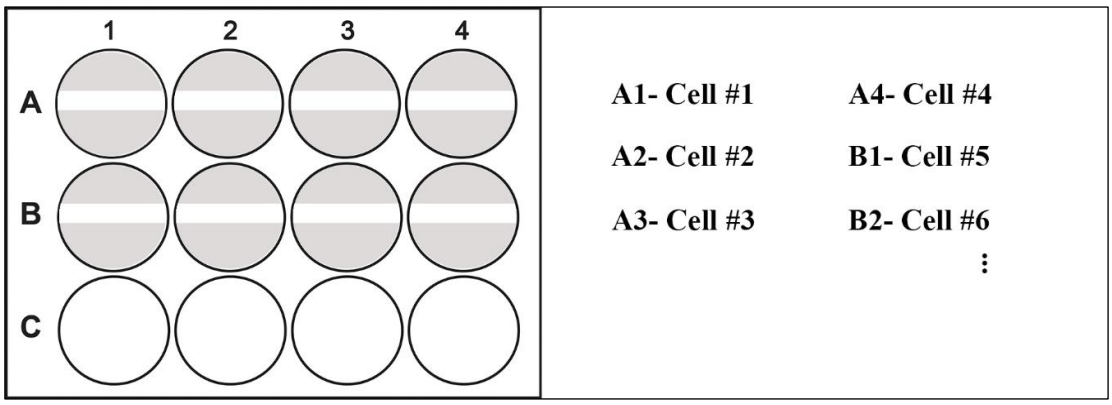


Figure 2.2 A sample assay plate for the scratch assay

After incubation, medium was removed from the group that reached confluency and cells were scratched with 200µl pipette tips. Wells were washed with PBS and a fresh culture medium is given to cells. Cells were observed and photographed in order to understand the change in migration.

2.6. TRANSMEMBRANE CELL MIGRATION ASSAY

Transmembrane migration capacity of genetically manipulated cells were analyzed by CytoSelect™ 24- Well Cell Migration and Invasion Assay (Cell Biolabs; CBA- 100- C). Briefly; migration plates were warmed up at room temperature before use. Cell suspensions containing 0.5-1.0x10^6 cells were prepared in 300µl serum free media and added to the insert of each well. 500µl of media containing 10 per cent FBS to lower well of the plate. After 24 hour incubation at 37°C media was aspirated from the insert and interior of insert was cleaned with cotton swabs to remove non-migratory cells. Inserts were then transferred to a clean wells containing 400µl cell stain solution and incubated for 10 minutes at room temperature. After incubation, inserts were washed with water. At least three fields of each inserts were counted. Migration ability was calculated by the counted average number of genetically manipulated cells over counted average number of shControl.

2.7. TRANSMEMBRANE CELL INVASION ASSAY

Transmembrane invasive capacity of genetically manipulated cells were analyzed by CytoSelect™ 24- Well Cell Migration and Invasion Assay (Cell Biolabs; CBA- 100- C).

Briefly; invasion plates were warmed up at room temperature before use. Insert was then rehydrated with warm serum free media for one hour. Cell suspensions containing 0.5-1.0x10^6 cells were prepared in 300µl serum free media and added to the insert of each well. 500µl of media containing 10 per cent FBS to lower well of the plate. After 24 hour incubation at 37°C media was aspirated from the insert and interior of insert was cleaned with cotton swabs to remove non-invasive cells. Inserts were then transferred to a clean wells containing 400µl cell stain solution and incubated for 10 minutes at room temperature. After incubation, inserts were washed with water. At least three fields of each inserts were counted.

Invasiveness was calculated by the counted average number of genetically manipulated cells over counted average number of shControl.

2.8. IN VITRO TUMOR SPHERE FORMATION

Tumor sphere formation assay was done to evaluate the changes in tumor forming ability from a clone and self-renewal capacity after gene therapy, tumor sphere formation assay was done. Genetically modified pancreas cells were cultured, trypsinized and counted. 250, 500, 1000 and 2500 cells were transferred to ultra-low attachment conditions. Cells were then treated with a special medium containing; 20ng/ml EGF (EGF Recombinant Human Protein, PHG0311, Thermo Scientific, USA), two per cent B27 supplement (B27, 17504044, Thermo Scientific, USA), 20ng/ml bFGF (Basic Fibroblast Growth Factor Recombinant Human Protein, 13256029, Thermo Scientific, USA), one per cent ITS (Insulin Transferrin Selenium supplement, 41400045, Thermo Scientific, USA), one per cent L-Glutamine (G3126, Sigma) and one per cent Penicillin Streptomycin Amphotericin. Cells were incubated in normal incubation conditions. Fresh medium was added onto the cells every other day for at least 15 days until spheres reach the diameter between 100 and 200 μM.

2.9. ATTACHMENT TO LAMININ

96-well plates were coated with Laminin proteins (one $\mu g/cm^2$) and incubated for 45 minutes at 37°C. Solution was then removed and plates were incubated at room temperature until they were dried. 1000 cells were seeded to each well in normal culture medium and incubated at 37°C for only 30 minutes. Cells were then removed and washed with PBS for once. Remaining attached cells were stained with crystal violet.

2.10. CANCER STEM CELL PROPERTIES

In order to understand how gene silencing effects cancer stem cell genes for pancreas cancer (CD24, CD44, CD133, CXCR4 and EPCAM) and embryonic stem cell characteristic genes (OCT3/4, Nanog, cMYC and Sox2) of pancreas cancer cell lines, realtime PCR analysis was done for evaluation of gene expression values. Changes in protein amounts of cancer stem

cell markers (CD44, CD24, CD133 and CXCR4) for pancreas cancer were tested with flow cytometry analysis.

For this test, qPCR was done with the primers given in Table one and flow cytometry analysis was done with the fluorescent conjugated antibodies given in the table below;

Table 2.3 Antibodies used in flow cytometry analysis

Name of the Antibody	Brand	Catalog number
CD44-FITC conjugated	Miltenyi	130-095-195
CD24-PE conjugated	Miltenyi	130-098-861
CD133-FITC conjugated	Miltenyi	130-105-226
CXCR4-PE conjugated	Thermo Scientific	12-9999-41

2.11. GENE EXPRESSION ANALYSIS FOR SELECTED EMT MARKERS

After gene therapy, gene expression analysis was done to evaluate the gene expression values of selected EMT markers including E-cadherin, Vimentin, N-cadherin, MMP1 and MET. Gene expression analysis was done by using Taqman Primer probes given in Table1.

2.12. DRUG RESISTANCE

After the assessment of all the tests above have done to the pancreas cancer cell lines, AsPc-1 cell line was chosen for further review.

In order to understand how the gene silencing has changed the drug resistance on AsPc-1 cell line, chosen chemotherapeutics (5-Fluorouracil, Docetaxel and Mitomycin C) was applied for 24 and 48 hours in the concentrations that are not toxic to healthy pancreas cell line (hTert-HPNE). All of the chemotherapeutics were purchased in dried form and dissolved with respect to the manufacturer's protocol. Supplier information and catalog numbers of the selected chemotherapeutics can be found in the table below.

Table 2.4 Chemoterapeutics

Name of the Chemical	Brand	Catalog Number
5-Fluorouracil	Sigma Aldrich	F6627 SIGMA
Docetaxel	Sigma Aldrich	01885 SIGMA-ALDRICH
Mitomycin C	Sigma Aldrich	M4287 SIGMA

Cells were seeded on 96 well plates as 5000 cells/well. After an incubation of 24 hours for cellular attachment, cells were treated with predetermined concentrations of chemotherapeutics. Cells were incubated with the chemotherapeutics for 24 and 48 hours and at the end of that period, cellular viability was analyzed with MTS assay. Absorbance was measured with Elisa Reader at 490 nm. MTS assay solution was prepared with using CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega).

After AsPc-1 shSnail, shSlug, shTwist and shControl cells were treated with selected drugs for 48 hours, gene expression values for drug resistance and survival genes (Survivin, Muc-1 and ABCG2) were evaluated. Realtime PCR analysis was done using the taqman probes given in the table below.

Table 2.5 Primer probes of drug resistance and survival

Name of the primer	Brand	Catalog Number
Survivin (Birc5)	Thermo Scientific	Hs04194392_s1
MUC1	Thermo Scientific	Hs00159357_m1
ABCG2	Thermo Scientific	Hs01053790_m1

2.13. COMBINATIONAL THERAPY

For combination therapy, SD208 and CX4945 was used. First MTS assay was done to evaluate the toxicity of the selected drugs (data not shown). The concentrations were chosen according to the IC_{50} values given in the respective papers and the chemicals websites.

For this study AsPc-1 cells were selected and used. Cells were (AsPc-1 shSnail, shSlug, shTwist and shControl) seeded to T25 flasks and incubated in humidified incubator overnight for attachment. The cells were then cultured with serum free RPMI (supplemented with one per cent PSA) for 24 hours. Cell culture medium was then replaced with serum free RPMI supplemented with five ng/ml TGFβ containing either 10μM CX4945, 200μM SD208 or in combination. After 48 hours of incubation cells were trypsinized, pelleted and RNA isolation was done. qPCR analysis was done to evaluate the changes in the gene expression values of selected EMT markers in order to understand the best combinational selection.

Afterwards the cells were subjected to invasion and migration assay, as mentioned in the previous sections.

2.14. STATISTICAL ANALYSIS

As measurement of statistical difference between groups; One-way ANOVA with Dunnett's post-test was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Graphics were directly copied from Graphpad Prism's graphic page and asterisks were added subsequent to statistical analysis. Single asterisk (*) indicates that the p value is less than 0.05 per cent.

2.15. IMAGE PROCESSING

Image processing operations; including cell counting and measurement of scratch was done by using appropriate plugins of Image J, an image processing program developed by National Institute of Health (NIH). Graphics and microscope images were collaged by Photoshop CS6 by Adobe. Scale bars in images are given automatically by each microscope's software. These software include; "Zen 2011, Zeiss"; "Zen Blue; Zeiss", "Zen Lite; Zeiss".

3. RESULTS

3.1. MICROSCOPE IMAGES

3.1.1. Microscope Images of Pancreas Cell lines

As shown in Figure 3.1, cellular phenotypes change between the cell lines. While Panc-1 and Mia Paca-2 having a mesenchymal phenotype, BxPc-3 and AsPc-1 shares a similar phenotype and they are more epithelial looking compared to the other two cell lines.

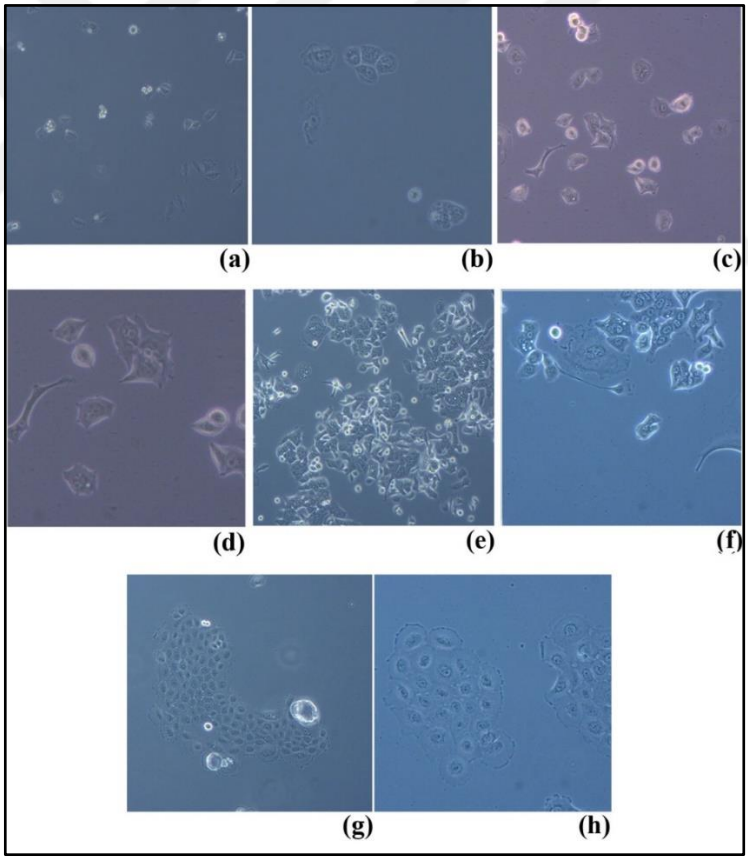


Figure 3.1 Microscope images of pancreas cancer cell line(a) Panc-1 10x magnification, (b) Panc-1 20x magnification, (c) MIA PaCa-2 10x magnification, (d) MIA PaCa-2 20x magnification, (e) BxPc-3 10x magnification, (f) BxPc-3 20x magnification, (g) AsPc-1 10x magnification, (h) AsPc-1 20x magnification

3.1.2. Microscope Images of Panc-1 Cell Line after Gene silencing

Figure 3.2 shows the cellular phenotypes of Snail silenced (a), Slug silenced (b), Twist silenced (c), Panc-1 shcontrol cells (d) and Panc-1 Parental cell lines. As cellular genotype changes, it is observed that, phenotype also changes towards being epithelial.

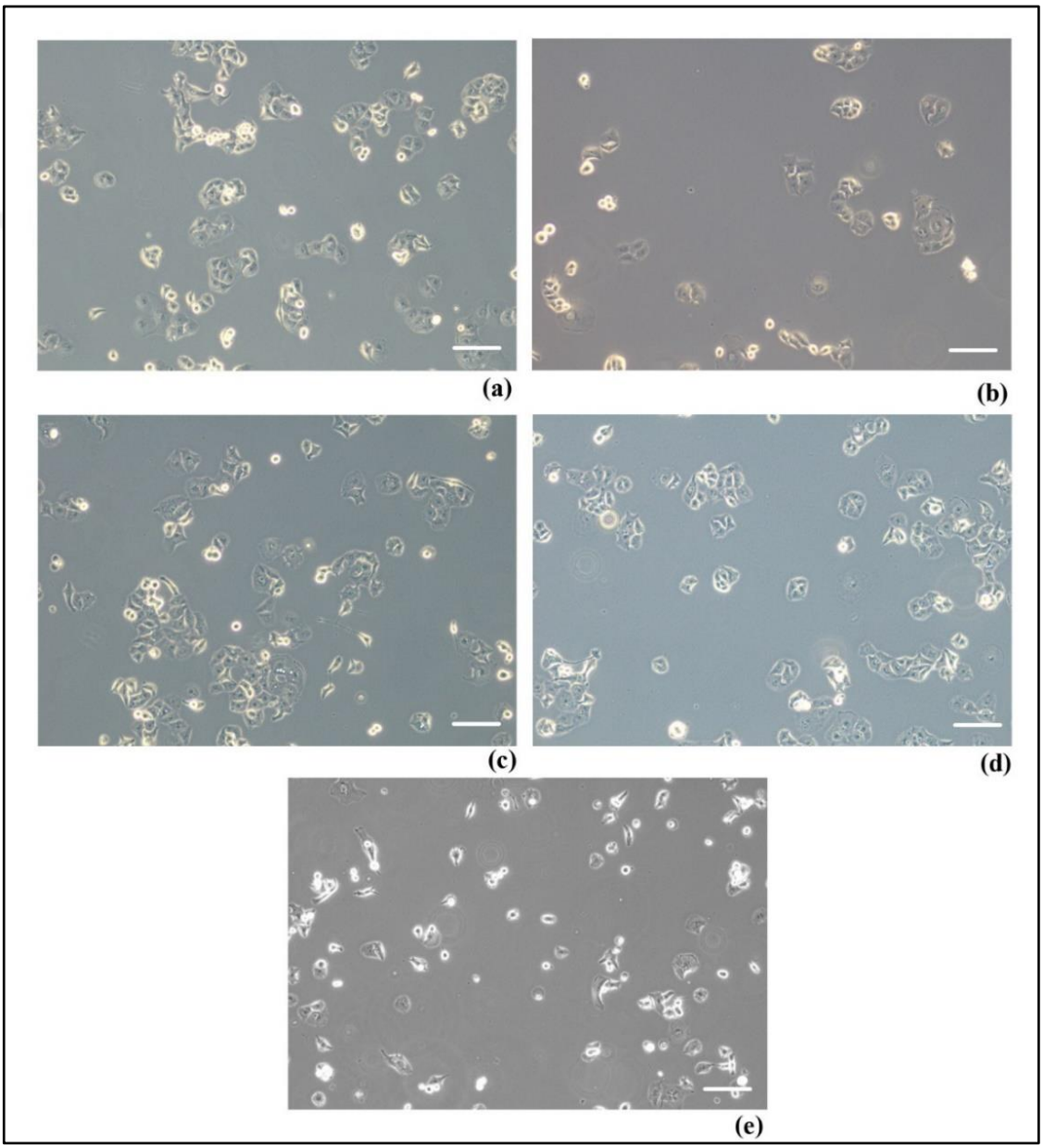


Figure 3.2 Microscope images of Panc-1 cells (a) Panc-1 shSnail Cells, (b) Panc-1 shSlug Cells, (c) Panc-1 shTwist Cells, (d) Panc-1 shControl Cells, (e) Panc-1 Parental cell line. The white line represents 100µm

3.1.3. Microscope Images of MIA PaCa-2 Cell Line after Gene silencing

Figure 3.3 shows the cellular phenotypes of Snail silenced (a), Slug silenced (b), Twist silenced (c), MIA PaCa-2 shcontrol cells (d) and MIA PaCa-2 Parental cell lines. As cellular genotype changes, it is observed that, phenotype also changes towards being epithelial.

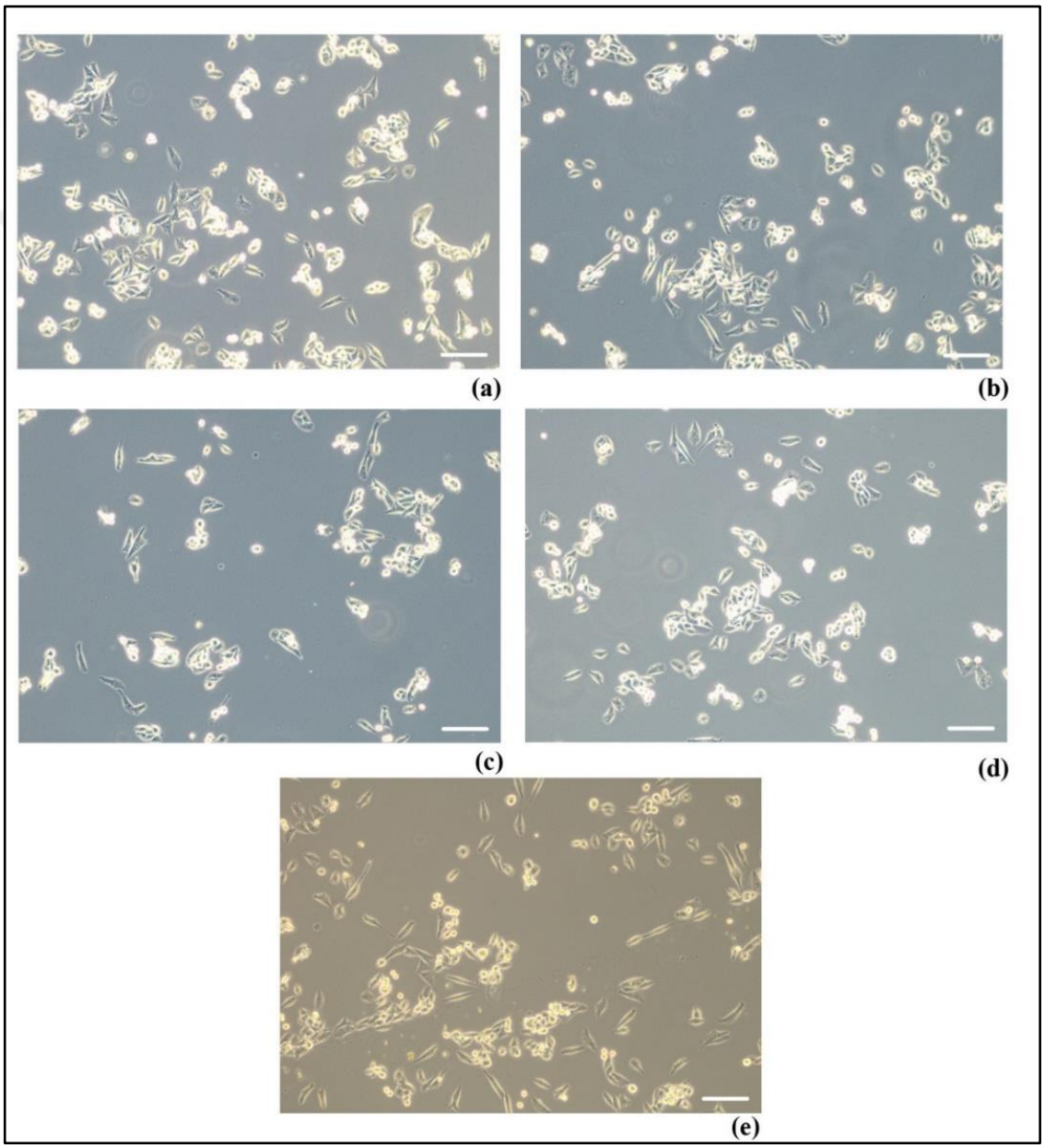


Figure 3.3 Microscope images of MIA PaCa-2 Cells; (a) MIA PaCa-2 shSnail Cells, (b) MIA PaCa-2 shSlug Cells, (c) MIA PaCa-2 shTwist Cells, (d) MIA PaCa-2 shControl Cells, (e) MIA PaCa-2 Parental cell line. The white line represents 100µm

3.1.4. Microscope Images of BxPc-3 Cell Line after Gene silencing

Figure 3.4 shows the cellular phenotypes of Snail silenced (a), Slug silenced (b), Twist silenced (c), BxPc-3 shcontrol cells (d) and BxPc-3 Parental cell lines. As cellular genotype changes, it is observed that, phenotype also changes towards being epithelial.

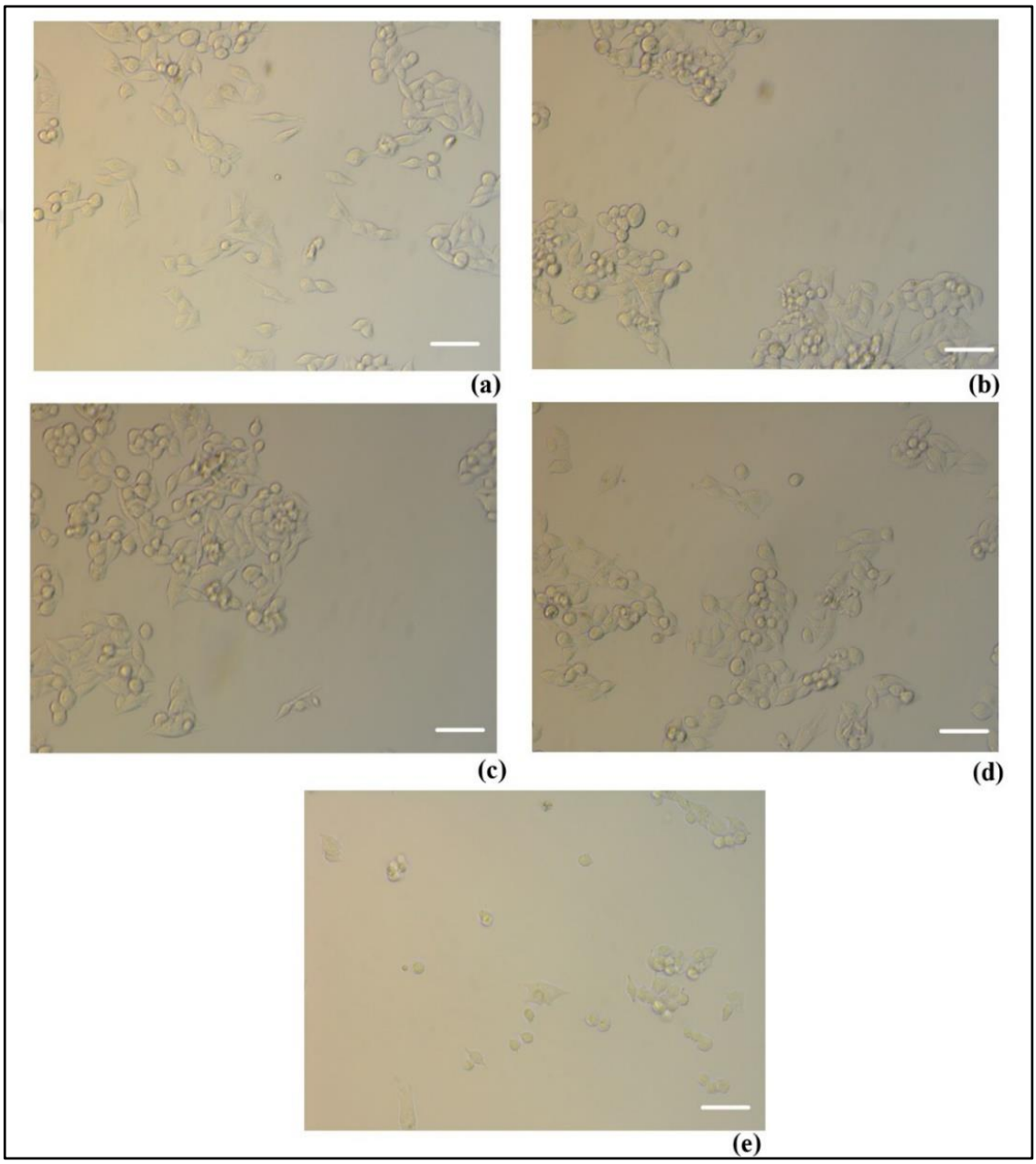


Figure 3.4 Microscope images of BxPc-3 Cells; (a) BxPc-3 shSnail Cells, (b) BxPc-3 shSlug Cells, (c) BxPc-3 shTwist Cells, (d) BxPc-3 shControl Cells, (e) BxPc-3 Parental cell line. The white line represents 100µm

3.1.5. Microscopic Images of AsPc-1 Cell Line after Gene silencing

Figure 3.5 shows the cellular phenotypes of Snail silenced (a), Slug silenced (b), Twist silenced (c), AsPc-2 shcontrol cells (d) and AsPc-2 Parental cell lines. As cellular genotype changes, it is observed that, phenotype also changes towards being more epithelial.

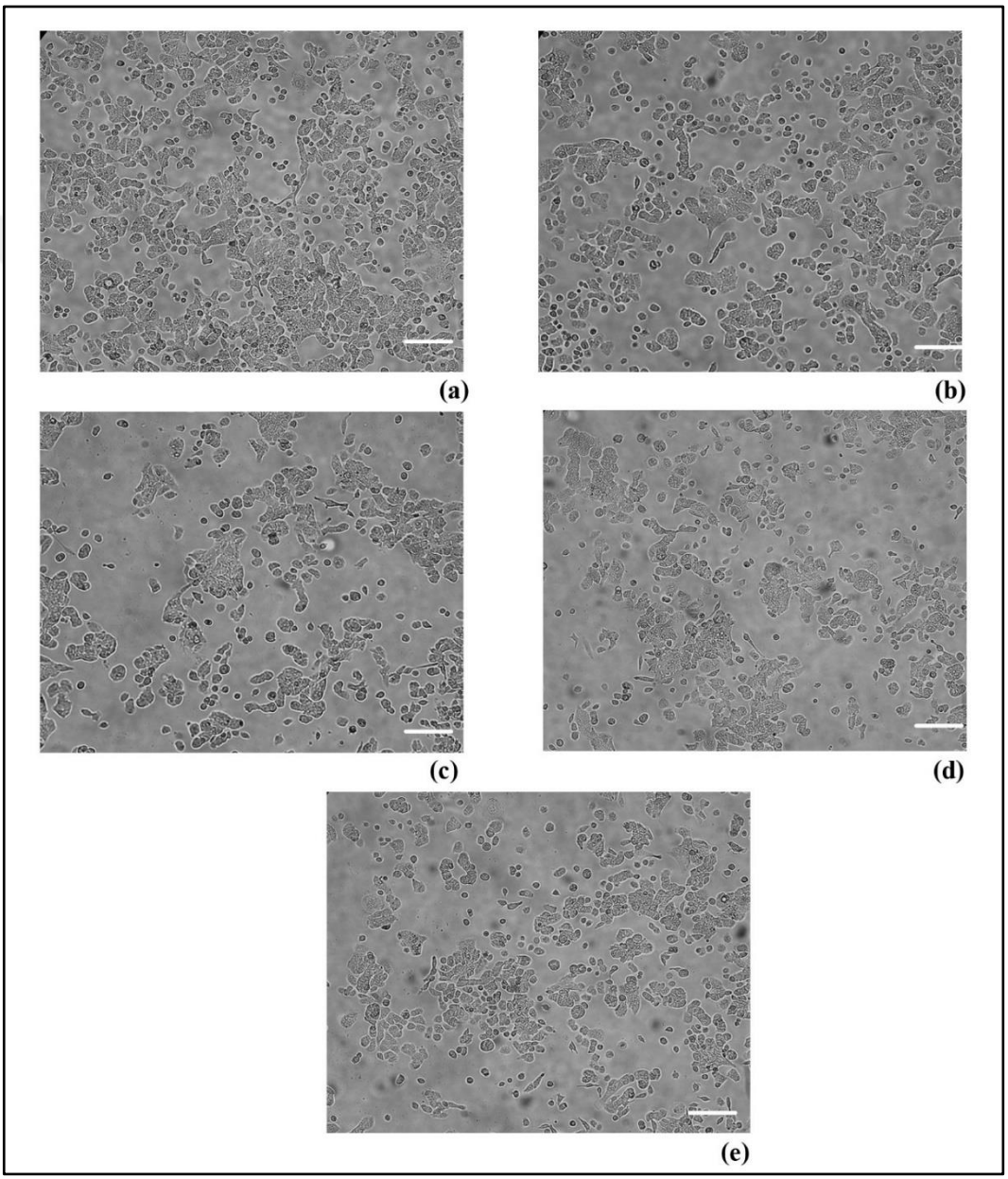


Figure 3.5 Microscope images of AsPc-1 Cells; (a) AsPc-1 shSnail Cells, (b) AsPc-1 shSlug Cells, (c) AsPc-1 shTwist Cells, (d) AsPc-1 shControl Cells, (e) AsPc-1 Parental cell line. The white line represents 100µm

3.2. GENE EXPRESSION ANALYSES

3.2.1. Changes in Gene Expressions of Snail, Slug and Twist Genes after Short Hairpin Silencing in Panc-1 Cell Line

After gene Snail, Slug and Twist silencing the changes in expression values of Snail, Slug and Twist in Panc-1 cell line were evaluated with realtime PCR. The results of gene expression values are given in the Figures 3.6, 3.7, 3.8 respectively.

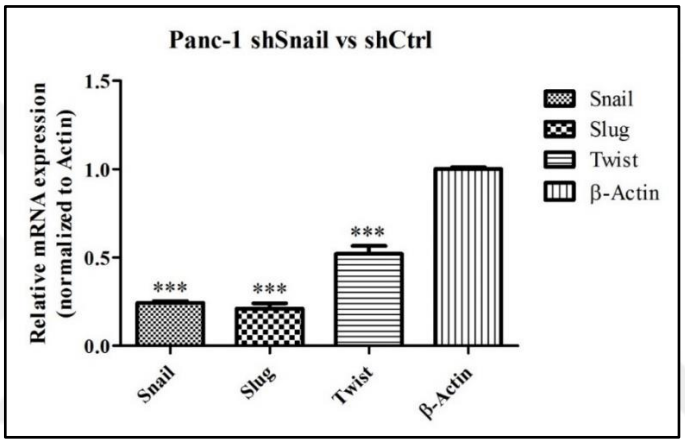


Figure 3.6 Graphical representation of gene expression decrease in Panc-1 cell line after short hairpin silencing of Snail gene compared to cells transfected with shControl lentiviral particle (*** indicates that the $p<0.001$)

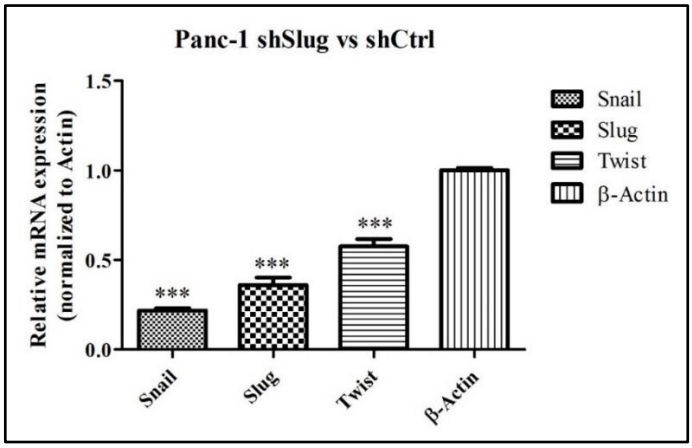


Figure 3.7 Graphical representation of gene expression decrease in Panc-1 cell line after short hairpin silencing of Slug gene compared to cells transfected with shControl lentiviral particle (*** indicates that $p<0.001$)

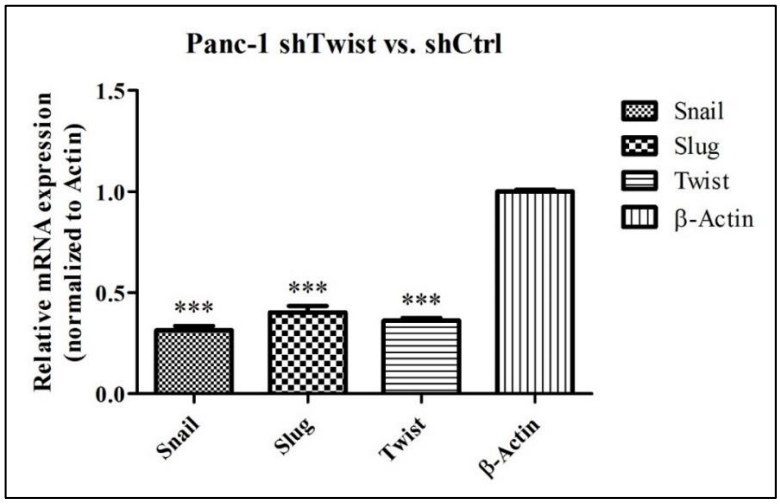


Figure 3.8 Graphical representation of gene expression decrease in Panc-1 cell line after short hairpin silencing of Twist gene compared to cells transfected with shControl lentiviral particle (*** indicates that $p<0.001$)

As it can be seen in the Table 3.1 it is observed that, after short hairpin silencing of Snail; a decrease in Snail, Slug and Twist significantly decreased the expression of snail gene up to 24 per cent compared to Panc-1 cells transfected with shControl plasmid. Additionally; together with Snail gene, expression of Slug gene is also decreased to 21 per cent and Twist expression is decreased to 52 per cent compared to Panc-1 cells transfected with shControl plasmid.

Similarly after short hairpin silencing of Slug gene it is also possible to say that expression of Slug gene is significantly decreased as well as the expression of snail and twist genes compared to Panc-1 cells transfected with shControl plasmid. As shown in Table 3.1 the expression of Snail, Slug and Twist has decreased to 21.7, 36 and 58 per cent respectively.

Transfection with twist short hairpin of Panc-1 cells was resulted with the significant decrease of twist gene together with snail and slug genes compared to Panc-1 cells transfected with shControl plasmid. As mentioned in Table 3.1, according to the results after Twist silencing, the expressions of Snail, Slug and Twist has decreased to 31, 40 and 36 per cent respectively.

Table 3.1 Relative gene expressions of Snail, Slug and Twist genes after short hairpin silencing compared to shControl transfected cells by using ΔΔCt method and normalized to β-Actin expression.

	Gene Expression Folds of shSnail Compared to shCtrl Panc-1 Cells (normalized to β-Actin)
Snail	0.243
Slug	0.210
Twist	0.520
	Gene Expression Folds of shSlug Compared to shCtrl Panc-1 Cells (normalized to β-Actin)
Snail	0.217
Slug	0.361
Twist	0.578
	Gene Expression Folds of shTwist Compared to shCtrl Panc-1 Cells (normalized to β-Actin)
Snail	0.314
Slug	0.403
Twist	0.361

The values given above are relative expressions of genes compared to cells transfected with shControl plasmid. Values were calculated with ΔΔCt method and normalized to β-Actin.

The Figures, 3.9, 3.10 and 3.11 includes the graphical representations of the fold changes after Snail, Slug and Twist silencing compared to the parental Panc-1 cell line.

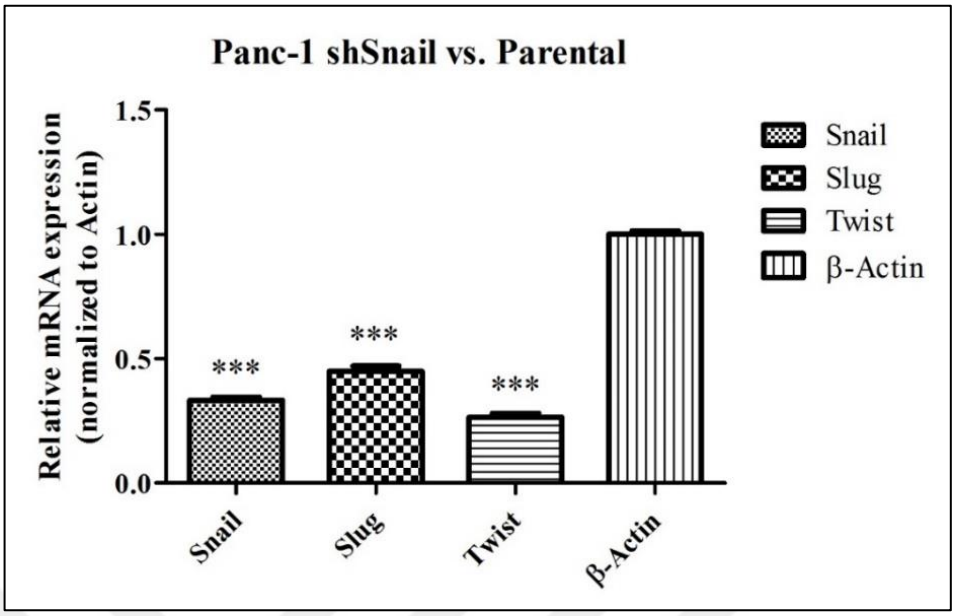


Figure 3.9 Graphical representation of gene expression decrease after short hairpin silencing of Snail gene compared to Panc-1 Parental cell line (*** indicates that $p<0.001$)

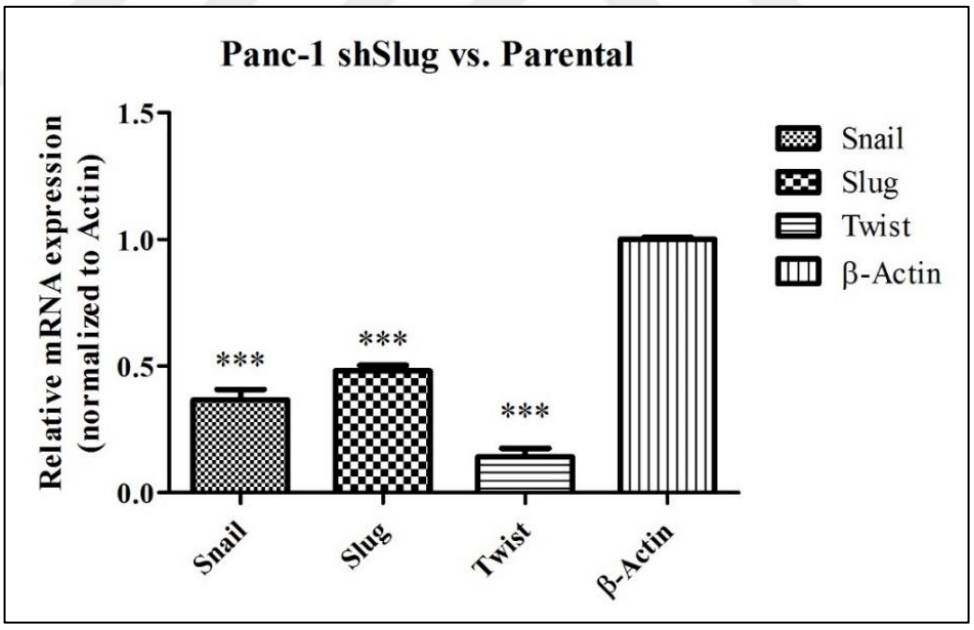


Figure 3.10 Graphical representation of gene expression decrease after short hairpin silencing of Slug gene compared to Panc-1 Parental cell line (*** indicates that $p<0.001$)

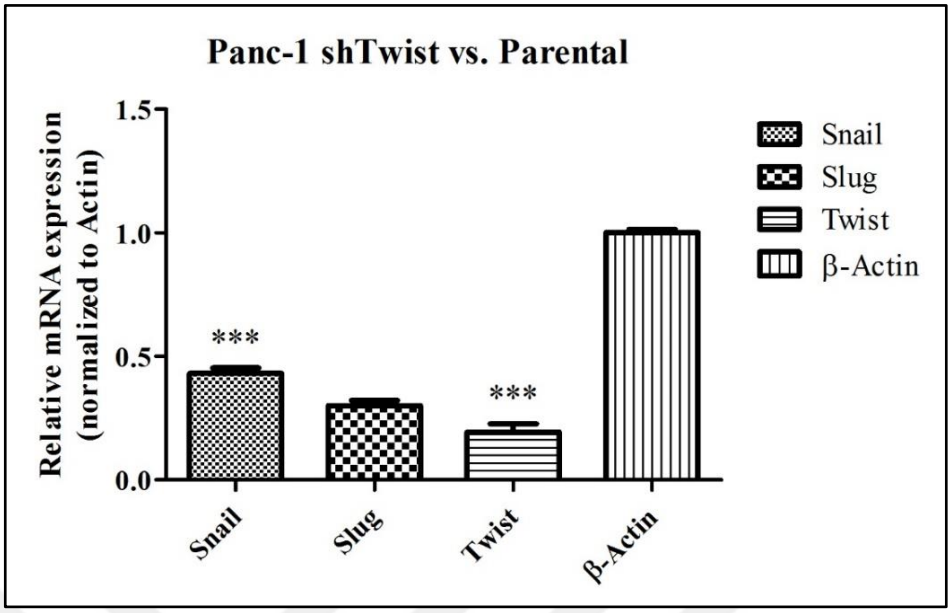


Figure 3.11 Graphical representation of gene expression decrease after short hairpin silencing of Twist gene compared to Panc-1 Parental cell line(*** indicates that $p<0.001$)

It is possible to say that after short hairpin silencing of Snail, expressions of snail, slug and twist genes were significantly decreased compared to Panc-1 parental cell line. As mentioned in the Table 3.2 after silencing Snail in Panc-1 cells the gene expression amount for Snail is decreased to 33.4 percent, expression of Slug is decreased to 44 per cent and expression of Twist has decreased to 2.3 per cent when the results are compared to Parental Panc-1 cells. After short hairpin silencing of Slug, expressions of Slug, Snail and Twist genes were significantly decreased compared to Panc-1 parental cell line and as mentioned in Table 3.2, the expressions values of Snail, Slug and Twist is decreased to 36, 48 and 14 percent when the expression values are compared to Parental Panc-1 cell line. Short hairpin silencing of Twist, downregulated expressions of Twist and Snail and Slug genes. As stated in the Table 3.2, the expression values of Snail, Slug and Twist has decreased to 43, 30 and 19 per cent when compared to the Parental Panc-1 cell line. As the result of the comparison against shControl and Parental Panc-1 cell line, it is possible to say that, silencing one of the selected genes controls the expressional levels of the other two genes in study. It was shown that, empty short hairpin plasmid transfection does not change the effect of gene silencing.

Table 3.2 Relative gene expressions of Snail, Slug and Twist genes after short hairpin silencing compared to Panc-1 Parental cell lineThe values given above are relative expressions of genes compared to Panc-1 parental cell line. Values were calculated with ΔΔCt method and normalized to β-Actin.

	Gene Expression Folds of shSnail Compared to Parental Panc-1 Cells (normalized to β-Actin)
Snail	0.334
Slug	0.449
Twist	0.265
	Gene Expression Folds of shSlug Compared to Parental Panc-1 Cells (normalized to β-Actin)
Snail	0.367
Slug	0.481
Twist	0.142
	Gene Expression Folds of shTwist Compared to Parental Panc-1 Cells (normalized to β-Actin)
Snail	0.430
Slug	0.301
Twist	0.193

The values given above are relative expressions of genes compared to Panc-1 parental cell line. Values were calculated with ΔΔCt method and normalized to β-Actin.

3.2.2. Changes in Gene Expressions of Snail, Slug and Twist Genes after Short Hairpin Silencing in MIA PaCa-2 Cell Line

The Figures 3.12, 3.13 and 3.14 are the graphical representations of the expressions of Snail, Slug and Twist genes after the short hairpin silencing of MIA PaCa-2 cell line. The resulted expression values are determined by comparing the gene silenced groups to shControl transfected cells.

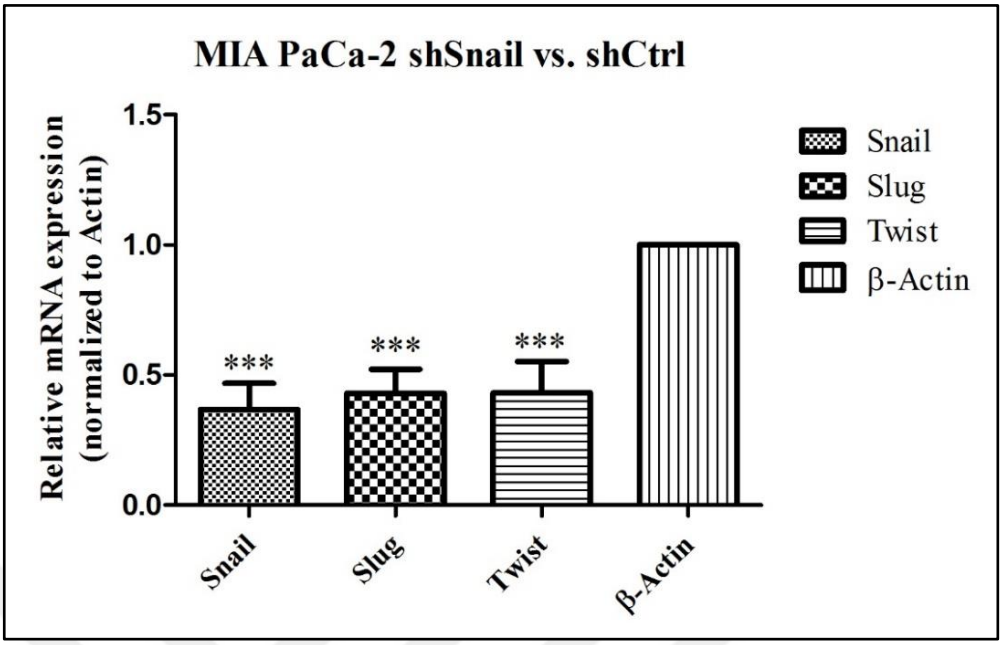


Figure 3.12 Graphical representation of gene expression decrease after short hairpin silencing of Snail genecompared to cells transfected with shControl lentiviral particle (*** indicates that $p<0.001$)

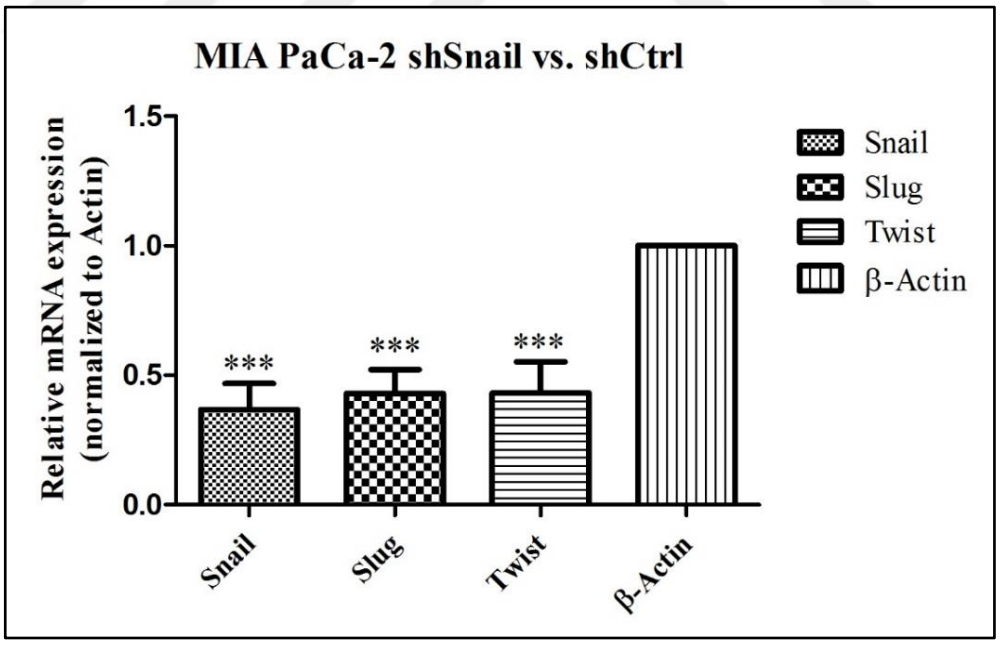


Figure 3.13 Graphical representation of gene expression decrease after short hairpin silencing of Slug genecompared to cells transfected with shControl lentiviral particle (*** indicates that $p<0.001$)

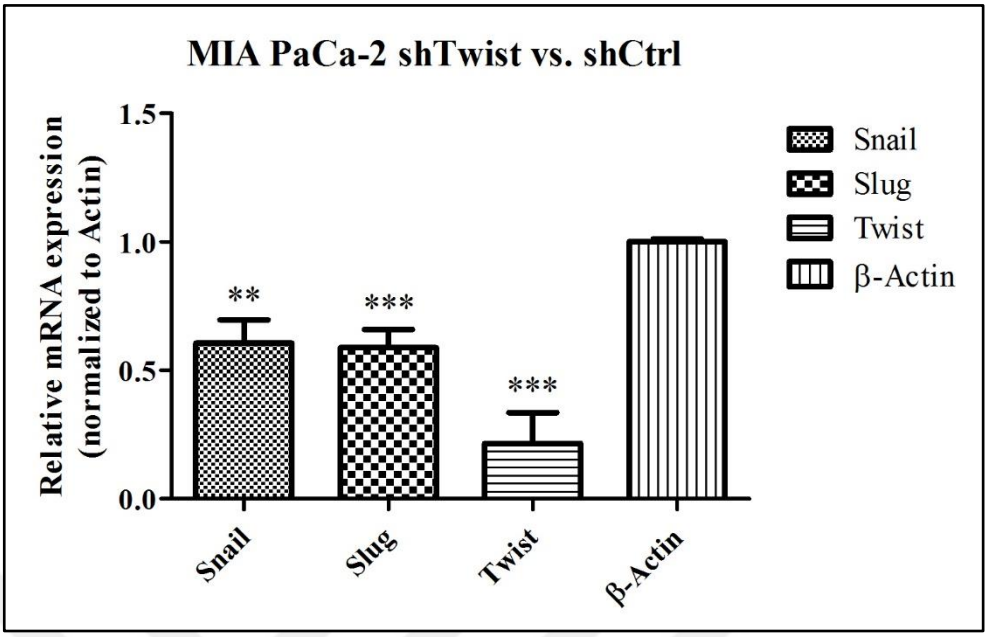


Figure 3.14 Graphical representation of gene expression decrease after short hairpin silencing of Twist genecompared to cells transfected with shControl lentiviral particle (*** indicates that $p<0.001$)

Real time PCR analysis after short hairpin silencing of MIA PaCa-2 cells has shown that; short hairpin silencing of Snail; significantly decreased the expression of Snail gene as well as Slug and Twist genes compared to MIA Paca-2 cells transfected with shControl plasmid. As indicated in Table 3.3 the expression of Snail, Slug and Twist genes is decreased to 36.9, 43 and 43.2 per cent respectively compared to shControl cell line. Short hairpin silencing of Slug, significantly decreased the expression of Slug gene as well as Snail and Twist genes compared to MIA Paca-2 cells transfected with shControl plasmid. As seen in the Table 3.3, expression values of Snail, Slug and Twist is decreased to 64, 30 and 52 per cent respectively compared to the shControl MIA PaCa-2 cell line. Silencing with Twist; significantly decreased the expression of Twist gene together with Snail and Slug genes compared to MIA Paca-2 cells transfected with shControl plasmid. As shown in Table 3.3, the expression values for Snail, Slug and Twist is decreased to 60, 58 and 21 per cent respectively when they are compared to the shControl cell line.

Table 3.3 Relative gene expressions of Snail, Slug and Twist genes after short hairpin silencing compared to shControl transfected cells The values given above are relative expressions of genes compared to MIA PaCa-2 parental cells. Values were calculated with ΔΔCt method and normalized to β-Actin.

	Gene Expression Folds of shSnail Compared to shControl MIA PaCa-2 Cells (normalized to β-Actin)
Snail	0.369
Slug	0.429
Twist	0.432
	Gene Expression Folds of shSlug Compared to shCtrl MIA PaCa-2 Cells (normalized to β-Actin)
Snail	0.643
Slug	0.298
Twist	0.523
	Gene Expression Folds of shTwist Compared to shCtrl MIA PaCa-2 Cells (normalized to β-Actin)
Snail	0.605
Slug	0.588
Twist	0.212

The values given above are relative expressions of genes compared to MIA PaCa-2 shControl cell line. Values were calculated with ΔΔCt method and normalized to β-Actin.

It is possible to say that when the expression of one of these three transcription factors decreases the expressions of the other two also decreases.

Figure 3.15, 3.16 and 3.17 are the graphical representations of the downregulation amount of Snail, Slug and Twist after short hairpin silencing, compared to the parental MIA PaCa-2 cell line.

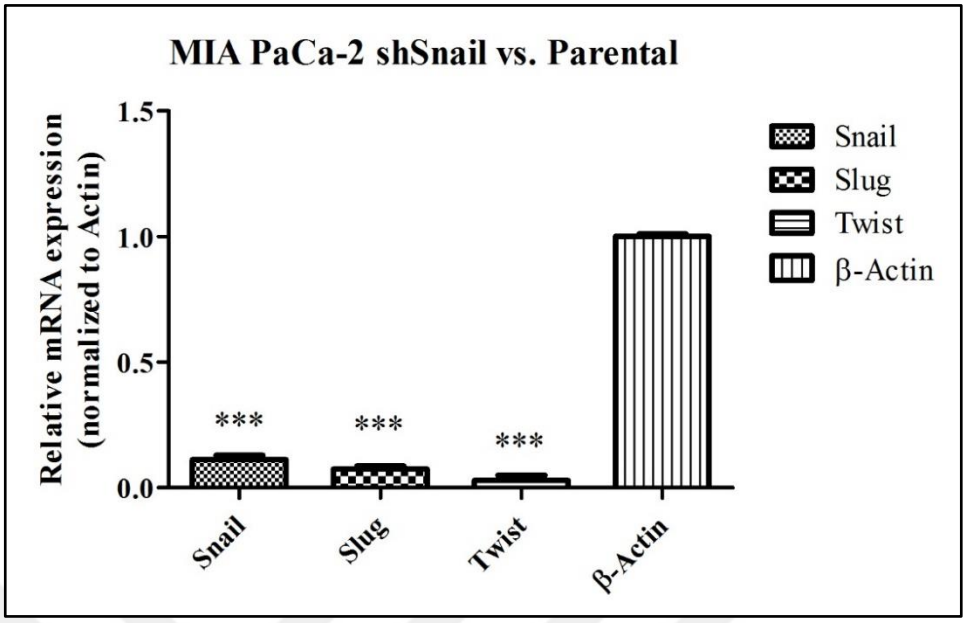


Figure 3.15 Graphical representation of gene expression decrease after short hairpin silencing of Snail gene compared to MIA PaCa-2 Parental cells (*** indicates that $p<0.001$)

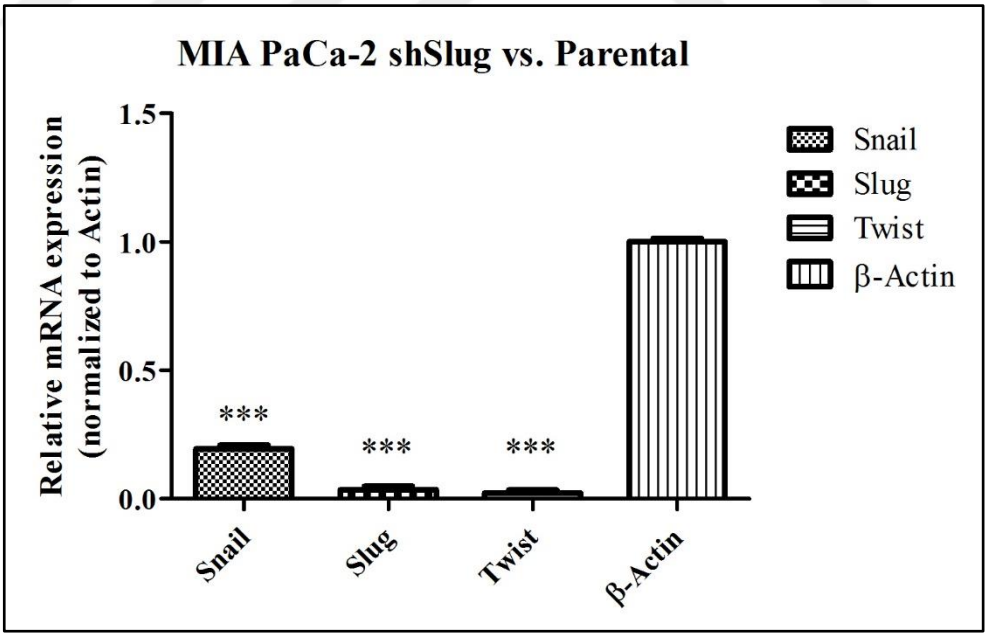


Figure 3.16 Graphical representation of gene expression decrease after short hairpin silencing of Slug gene compared to MIA PaCa-2 Parental cells(*** indicates that $p<0.001$)

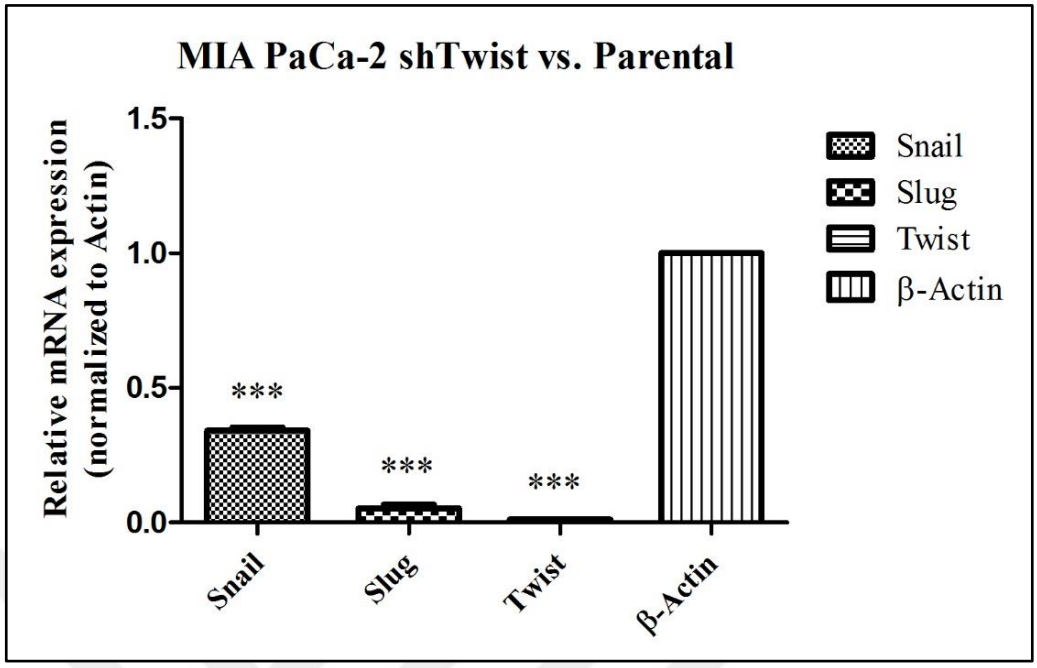


Figure 3.17 Graphical representation of gene expression decrease after short hairpin silencing of Twist gene compared to MIA PaCa-2 Parental cells(*** indicates that $p<0.001$)

Real-time PCR analysis after short hairpin silencing of MIA PaCa-2 cells has shown that; short hairpin silencing of Snail significantly decreased the expression of Snail, Slug and Twist genes significantly compared to MIA PaCa-2 parental cells. As seen in Table 3.4, the expression values of Snail, Slug and Twist to 11, 7.4 and 2.9 per cent respectively when the expressional results are compared to the expression values of MIA PaCa-2 parental cell line.

Short hairpin silencing of Slug has shown to decrease the expression of Slug, Snail and Twist genes significantly compared to MIA PaCa-2 parental cells. As mentioned in the Table 3.4 the expression values of Snail, Slug and Twist are decreased to 19.5, 3.5 and 2.3 per cent compared to the parental MIA PaCa-2 group.

Silencing with Twist; significantly decreased the expression of Twist, Snail and Slug genes compared to MIA PaCa-2 parental cells. As given in the Table 3.4, the expression values for Snail, Slug and Twist are decreased to 34, 0.5 and 0.1 per cent respectively when the results are compared to the Parental MIA PaCa-2 group.

Table 3.4 Relative gene expressions of Snail, Slug and Twist genes after short hairpin silencing compared to MIA PaCa-2 Parental cells The values given above are relative expressions of genes compared to MIA PaCa-2 Parental cell line. Values were calculated with ΔΔCt method and normalized to β-Actin.

	Gene Expression Folds of shSnail Compared to MIA PaCa-2 Parental Cells (normalized to β-Actin)
Snail	0.112
Slug	0.074
Twist	0.029
	Gene Expression Folds of shSlug Compared to MIA PaCa-2 Parental Cells (normalized to β-Actin)
Snail	0.195
Slug	0.035
Twist	0.023
	Gene Expression Folds of shTwist Compared to MIA PaCa-2 Parental Cells (normalized to β-Actin)
Snail	0.342
Slug	0.0525
Twist	0.0116

The values given above are relative expressions of genes compared to MIA PaCa-2 parental cell line. Values were calculated with ΔΔCt method and normalized to β-Actin. The results indicate that there is a great amount of decrease in the gene expression values of Snail, Slug and Twist, when the results are compared to the parental MIA PaCa-2 group. This indicates that the expressional values for these genes have also decreased by empty short hairpin plasmid transfection.

3.2.3. Changes in Gene Expressions of Snail, Slug and Twist Genes after Short Hairpin Silencing in BxPc-3 Cell Line

Figure 3.18, 3.19 and 3.20 are the graphical representations of the changed gene expression values after the treatment with short hairpins. The values that are presented in graphs are the expression values of genes in folds when they are compared to shControl transfected BxPc-3 cell line.

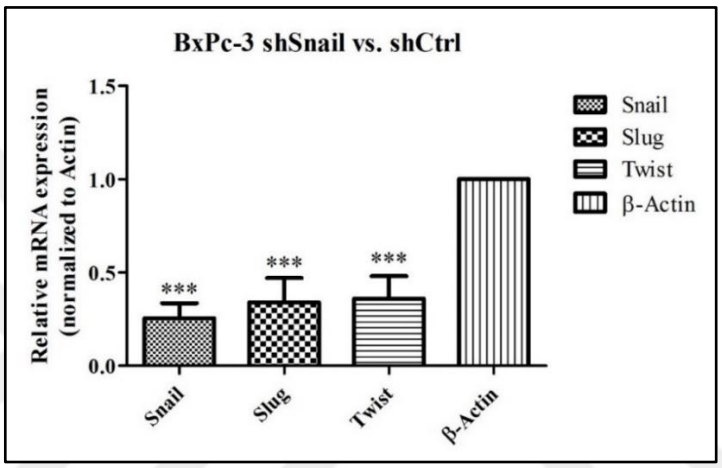


Figure 3.18 Graphical representation of gene expression decrease after short hairpin silencing of Snail genecompared to cells transfected with shControl lentiviral particle (*** indicates that p<0.001)

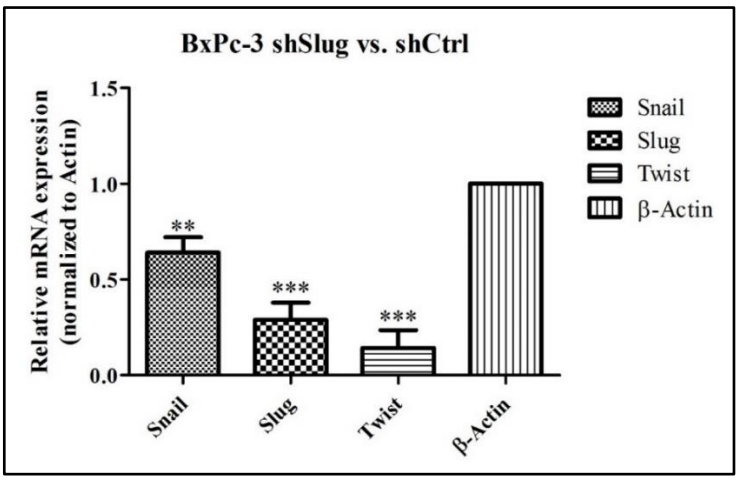


Figure 3.19 Graphical representation of gene expression decrease after short hairpin silencing of Slug genecompared to cells transfected with shControl lentiviral particle(*** indicates that p<0.001)

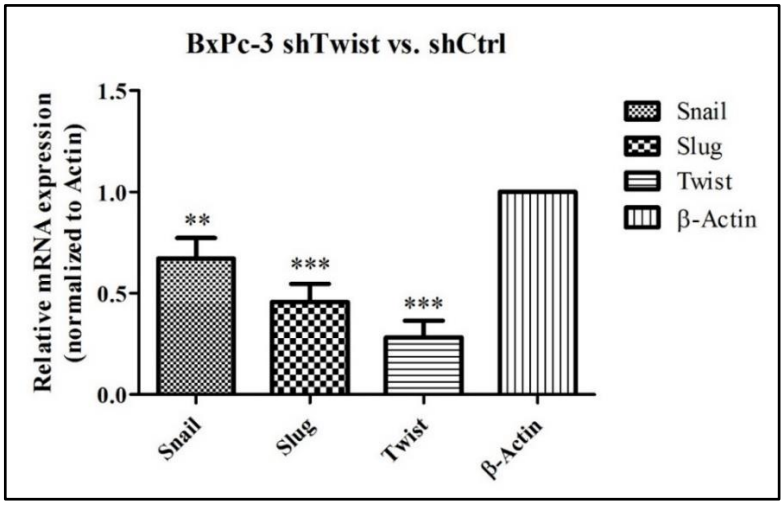


Figure 3.20 Graphical representation of gene expression decrease after short hairpin silencing of Twist genecompared to cells transfected with shControl lentiviral particle (*** indicates that $p<0.001$)

It is observed that, after short hairpin silencing of Snail; significantly decreased the expression of snail gene compared to BxPc-3 cells transfected with shControl plasmid. Additionally; together with snail gene, expression of slug gene and twist gene are also decreased compared to BxPc-3 cells transfected with shControl plasmid. As given in the Table 3.5, the expression values for Snail, Slug and Twist were decreased to 25.6, 34 and 36 per cent respectively when compared to the cells transfected with shControl plasmid.

Similarly after short hairpin silencing of slug gene it is also possible to say that expression of Slug gene is significantly decreased as well as the expression of snail gene and twist gene compared to BxPc-3 cells transfected with shControl plasmid. As shown in Table 3.5 the expression values of Snail, Slug and Twist was decreased to 64, 29 and 14 per cent after short hairpin treatment compared to shControl cell line.

Transfection with Twist short hairpin of BxPc-3 cells was resulted with the significant decrease of twist gene together with snail and slug genes compared to BxPc-3 cells transfected with shControl plasmid. As mentioned in Table 3.5, the expression values of Snail, Slug and Twist was decreased to 67, 45 and 28 per cent after short hairpin treatment compared to shControl cell line.

Table 3.5 Relative gene expressions of Snail, Slug and Twist genes after short hairpin silencing compared to shControl transfected cells The values given above are relative expressions of genes compared to BxPc-3 shControl cells. Values were calculated with ΔΔCt method and normalized to β-Actin.

	Gene Expression Folds of shSnail Compared to shCtrl BxPc-3 Cells (normalized to β-Actin)
Snail	0.256
Slug	0.341
Twist	0.361
	Gene Expression Folds of shSlug Compared to shCtrl BxPc-3 Cells (normalized to β-Actin)
Snail	0.640
Slug	0.289
Twist	0.143
	Gene Expression Folds of shTwist Compared to shCtrl BxPc-3 Cells (normalized to β-Actin)
Snail	0.671
Slug	0.456
Twist	0.282

The values given above are relative expressions of genes compared to cells transfected with BxPc-3 shControl plasmid. Values were calculated with ΔΔCt method and normalized to β-Actin.

It is possible to say that, when one of the mentioned genes is silenced, it influences the expression levels of the other two toward decreasing their expression values too.

The Figures 3.21, 3.22, 3.23 are the graphical representations of the expressional changes of Snail, Slug and Twist genes after short hairpin silencing. The graphics are prepared comparing the expression values to the expressions of the corresponding genes of Parental BxPc-3 cells.

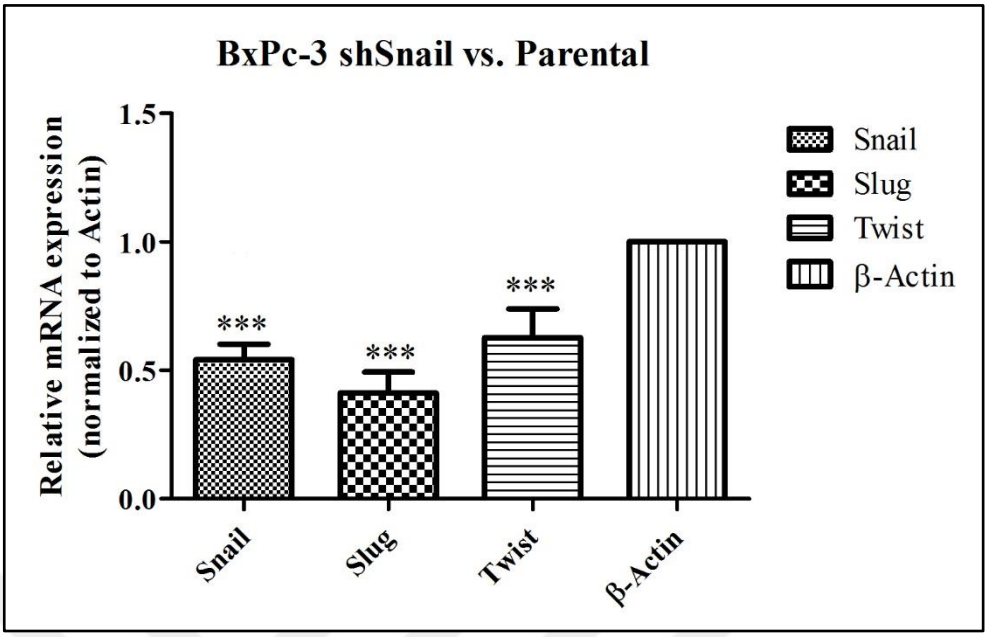


Figure 3.21 Graphical representation of gene expression decrease after short hairpin silencing of Snail gene compared to BxPc-3 Parental cell line(*** indicates that p<0.001)

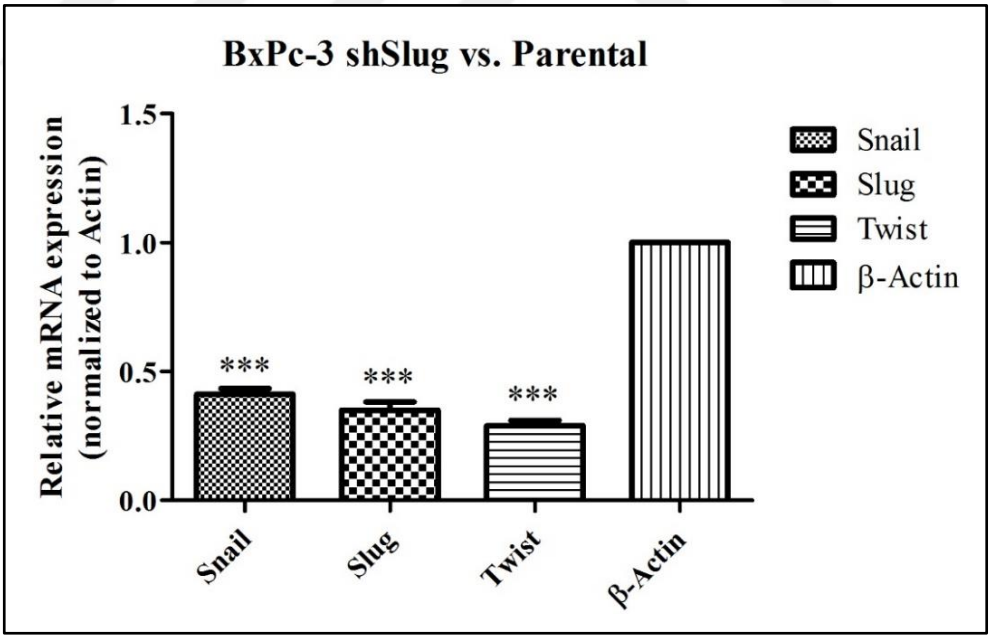


Figure 3.22 Graphical representation of gene expression decrease after short hairpin silencing of Slug gene compared to BxPc-3 Parental cell line(*** indicates that p<0.001)

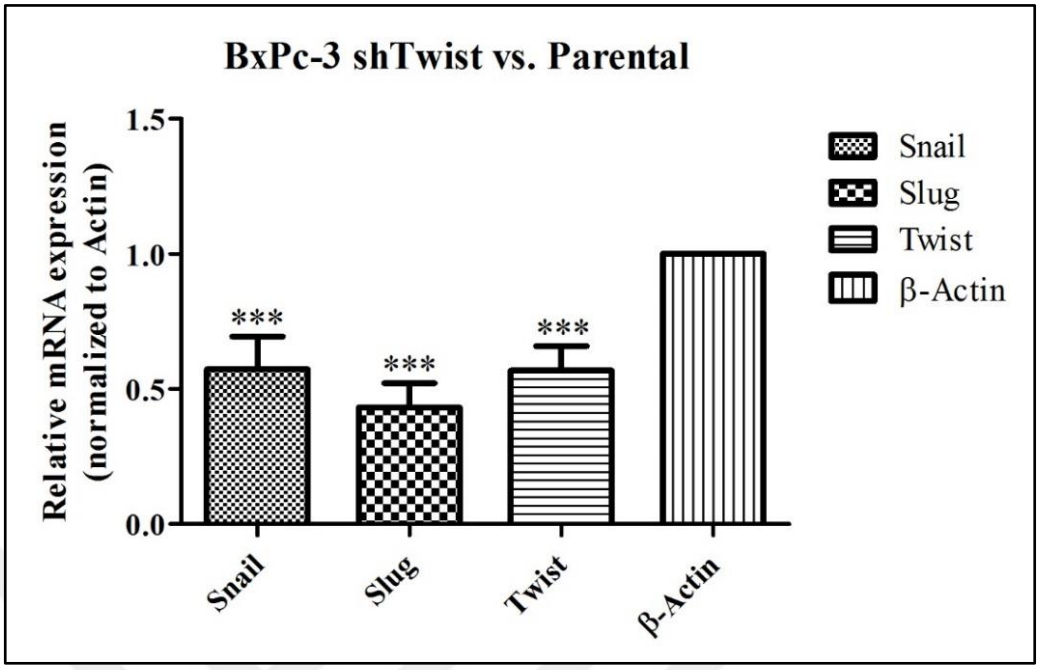


Figure 3.23 Graphical representation of gene expression decrease after short hairpin silencing of Twist gene compared to BxPc-3 Parental cell line(*** indicates that $p<0.001$)

It is possible to say that after short hairpin silencing of Snail, expressions of Snail, Slug and Twist genes were significantly decreased compared to BxPc-3 parental cell line. As given in the Table 3.6, the expression levels for Snail, Slug and Twist was decreased to 54, 41 and 62 per cent with short hairpin silencing, compared to the Parental BxPc-3 cell line.

After short hairpin silencing of Slug, expressions of Slug, Snail and Twist genes were significantly decreased compared to BxPc-3 parental cell line. The expression values of Snail, Slug and Twist, as shown in Table 3.6, was decreased to 41, 35 and 28 per cent compared to the Parental BxPc-3 cell line.

After short hairpin silencing of Twist, expressions of Twist, Snail and Slug genes were significantly decreased. As mentioned in Table 3.6, the expression values of Snail, Slug and Twist was decreased to 57, 43 and 56 respectively when compared to the Parental BxPc-3 cell line.

Table 3.6 Relative gene expressions of Snail, Slug and Twist genes after short hairpin silencing compared to Parental BxPc-3 cells The values given above are relative expressions of genes compared to BxPc-3 parental cells. Values were calculated with ΔΔCt method and normalized to β-Actin.

	Gene Expression Folds of shSnail Compared to Parental BxPc-3 Cells (normalized to β-Actin)
Snail	0.542
Slug	0.411
Twist	0.627
	Gene Expression Folds of shSlug Compared to Parental BxPc-3 Cells (normalized to β-Actin)
Snail	0.411
Slug	0.350
Twist	0.289
	Gene Expression Folds of shTwist Compared to Parental BxPc-3 Cells (normalized to β-Actin)
Snail	0.570
Slug	0.431
Twist	0.567

The values given above are relative expressions of genes compared to BxPc-3 parental cell line. Values were calculated with ΔΔCt method and normalized to β-Actin.

When all of the expression values are compared, it is possible to say that, short hairpin silencing successfully decreases the expression of their target gene along with the other two genes.

3.2.4. Changes in Gene Expressions of Snail, Slug and Twist Genes after Short Hairpin Silencing in AsPC-1 Cell Line

The Figures 3.24, 3.25 and 3.26 are the graphical representations of the changes in gene expression values after Snail, Slug and Twist downregulation via short hairpin silencing, compared to the expression values of cells transfected with the empty control lentiviral particles as control

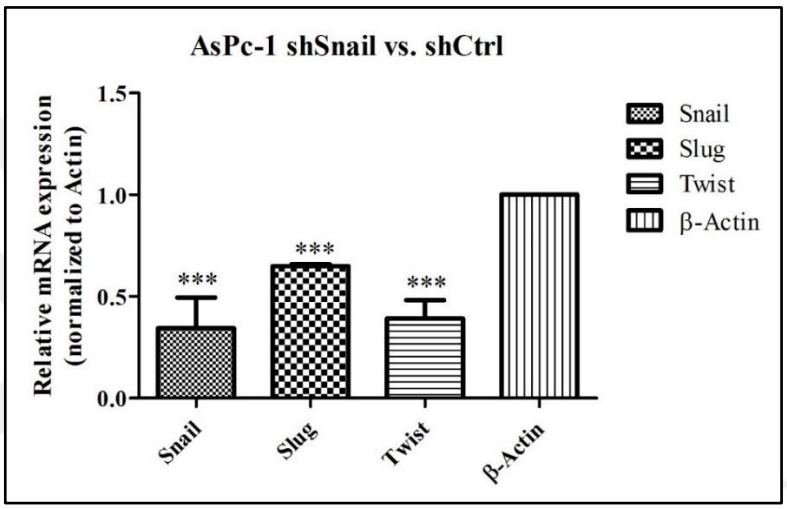


Figure 3.24 Graphical representation of gene expression decrease after short hairpin silencing of Snail genecompared to AsPc-1 shCtrl cell line (*** indicates that p<0.001)

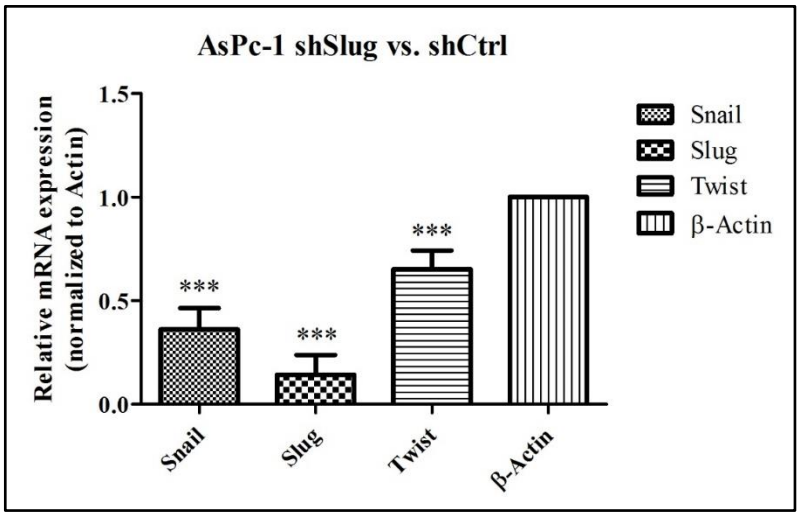


Figure 3.25 Graphical representation of gene expression decrease after short hairpin silencing of Slug genecompared to AsPc-1 shCtrl cell line (*** indicates that p<0.001)

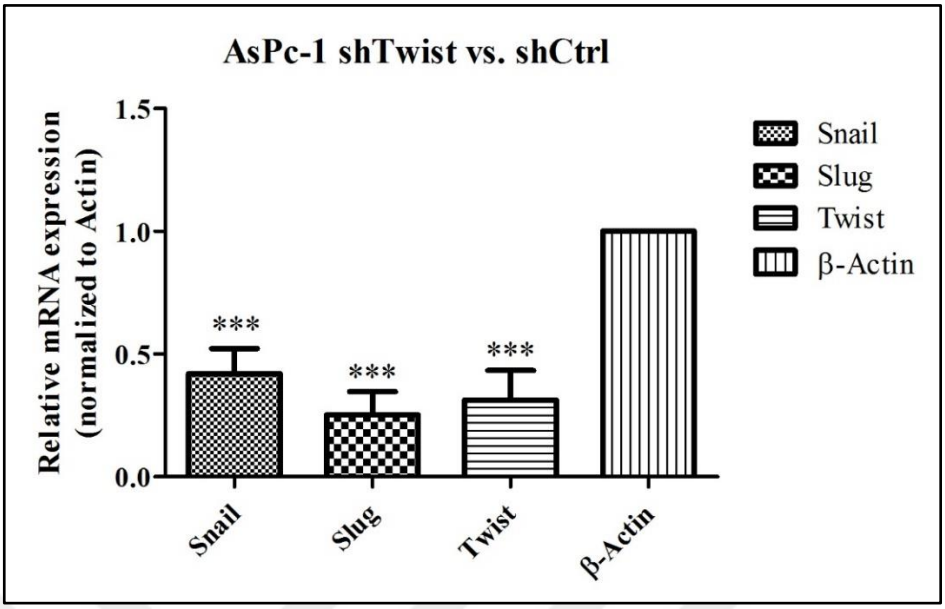


Figure 3.26 Graphical representation of gene expression decrease after short hairpin silencing of Twist genecompared to AsPc-1 shCtrl cell line (*** indicates that $p<0.001$)

After downregulation of Snail, the expression of Snail, Slug and Twist genes were found to be decreased significantly when compared to AsPc-1 shControl cell line. As given in the Table 3.7 expression values of Snail, Slug and Twist has decreased to 34, 65 and 39 per cent respectively.,

Slug short hairpin silencing has shown a significant decreased in Snail, Slug and Twist genes compared to AsPc-1 shControl cell line. As given in the Table 3.7 expression values of Snail, Slug and Twist has decreased to 36, 14 and 65 per cent respectively.,

It is possible to say that Twist silencing with short hairpin has resulted with a significant decrease of Snail, Slug and Twist genes, compared to AsPc-1 shControl cell line. As given in the Table 3.7 expression values of Snail, Slug and Twist has decreased to 42, 25 and 31 per cent respectively.

Table 3.7 Relative gene expressions of Snail, Slug and Twist genes after short hairpin silencing compared to shControl transfected cells The values given above are relative expressions of genes compared to AsPc-1 shControl cells. Values were calculated with ΔΔCt method and normalized to β-Actin.

	Gene Expression Folds of shSnail Compared to AsPc-1 shControl Cells (normalized to β-Actin)
Snail	0.344
Slug	0.65
Twist	0.392
	Gene Expression Folds of shSlug Compared to AsPc-1 shControl Cells (normalized to β-Actin)
Snail	0.362
Slug	0.142
Twist	0.652
	Gene Expression Folds of shTwist Compared to AsPc-1 shControl Cells (normalized to β-Actin)
Snail	0.420
Slug	0.252
Twist	0.313

The values given above are relative expressions of genes compared to AsPc-1 shControl cell line. Values were calculated with ΔΔCt method and normalized to β-Actin.

Figures 3.27, 3.28 and 3.29 are the graphical representation of compared gene expression values of Snail, Slug and Twist to the parental AsPc-1 cells.

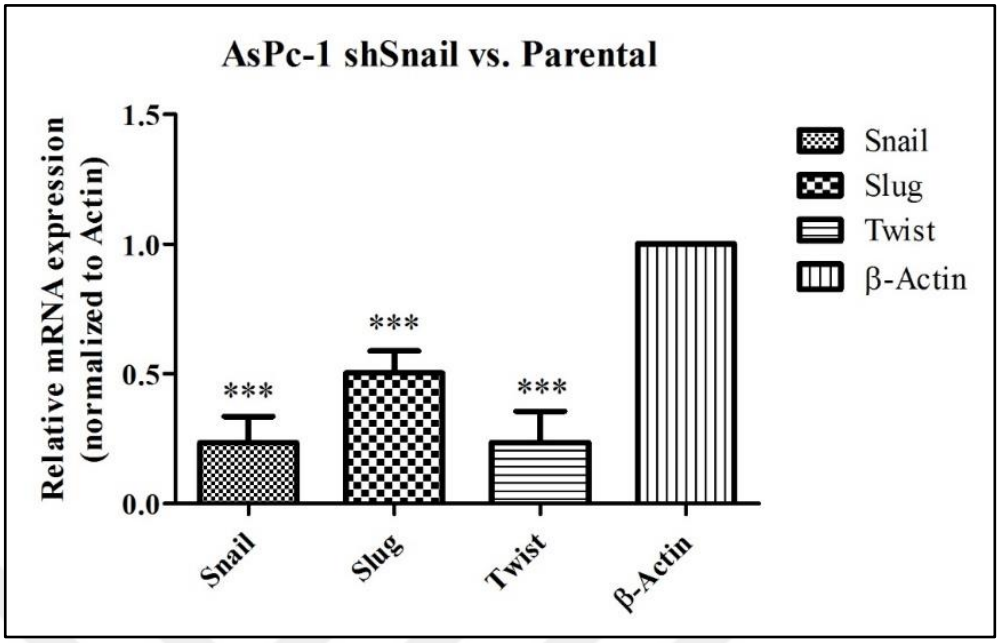


Figure 3.27 Graphical representation of gene expression decrease after short hairpin silencing of Snail gene compared to AsPc-1 Parental cell line compared to AsPc-1 shCtrl cell line (*** indicates that p<0.001)

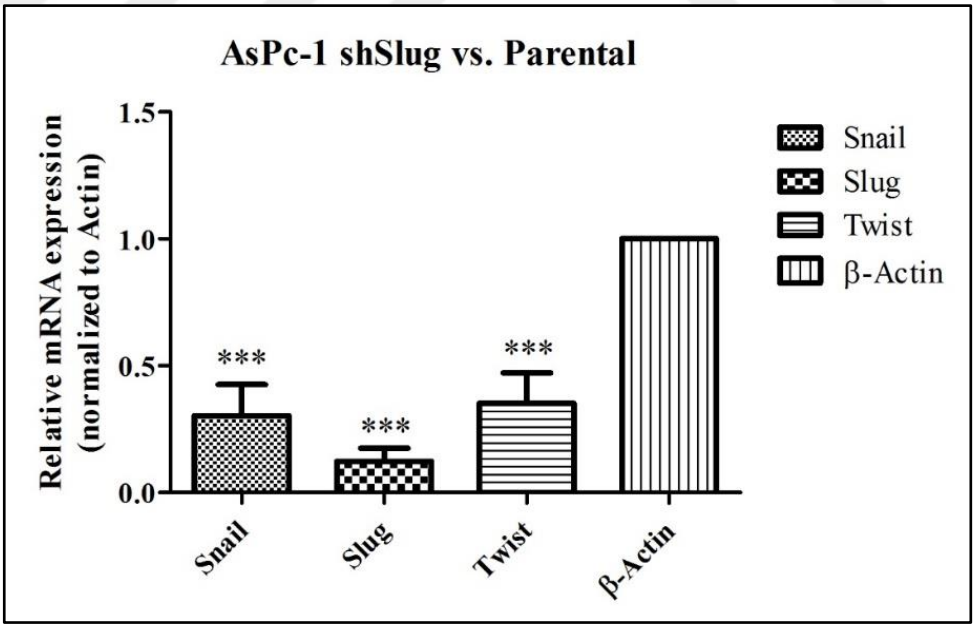


Figure 3.28 Graphical representation of gene expression decrease after short hairpin silencing of Slug gene compared to AsPc-1 Parental cell linecompared to AsPc-1 shCtrl cell line (*** indicates that p<0.001)

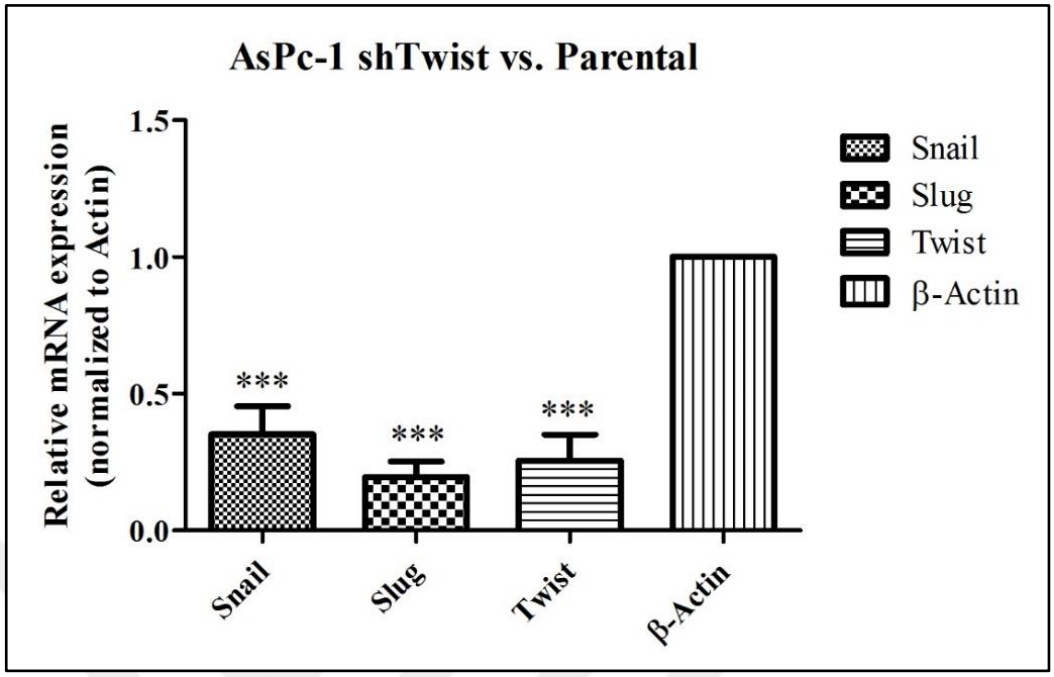


Figure 3.29 Graphical representation of gene expression decrease after short hairpin silencing of Twist gene compared to AsPc-1 Parental cell linecompared to AsPc-1 shCtrl cell line (*** indicates that $p<0.001$)

It is possible to say that after short hairpin silencing of Snail, expressions of Snail, Slug and Twist genes were significantly decreased compared to AsPc-1 parental cell line. As given in the Table 3.8 the expression values for Snail, Slug and Twist are decreased to 23, 50 and 23.6 per cent compared to the parental AsPc-1 cells.

After short hairpin silencing of Slug, expressions of Slug, Snail and Twist genes were significantly decreased compared to AsPc-1 parental cell line. As stated in the Table 3.8 the expression values for Snail, Slug and Twist are decreased to 30, 12 and 35 per cent compared to the parental AsPc-1 cells.

After short hairpin silencing of Twist, expressions of Twist, Snail and Slug genes were significantly decreased compared to AsPc-1 Parental cell line. As given in the Table 3.8 the expression values for Snail, Slug and Twist are decreased to 35, 19 and 25,6 per cent respectively when compared to the parental AsPc-1 cells.

Table 3.8 Relative gene expressions of Snail, Slug and Twist genes after short hairpin silencing compared to parental AsPc-1 cells The values given above are relative expressions of genes compared to AsPc-1 shControl cells. Values were calculated with ΔΔCt method and normalized to β-Actin.

	Gene Expression Folds of shSnail Compared to Parental AsPc-1 Cells (normalized to β-Actin)
Snail	0.234
Slug	0.503
Twist	0.236
	Gene Expression Folds of shSlug Compared to Parental AsPc-1 Cells (normalized to β-Actin)
Snail	0.303
Slug	0.122
Twist	0.352
	Gene Expression Folds of shTwist Compared to Parental AsPc-1 Cells (normalized to β-Actin)
Snail	0.352
Slug	0.195
Twist	0.256

The values given above are relative expressions of genes compared to AsPc-1 Parental cell line. Values were calculated with ΔΔCt method and normalized to β-Actin.

3.3. EVALUATION OF PROTEIN AMOUNT

3.3.1. Changes in Protein Synthesis Levels of Snail, Slug and Twist Genes after Short Hairpin Silencing in Panc-1 Cell Line by Elisa

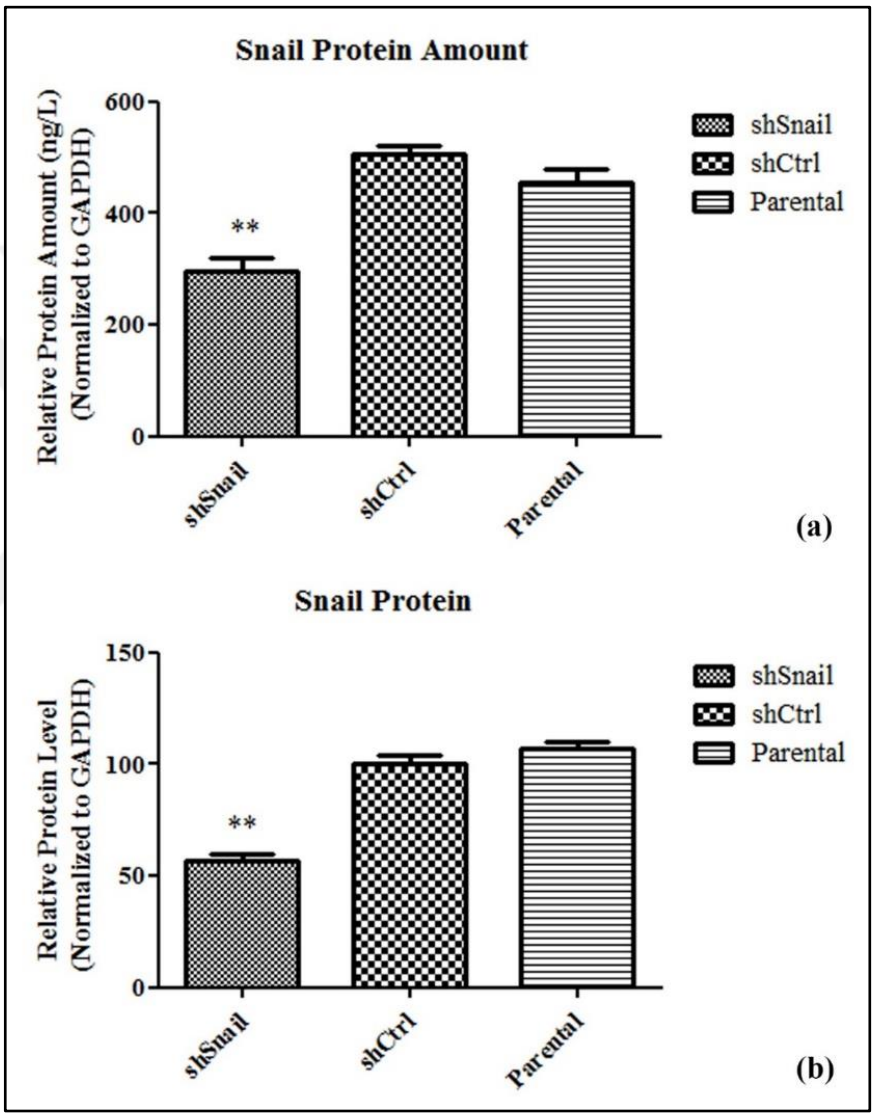


Figure 3.30 Graphical representation of Panc-1 Snail protein (a) Snail protein amount (b) relative snail protein levels in shSnail, shControl and Panc-1 Parental cellsGraphic on top (a) shows the relative calculated snail protein amount in shSnail, shControl and parental Panc-1 cells. Protein amount was calculated by using the equation of the standard curve. Bottom graphic (b) shows the relative percentage of snail protein compared to shControl cells and parental Panc-1 cell line

The results from figure 3.30 indicates that snail protein amount was significantly decreased compared to both shControl and Parental Panc-1 cells.. It is possible to observe that snail protein synthesis in shSnail cell is significantly less compared to both shControl and Parental Panc-1 cells.

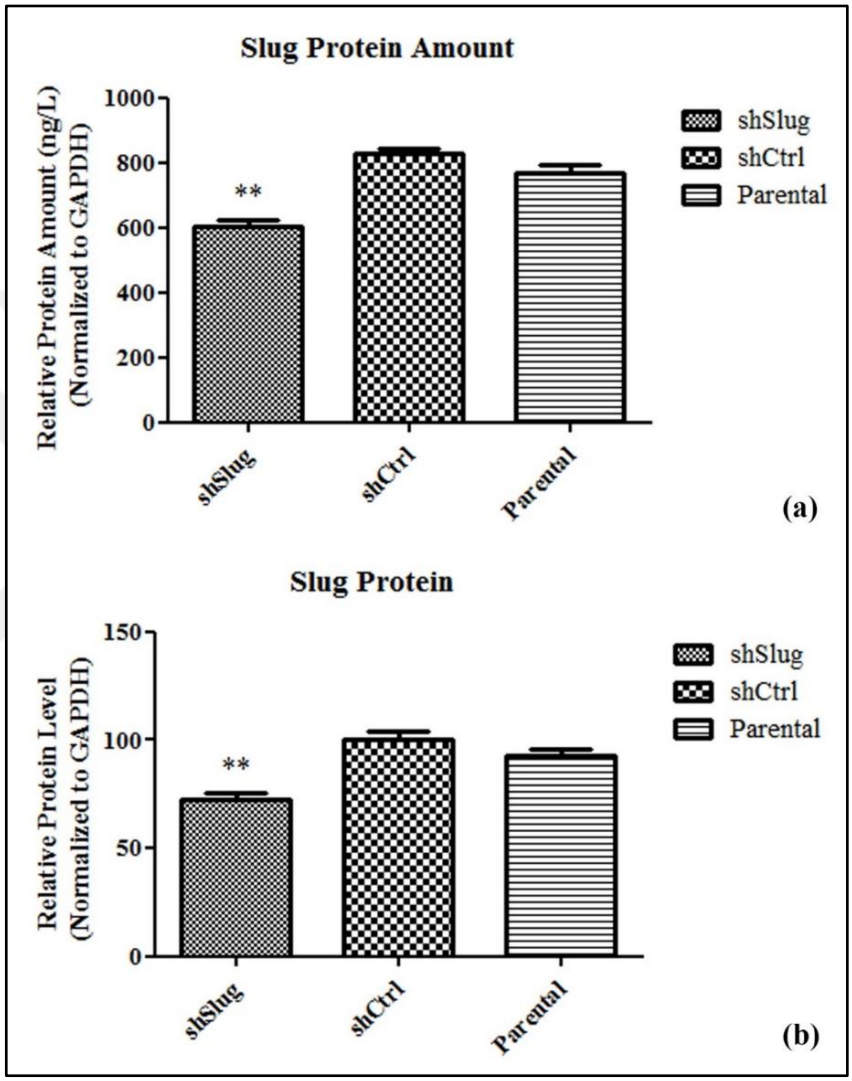


Figure 3.31 Graphical representation of Panc-1 Slug protein (a) Slug protein amount (b) relative slug protein levels in shSlug shControl and Panc-1 Parental cellsGraphic on top (a) shows the relative calculated slug protein amount in shSlug, shControl and parental Panc-1 cells. Protein amount was calculated by using the equation of the standard curve. Bottom graphic (b) shows the relative percentage of slug protein compared to shControl cells and parental Panc-1 cell line.

The results from figure 3.31 indicates that slug protein amount was significantly decreased compared to both shControl and Parental Panc-1 cells. It is possible to observe that slug protein synthesis in shTwist cell is significantly less compared to both shControl and Parental Panc-1 cells.

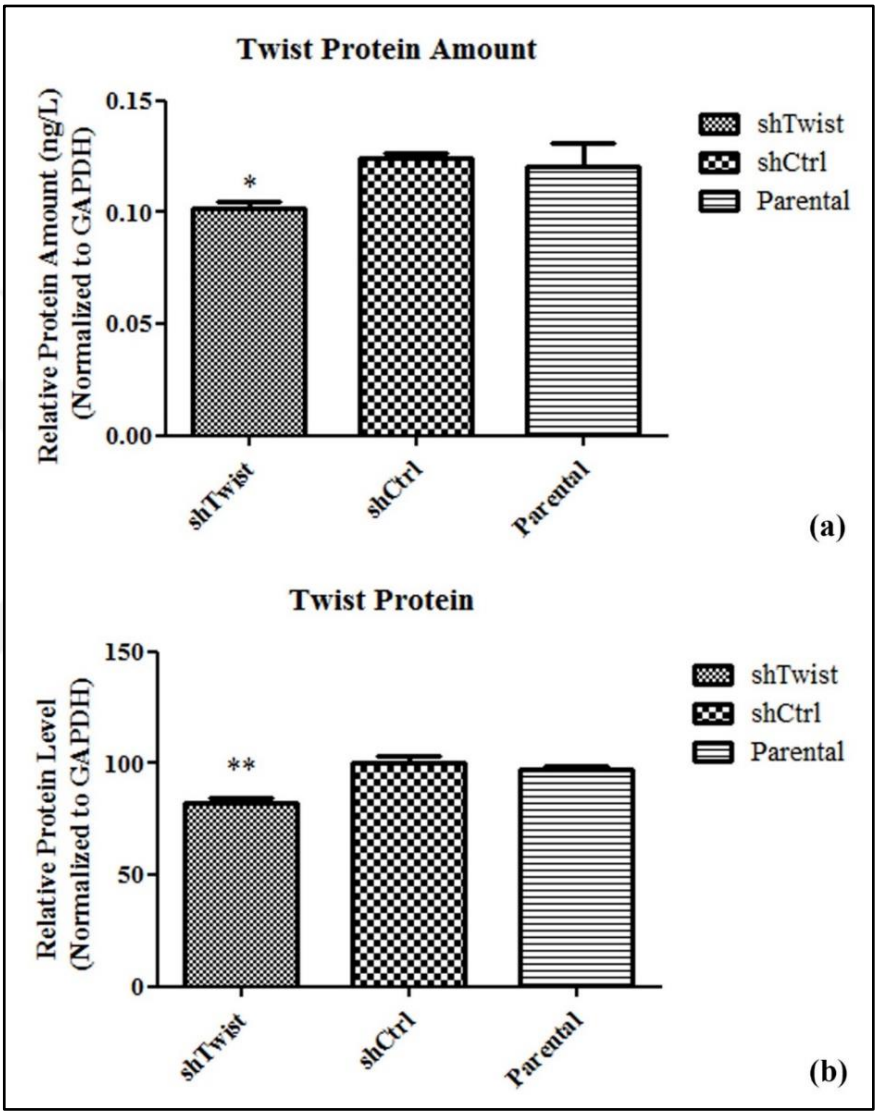


Figure 3.32 Graphical representation of Panc-1 Twist Protein (a) Twist protein amount (b) relative twist protein levels in shTwist, shControl and Panc-1 Parental cellsGraphic on top (a) shows the relative calculated slug protein amount in shTwist, shControl and parental Panc-1 cells. Protein amount was calculated by using the equation of the standard curve. Bottom graphic (b) shows the relative percentage of twist protein compared to shControl cells and parental Panc-1 cell line

The results from Figure 3.32 indicates that twist protein amount was significantly decreased compared to both shControl and Parental Panc-1 cells.. It is possible to observe that twist protein synthesis in shTwist cells is significantly less compared to both shControl and Parental Panc-1 cells.

Table 3.9 Calculated protein amounts and relative percentage values of Snail, Slug and Twist proteins.Table above shows the calculated protein amounts and relative protein percentages (as protein amount of shControl cells was accepted as 100 per cent

Panc-1	**shSnail** (ng/l) / per cent	**shSlug** (ng/l) / per cent	**shTwist** (ng/l) / per cent	**shCtrl** (ng/l) /per cent	**Parental** (ng/l) / per cent
Snail Protein	297.32 58.88%	----------	----------	504.88 100%	454.31 89.98%
Slug Protein	----------	603.41 72.69%	----------	859.40 100%	801.30 93.23%
Twist Protein	----------	----------	0.102 81.92%	0.118 100%	0.116 98.27%

As summarized in Table 3.9 the amounts of Snail was decreased to 58 per cent, the amount of Slug was decreased to 72 per cent and the amount of Twist was decreased to 82 per cent in shSnail, shSlug and shTwist cells respectively. Compared to control and parental Panc-1, Snail, Slug and Twist downregulation decrease is statistically significant according to one-way ANOVA and Tukey’s posttest.

3.3.2. Changes in Protein Synthesis Levels of Snail, Slug and Twist Genes after Short Hairpin Silencing in MIA PaCa-2 Cell Line by Elisa

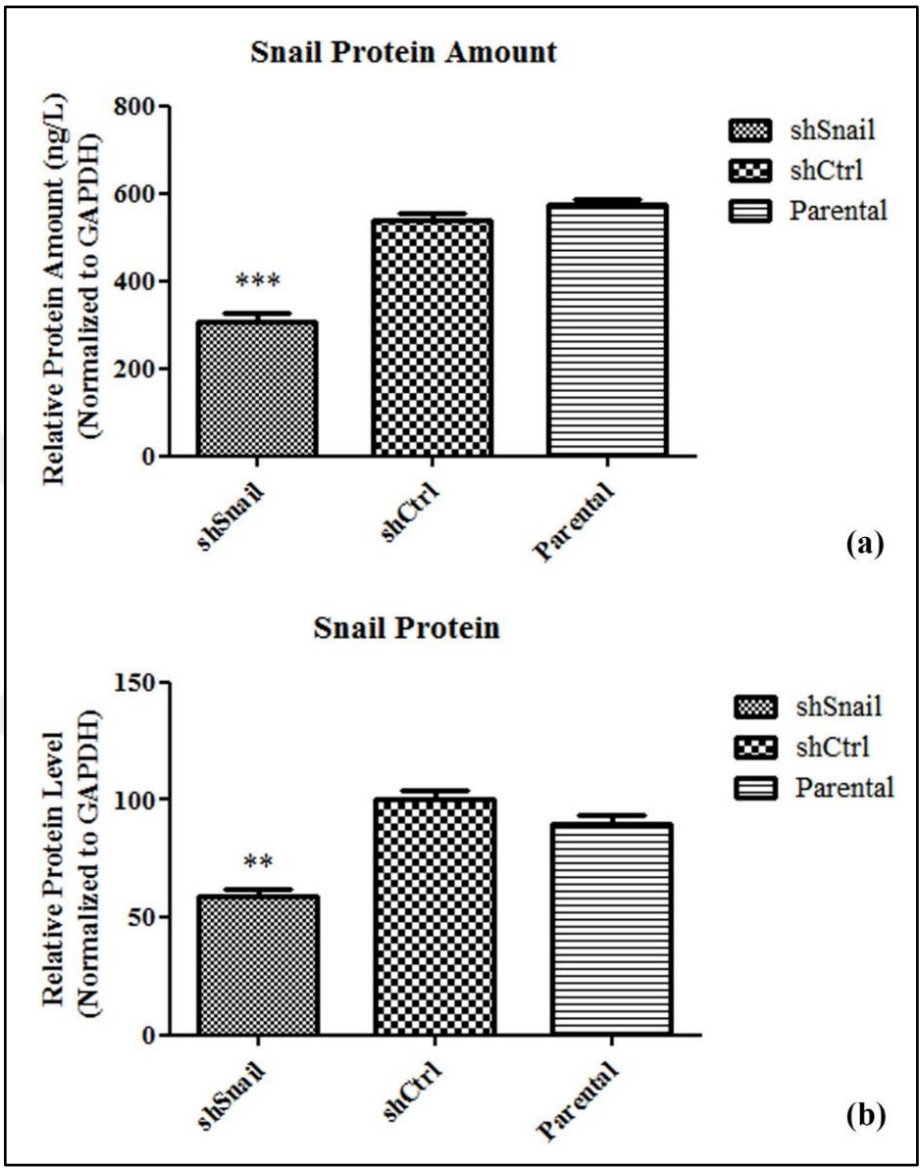


Figure 3.33 Graphical representation of MIA PaCa Snail Protein (a) Snail protein amounts (b) relative snail protein levels in shSnail, shControl and MIA PaCa-2 Parental cells Graphic on top (a) shows the relative calculated snail protein amount in shSnail, shControl and parental MIA PaCa-2 cells. Protein amount was calculated by using the equation of the standard curve. Bottom graphic (b) shows the relative percentage of snail protein compared to shControl cells and parental MIA PaCa-2 cell line.(** indicates that $p<0.005$ and *** indicates that $p<0.001$)

The results in Figure 3.33 indicates that snail protein amount was significantly decreased compared to both shControl and Parental MIA PaCa-2 cells. It is possible to observe that snail protein synthesis in shSnail cell is significantly less compared to both shControl and Parental MIA PaCa-2 cells.

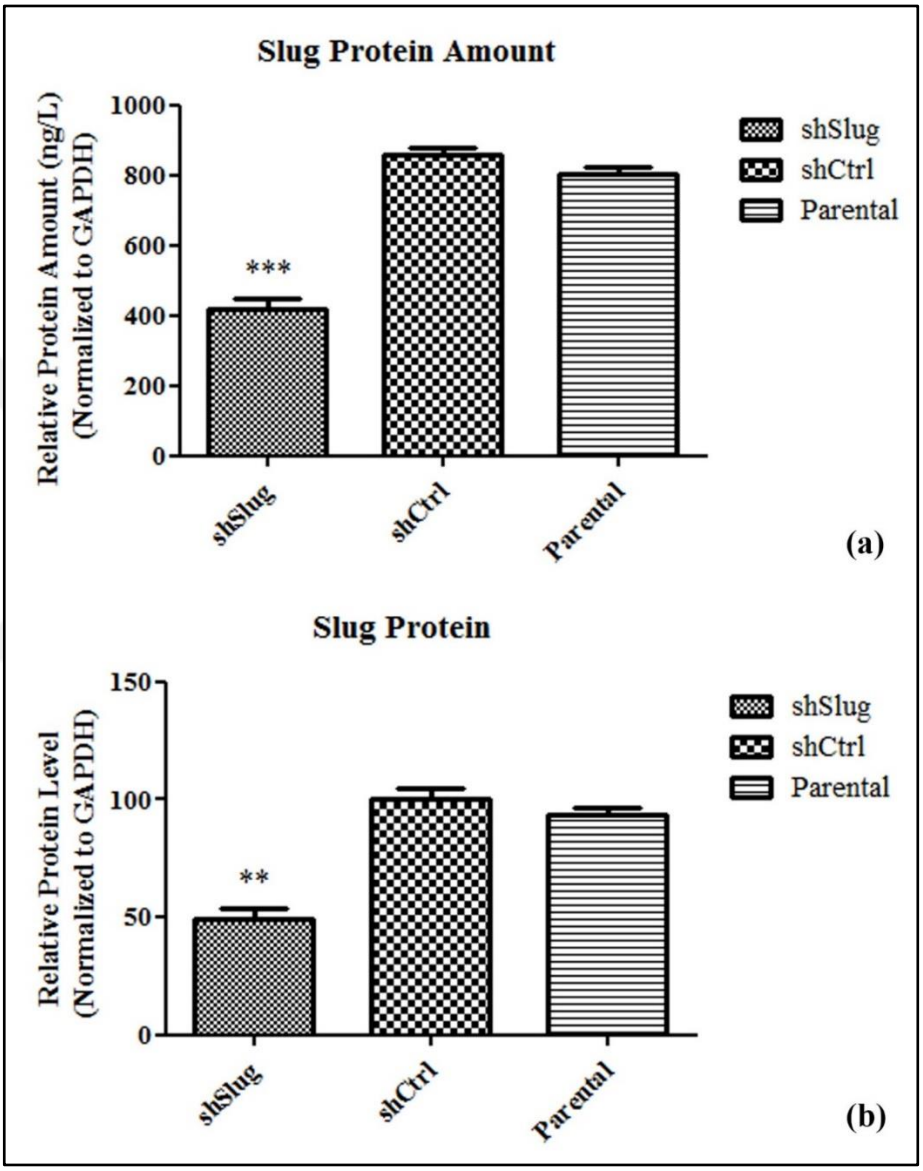


Figure 3.34 Graphical representation of MIA PaCa-2 Slug Protein (a) Slug protein amounts (b) relative slug protein levels in shSlug, shControl and MIA PaCa-2 Parental cells.Graphic on top (a) shows the relative calculated slug protein amount in shSlug, shControl and parental MIA PaCa-2 cells. Protein amount was calculated by using the equation of the standard curve. Bottom graphic (b) shows the relative percentage of slug protein compared to shControl cells and parental MIA PaCa-2 cell line .(** indicates that $p<0.005$ and *** indicates that $p<0.001$)

The results from Figure 3.34 indicates that slug protein amount was significantly decreased compared to both shControl and Parental MIA PaCa-2 cells.. It is possible to observe that slug protein synthesis in shTwist cell is significantly less compared to both shControl and Parental MIA PaCa-2 cells.

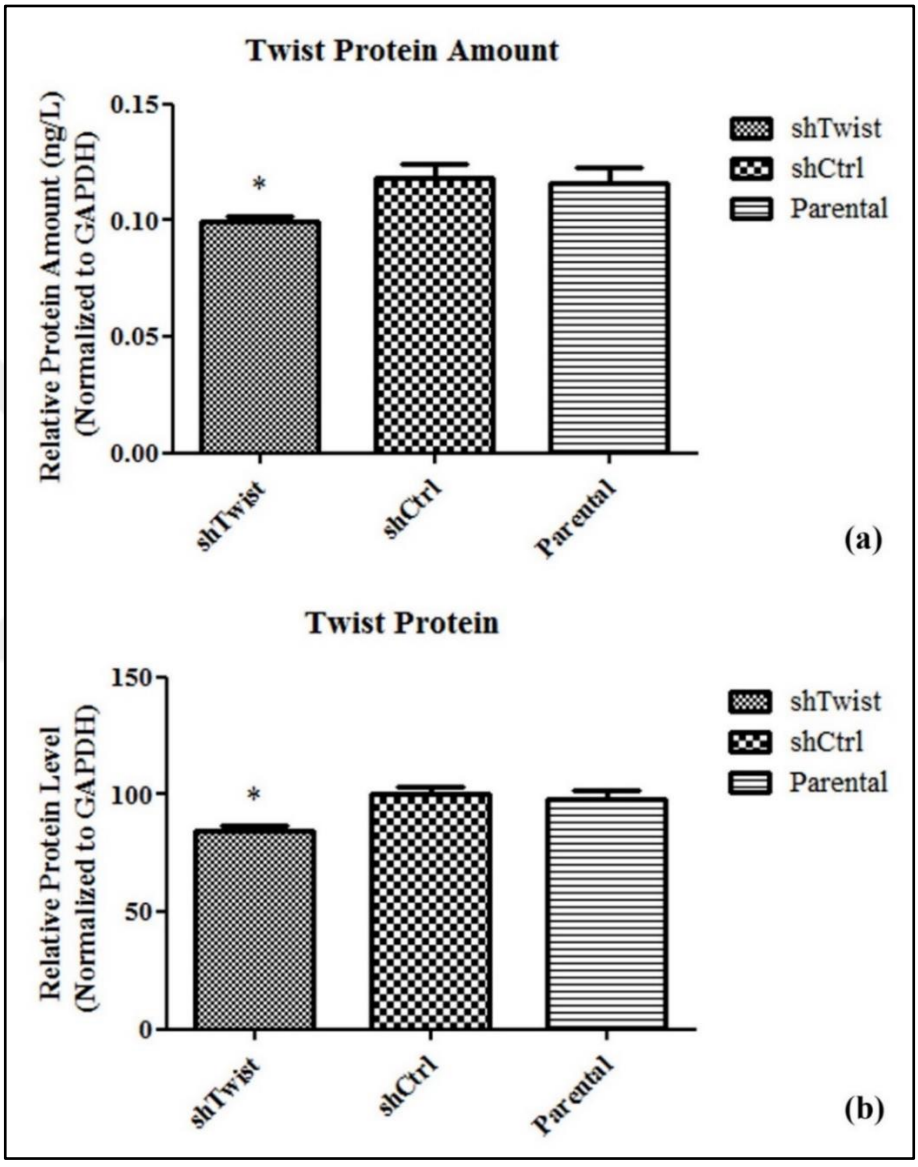


Figure 3.35 Graphical representation of MIA PaCa-2 Twist protein (a) Twist protein amounts (b) relative twist protein levels in shTwist, shControl and MIA PaCa-2 Parental cells Graphic on top (a) shows the relative calculated slug protein amount in shTwist, shControl and parental MIA PaCa-2 cells. Protein amount was calculated by using the equation of the standard curve. Bottom graphic (b) shows the relative percentage of twist protein compared to shControl cells and parental MIA PaCa-2 cell line.(* indicates that $p<0.05$)

The results from Figure 3.35 indicate that twist protein amount was significantly decreased compared to both shControl and Parental MIA PaCa-2 cells.. It is possible to observe that twist protein synthesis in shTwist cells is significantly less compared to both shControl and Parental MIA PaCa-2 cells.

Table 3.10 Calculated protein amounts and relative percentage values of Snail, Slug and Twist proteinsTable below shows the calculated protein amounts and relative protein percentages (as protein amount of shControl cells was accepted as 100 per cent)

MIA PaCa-2	**shSnail** (ng/l) / per cent	**shSlug** (ng/l) / per cent	**shTwist** (ng/l) / per cent	**shCtrl** (ng/l) / per cent	**Parental** (ng/l) / per cent
Snail Protein	304.96 56.73%	----------	----------	537.55 100%	574.29 106.86%
Slug Protein	----------	420.58 48.93%	----------	859.40 100%	801.30 95.25%
Twist Protein	----------	----------	0.099 84.26%	0.118 100%	0.116 98.27%

As summarized in Table 3.10 the amounts of Snail was decreased to 56 per cent, the amount of Slug was decreased to 49 per cent and the amount of Twist was decreased to 84 per cent in shSnail, shSlug and shTwist cells respectively

3.3.3. Changes in Protein Synthesis Levels of Snail, Slug and Twist Genes after Short Hairpin Silencing in BxPc-3 Cell Line by Elisa

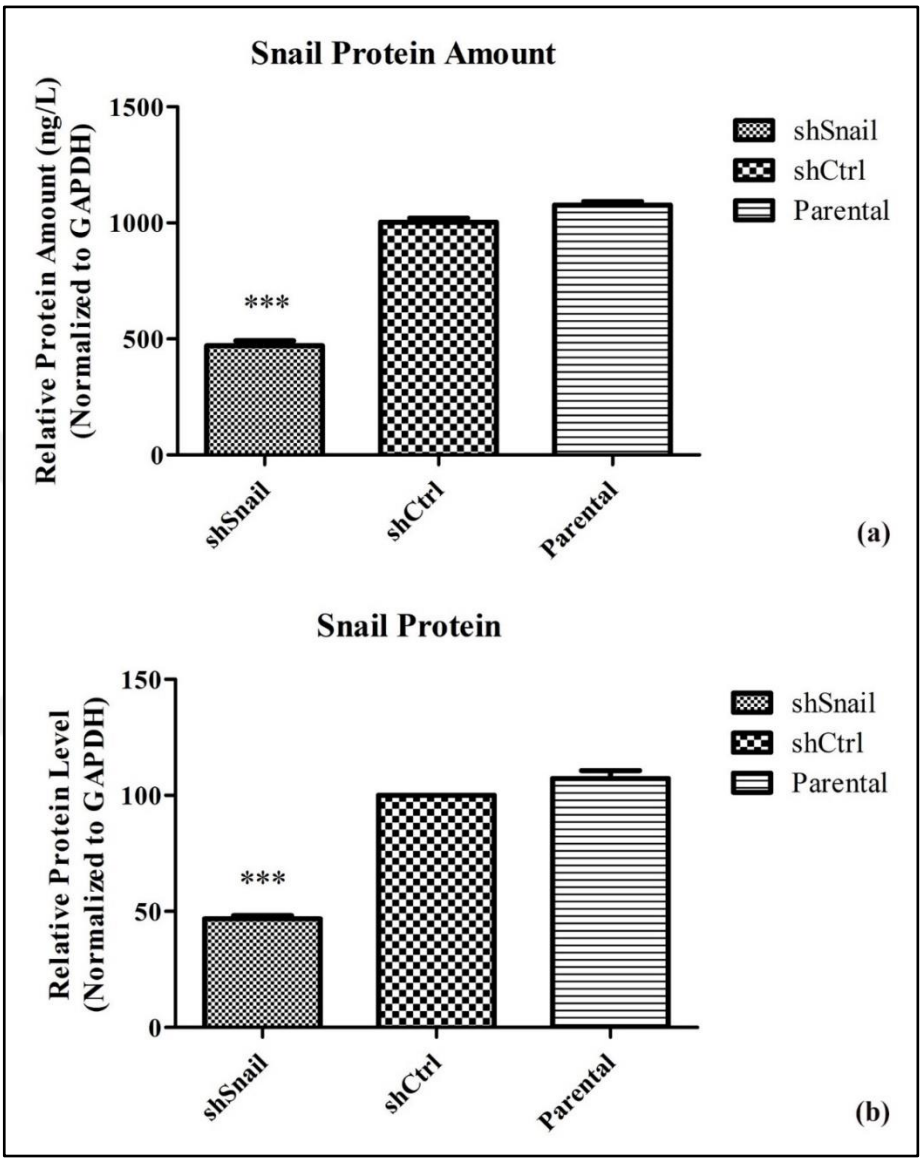


Figure 3.36 Graphical representation of BxPc-3 Snail Protein (a) Snail protein amounts (b) relative snail protein levels in shSnail, shControl and BxPc-3 Parental cells.Graphic on top (a) shows the relative calculated snail protein amount in shSnail, shControl and parental BxPc-3 cells. Bottom graphic (b) shows the relative percentage of snail protein compared to shControl cells and parental BxPc-3 cell line. Protein amount was calculated by using the equation of the standard curve.

The results from figure 3.36 indicates that snail protein amount was significantly decreased compared to both shControl and Parental BxPc-3 cells. It is possible to observe that snail protein synthesis in shSnail cell is significantly less compared to both shControl and Parental BxPc-3 cells.

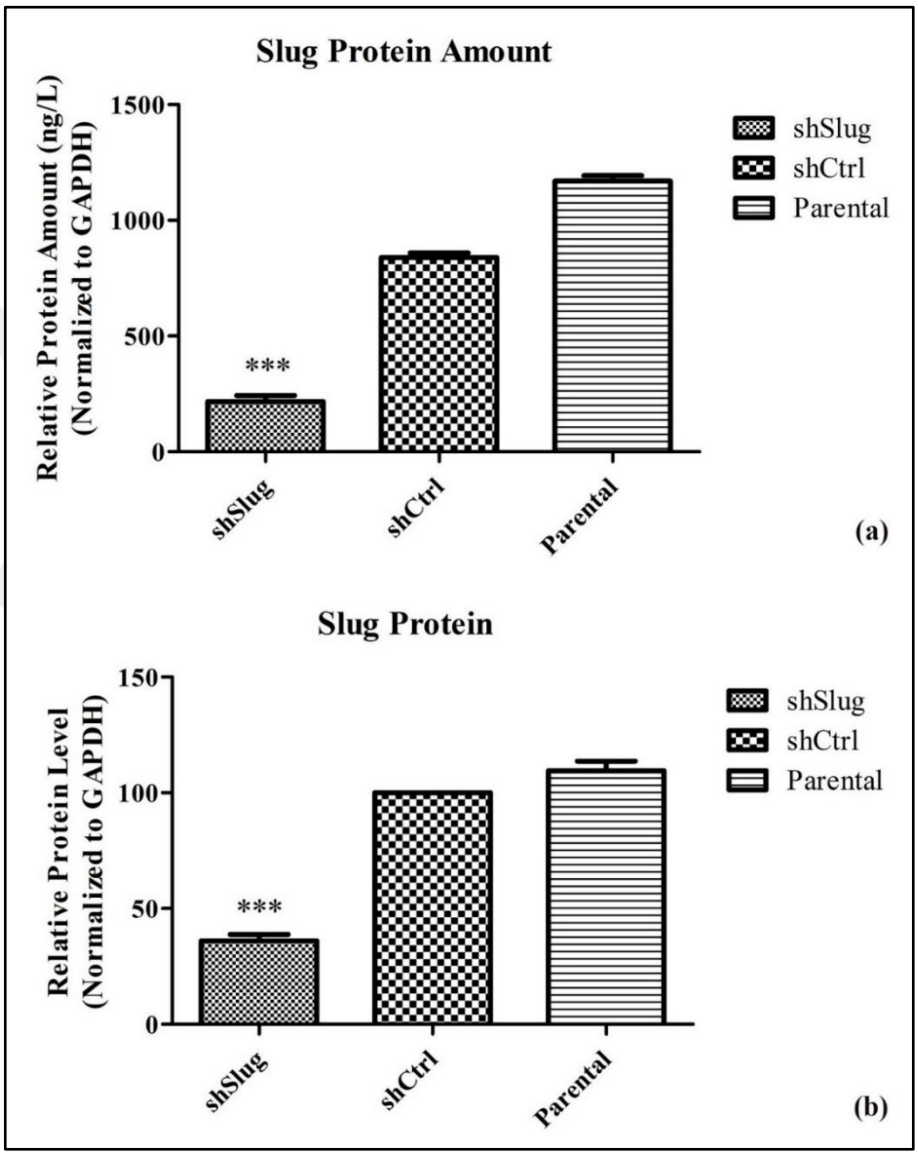


Figure 3.37 Graphical representation of BxPc-3 Slug Protein (a) Slug protein amounts (b) relative slug protein levels in shSlug, shControl and BxPc-3 Parental cellsGraphic on top (a) shows the relative calculated slug protein amount in shSlug, shControl and parental BxPc-3 cells. Bottom graphic (b) shows the relative percentage of slug protein compared to shControl cells and parental BxPc-3 cell line. Protein amount was calculated by using the equation of the standard curve

The results from Figure 3.37 indicates that slug protein amount was significantly decreased compared to both shControl and Parental BxPc-3 cells.. It is possible to observe that slug protein synthesis in shTwist cell is significantly less compared to both shControl and Parental BxPc-3 cells.

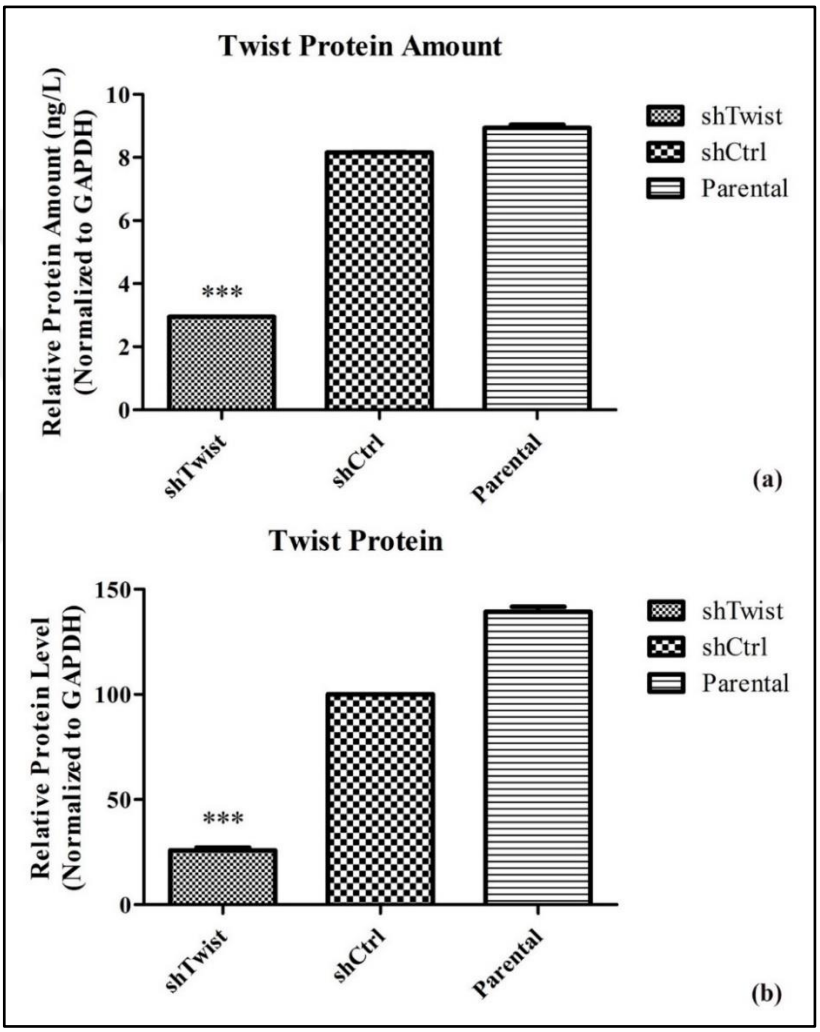


Figure 3.38 Graphical representation of BxPc-3 Twist Protein (a) Twist protein amounts (b) relative twist protein levels in shTwist, shControl and BxPc-3 Parental cellsGraphic on top (a) shows the relative calculated Twist protein amount in shTwist, shControl and parental BxPc-3 cells. Protein amount was calculated by using the equation of the standard curve. Bottom graphic (b) shows the relative percentage of twist protein compared to shControl cells and parental BxPc-3 cell line.

The results from Figure 3.38 indicated that twist protein amount was significantly decreased compared to both shControl and Parental BxPc-3 cells. It is possible to observe that twist protein synthesis in shTwist cells is significantly less compared to both shControl and Parental BxPc-3 cells.

Table 3.11 Calculated protein amounts and relative percentage values of Snail, Slug and Twist proteins.Table below shows the calculated protein amounts and relative protein percentages (as protein amount of shControl cells was accepted as 100 per cent)

BxPc-3	**shSnail** (ng/l)/ per cent	**shSlug** (ng/l)/ per cent	**shTwist** (ng/l)/ per cent	**shCtrl** (ng/l)/ per cent	**Parental** (ng/l)/ per cent
Snail Protein	470.15	----------	----------	1003.72	1077.29
(Percentage)	46.84			100	107.3
Slug Protein	----------	217.17	----------	839.62	1169.56
(Percentage)		25.86		100	139.29
Twist Protein	----------	----------	2.94	8.155	8.93
(Percentage)			36.08	100	109.56

As summarized in Table 3.11 the amounts of Snail was decreased to 46 per cent, the amount of Slug was decreased to 26 per cent and the amount of Twist was decreased to 36 per cent in shSnail, shSlug and shTwist cells respectively

3.3.4. Changes in Protein Synthesis Levels of Snail, Slug and Twist Genes after Short Hairpin Silencing in AsPC-1 Cell Line by Elisa

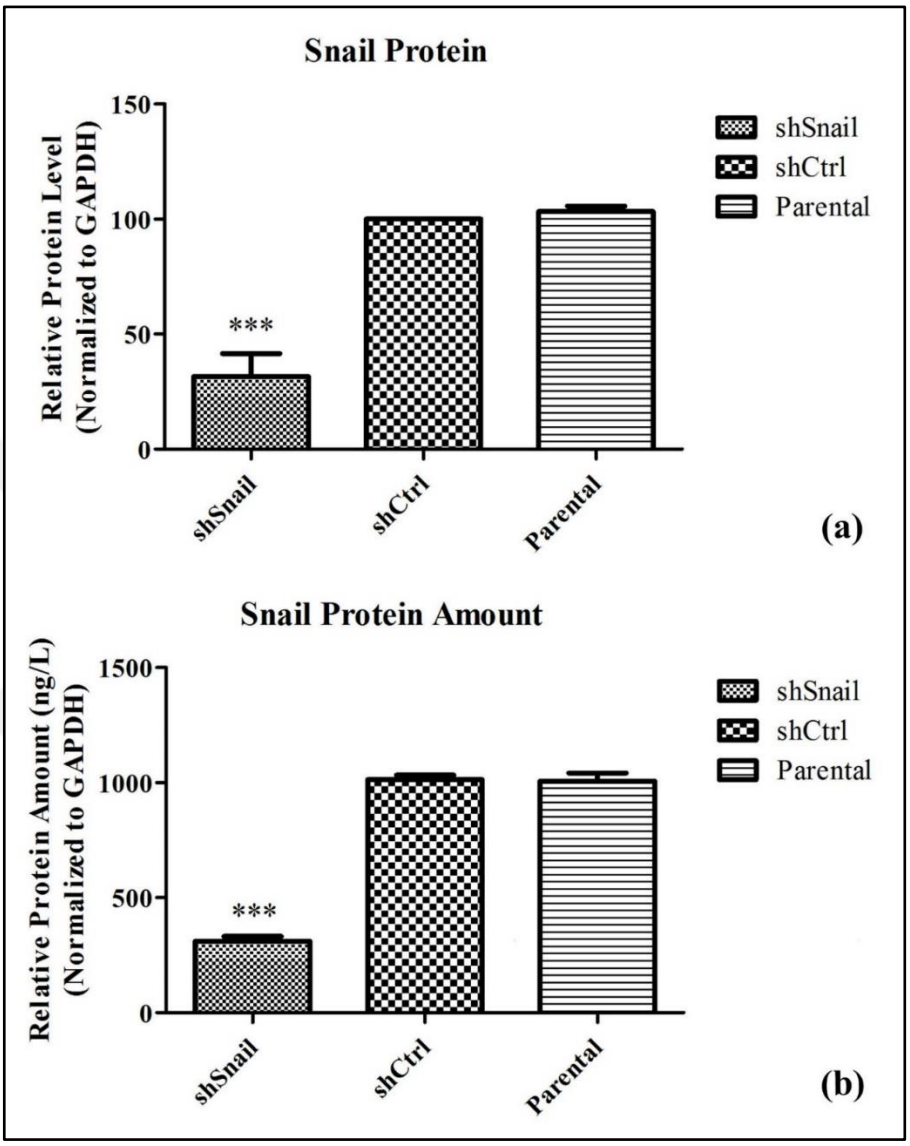


Figure 3.39 Graphical representation of AsPc-1 Snail Protein (a) Snail protein amounts (b) relative snail protein levels in shSnail, shControl and AsPc-1 Parental cellsGraphic on top (a) shows the relative calculated Snail protein amount in shSnail, shControl and parental AsPc-1. Protein amount was calculated by using the equation of the standard curve. Bottom graphic (b) shows the relative percentage of snail protein compared to shControl cells and parental AsPc-1 cell line

The results from Figure 3.39 indicates that snail protein amount was significantly decreased compared to both shControl and Parental AsPc-1 cells. It is possible to observe that snail protein synthesis in shSnail cells is significantly less compared to both shControl and parental AsPc-1 cells.

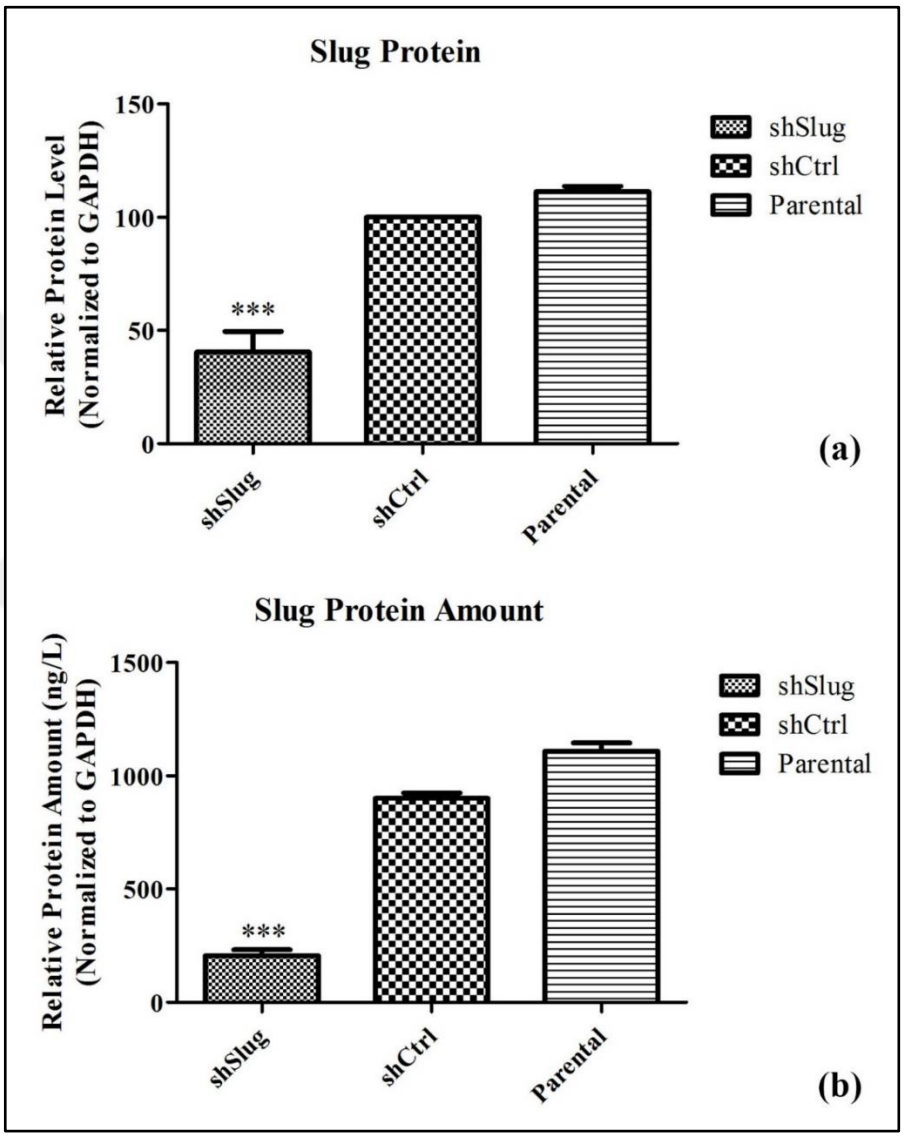


Figure 3.40 Graphical representation of AsPc-1 Slug Protein (a) Slug protein amounts (b) relative slug protein levels in shSlug, shControl and AsPc-1 Parental cellsGraphic on top (a) shows the relative calculated Slug protein amount in shSlug, shControl and parental AsPc-1. Protein amount was calculated by using the equation of the standard curve. Bottom graphic (b) shows the relative percentage of slug protein compared to shControl cells and parental AsPc-1 cell line

The results from 3.40 indicates that slug protein amount was significantly decreased compared to both shControl and Parental AsPc-1 cells.. It is possible to observe that slug protein synthesis in shSlug cells is significantly less compared to both shControl and parental AsPc-1 cells.

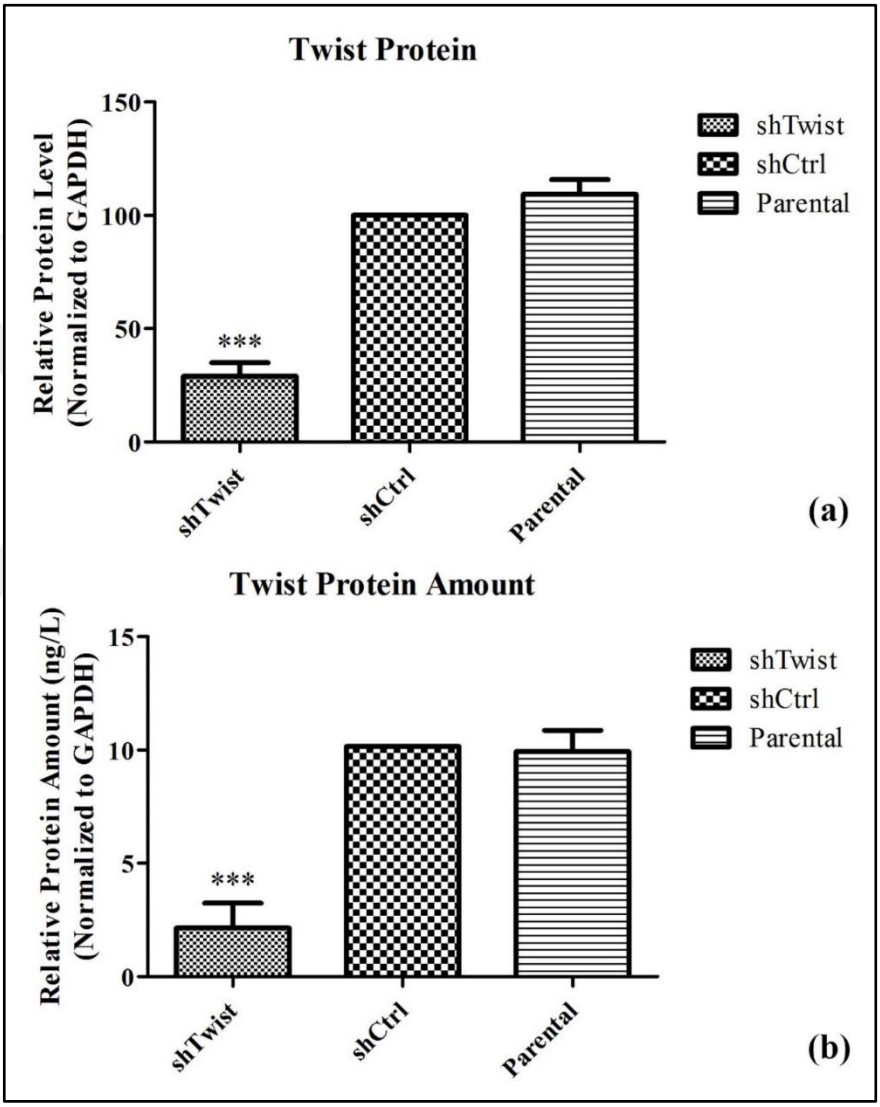


Figure 3.41 Graphical representation of AsPc-1 Twist Protein (a) Twist protein amounts (b) relative Twist protein levels in shTwist, shControl and AsPc-1 Parental cells.Graphic on top (a) shows the relative calculated Twist protein amount in shTwist, shControl and parental AsPc-1 cells. Protein amount was calculated by using the equation of the standard curve Bottom graphic (b) shows the relative percentage of twist protein compared to shControl cells and parental AsPc-1 cell line.

The results from 3.41 indicates that twist protein amount was significantly decreased compared to both shControl and Parental AsPc-1 cells. It is possible to observe that twist protein synthesis in shTwist cells is significantly less compared to both shControl and Parental AsPc-1 cells.

Table 3.12 Calculated protein amounts and relative percentage values of Snail, Slug and Twist proteins.Table below shows the calculated protein amounts and relative protein percentages (as protein amount of shControl cells was accepted as 100 per cent)

AsPc-1	**shSnail** (ng/l) / per cent	**shSlug** (ng/l) / per cent	**shTwist** (ng/l)/ per cent	**shCtrl** (ng/l) / per cent	**Parental** (ng/l) / per cent
Snail Protein (Percentage)	310.15 31.5	----------	----------	1013.72 100	1007.15 103.2
Slug Protein (Percentage)	----------	205.14 40.48	----------	900.62 100	1107.03 111.26
Twist Protein (Percentage)	----------	----------	2.19 29.9	10.15 100	9.51 109.29

As summarized in Table 3.12 the amounts of Snail was decreased to 31.5 per cent, the amount of Slug was decreased to 40.48 per cent and the amount of Twist was decreased to 29.9 per cent in shSnail, shSlug and shTwist cells respectively

3.4. CHANGES IN PROLIFERATION RATES

3.4.1. Changes in Proliferation Rate of Panc-1 after Short Hairpin Silencing of Snail, Slug and Twist

Changes in the proliferation rate is examined with the method given in the section 2.4. After having the proliferative curve of each silenced cell, shControl and Panc-1 parental, proliferative rates were calculated with proliferative equations.

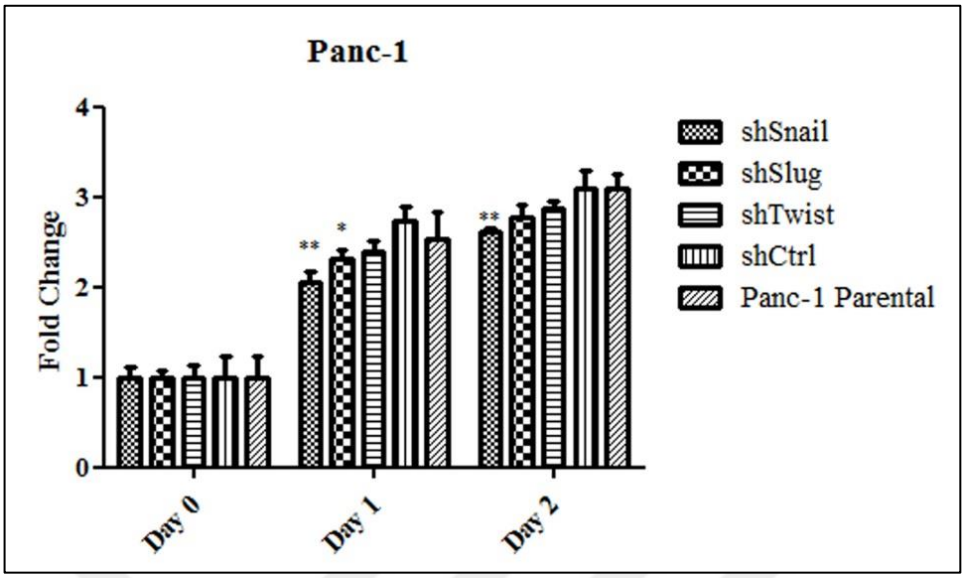


Figure 3.42 Graphical representation of cell number fold change during 48 hour periodOne –way ANOVA assay is used to calculate the statistical differences between groups. (* indicates that $p<0.05$ and ** indicates that $p<0.005$)

After 24 hours of incubation; proliferation rates of shSnail, shSlug cells were significantly less than shControl cells. According to the fold changes given in the Table 3.13, after 48 hours of incubation proliferation rate of shSnail was significantly less than shControl cells due to the results of One-way ANOVA. It is possible to say that short hairpin silencing of Snail gene decreased cellular proliferation rate compared to shControl cells.

Table 3.13 Cell number folds in 24 hours and in 48 hoursFold changes were calculated by accepting the day zero values as 1

	shSnail	**shSlug**	**shTwist**	**shCtrl**	**Parental**
Day 1	2.04	2.3	2.4	2.7	2.5
Day 2	2.6	2.7	2.8	3.0	3.1

After 24 hours of incubation; proliferation rates of shSnail, shSlug cells were significantly less than shControl cells. After 48 hours of incubation proliferation rate of shSnail was significantly less than shControl cells.

3.4.2. Changes in Proliferation Rate of MIA PaCa-2 after Short Hairpin Silencing of Snail, Slug and Twist

After having the proliferative curve of each silenced cell, shControl and MIA PaCa-2 parental, proliferative rates were calculated with proliferative equations.

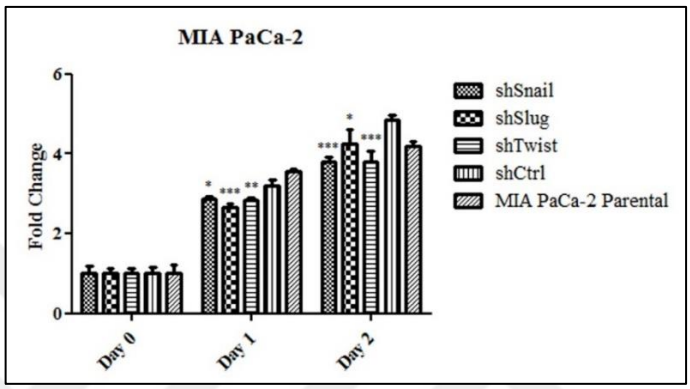


Figure 3.43 Graphical representation of cell number fold change during 48 hour periodOne –way ANOVA assay is used to calculate the statistical differences between groups. (* indicates that $p<0.05$ and ** indicates that $p<0.005$ and *** indicates that $p<0.001$)

After 24 hours of incubation; proliferation rates of shSnail, shSlug and shTwist cells were significantly less than shControl cells. According to the fold changes given in the Table 3.14 after 48 hours of incubation proliferation rate of shSnail, shSlug and shTwist cells were significantly less than shControl cells by looking at the statistical differences between groups according to One-way ANOVA test.

It is possible to say that short hairpin silencing of Snail, Slug and Twist genes decreased cellular proliferation rate compared to shControl cells.

Table 3.14 Cell number folds in 24 hours and in 48 hoursFold changes were calculated by accepting the day zero values as 1

	shSnail	**shSlug**	**shTwist**	**shControl**	**Parental**
Day 1	2.8	2.6	2.8	3.18	3.55
Day 2	3.8	4.2	3.8	4.8	4.2

After 24 hours of incubation; proliferation rates of shSnail, shSlug cells were significantly less than shControl cells. According to the fold changes given in the Table 3.14 after 48 hours of incubation proliferation rate of shSnail was significantly less than shControl cells.by looking at the statistical differences between groups according to One-way ANOVA test.

3.4.3. Changes in Proliferation Rate of BxPc-3 after Short Hairpin Silencing of Snail, Slug and Twist

After having the proliferative curve of each silenced cell, shControl and BxPc-3 parental, proliferative rates were calculated with proliferative equations.

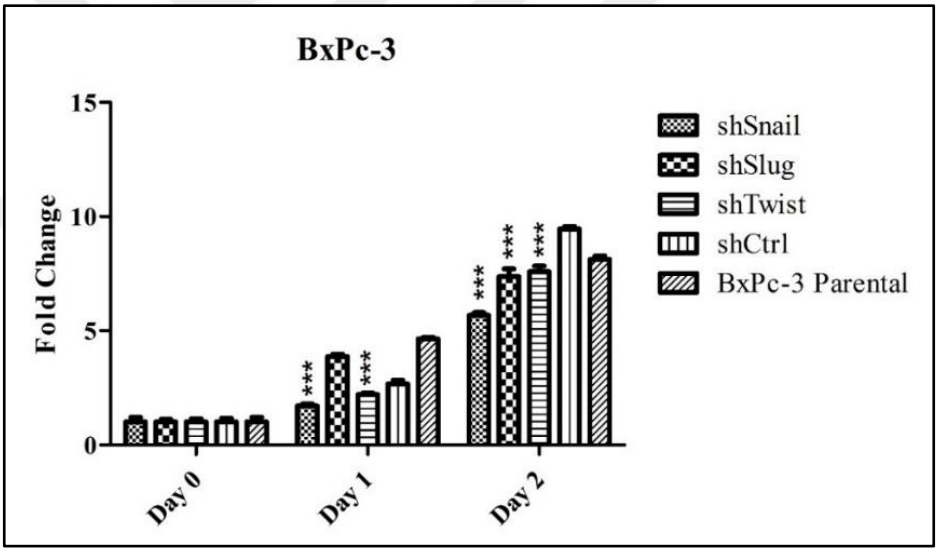


Figure 3.44 Graphical representation of cell number fold change during 48 hour periodOne –way ANOVA assay is used to calculate the statistical differences between groups. (* indicates that $p<0.05$ and ** indicates that $p<0.005$, *** indicates $p<0.001$)

After 24 hours of incubation; proliferation rates of shSnail and shTwist cells were significantly less than shControl cells. After 48 hours of incubation proliferation rate of shSnail, shSlug and shTwist were significantly less than shControl cells. It is possible to say that short hairpin silencing of Snail gene decreased cellular proliferation rate compared to shControl cells.

Table 3.15 Cell number folds in 24 hours and in 48 hoursFold changes were calculated by accepting the day zero values as 1

	shSnail	**shSlug**	**shTwist**	**shCtrl**	**Parental**
Day 1	1.7	3.8	2.2	2.7	4.6
Day 2	5.7	7.4	7.6	9.45	8.12

Table above shows changes in total cell numbers after 24 hours and 48 hours of incubation. Results indicate that short hairpin silenced cells proliferate slower (for day one shSnail and shSlug cells were significantly less proliferative and for day two shSnail cells were significantly less proliferative) than shControl cells by looking at the results of one way ANOVA statistical analysis.

3.4.4. Changes in Proliferation Rate of AsPC-1 after Short Hairpin Silencing of Snail, Slug and Twist

After having the proliferative curve of each silenced cell, shControl and AsPc-1 parental, proliferative rates were calculated with proliferative equations.

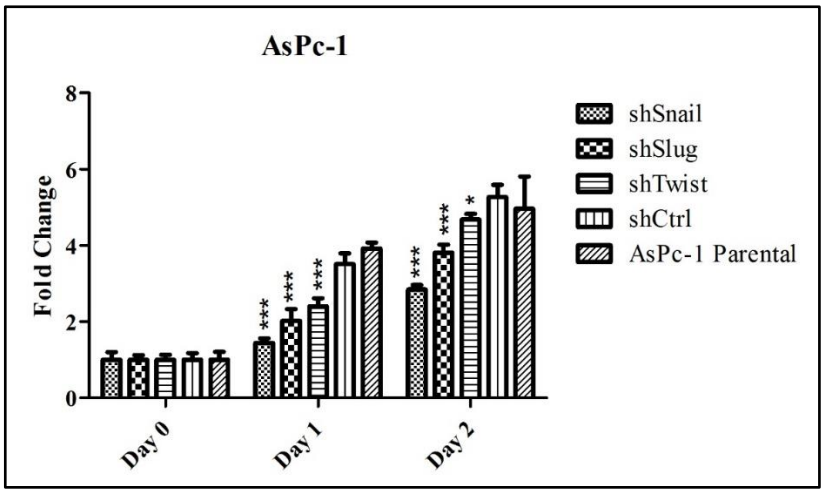


Figure 3.45 Graphical representation of cell number fold change during 48 hour periodOne –way ANOVA assay is used to calculate the statistical differences between groups. (* indicates that $p<0.05$ and ** indicates that $p<0.005$, *** indicates $p<0.001$)

According to the fold changes given in the Table 3.16 after 24 hours of incubation; proliferation rates of shSnail, shSlug and shTwist cells were significantly less than shControl cells. After 48 hours of incubation proliferation rate of shSnail, shSlug and shTwist were significantly less than shControl cells. It is possible to say that short hairpin silencing of Snail, Slug and Twist genes decreased cellular proliferation rate compared to shControl cells.

Table 3.16 Cell number folds in 24 hours and in 48 hoursFold changes were calculated by accepting the day zero values as 1

	shSnail	**shSlug**	**shTwist**	**shCtrl**	**Parental**
Day 1	1.44	2.02	2.40	3.51	3.80
Day 2	2.84	3.81	4.38	4.97	4.95

Table above shows changes in total cell numbers after 24 hours and 48 hours of incubation. Results indicate that short hairpin silenced cells proliferate slower than shControl cells.

3.5. SCRATCH ASSAY

3.5.1. Change in wound healing ability of Panc-1 Cells after short hairpin silencing of Snail, Slug and Twist

Figure 3.46 contains the microscope images of scratch assay from day zero and up to day three.

As indicated in the Figure 3.46 it was harder for Snail, Slug and Twist silenced Panc-1 cells to close the scratch wound compared to Control Panc-1 cells.

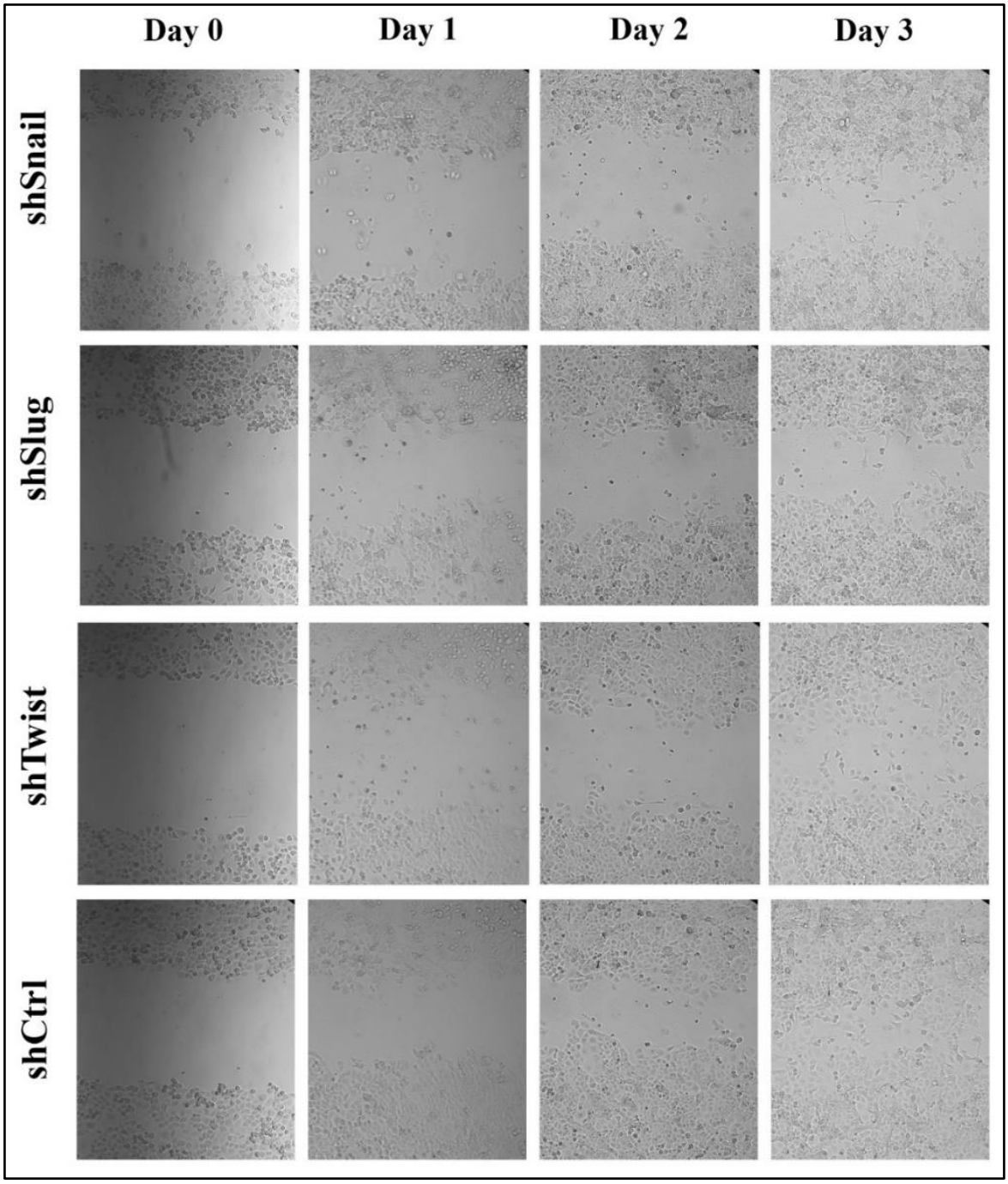


Figure 3.46 Images of short hairpin silenced cells and shControl cell for 72 hour incubation with culture medium containing only two per cent FBS.All of the images were taken in 10x magnification.

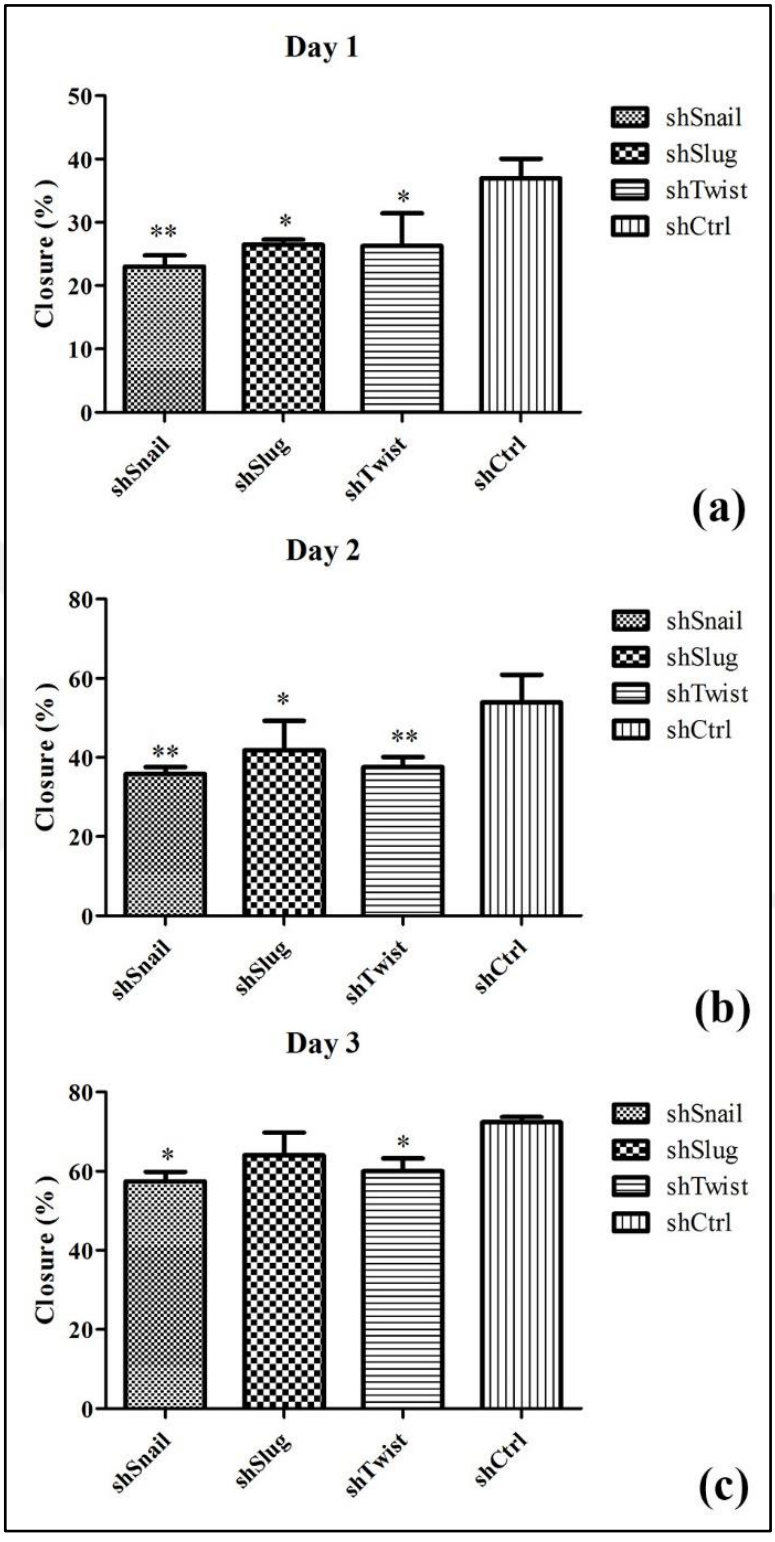


Figure 3.47 Graphical representation of wound healing percentages of shSnail, shSlug, shTwist, shControl transfected cells after (a) one day of incubation with the medium containing two per cent FBS, (b) two days of incubation with the medium containing two per cent (*indicates that $p<0.05$ and ** indicates that $p<0.005$)

Scratch assay has revealed that short hairpin silencing of Snail and Slug genes has significantly decreased the wound healing ability of Panc-1 cells in a 72 hour period while Twist gene silencing significantly decreased wound healing ability of Panc-1 cells only for 48 hour period compared to shControl cells.

As given in Table 3.17, the amount of wound closure of shSnail cells was 23 per cent in day one, 35 per cent in day two and 54 per cent in day three.

The amount of wound closure of shSlug cells was 26 per cent in day one, 41 per cent in day two and 64 per cent in day three.

The amount of wound closure of shTwist cells was 26 per cent in day one, 37 per cent in day two and 60 per cent in day three. The amount of wound closure of shControl cells on the other hand was 37 per cent in day one, 54 per cent in day two and 72 per cent in day three.

The differences between the closure percentages were statistically significant according to the one-way ANOVA accompanied by Tukey posttest.

Table 3.17 Wound healing percentages of shSnail, shSlug, shTwist, shCtrl Panc-1 cells in 3 days

	shSnail	**shSlug**	**shTwist**	**shCtrl**
Day1	23.02%	26.48%	26.25%	36.91%
Day2	35.83%	41.84%	37.58%	53.92%
Day3	54.46%	64.01%	60.05%	72.33%

3.5.2. Change in wound healing ability of MIA PaCa-2 Cells after short hairpin silencing of Snail, Slug and Twist

Figure 3.48 contains the microscopic images of Snail, Slug, Twist silenced MIA PaCa-2 cells and their negative control’s wound closure.

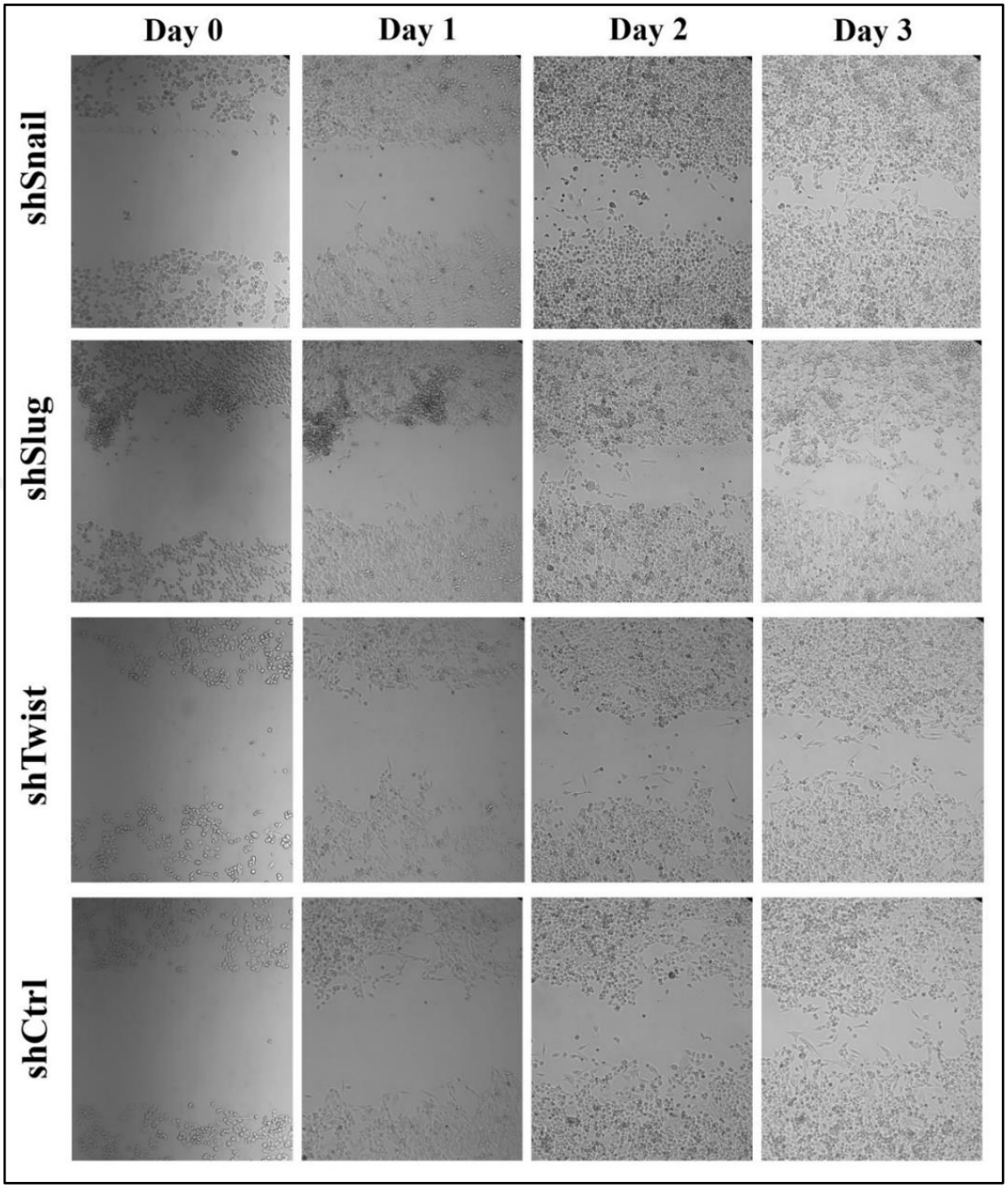


Figure 3.48 Images of short hairpin silenced cells and shControl cell for 72 hour incubation with culture medium containing two per cent FBS.All of the images were taken in 10x magnification.

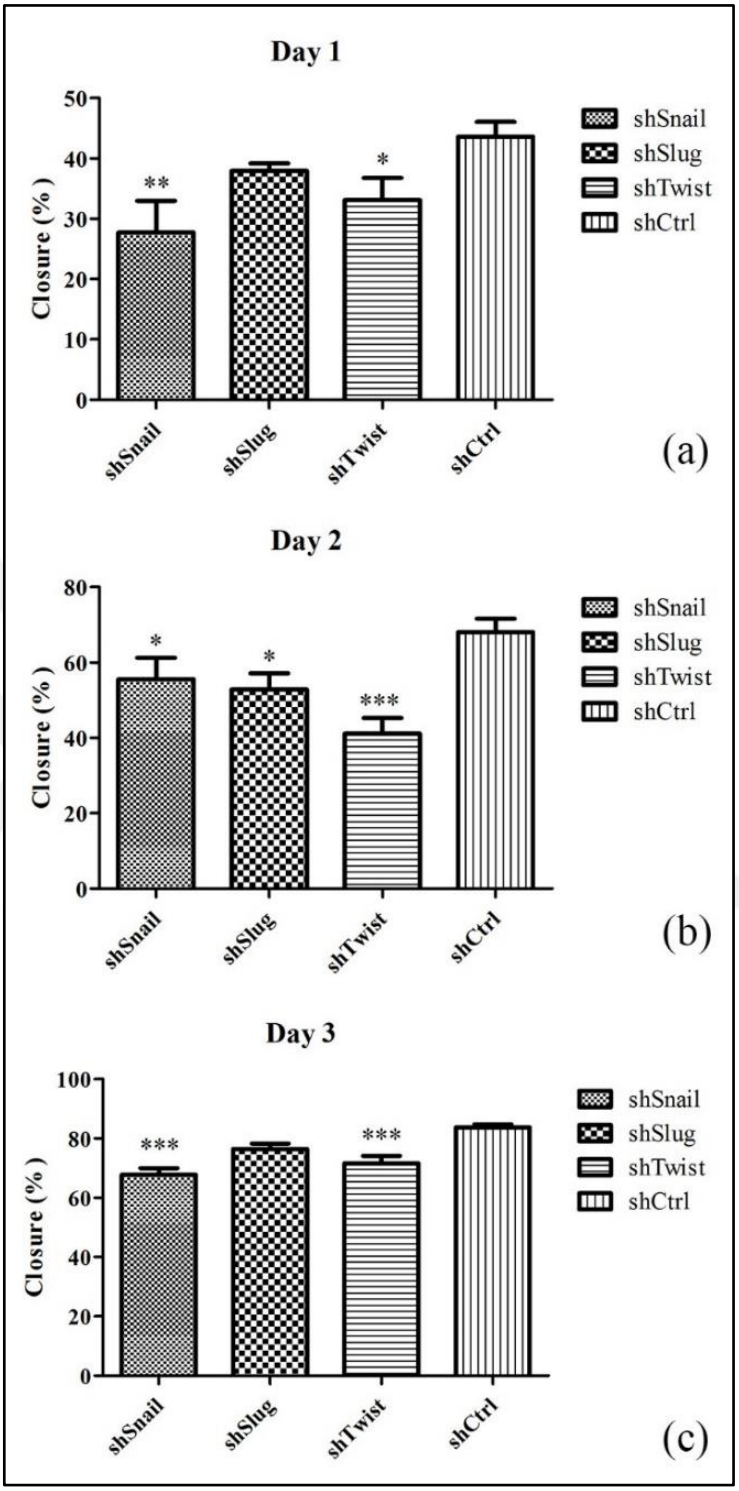


Figure 3.49 Graphical representation of wound healing percentages of shSnail, shSlug, shTwist, shControl transfected cells after (a) one day of incubation with the medium containing two per cent FBS, (b) two days of incubation with the medium containing two per cent. (* indicates that $p<0.05$, ** indicates that $p<0.005$ and *** indicates that $p<0.001$)

Wound healing assay (scratch assay) has revealed that wound healing ability of MIA PaCa-2 cells was significantly decreased with short hairpin silencing of Snail and Slug genes Wound healing ability was decreased significantly in shTwist cells only at 48th hour.

As given in Table 3.18, the amount of wound closure of shSnail cells was 27 per cent in day one, 55 per cent in day two and 71 per cent in day three. The amount of wound closure of shSlug cells was 37 per cent in day one, 52 per cent in day two and 82 per cent in day three. The amount of wound closure of shTwist cells was 33 per cent in day one, 41 per cent in day two and 71 per cent in day three. The amount of wound closure of shControl cells on the other hand was 43.61 per cent in day one, 68 per cent in day two and 83 per cent in day three. The differences between the closure percentages were statistically significant according to the one-way ANOVA accompanied by Tukey posttest.

Table 3.18 Wound closure percentages of shSnail, shSlug, shTwist, shctrl MIA PaCa-2 Cells in 3 days

	shSnail	**shSlug**	**shTwist**	**shCtrl**
Day1	27.72%	36.92%	33.09%	43.61%
Day2	55.56%	52.83%	41.07%	68.02%
Day3	70.81%	82.44%	71.58%	83.69%

As indicated in the Table 3.18, it is possible to say that Snail, Slug and Twist short hairpin treatment on MIA PaCa-2 cells have decreased the wound healing abilities of this cell line compared to shControl cells

It is possible to say that after 3 days of experiment shSnail silencing deceased the wound healing amount more than shSlug and shTwist treatments.

3.5.3. Change in wound healing ability of BxPc-3 cells after short hairpin silencing of Snail, Slug and Twist

Figure 3.50 contains the microscopic images of Snail, Slug, Twist silenced BxPc-3 cells and their negative control's wound closure

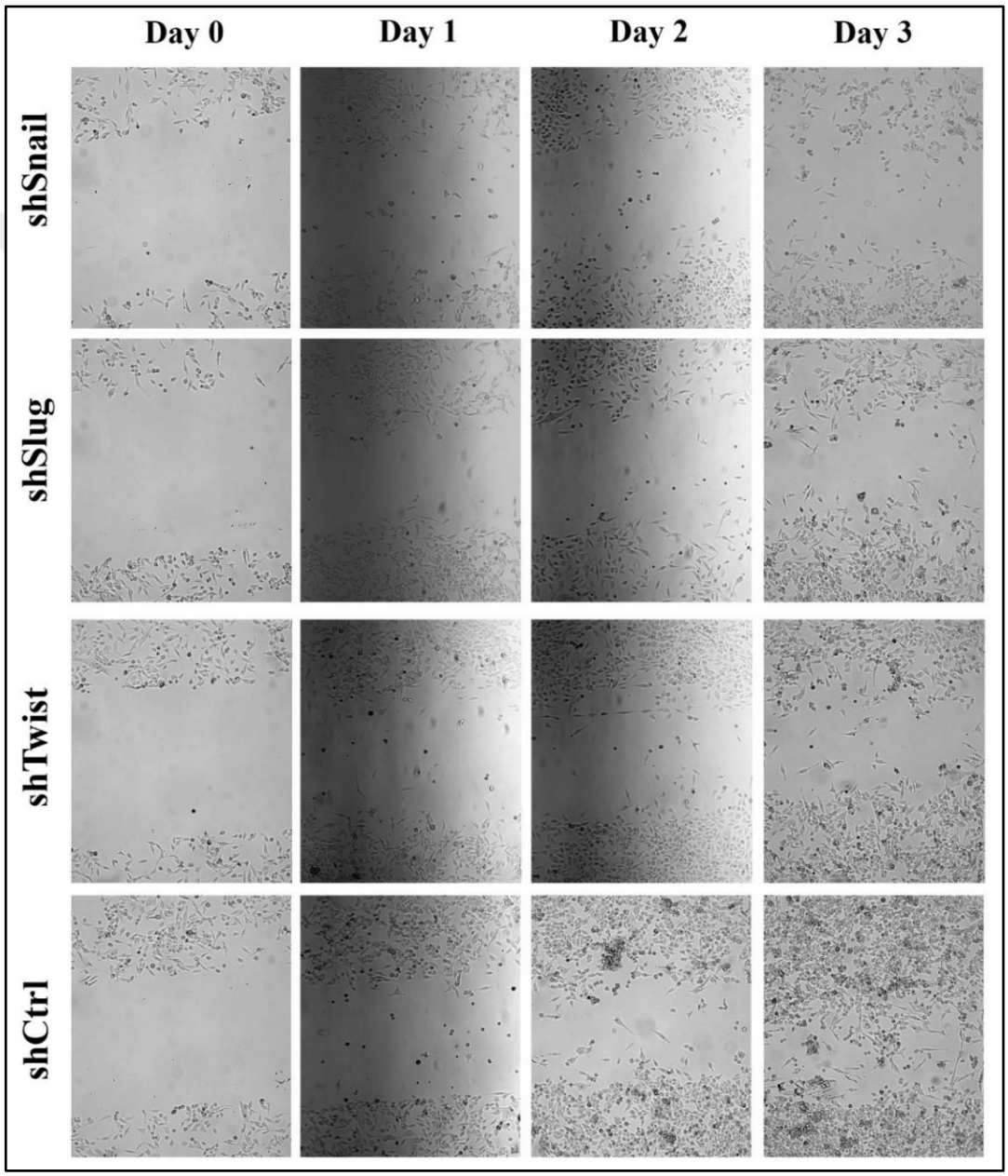


Figure 3.50 Images of short hairpin silenced cells and shControl cell for 48 hour incubation with culture medium containing two per cent FBS.All of the images were taken in 10x magnification.

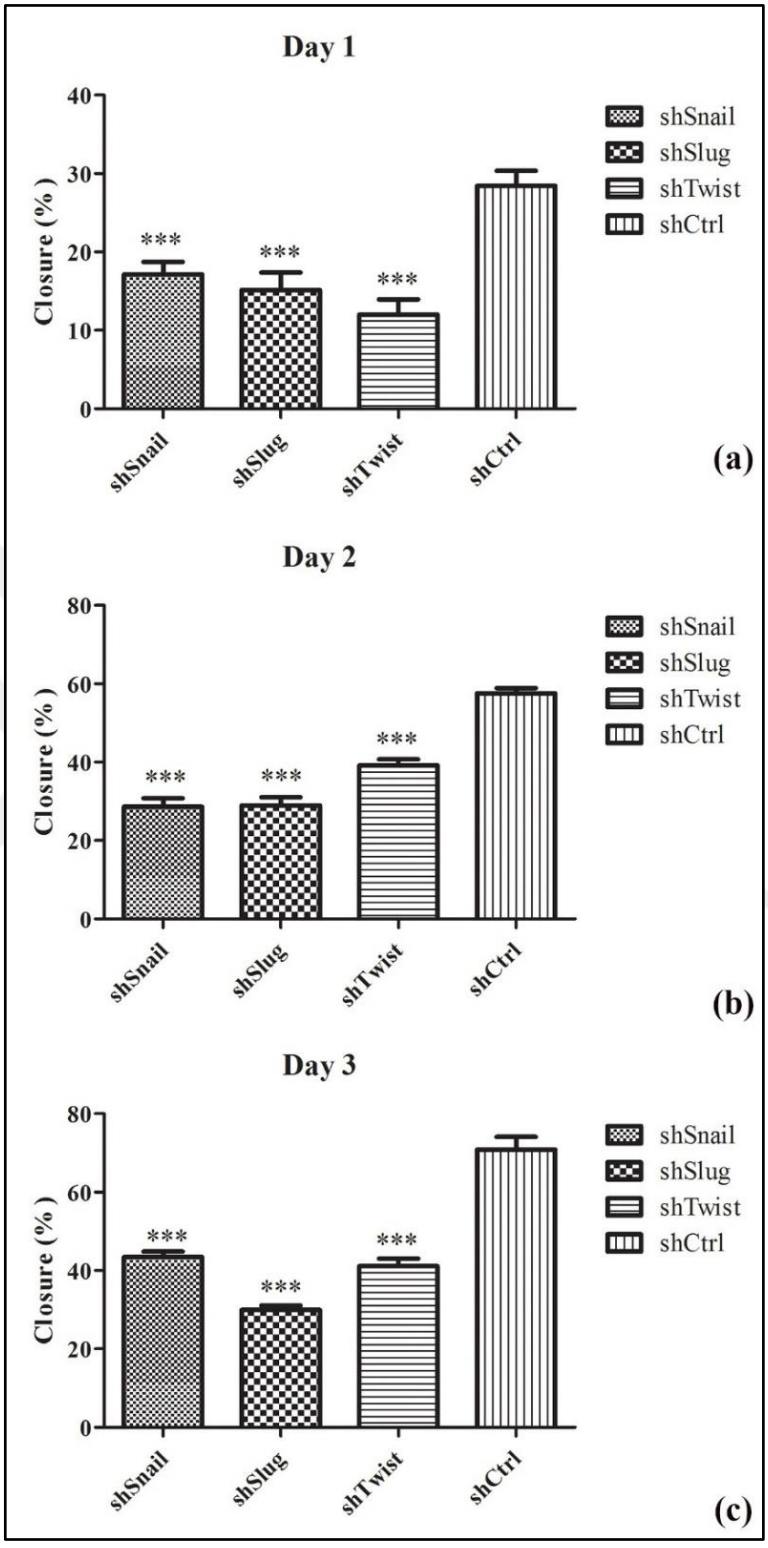


Figure 3.51 Graphical representation of wound healing percentages of shSnail, shSlug, shTwist, shControl transfected cells after (a) one day of incubation, (b) two days of incubation in culture medium containing two per cent FBS (*** indicates that $p<0.001$)

Scratch assay has revealed that short hairpin silencing of Snail, Slug and Twist gene has decreased the wound healing ability of BxPc-3 cells compared to control short hairpin silenced cells.

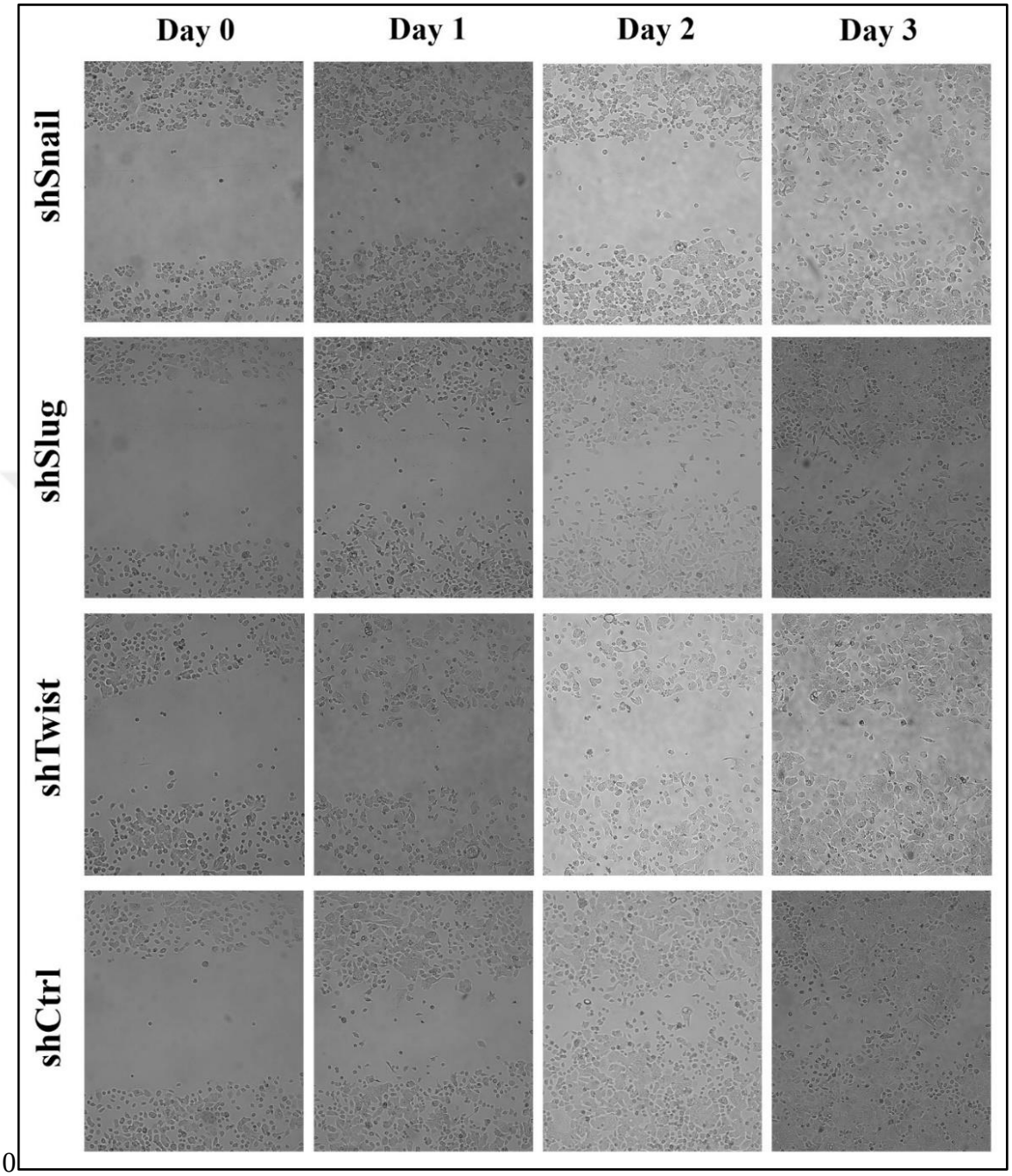
As given in Table 3.19, the amount of wound closure of shSnail cells was 17 per cent in day one, 28 per cent in day two and 48 per cent in day three. The amount of wound closure of shSlug cells was 15 per cent in day one, 29 per cent in day two and 30 per cent in day three. The amount of wound closure of shTwist cells was 12 per cent in day one, 39 per cent in day two and 41 per cent in day three. The amount of wound closure of shControl cells on the other hand was 28.4 per cent in day one, 57.6 per cent in day two and 71 per cent in day three. The differences between the closure percentages were statistically significant according to the one-way ANOVA accompanied by Tukey posttest.

Table 3.19 Wound closure percentages of shSnail, shSlug, shTwist, shctrl cells in 3 days

	shSnail	**shSlug**	**shTwist**	**shCtrl**
Day1	17.13%	15.13%	12.00%	28.40%
Day2	28.66%	28.96%	39.14%	57.58%
Day3	48.45%	29.94%	41.10%	70.82%

3.5.4. Change in wound healing ability of AsPc-1 Cells after short hairpin silencing of Snail, Slug and Twist

Figure 3.50 contains the microscopic images of Snail, Slug, Twist silenced AsPc-1 cells and their negative control’s wound closure



0

Figure 3.52 Images of short hairpin silenced cells and shControl cell for 48 hour incubation with culture medium containing two per cent FBS.All of the images were taken in 10x magnification.

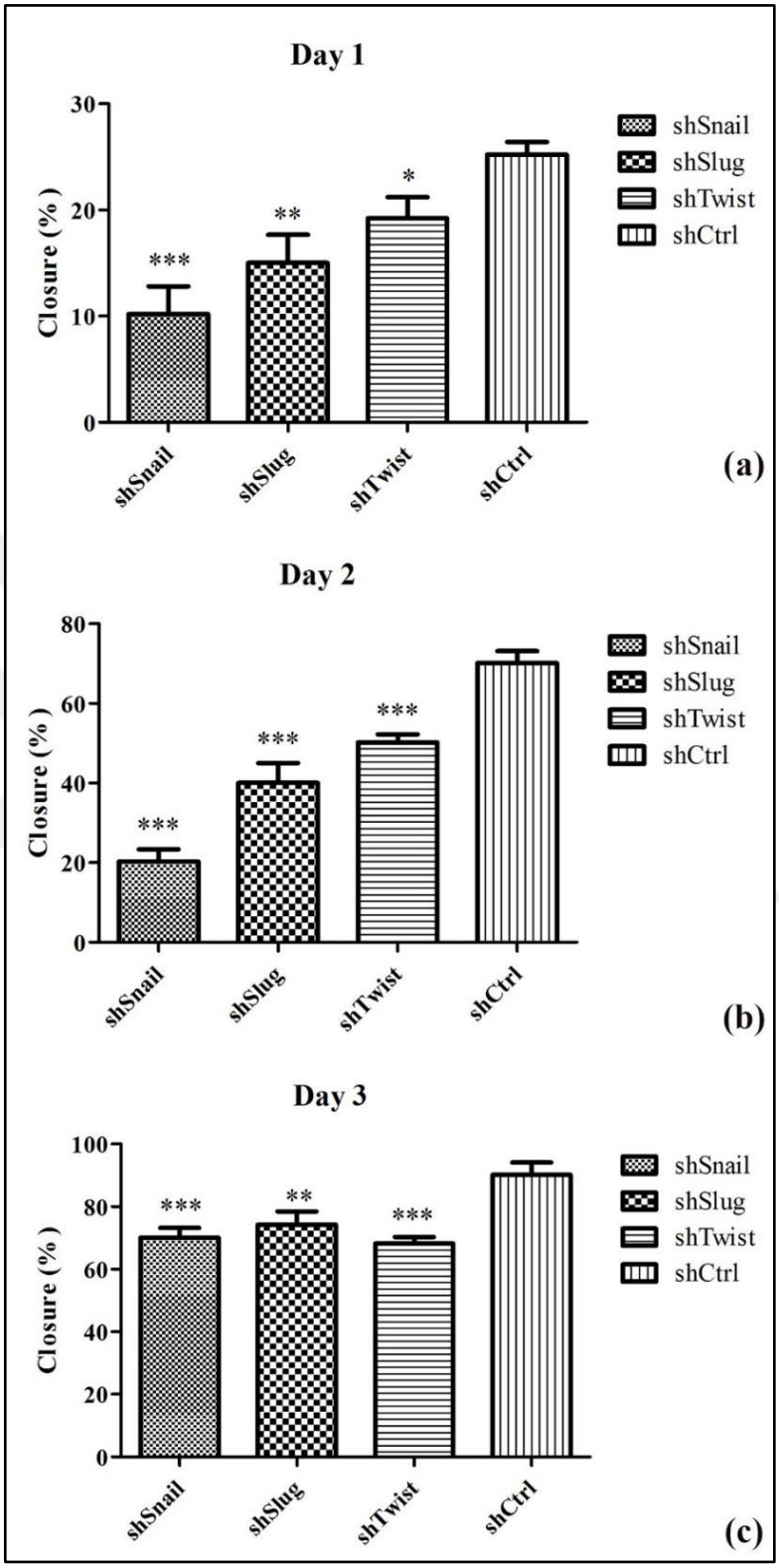


Figure 3.53 Graphical representation of wound healing percentages of shSnail, shSlug, shTwist, shControl transfected cells after (a) one day of incubation, (b) two days of incubation in culture medium containing two per cent FBS(* indicates that $p<0.05$, ** indicates that $p<0.005$ and *** indicates that $p<0.001$)

As given in Table 3.20, the amount of wound closure of shSnail cells was 10 per cent in day one, 20 per cent in day two and 70 per cent in day three. The amount of wound closure of shSlug cells was 15 per cent in day one, 40 per cent in day two and 74 per cent in day three. The amount of wound closure of shTwist cells was 19 per cent in day one, 50 per cent in day two and 68 per cent in day three. The amount of wound closure of shControl cells on the other hand was 25 per cent in day one, 70 per cent in day two and 90 per cent in day three. The differences between the closure percentages were statistically significant according to the one-way ANOVA accompanied by Tukey posttest

Table 3.20 Wound closure percentages of shSnail, shSlug, shTwist, shctrl cells in 3 days

	shSnail	**shSlug**	**shTwist**	**shCtrl**
Day1	10.20%	15.02%	19.20%	25.20%
Day2	20.30%	40.02%	50.23%	70.15%
Day3	70.12%	74.23%	68.23%	90.14%

3.6. TRANSMEMBRANE CELL MIGRATION ASSAY

3.6.1. Changes in Migration Capacity of Panc-1 Cells after Gene Therapy

Figure 3.54 contains the microscope images of Snail, Slug and Twist silenced Panc-1 cells along with their negative control groups that passed through the micro-porous membrane of the insert.

The purple colored dots in the images shows the cells passed through the membrane. Migrated cells were dyed with a special cell stain in order to increase the visibility. Microscope images are taken from five different insert locations and calculations (statistic analysis with one-way ANOVA with Tukey’s post test) was done with the means of counted cell numbers from the 5 images for each group.

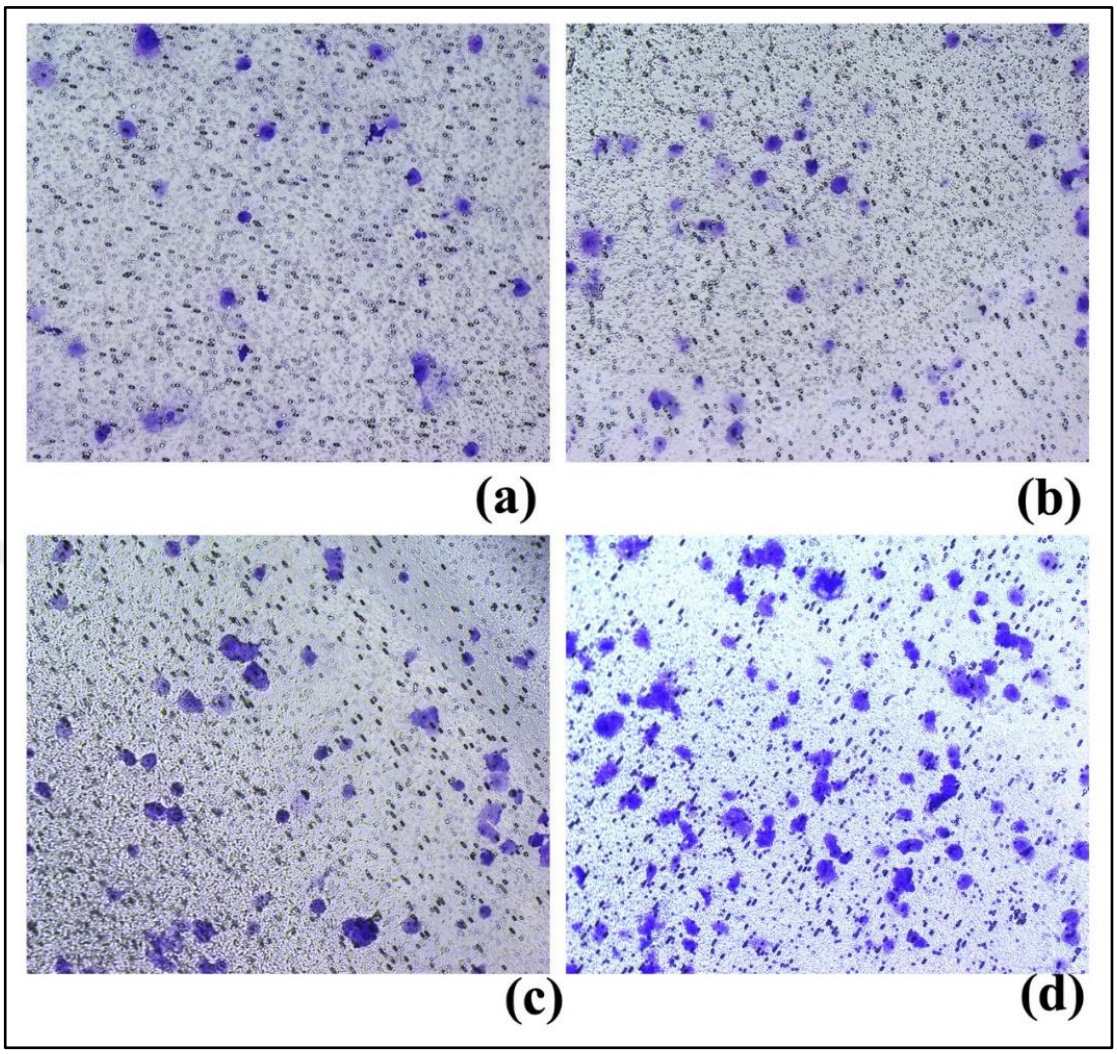


Figure 3.54 Microscopical images of Panc-1 migration (a) shSnail Cells (b) shSlug Cells (c) shTwist Cells (d) shControl Cells after 24 hours of incubation in transmembrane insert with 20x magnification

Even from microscope images given in the Figure 3.54 it is possible to observe that transmembrane migration ability of short hairpin silenced cells (in shSnail, shSlug and shTwist silenced groups) are less when the number of cells were compared to number of cells have migrated in shControl group

There are more cells in the shControl group's insert (d) compared to shSnail (a), shSlug (b), shTwist (c) cells.

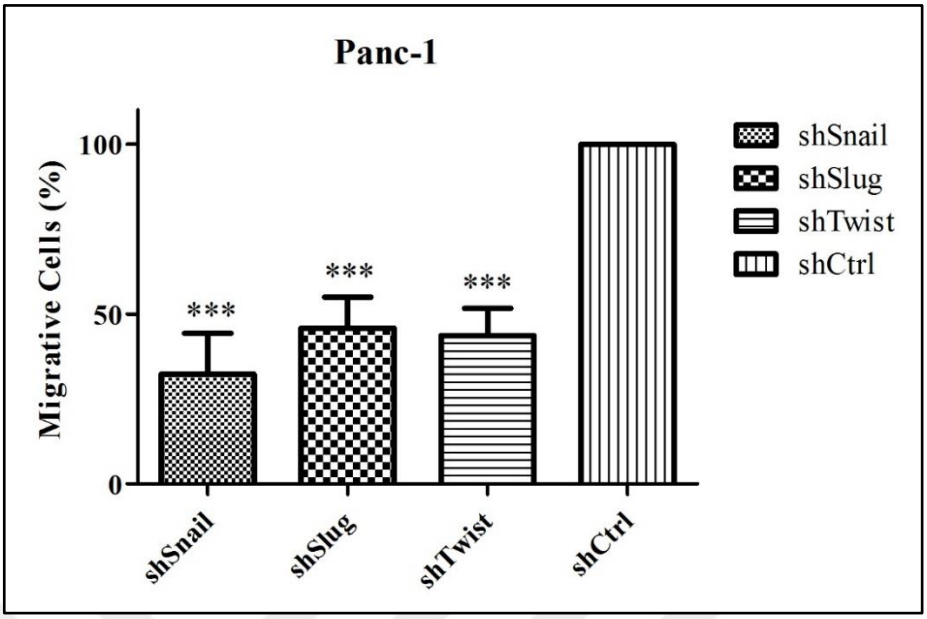


Figure 3.55 Graphical representation of migrated cell percentage compared to shControl cells One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (*** indicates $p<0.001$)

It is possible to say that migrated cell percentage was significantly decreased with short hairpin silencing of Snail, Slug and Twist genes when compared to shControl cells after the results are statistically analyzed with One-way ANOVA with Tukey's posttest. As given in the Table 3.21, compared to the shControl Panc-1 cells the migrated cell percentages of shSnail cells were decreased to 60 per cent, shSlug cells were decreased to 84 per cent and shTwist cells were decreased to 73 per cent when the number of migrated cells in the shControl was accepted as 100 per cent

Table 3.21 Migrated cell percentage compared to shControl cells

Migrated Cells Compared to shControl Cells (per cent)		
shSnail	shSlug	shTwist
60.48	84.19	72.85

3.6.2. Changes in Migration Capacity of MIA PaCa-2 Cells after Gene Therapy

Figure 3.55 contains the microscope images of Snail, Slug and Twist silenced MIA PaCa-2 cells along with their negative control groups that passed through the micro-porous membrane of the insert.

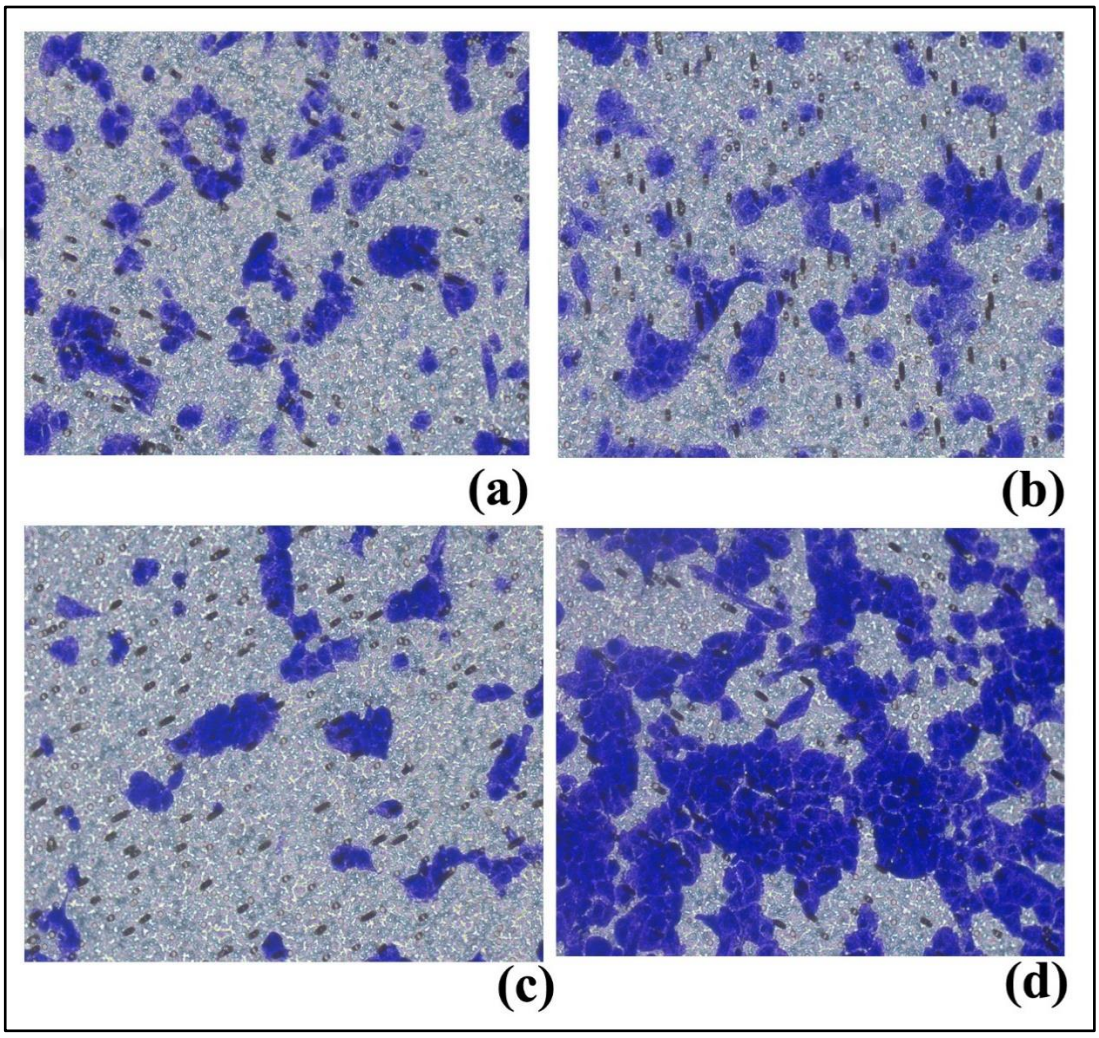


Figure 3.56 Microscopic images of MIA PaCa-2 migration (a) shSnail Cells (b) shSlug Cells (c) shTwist Cells (d) shControl Cells
after 24 hours of incubation in transmembrane insert with 20x magnification

Even from the microscopic images given in the Figure 3.36, it is possible to say that short hairpin silencing has decreased the transmembrane migration ability of MIA PaCa-2 cells.

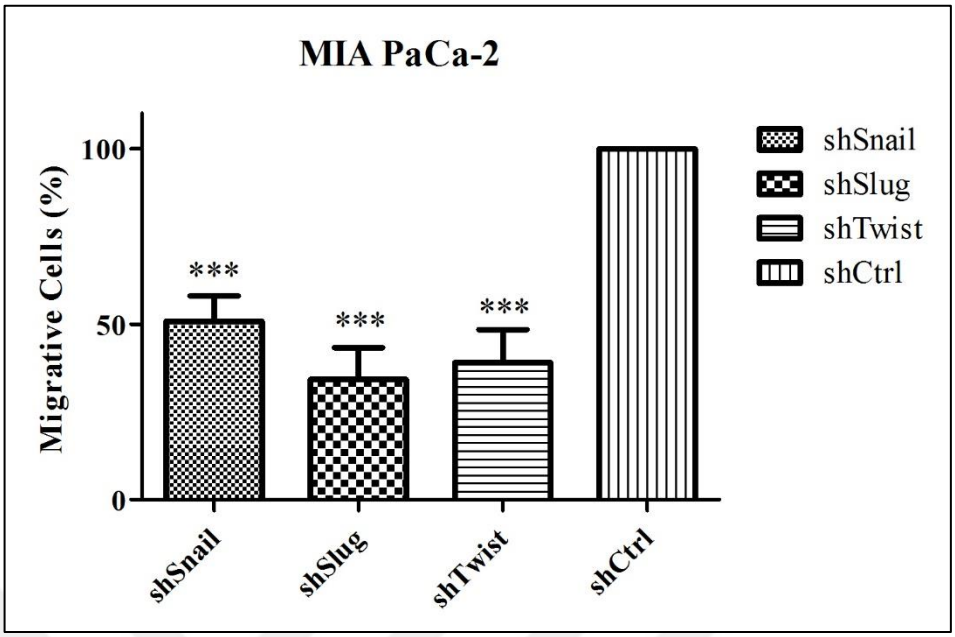


Figure 3.57 Graphical representation of migrated cell percentage compared to shControl cells One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (*** indicates $p<0.001$)

It is possible to say that migrated cell percentage was significantly decreased with short hairpin silencing of Snail, Slug and Twist genes when compared to shControl cells after the results are statistically analyzed with One-way ANOVA with Tukey's posttest. As given in the Table 3.22, compared to the shControl MIA PaCa-2 cells the migrated cell percentages of shSnail cells were decreased to 67.72 per cent, shSlug cells were decreased to 67.43 per cent and shTwist cells were decreased to 64.26 per cent when the number of migrated cells in the shControl was accepted as 100 per cent

Table 3.22 Migrated cell percentage compared to shControl cells

Migrated Cells Compared to shControl Cells (per cent)		
shSnail	shSlug	shTwist
67.72	67.43	64.26

3.6.3. Changes in Migration Capacity of BxPc-3 Cells after Gene Therapy

Figure 3.58 contains the microscope images of Snail, Slug and Twist silenced BxPc-3 cells along with their negative control groups that passed through the micro-porous membrane of the insert.

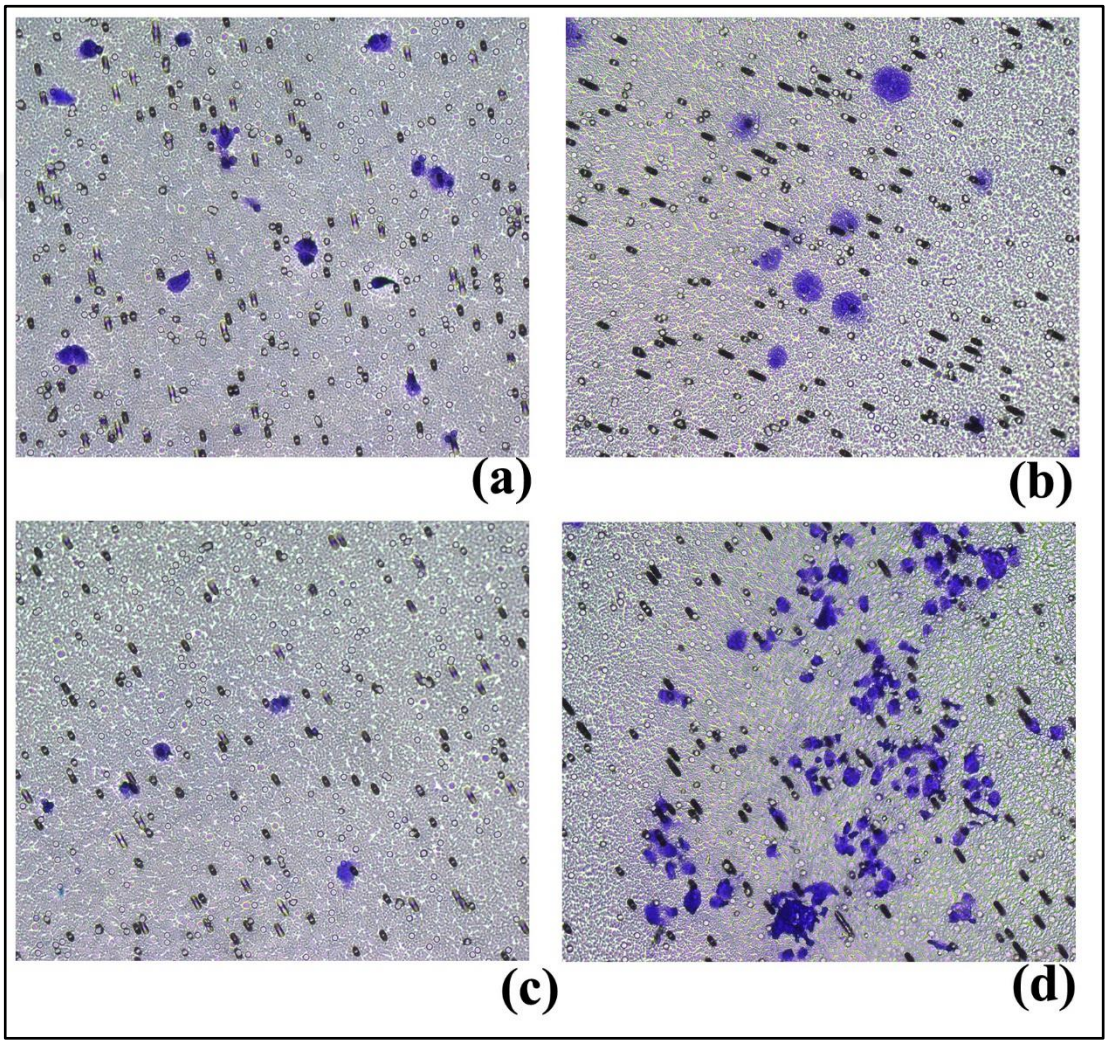


Figure 3.58 Microscope images of BxPc-3 Migration (a) shSnail Cells (b) shSlug Cells (c) shTwist Cells (d) shControl Cells after 24 hours of incubation in transmembrane insert with 20x magnification

Even from microscopic images given in Figure 3.58 it is possible to observe that transmembrane migration ability of short hairpin silenced cells are less compared to shControl cells

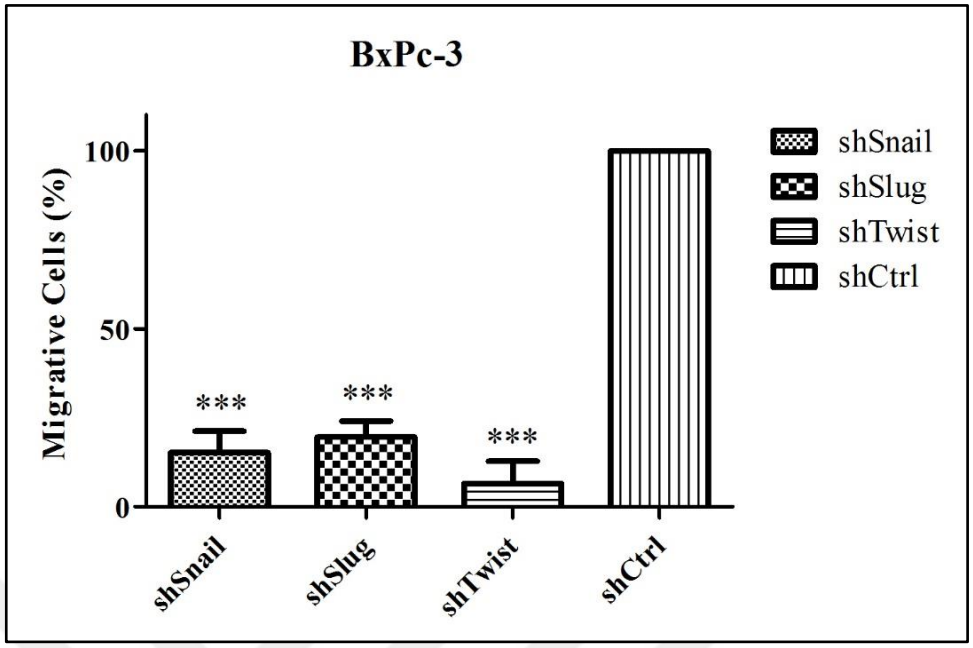


Figure 3.59 Graphical representation of migrated cell percentage compared to shControl cellsOne-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (*** indicates $p<0.001$)

It is possible to say that migrated cell percentage was significantly decreased with short hairpin silencing of Snail, Slug and Twist genes when compared to shControl cells after the results are statistically analyzed with One-way ANOVA with Tukey's posttest. As given in the Table 3.23, compared to the shControl BxPc-3 cells the migrated cell percentages of shSnail cells were decreased to 11.28 per cent, shSlug cells were decreased to 13.2 per cent and shTwist cells were decreased to 5.8 per cent when the number of migrated cells in the shControl was accepted as 100 per cent

Table 3.23 Migrated cell percentage compared to shControl cells

Migrated Cells Compared to shControl Cells (per cent)		
shSnail	shSlug	shTwist
11.28	13.2	5.8

3.6.4. Changes in Migration Capacity of AsPc-1 Cells after Gene Therapy

Figure 3.58 contains the microscope images of Snail, Slug and Twist silenced AsPc-1 cells along with their negative control groups that passed through the micro-porous membrane of the insert.

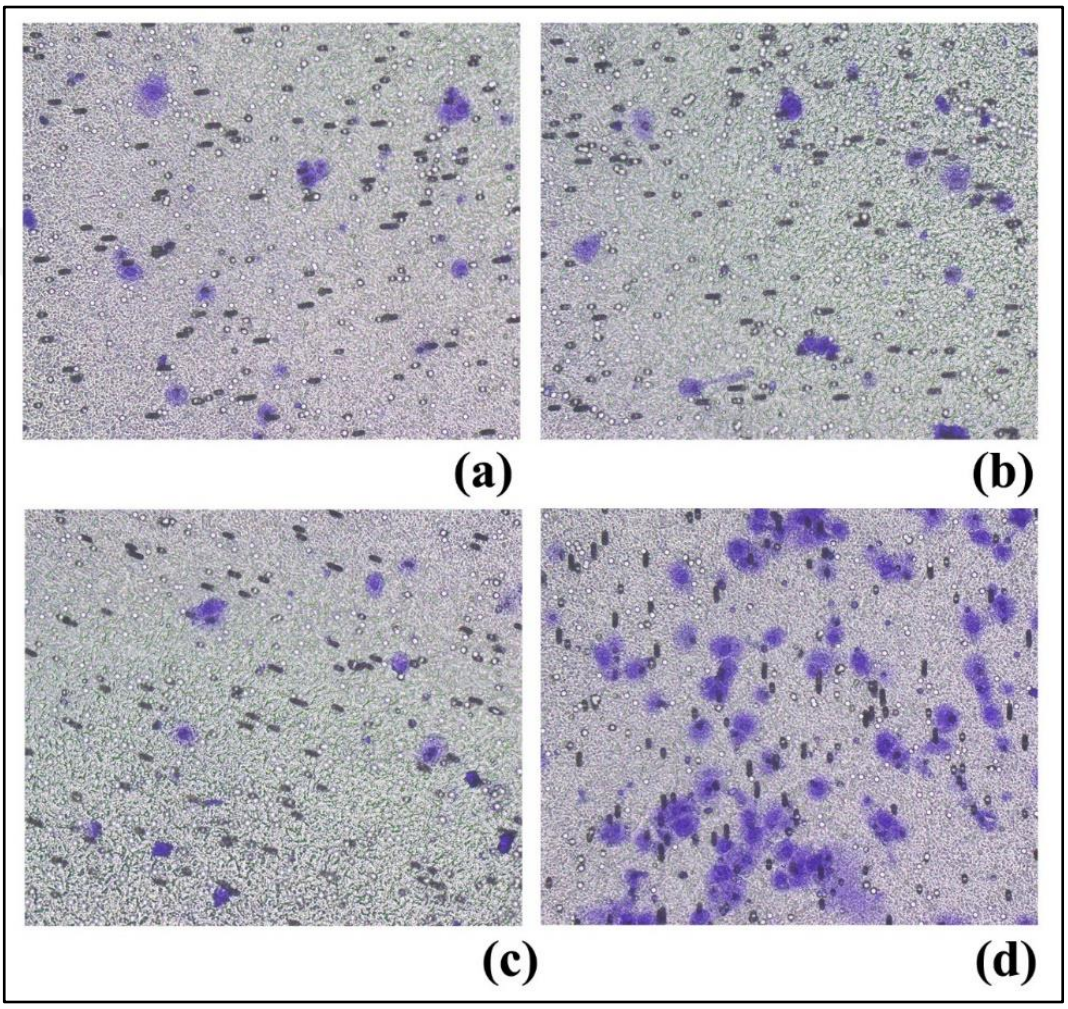


Figure 3.60 Microscope images of AsPc-1 migration (a) shSnail Cells (b) shSlug Cells (c) shTwist Cells (d) shControl Cells after 24 hours of incubation in transmembrane insert with 20x magnification

It is observed that, even from the microscopic images given in the Figure 3.60, lesser number of cells have passed in gene silenced groups compared to shControl cells.

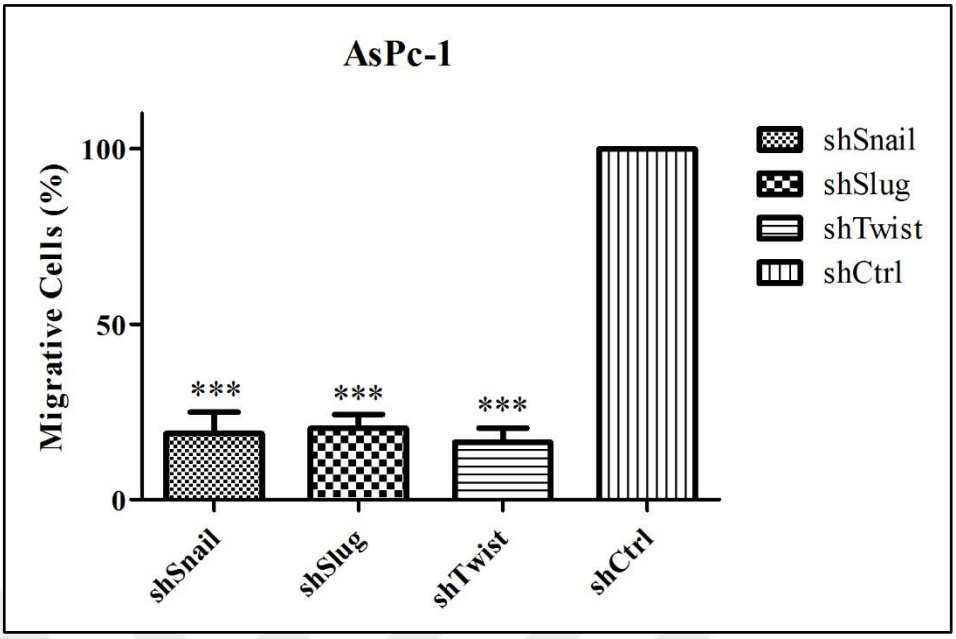


Figure 3.61 Graphical representation of migrated cell percentage compared to shControl cellsOne-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (*** indicates $p<0.001$)

It is possible to say that migrated cell percentage was significantly decreased with short hairpin silencing of Snail, Slug and Twist genes when compared to shControl cells after the results are statistically analyzed with One-way ANOVA with Tukey's posttest. As given in the Table 3.24, compared to the shControl AsPc-1 cells the migrated cell percentages of shSnail cells were decreased to 19 per cent, shSlug cells were decreased to 20.27 per cent and shTwist cells were decreased to 16.4 per cent when the number of migrated cells in the shControl was accepted as 100 per cent

Table 3.24 Migrated cell percentage compared to shControl cells

Migrated Cells Compared to shControl Cells (per cent)		
shSnail	shSlug	shTwist
18.8	20.27	16.4

3.7. TRANSMEMBRANE CELL INVASION ASSAY

3.7.1. Changes in Invasive Capacity of Panc-1 Cells after Gene Therapy

Figure 3.62 contains the microscope images of Snail, Slug and Twist silenced Panc-1 cells and their negative control at the 48th hour of invasion assay.

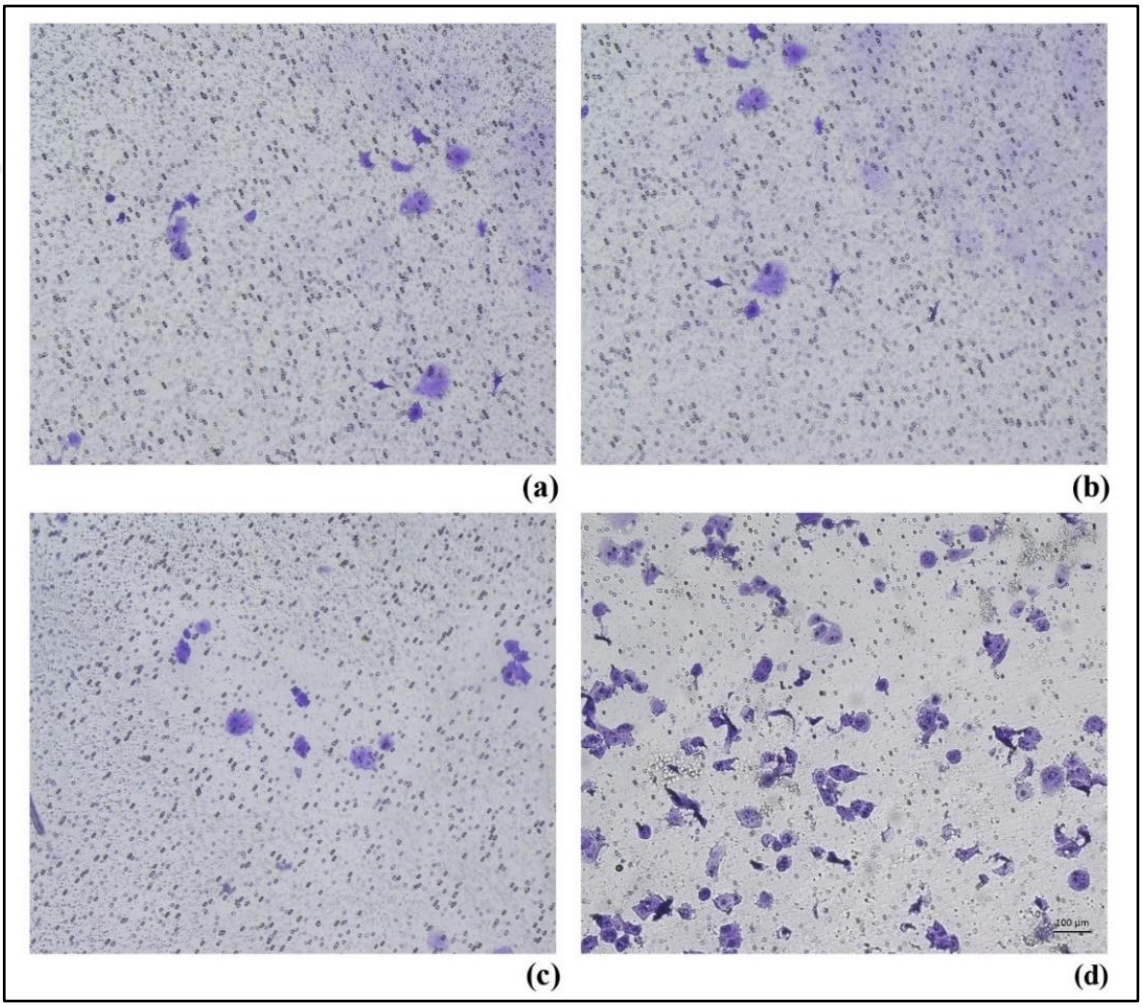


Figure 3.62 Microscopic images of Panc-1 invasion (a) shSnail Cells (b) shSlug Cells (c) shTwist Cells (d) shControl Cells after 48 hours of incubation in invasion (matrigel) insert with 20x magnification

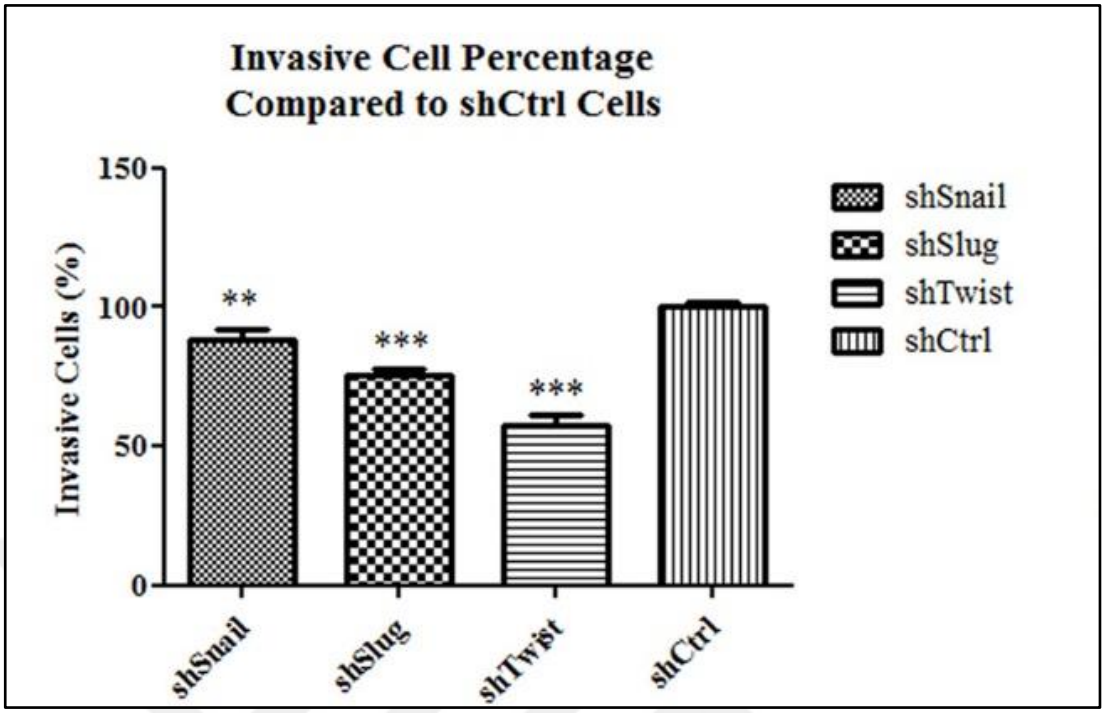


Figure 3.63 Graphical representation of invasive cell percentage compared to shControl cellsOne-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (** indicates that $p<0.005$ and *** indicates that $p<0.001$)

It is possible to say that invasive cell percentage was significantly decreased with short hairpin silencing of Snail, Slug and Twist genes when compared to shControl cells after the results are statistically analyzed with One-way ANOVA with Tukey's posttest. As given in the Table 3.25, compared to the shControl Panc-1 cells, the invaded cell percentages of shSnail cells were decreased to 88 per cent, shSlug cells were decreased to 75 per cent and shTwist cells were decreased to 57.81 per cent when the number of invaded cells in the shControl was accepted as 100 per cent

Table 3.25 Invasive cell percentage compared to shControl cells

Invasive Cells Compared to shControl Cells (per cent) (Invasive index)		
shSnail	shSlug	shTwist
88.47	75.5	57.81

Invasive ability was decreased to 88.5 percent in shSnail cells, 75.5 per cent in shSlug cells, and 57.81 per cent in shTwist cells when the values of shControl cells were accepted as 100 per cent.

3.7.2. Changes in Invasive Capacity of MIA PaCa-2 Cells after Gene Therapy

Figure 3.64 contains the microscope images of Snail, Slug and Twist silenced MIA PaCa-2 cells and their negative control at the 48th hour of invasion assay.

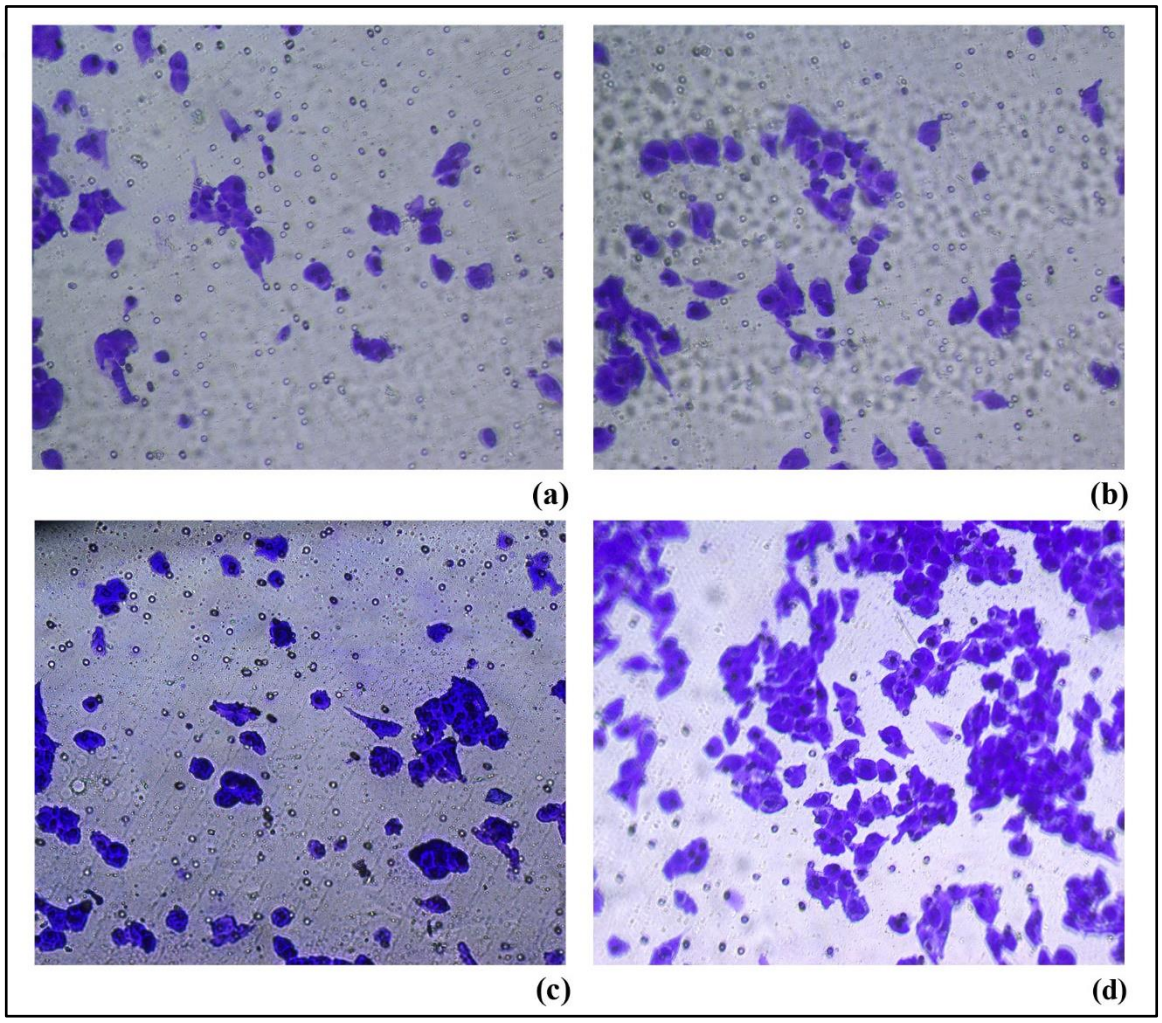


Figure 3.64 Microscopical images of Mia PaCa-2 invasion (a) shSnail Cells (b) shSlug Cells (c) shTwist Cells (d) shControl Cells after 48 hours of incubation in invasion (matrigel) insert with 20x magnification

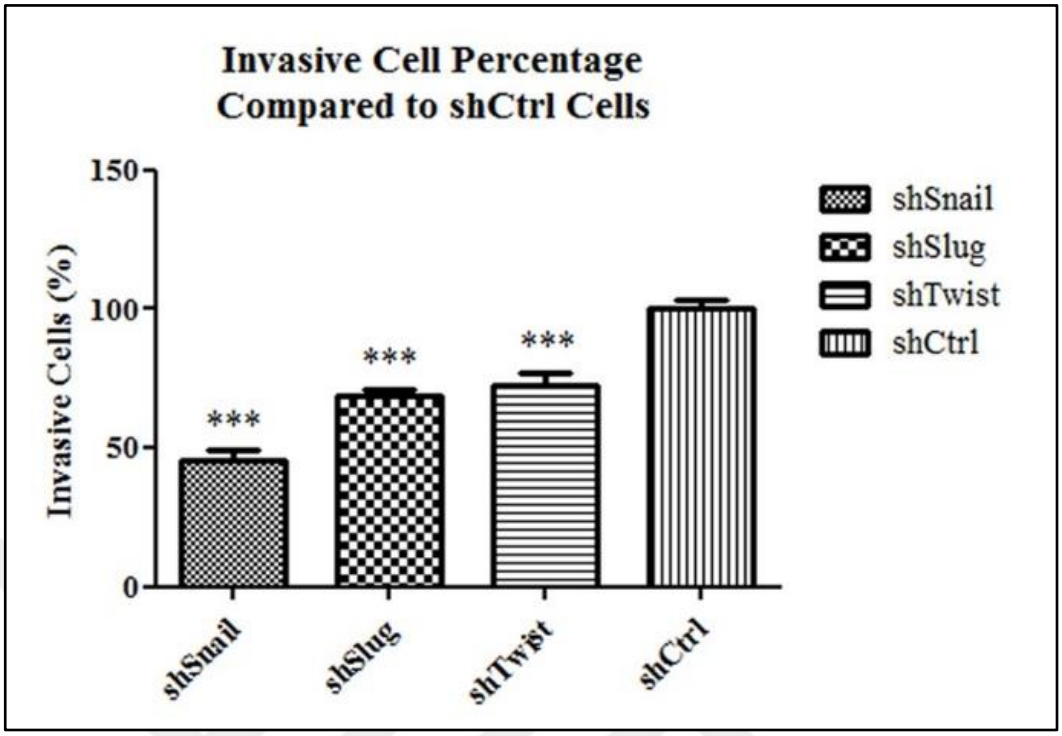


Figure 3.65 Graphical representation of invasive cell percentage after short hairpin silencing (a) compared to shControl cellsOne-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (** indicates that $p<0.005$ and *** indicates that $p<0.001$)

It is possible to say that invasive cell percentage was significantly decreased with short hairpin silencing of Snail, Slug and Twist genes when compared to shControl cells after the results are statistically analyzed with One-way ANOVA with Tukey's posttest. As given in the Table 3.26, compared to the shControl MIA PaCa-2 cells, the invaded cell percentages of shSnail cells were decreased to 45 per cent, shSlug cells were decreased to 69 per cent and shTwist cells were decreased to 73 per cent when the number of invaded cells in the shControl was accepted as 100 per cent

Table 3.26 Invasive cell percentage compared to shControl cells

Invasive Cells Compared to shControl Cells (per cent) (Invasive index)		
shSnail	shSlug	shTwist
45.34	68.82	72.8

3.7.3. Changes in Invasive Capacity of BxPc-3 Cells after Gene Therapy

Figure 3.66 contains the images of Snail, Slug and Twist silenced BxPc-3 cells and their negative control at the 48th hour of invasion assay.

As seen in the Figure 3.66, it is possible to say that, more cells have passed from the matrigel layers in shControl group compared to shSnail, shSlug and shTwist cells.

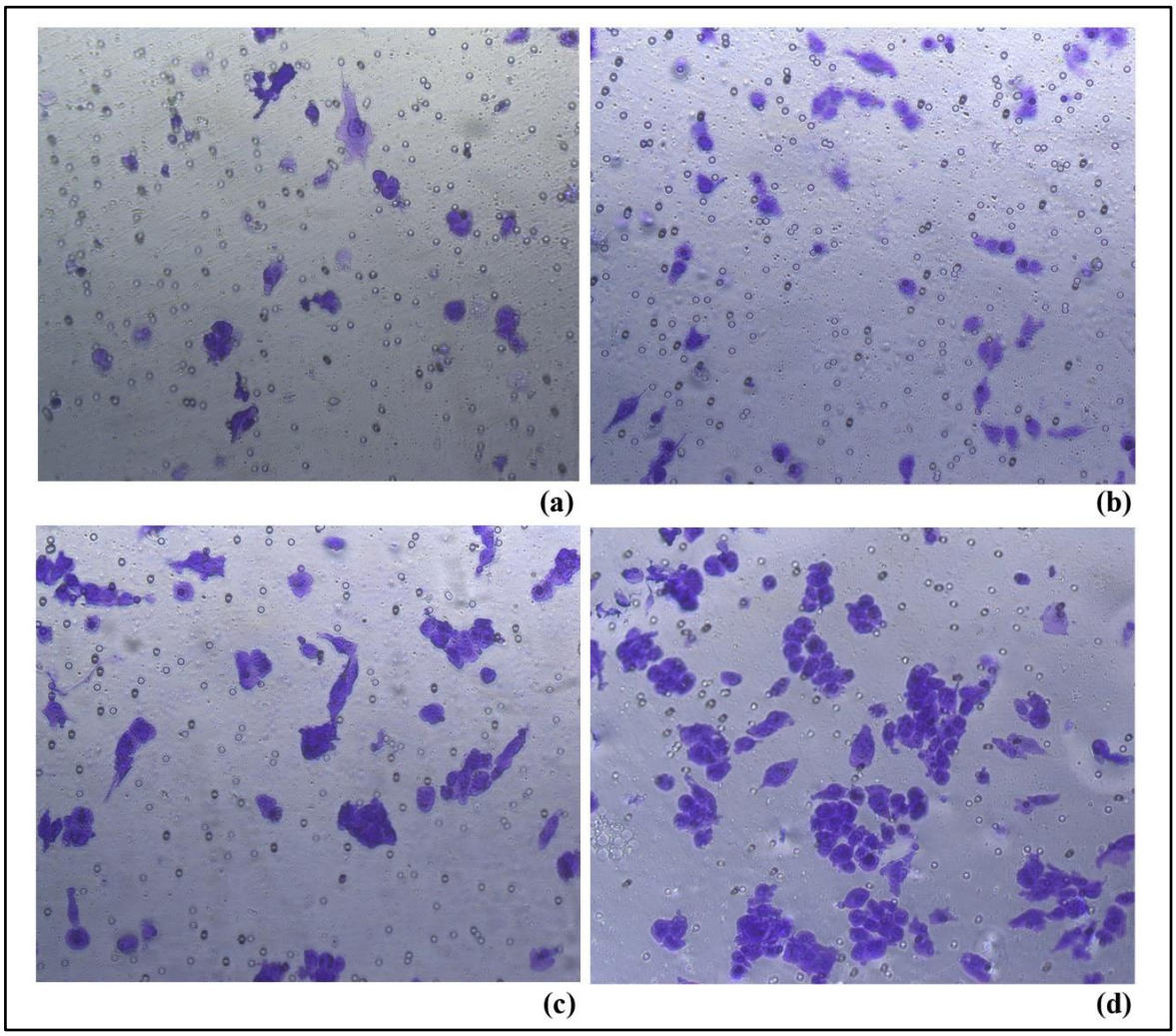


Figure 3.66 Microscopic images of BxPc-3 invasion (a) shSnail Cells (b) shSlug Cells (c) shTwist Cells (d) shControl Cells after 48 hours of incubation in invasion (matrigel) insert with 20x magnification

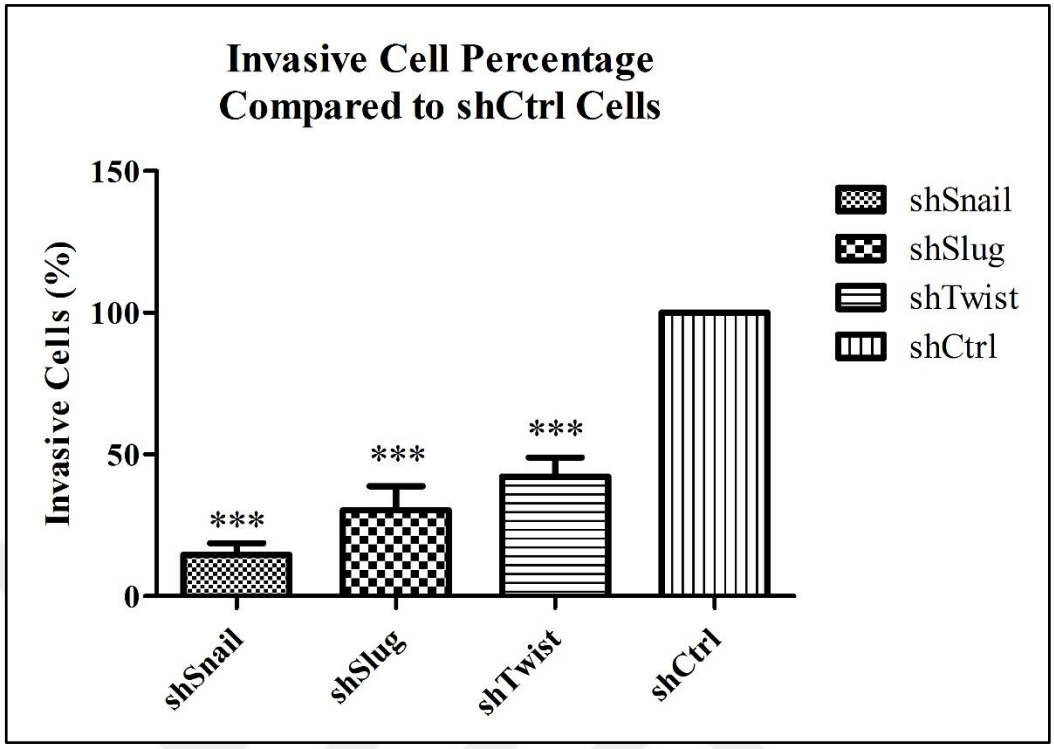


Figure 3.67 Graphical representation of invasive cell percentage compared to shControl cellsOne-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (** indicates that $p<0.005$ and *** indicates that $p<0.001$)

It is possible to say that invasive cell percentage was significantly decreased with short hairpin silencing of Snail, Slug and Twist genes when compared to shControl cells after the results are statistically analyzed with One-way ANOVA with Tukey's posttest. As given in the Table 3.27, compared to the shControl BxPc-3 cells, the invaded cell percentages of shSnail cells were decreased to 14.6 per cent, shSlug cells were decreased to 30.3 per cent and shTwist cells were decreased to 42.2 per cent when the number of invaded cells in the shControl was accepted as 100 per cent

Table 3.27 Invasive cell percentage compared to shControl cells

Invasive Cells Compared to shControl Cells (per cent) (Invasive index)		
shSnail	shSlug	shTwist
14.6	30.3	42.2

3.7.4. Changes in Invasive Capacity of AsPc-1 Cells after Gene Therapy

Figure 3.68 contains the microscope images of Snail, Slug and Twist silenced AsPc-1 cells and their negative control at the 48th hour of invasion assay.

As seen in the Figure 3.68, it is possible to say that, more cells have passed from the matrigel layers in shControl group compared to shSnail, shSlug and shTwist cells. . It could be said that, Snail, Slug and Twist silencing decreases the invasion rate of AsPc-1 pancreas cancer cell line.

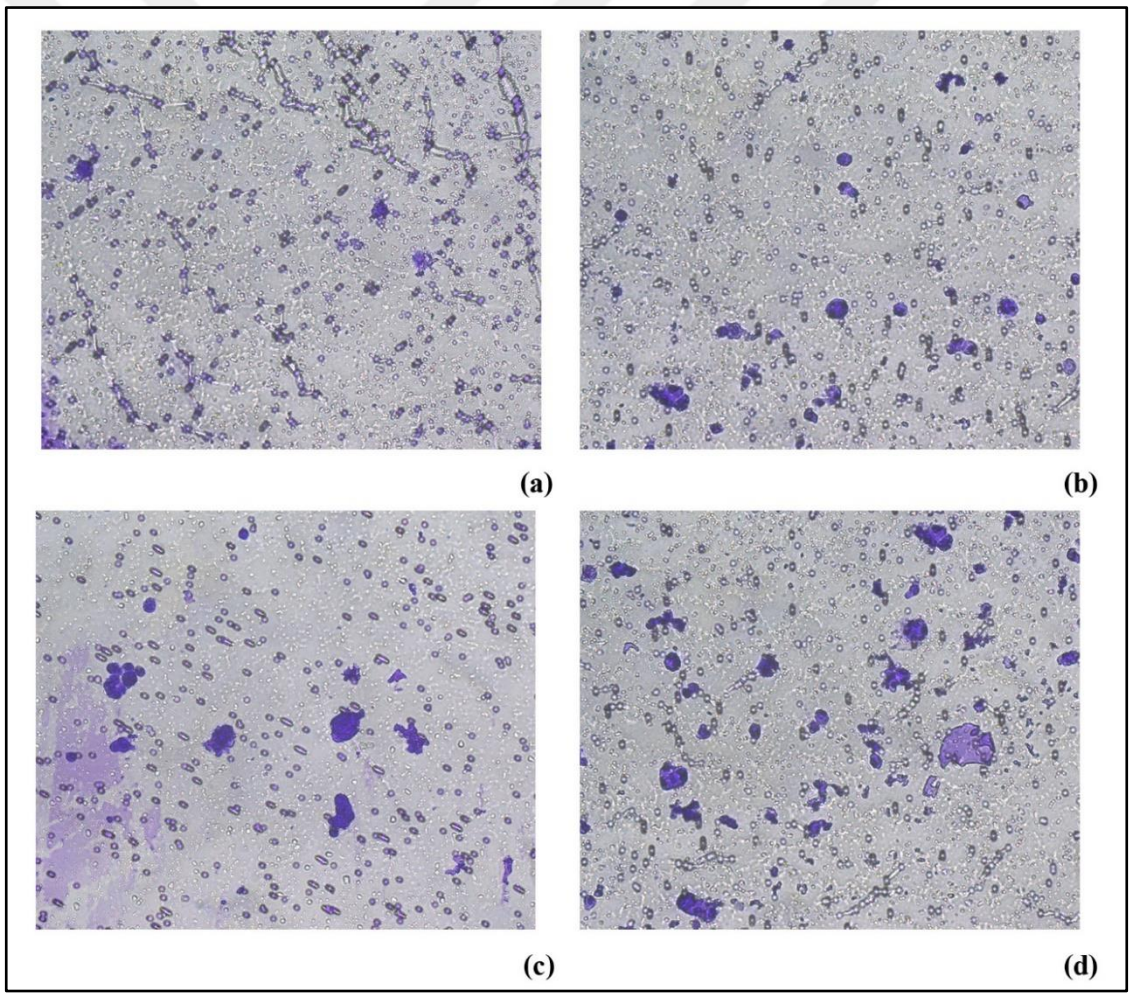


Figure 3.68 Microscopic images of AsPc-1 invasion (a) shSnail Cells (b) shSlug Cells (c) shTwist Cells (d) shControl Cells after 48 hours of incubation in invasion (matrigel) insert with 20x magnification

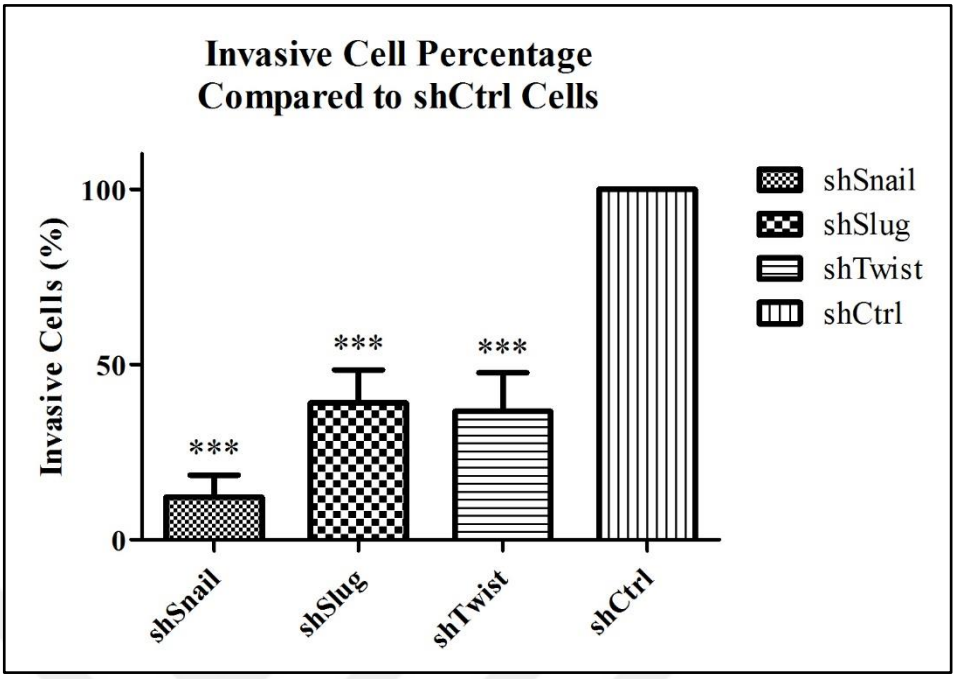


Figure 3.69 Graphical representation of invasive cell percentage compared to shControl cellsOne-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (*** indicates that $p<0.001$)

It is possible to say that invasive cell percentage was significantly decreased with short hairpin silencing of Snail, Slug and Twist genes when compared to shControl cells after the results are statistically analyzed with One-way ANOVA with Tukey's posttest. As given in the Table 3.27, compared to the shControl AsPc-1 cells, the invaded cell percentages of shSnail cells were decreased to 12 per cent, shSlug cells were decreased to 39 per cent and shTwist cells were decreased to 36.5 per cent when the number of invaded cells in the shControl was accepted as 100 per cent

Table 3.28 Invasive cell percentage compared to shControl cells

Invasive Cells Compared to shControl Cells (per cent) (Invasive index)		
shSnail	shSlug	shTwist
12.19	39	36.5

3.8. IN VITRO TUMOR SPHERE FORMATION

3.8.1. Tumor formation ability of Panc-1 cells after Gene Therapy

Figure 3.70 contains the images of tumor spheres of Panc-1 cells after the downregulation of Snail, Slug and Twist genes and their negative control.

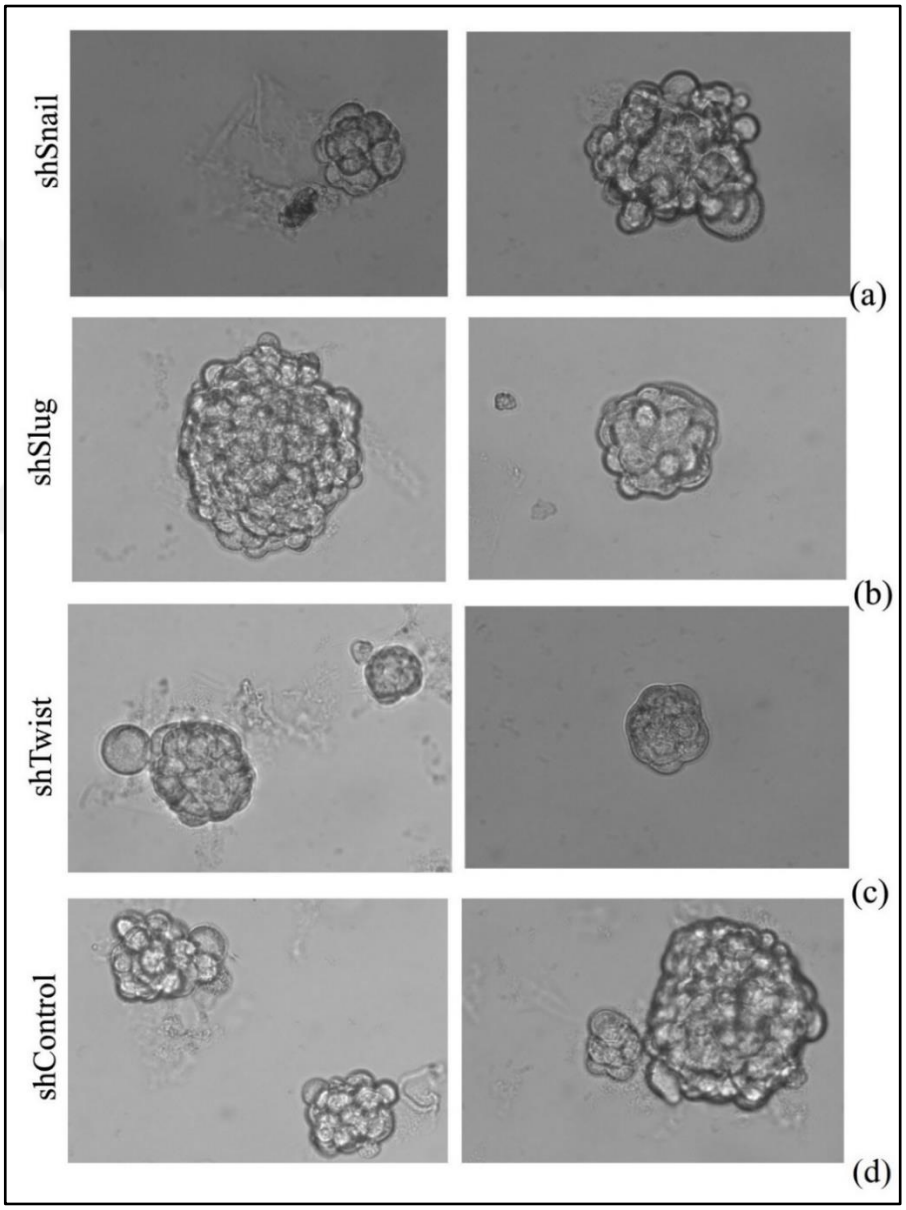


Figure 3.70 Formation of tumor spheres due to cancer stem cells of Panc-1 cells (a) wells containing shSnail cells, (b) wells containing shSlug cells, (c) wells containing shTwist cells (d) wells containing shControl cells after 10 days of incubation in sphere forming medium

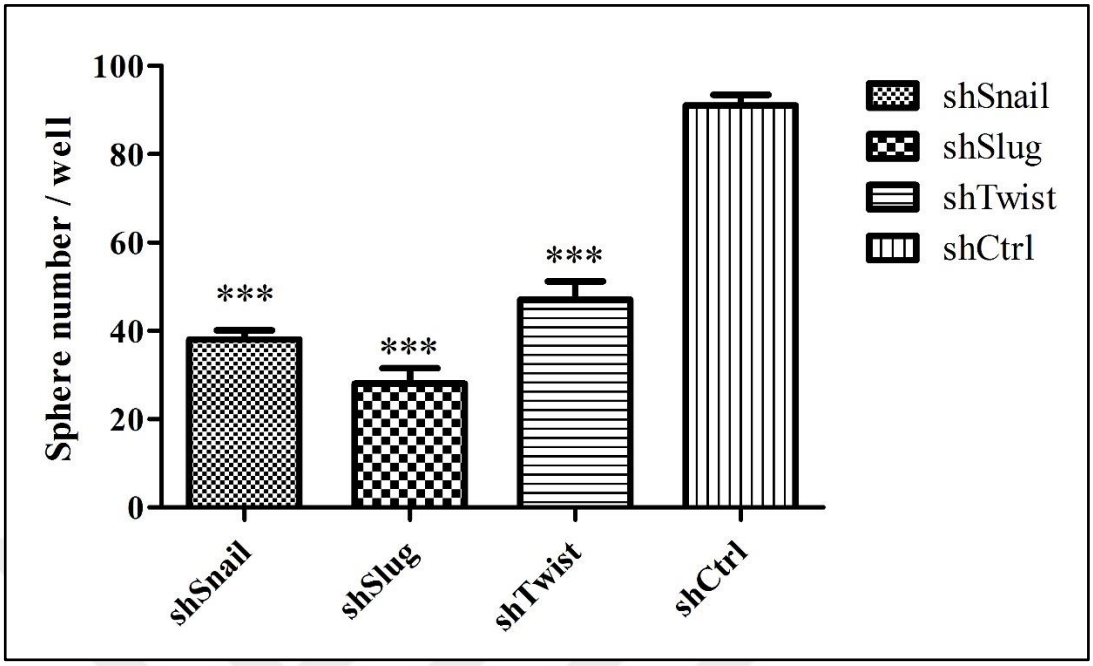


Figure 3.71 Graphical representation of tumor spheres number per well after 10 days of incubation in sphere forming mediumOne-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (*** indicates that $p<0.001$)

After incubation in sphere forming medium it is observed that when Snail, Slug and Twist gene was silenced in Panc-1 cells, tumor sphere formation significantly decreased compared to control plasmid transformed Panc-1 cells. As indicated in the Table 3.29, the average number of tumor spheres of shSnail cells were 38, the average number of tumor spheres of shSlug were 28, the average number of tumor spheres of shTwist were 47 while the average number of tumor spheres of shControl was 91. The difference between the average sphere numbers were analyzed by One-way ANOVA with Tukey's test as the post test.

Table 3.29 Average sphere numbers of wells containing short hairpin silenced cells, and shControl cells

	shSnail	**shSlug**	**shTwist**	**shCtrl**
Average Sphere number/well	38	28	47	91

3.8.2. Tumor formation ability of MIA PaCa-2 cells after Gene Therapy

Figure 3.72 contains the images of tumor spheres in MIA PaCa-2 cells after the downregulation of Snail, Slug and Twist genes and their negative control.

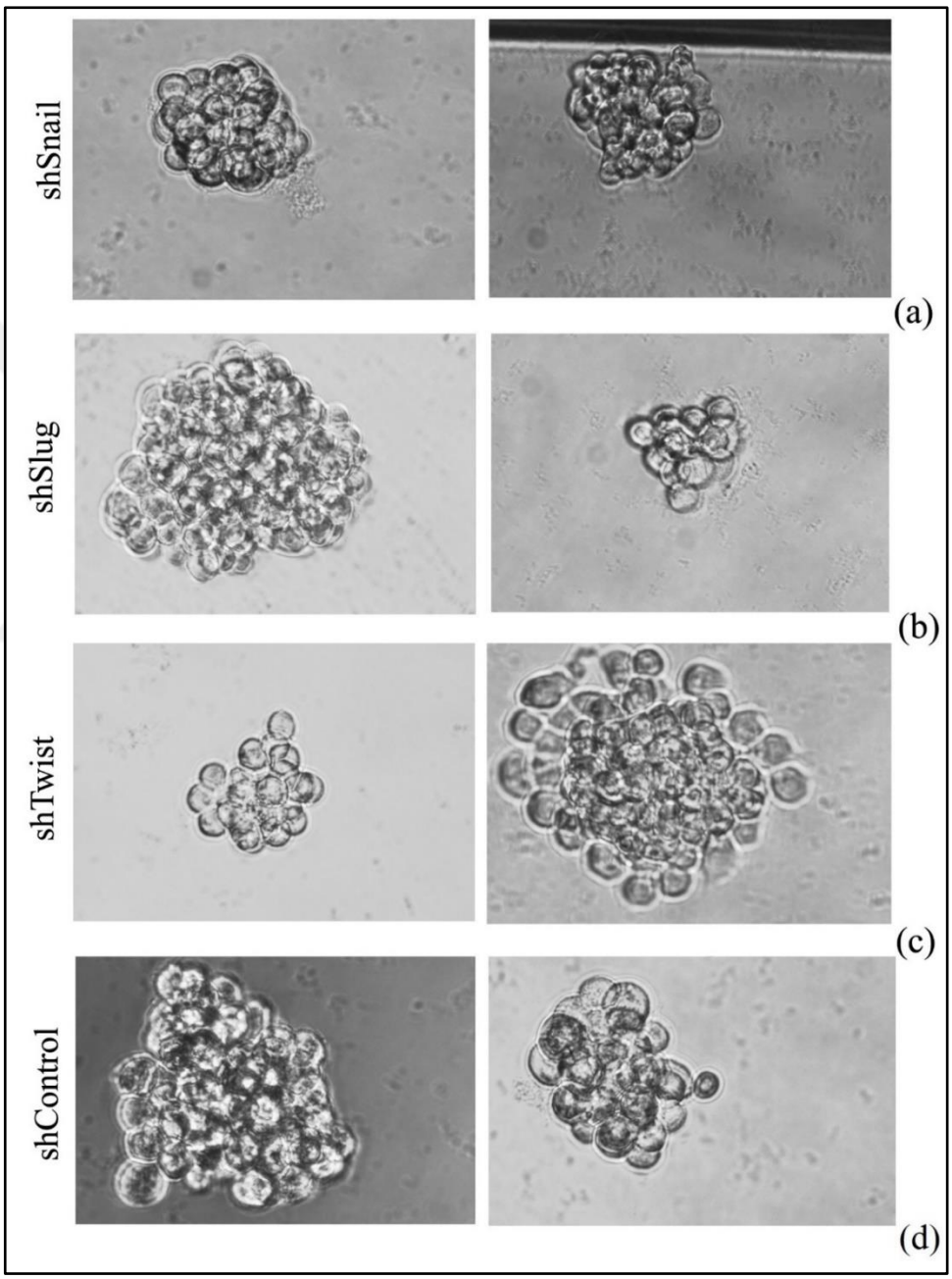


Figure 3.72 Formation of tumor spheres due to cancer stem cells of MIA PaCa-2 cells (a) wells containing shSnail cells, (b) wells containing shSlug cells, (c) wells containing shTwist cells (d) wells containing shControl cells after 10 days of incubation in sphere forming medium

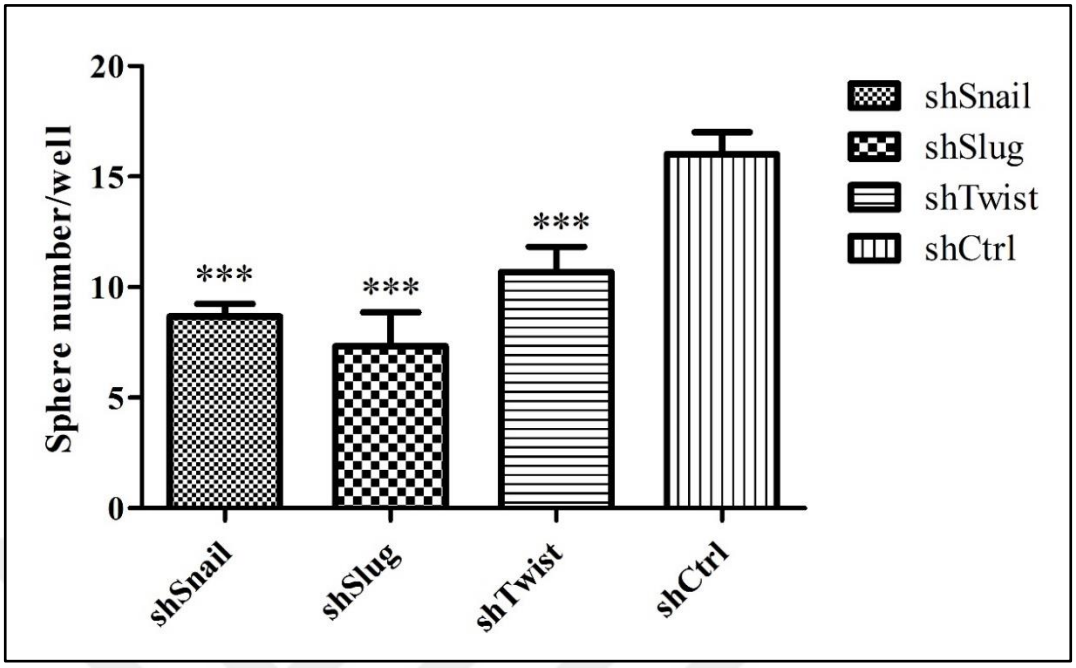


Figure 3.73 Graphical representation of tumor spheres number per well after 10 days of incubation in sphere forming mediumOne-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (*** indicates that $p<0.001$)

After incubation in sphere forming medium it is observed that when Snail, Slug and Twist gene was silenced in MIA PaCa-2 cells, tumor sphere formation significantly decreased compared to control plasmid transformed MIA PaCa-2 cells. As indicated in the Table 3.30, the average number of tumor spheres of shSnail cells were 9 the average number of tumor spheres of shSlug were 7.33, the average number of tumor spheres of shTwist were 10.66 while the average number of tumor spheres of shControl was 16. The difference between the average sphere numbers were analyzed by One-way ANOVA with Tukey's test as the post test.

Table 3.30 Average sphere numbers of wells containing short hairpin silenced cells and shControl cells

	shSnail	**shSlug**	**shTwist**	**shControl**
Average Sphere number/well	9	7.33	10.66	16

3.8.3. Tumor formation ability of BxPc-3 cells after Gene Therapy

Figure 3.74 contains the images of tumor spheres in BxPc-3 cells after the downregulation of Snail, Slug and Twist genes and their negative control.

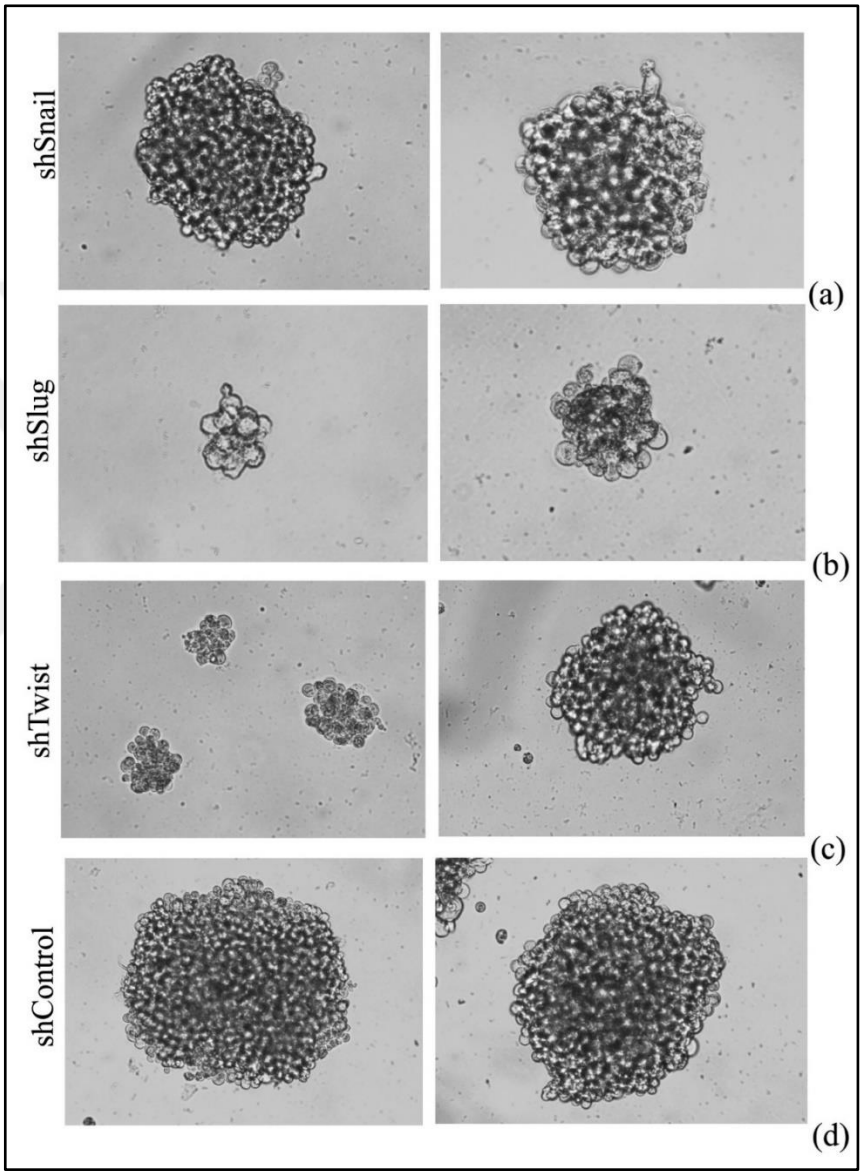


Figure 3.74 Formation of tumor spheres due to cancer stem cells of BxPc-3 cells (a) wells containing shSnail cells, (b) wells containing shSlug cells, (c) wells containing shTwist cells (d) wells containing shControl cells after 10 days of incubation in sphere forming medium

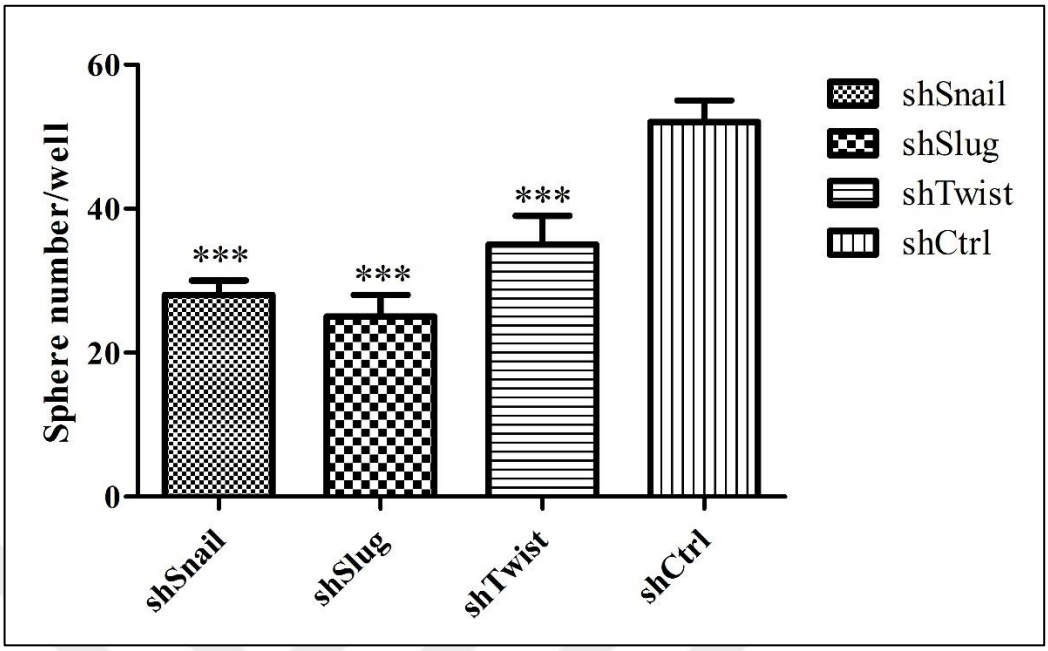


Figure 3.75 Graphical representation of tumor spheres number per well after 10 days of incubation in sphere forming medium One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (*** indicates that $p<0.001$)

After incubation in sphere forming medium it is observed that when Snail, Slug and Twist gene was silenced in BxPc-3 cells, tumor sphere formation significantly decreased compared to control plasmid transformed BxPc-3 cells. As indicated in the Table 3.31 the average number of tumor spheres of shSnail cells were 28, the average number of tumor spheres of shSlug were 25, the average number of tumor spheres of shTwist were 35 while the average number of tumor spheres of shControl was 52. The difference between the average sphere numbers were analyzed by One-way ANOVA with Tukey's test as the post test.

Table 3.31 Average sphere numbers of wells containing short hairpin silenced cells, shControl cells

	shSnail	**shSlug**	**shTwist**	**shCtrl**
Average Sphere number/well	28	25	35	52

3.8.4. Tumor formation ability of AsPc-1 cells after Gene Therapy

Figure 3.76 contains the images of tumor spheres in AsPc-1 cells after the downregulation of Snail, Slug and Twist genes and their negative control.

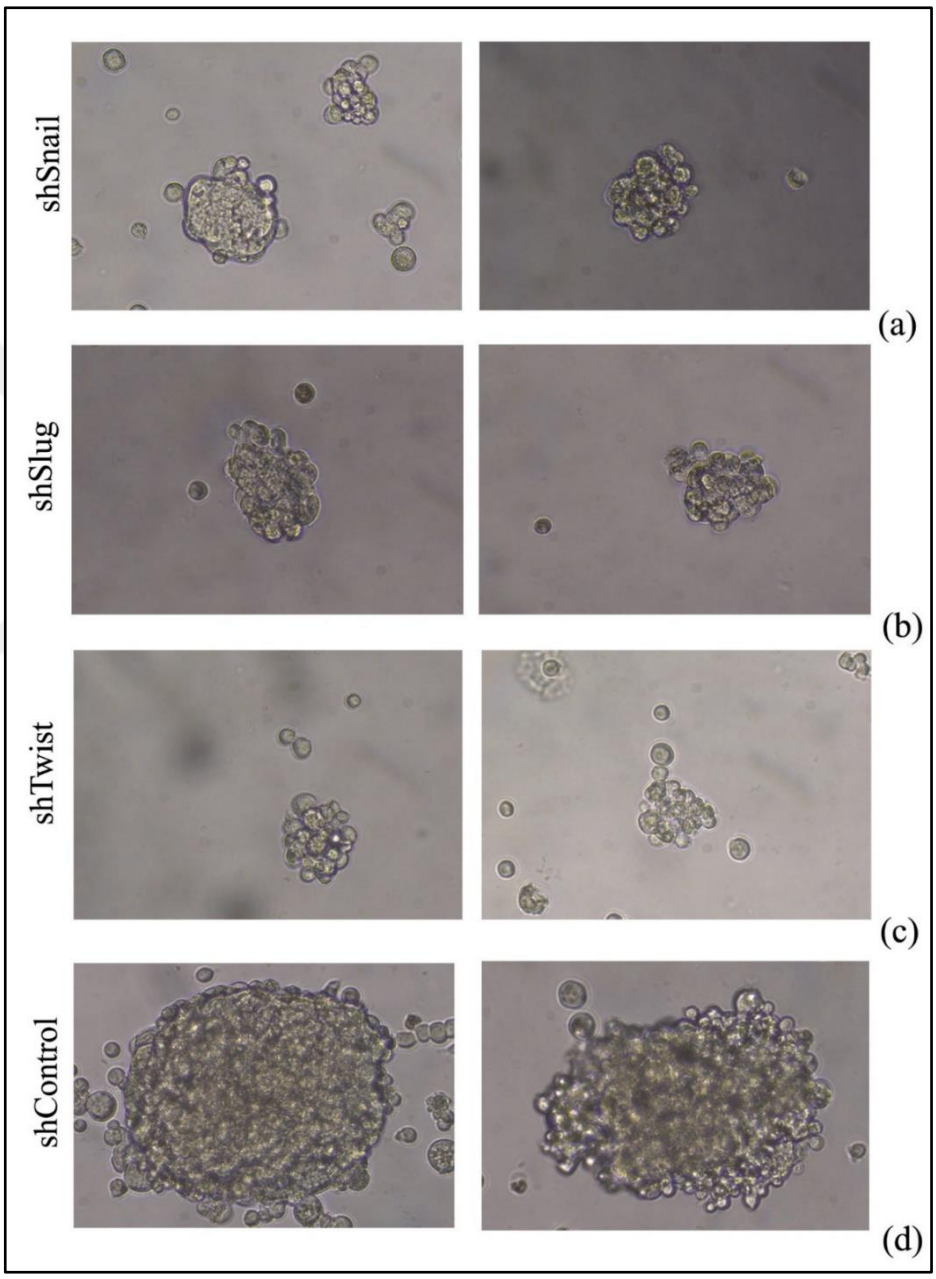


Figure 3.76 Formation of tumor spheres due to cancer stem cells of AsPc-1 cells (a) wells containing shSnail cells, (b) wells containing shSlug cells, (c) wells containing shTwist cells (d) wells containing shControl cells after 10 days of incubation in sphere forming medium

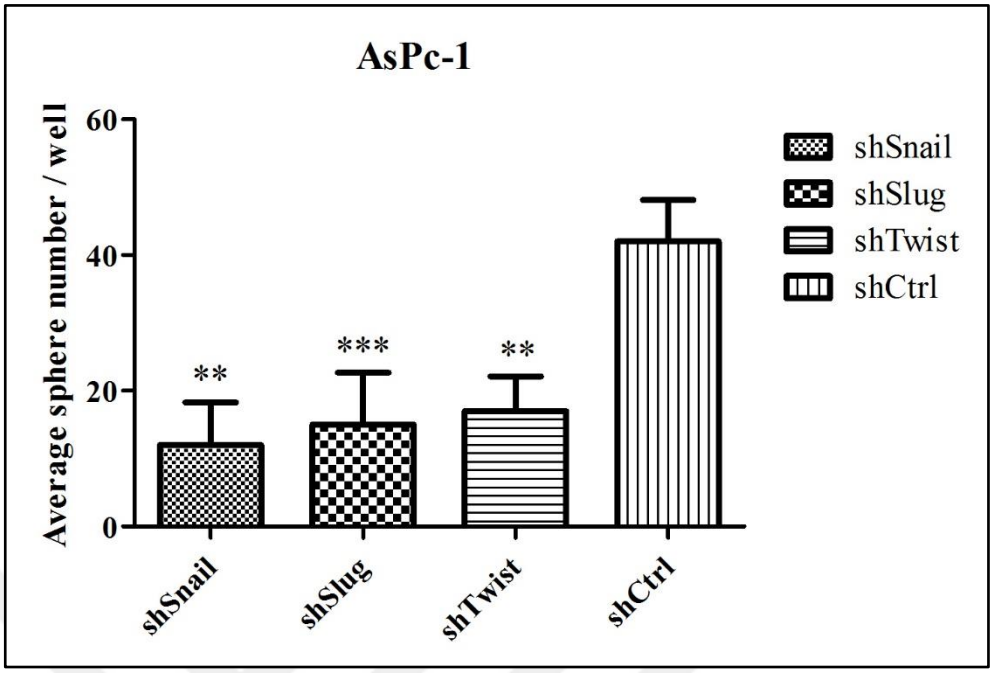


Figure 3.77 Graphical representation of tumor spheres number per well after 10 days of incubation in sphere forming mediumOne-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (** indicates that $p<0.005$ and *** indicates that $p<0.001$)

After incubation in sphere forming medium it is observed that when Snail, Slug and Twist gene was silenced in AsPc-1 cells, tumor sphere formation significantly decreased compared to control plasmid transformed AsPc-1 cells. As indicated in the Table 3.32 the average number of tumor spheres of shSnail cells were 12 the average number of tumor spheres of shSlug were 15, the average number of tumor spheres of shTwist were 17 while the average number of tumor spheres of shControl was 42. The difference between the average sphere numbers were analyzed by One-way ANOVA with Tukey's test as the post test.

Table 3.32 Average sphere numbers of wells containing short hairpin silenced cells, shControl cells

	shSnail	**shSlug**	**shTwist**	**shCtrl**
Average Sphere number/well	12	15	17	42

3.9. ATTACHMENT TO LAMININ

3.9.1. Amount of Laminin Attachment of Panc-1 cells after Gene Therapy

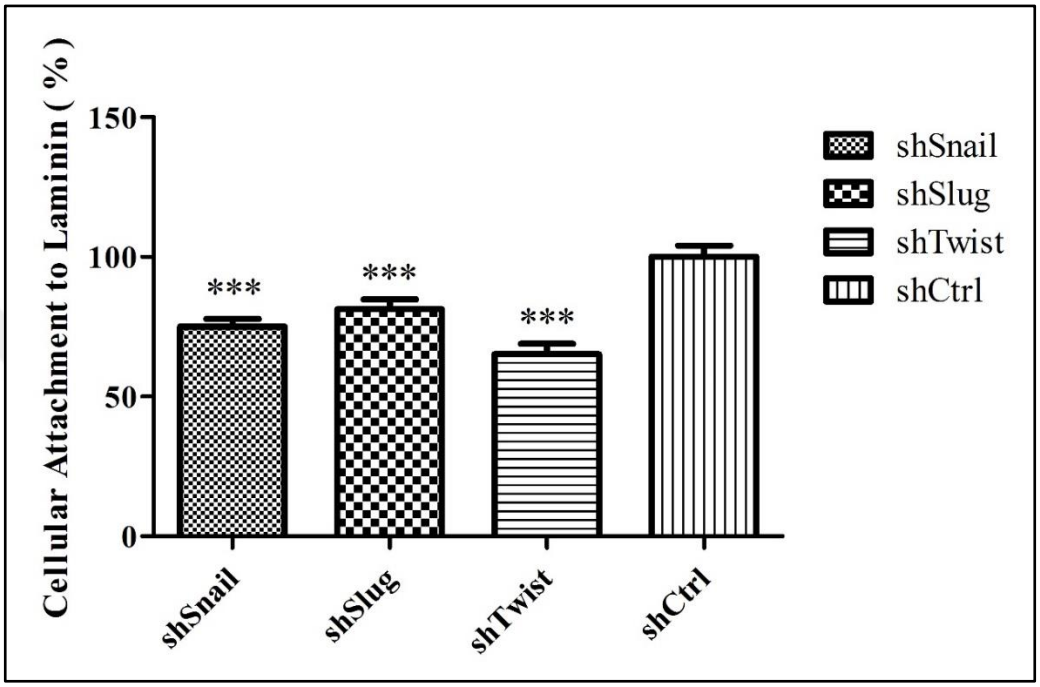


Figure 3.78 Graphical representation of attached cell percentages compared to shControl cellsOne-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (*** indicates that $p<0.001$)

It is observed that, short hairpin silencing of Snail, Slug and Twist gene significantly decreased attachment ability to laminin in Panc-1 cell line. As indicated in the Table 3.33, the cellular attachment percentage was decreased to 75 per cent in shSnail cells, 81.2 in shSlug cells and 65.16 in shTwist cells when the cellular attachment to laminin is accepted as 100 per cent in shControl cell groups

Table 3.33 Cellular attachment percentages compared to shControl cells

	shSnail	**shSlug**	**shTwist**	**shControl**
Cellular Attachment Percentage	75	81.25	65.16	100

3.9.2. Amount of Laminin Attachment of MIA PaCa-2 Cells after Gene Therapy

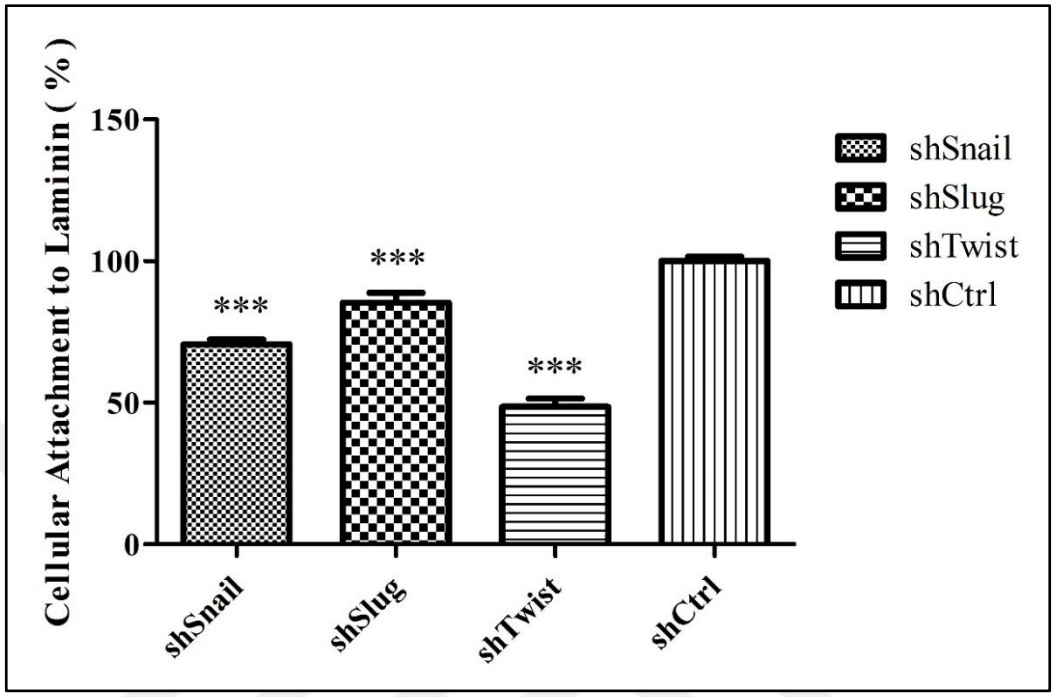


Figure 3.79 Graphical representation of attached cell percentages compared to shControl cellsOne-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (*** indicates that $p<0.001$)

It is observed that, short hairpin silencing of Snail, Slug and Twist gene significantly decreased attachment ability to laminin in MIA PaCa-2 cell line. As indicated in the Table 3.34, the cellular attachment percentage was decreased to 70 per cent in shSnail cells, 85.3 in shSlug cells and 48.53 in shTwist cells when the cellular attachment to laminin is accepted as 100 per cent in shControl cell groups

Table 3.34 Cellular attachment percentages compared to shControl cells

	shSnail	**shSlug**	**shTwist**	**shControl**
Cellular Attachment Percentage	70	85.29	48.53	100

3.9.3. Amount of Laminin Attachment of BxPc-3 Cells after Gene Therapy

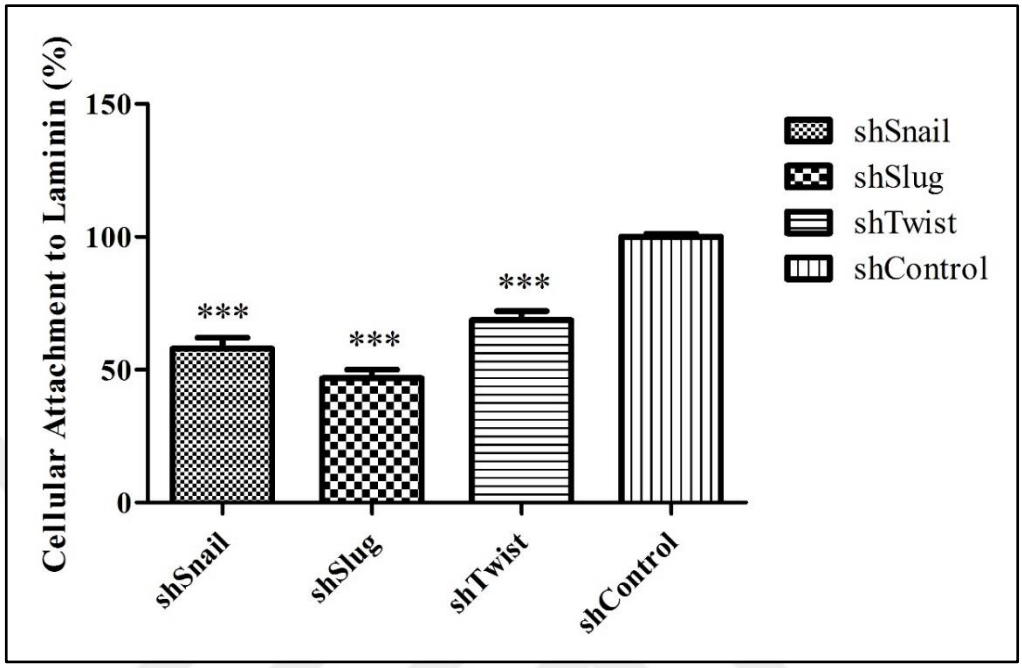


Figure 3.80 Graphical representation of attached cell percentages compared to shControl cellsOne-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (*** indicates that $p<0.001$)

It is observed that, short hairpin silencing of Snail, Slug and Twist gene significantly decreased attachment ability to laminin in BxPc-3 cell line. As indicated in the Table 3.35, the cellular attachment percentage was decreased to 58 per cent in shSnail cells, 46.8 per cent in shSlug cells and 68.7 per cent in shTwist cells when the cellular attachment to laminin is accepted as 100 per cent in shControl cell groups

Table 3.35 Cellular attachment percentages compared to shControl cells

	shSnail	**shSlug**	**shTwist**	**shControl**
Cellular Attachment Percentage	57.9	46.8	68.7	100

3.9.4. Amount of Laminin Attachment of AsPc-1 Cells after Gene Therapy

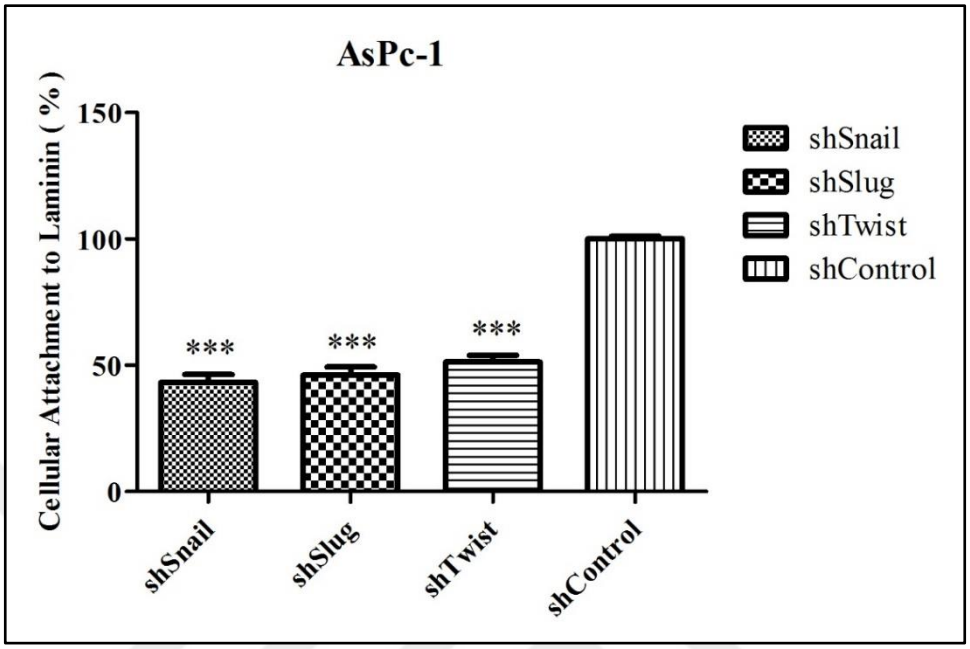


Figure 3.81 Graphical representation of attached cell percentages compared to shControl cellsOne-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (*** indicates that $p<0.001$)

It is observed that, short hairpin silencing of Snail, Slug and Twist gene significantly decreased attachment ability to laminin in AsPc-1 cell line. As indicated in the Table 3.36, the cellular attachment percentage was decreased to 43 per cent in shSnail cells, 46 per cent in shSlug cells and 51 per cent in shTwist cells when the cellular attachment to laminin is accepted as 100 per cent in shControl cell groups

Table 3.36 Cellular attachment percentages compared to shControl cells

	shSnail	**shSlug**	**shTwist**	**shControl**
Cellular Attachment Percentage	43	46	51	100

3.10. CANCER STEM CELL PROPERTIES

3.10.1. Changes in Gene Expression of Cancer Stem Cell Characteristic Genes after Gene Therapy

In order to evaluate the changes in the expressions of pancreas cancer stem cell markers, cells after gene therapy were subjected to realtime PCR analysis for related genes.

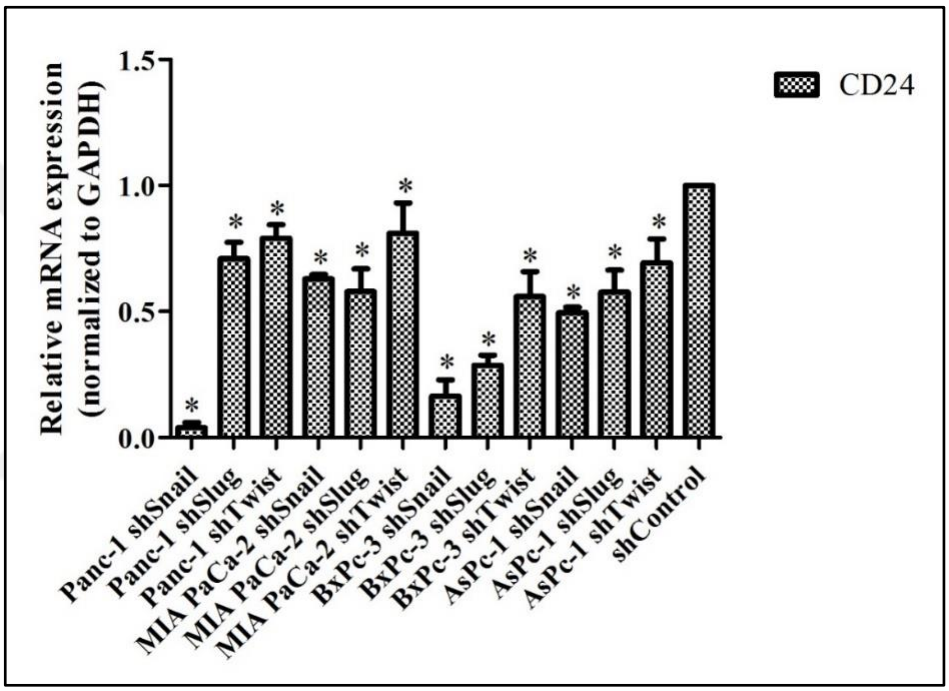


Figure 3.82 Graphical representation of CD24 expression One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$)

It is found that, downregulation of Snail, Slug and Twist significantly downregulate the gene expression of CD24, pancreas cancer stem cell marker. Downregulation of EMT has decreased the expression of CD24 to four per cent in Panc-1 shSnail cells, 70 per cent in Panc-1 shSlug cells, 79 per cent in Panc-1 shTwist cells, 63 per cent in MIA PaCa-2 shSnail cells, 58 per cent in MIA PaCa-2 shSlug cells, 81 per cent in MIA PaCa-2 shTwist cells, 16 per cent in BxPc-3 shSnail cells, 28 per cent in BxPc-3 shSlug cells, 55 per cent in BxPc-3 shTwist cells, 49 per cent in AsPc-1 shSnail cells, 57 per cent in AsPc-1 shSlug cells and 69 per cent in AsPc-1 shTwist cells when the expression values for their shControl cells were

normalized to one. The expression values were analyzed by ΔΔCt method and the statistical evaluation of the differences between groups were analyzed with One-way ANOVA with Tukey's posttest.

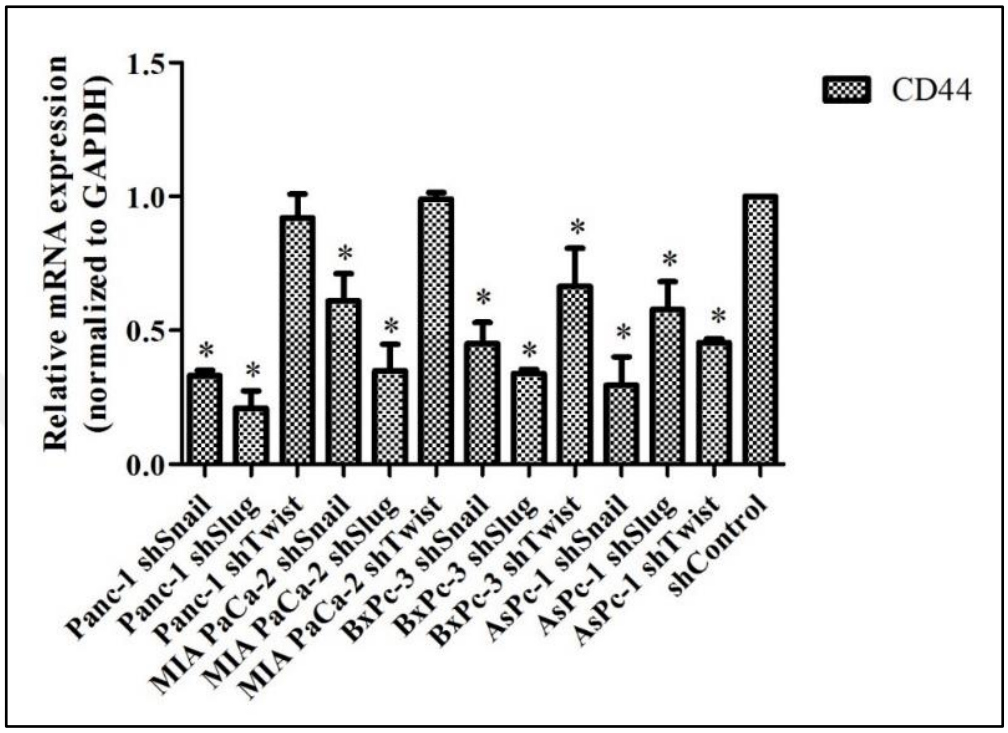


Figure 3.83 Graphical representation of changes in CD44 expression.One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$)

It was found that, silencing Snail, Slug and Twist in pancreas cancer cell lines has significantly downregulated the expression of CD44, a cancer stem cell marker for PC. Downregulation of EMT has decreased the expression of CD44 to 33 per cent in Panc-1 shSnail cells, 21 per cent in Panc-1 shSlug cells, 79 per cent in Panc-1 shTwist cells, 61 per cent in MIA PaCa-2 shSnail cells, 35 per cent in MIA PaCa-2 shSlug cells, 99 per cent in MIA PaCa-2 shTwist cells, 45 per cent in BxPc-3 shSnail cells, 33 per cent in BxPc-3 shSlug cells, 66 per cent in BxPc-3 shTwist cells, 29 per cent in AsPc-1 shSnail cells, 57 per cent in AsPc-1 shSlug cells and 45 per cent in AsPc-1 shTwist cells when the expression values for their shControl cells were normalized to one. The expression values were analyzed by ΔΔCt method and the statistical evaluation of the differences between groups were analyzed with One-way ANOVA with Tukey's posttest.

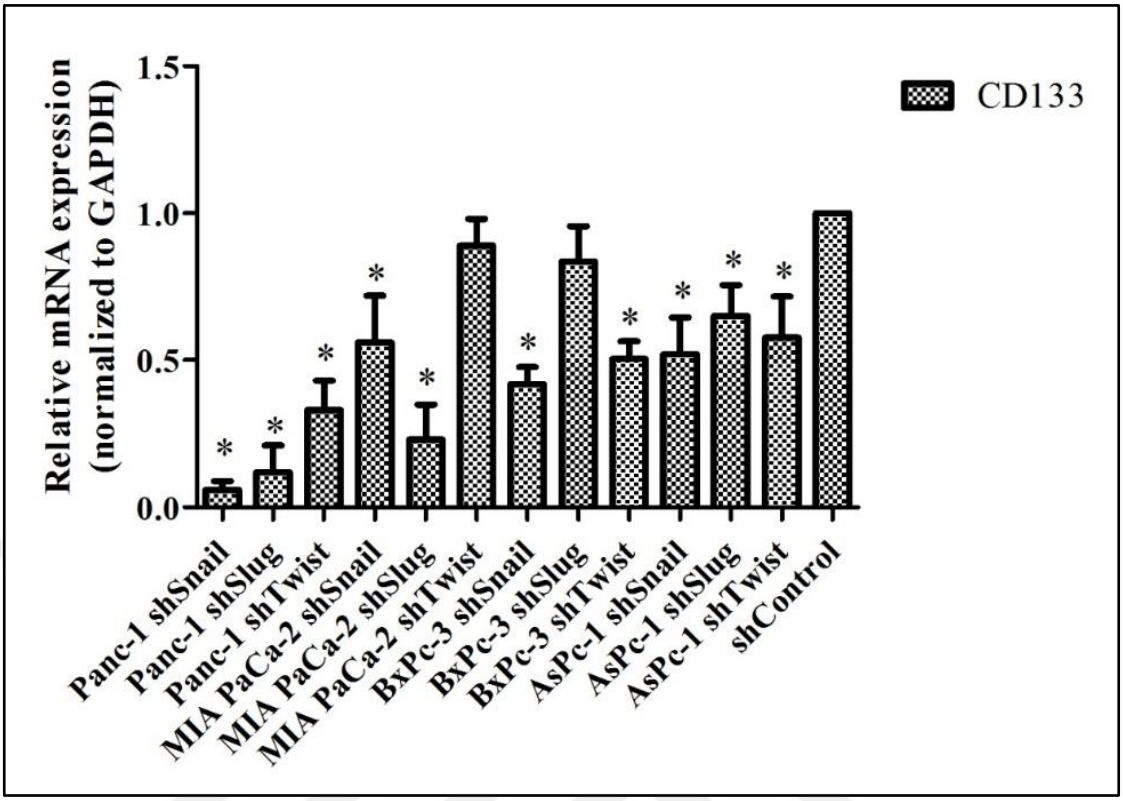


Figure 3.84 Graphical representation of changes in CD133 expression.One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$)

It was found that, downregulation of EMT transcription factors Snail, Slug and Twist also significantly downregulate the cancer stem cell marker CD133 in PC cell lines. Downregulation of EMT has decreased the expression of CD133 to six per cent in Panc-1 shSnail cells, 12 per cent in Panc-1 shSlug cells, 33 per cent in Panc-1 shTwist cells, 56 per cent in MIA PaCa-2 shSnail cells, 23 per cent in MIA PaCa-2 shSlug cells, 89 per cent in MIA PaCa-2 shTwist cells, 41 per cent in BxPc-3 shSnail cells, 83 per cent in BxPc-3 shSlug cells, 50 per cent in BxPc-3 shTwist cells, 52 per cent in AsPc-1 shSnail cells, 66 per cent in AsPc-1 shSlug cells and 57 per cent in AsPc-1 shTwist cells when the expression values for their shControl cells were normalized to one. The expression values were analyzed by $\Delta\Delta$Ct method and the statistical evaluation of the differences between groups were analyzed with One-way ANOVA with Tukey's posttest.

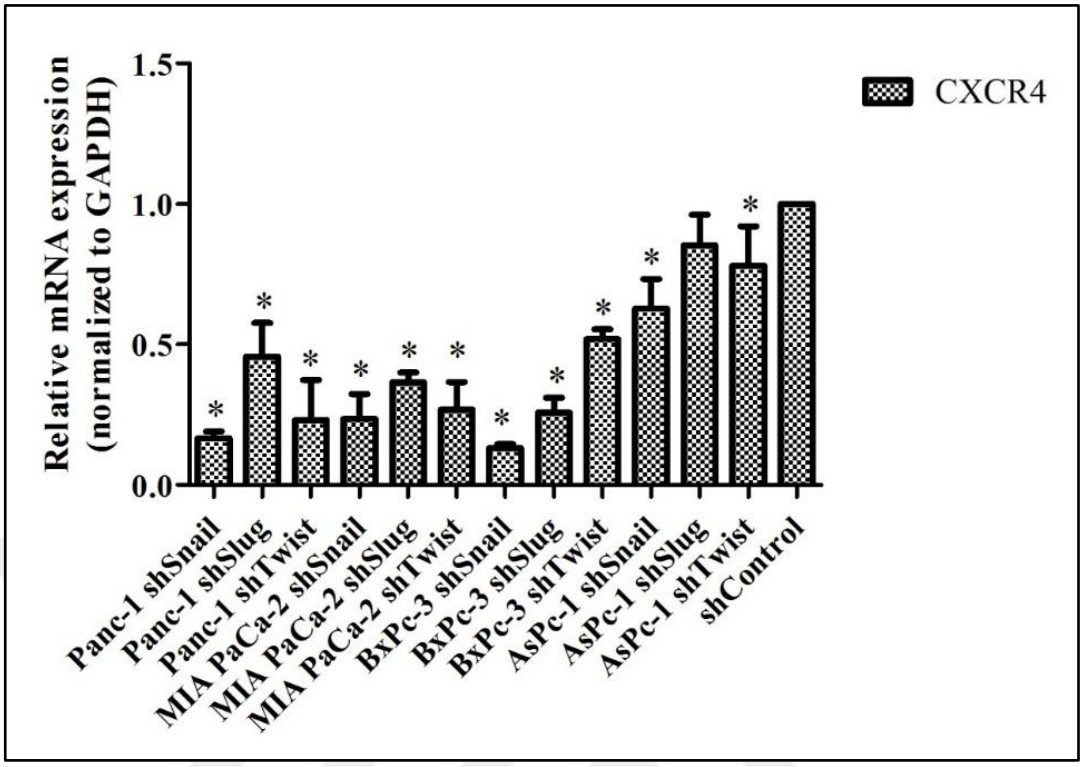


Figure 3.85 Graphical representation of changes in CXCR4 expression
Snail, Slug and Twist downregulation has decreased the expression of CXCR4 in pancreas cancer cell lines..One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$)

It was found that, downregulation of EMT transcription factors Snail, Slug and Twist also significantly downregulate the cancer stem cell marker CXCR4 in PC cell lines. Downregulation of EMT has decreased the expression of CXCR4 to 16 per cent in Panc-1 shSnail cells, 45 per cent in Panc-1 shSlug cells, 23 per cent in Panc-1 shTwist cells, 23 per cent in MIA PaCa-2 shSnail cells, 36 per cent in MIA PaCa-2 shSlug cells, 26 per cent in MIA PaCa-2 shTwist cells, 13 per cent in BxPc-3 shSnail cells, 25 per cent in BxPc-3 shSlug cells, 51 per cent in BxPc-3 shTwist cells, 62 per cent in AsPc-1 shSnail cells, 85 per cent in AsPc-1 shSlug cells and 78 per cent in AsPc-1 shTwist cells when the expression values for their shControl cells were normalized to one. The expression values were analyzed by $\Delta\Delta$Ct method and the statistical evaluation of the differences between groups were analyzed with One-way ANOVA with Tukey's posttest.

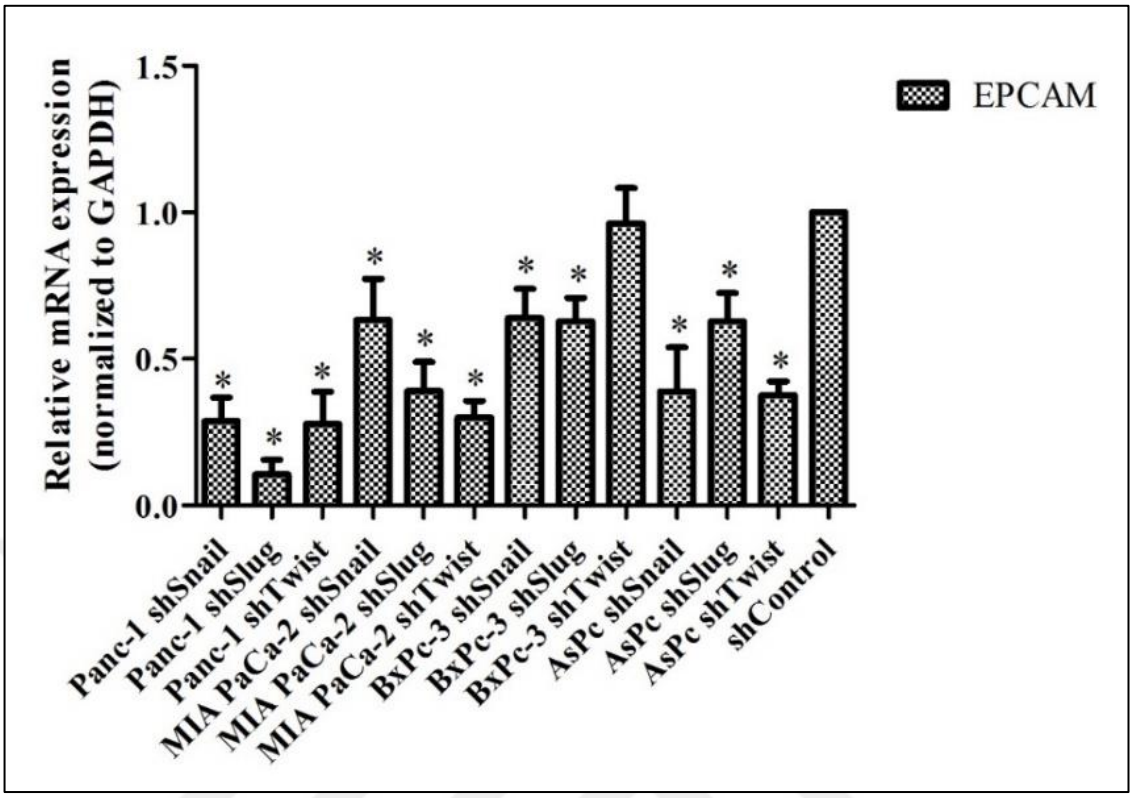


Figure 3.86 Graphical representation of changes in EpCAM expression.One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$)

It is found that, the gene silencing of Snail, Slug and Twist downregulates the cancer stem cell marker EpCAM in pancreas cancer cell lines. Downregulation of EMT has decreased the expression of EPCAM to 28 per cent in Panc-1 shSnail cells, 10 per cent in Panc-1 shSlug cells, 27 per cent in Panc-1 shTwist cells, 63 per cent in MIA PaCa-2 shSnail cells, 39 per cent in MIA PaCa-2 shSlug cells, 30 per cent in MIA PaCa-2 shTwist cells, 68 per cent in BxPc-3 shSnail cells, 62 per cent in BxPc-3 shSlug cells, 96 per cent in BxPc-3 shTwist cells, 38 per cent in AsPc-1 shSnail cells, 62 per cent in AsPc-1 shSlug cells and 37 per cent in AsPc-1 shTwist cells when the expression values for their shControl cells were normalized to one. The expression values were analyzed by $\Delta\Delta$Ct method and the statistical evaluation of the differences between groups were analyzed with One-way ANOVA with Tukey's posttest.

3.10.2. Changes in Protein amounts of Cancer Stem Cell Characteristic Surface Markers after Gene Therapy

In order to evaluate the decrease in the pancreas cancer stem cell characteristic markers in protein level, flow cytometry analysis was done

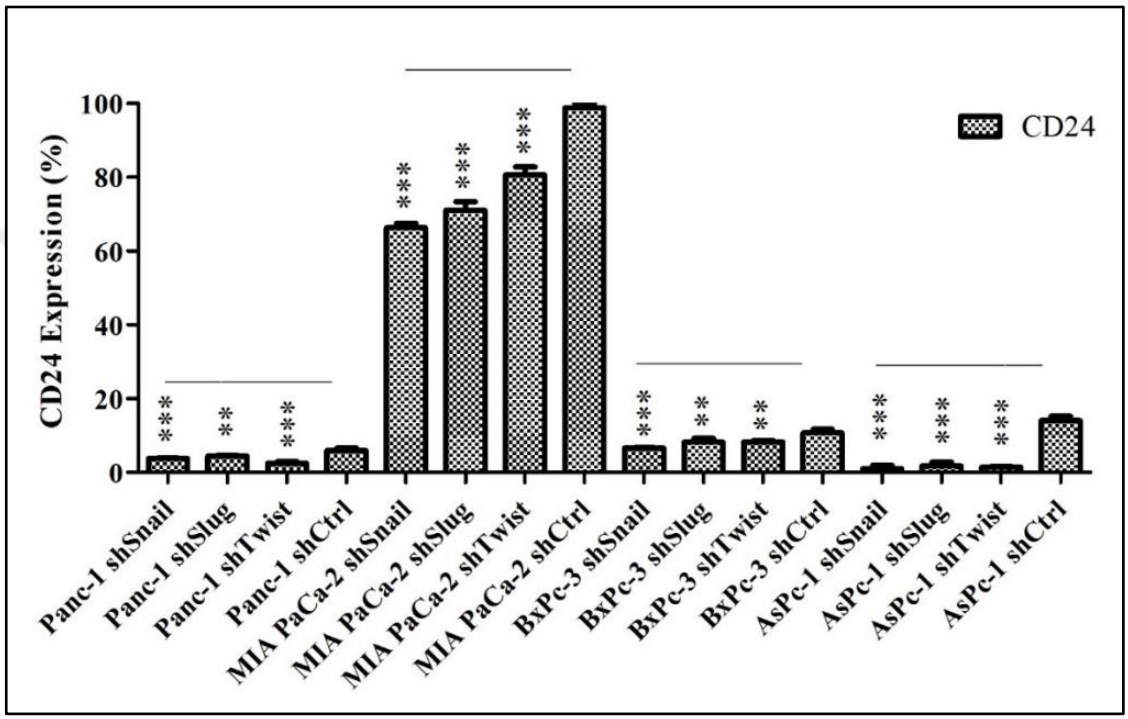


Figure 3.87 Graphical representation of the changes in CD24 amount.One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$)

The Figure 3.87 represents the protein amount changes of CD24 after downregulation of Snail, Slug and Twist. It is found that, gene therapy significantly decreases the amount of CD24 in pancreas cancer cell lines. Downregulation of EMT has decreased the amount of CD24 to 3.8 per cent in Panc-1 shSnail cells, 4.47 per cent in Panc-1 shSlug cells, 2.43 per cent in Panc-1 shTwist cells when the amount was 5.96 in Panc-1 shControl cells, 66 per cent in MIA PaCa-2 shSnail cells, 71 per cent in MIA PaCa-2 shSlug cells, 80 per cent in MIA PaCa-2 shTwist cells when the amount was 99 per cent in MIA PaCa-2 shControl cells, 6.6 per cent in BxPc-3 shSnail cells, 8.2 per cent in BxPc-3 shSlug cells, 8.3 per cent in BxPc-3 shTwist cells when the amount was 10.7 in BxPc-3 shControl cells and, 1.07 per cent in AsPc-1 shSnail cells, 1.8 per cent in AsPc-1 shSlug cells and 1.4 per cent in AsPc-1

shTwist cells when the amount was 14 per cent in AsPc-1 shControl cells. The statistical evaluation of the differences between groups were analyzed with One-way ANOVA with Tukey's posttest. These changes indicate that EMT silencing (Snail, Slug and Twist downregulation) also decreases the expressions of cancer stem cell marker CD24.

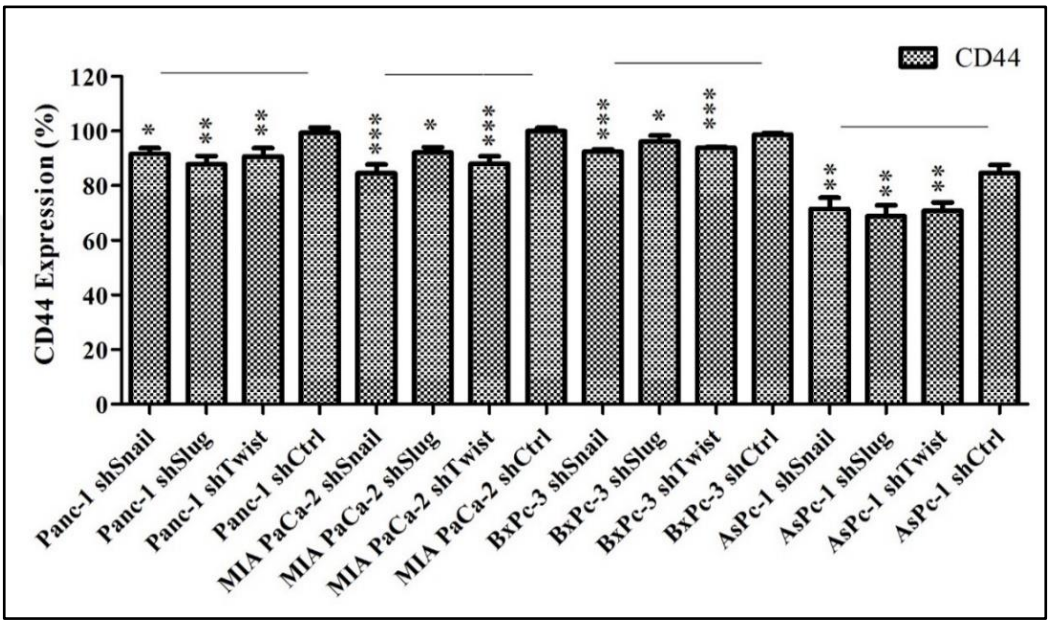


Figure 3.88 Graphical representation of the changes in CD44 amount.One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$)

Figure 3.88 represents the protein amount changes of CD44 after downregulation of Snail, Slug and Twist. It is found that, gene therapy significantly decreases the amount of CD44 in pancreas cancer cell lines. Downregulation of EMT has decreased the amount of CD44 to 91 per cent in Panc-1 shSnail cells, 88 per cent in Panc-1 shSlug cells, 90.6 per cent in Panc-1 shTwist cells when the amount was 99.33 in Panc-1 shControl cells, 84.5 per cent in MIA PaCa-2 shSnail cells, 92 per cent in MIA PaCa-2 shSlug cells, 88 per cent in MIA PaCa-2 shTwist cells when the amount was 99.9 per cent in MIA PaCa-2 shControl cells, 92.5 per cent in BxPc-3 shSnail cells, 96.12 per cent in BxPc-3 shSlug cells, 93.85 per cent in BxPc-3 shTwist cells when the amount was 98.68 in BxPc-3 shControl cells and, 71 per cent in AsPc-1 shSnail cells, 69 per cent in AsPc-1 shSlug cells and 71 per cent in AsPc-1 shTwist cells when the amount was 85 per cent in AsPc-1 shControl cells. The statistical evaluation

of the differences between groups were analyzed with One-way ANOVA with Tukey's posttest. These changes indicate that EMT silencing (Snail, Slug and Twist downregulation) also decreases the expressions of cancer stem cell marker CD44.

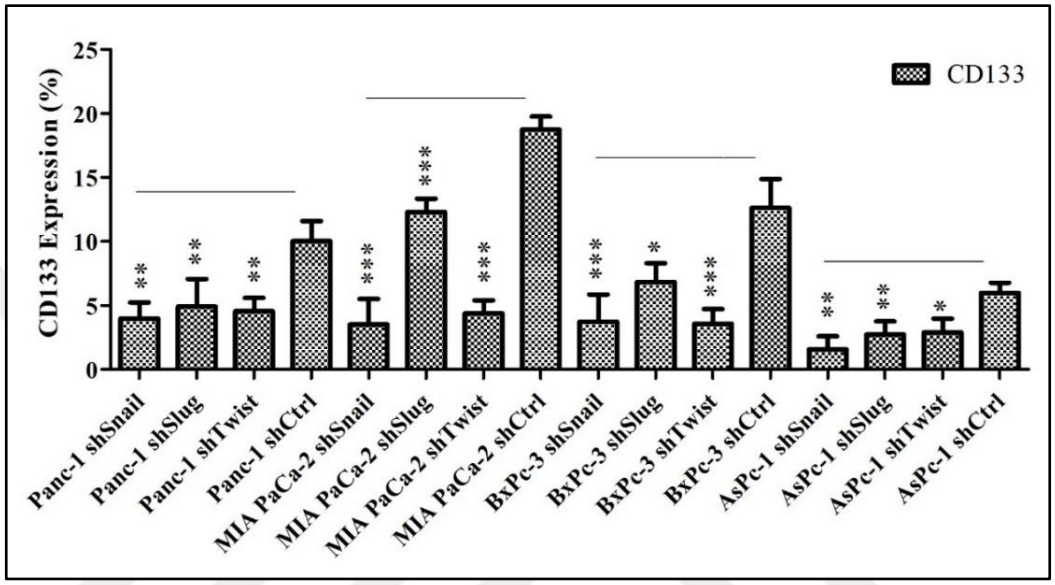


Figure 3.89 Graphical representation of the changes in CD133 amount.One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$)

Figure 3.89 represents the protein amount changes of CD133 after downregulation of Snail, Slug and Twist. It is found that, gene therapy significantly decreases the amount of CD133 in all of the pancreas cancer cell lines. Downregulation of EMT has decreased the amount of CD133 to 3.99 per cent in Panc-1 shSnail cells, 4.93 per cent in Panc-1 shSlug cells, 4.55 per cent in Panc-1 shTwist cells when the amount was 10 per cent in Panc-1 shControl cells, 3.5 per cent in MIA PaCa-2 shSnail cells, 12 per cent in MIA PaCa-2 shSlug cells, 4.3 per cent in MIA PaCa-2 shTwist cells when the amount was 18.7 per cent in MIA PaCa-2 shControl cells, 3.7 per cent in BxPc-3 shSnail cells, 6.83 per cent in BxPc-3 shSlug cells, 3.57 per cent in BxPc-3 shTwist cells when the amount was 12.6 in BxPc-3 shControl cells and, 1.6 per cent in AsPc-1 shSnail cells, 2.71 per cent in AsPc-1 shSlug cells and 2.88 per cent in AsPc-1 shTwist cells when the amount was 6 per cent in AsPc-1 shControl cells. The statistical evaluation of the differences between groups were analyzed with One-way

ANOVA with Tukey's posttest. These changes indicate that EMT silencing (Snail, Slug and Twist downregulation) also decreases the expressions of cancer stem cell marker CD133.

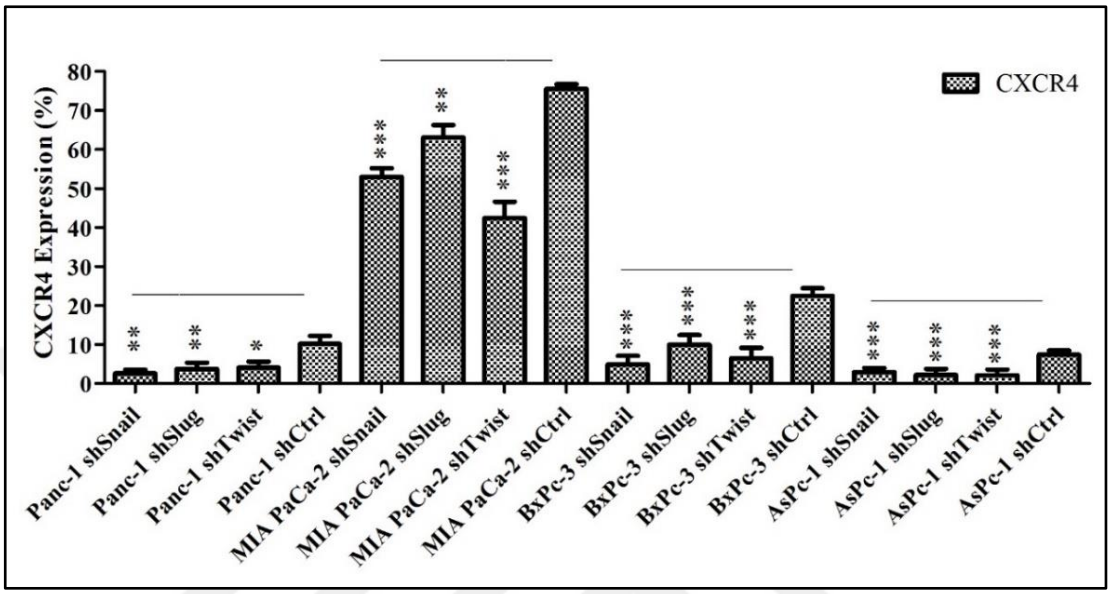


Figure 3.90 Graphical representation of the changes in CXCR4 amount.One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$)

Figure 3.90 represents the protein amount changes of CXCR4 after downregulation of Snail, Slug and Twist. It is found that, gene therapy significantly decreases the amount of CXCR4 in all of the pancreas cancer cell lines. Downregulation of EMT has decreased the amount of CXCR4 to 2.6 per cent in Panc-1 shSnail cells, 3.7 per cent in Panc-1 shSlug cells, 4 per cent in Panc-1 shTwist cells when the amount was 10.2 per cent in Panc-1 shControl cells, 53 per cent in MIA PaCa-2 shSnail cells, 63 per cent in MIA PaCa-2 shSlug cells, 43 per cent in MIA PaCa-2 shTwist cells when the amount was 75.6 per cent in MIA PaCa-2 shControl cells, 4.8 per cent in BxPc-3 shSnail cells 9.9 per cent in BxPc-3 shSlug cells, 6.48 per cent in BxPc-3 shTwist cells when the amount was 22.5 in BxPc-3 shControl cells and, 2.99 per cent in AsPc-1 shSnail cells, 2.25 per cent in AsPc-1 shSlug cells and 2.19 per cent in AsPc-1 shTwist cells when the amount was 7.45 per cent in AsPc-1 shControl cells. The statistical evaluation of the differences between groups were analyzed with One-way ANOVA with Tukey's posttest. These changes indicate that EMT silencing (Snail, Slug and Twist downregulation) also decreases the expressions of cancer stem cell marker CXCR4.

3.11. EMBRYONIC STEM CELL PROPERTIES

3.11.1. Changes in Gene Expression of Embryonic Stem Cell Characteristic Genes after Gene Therapy

To analyze the effect of EMT silencing on pluripotency markers, cells were subjected to realtime PCR.

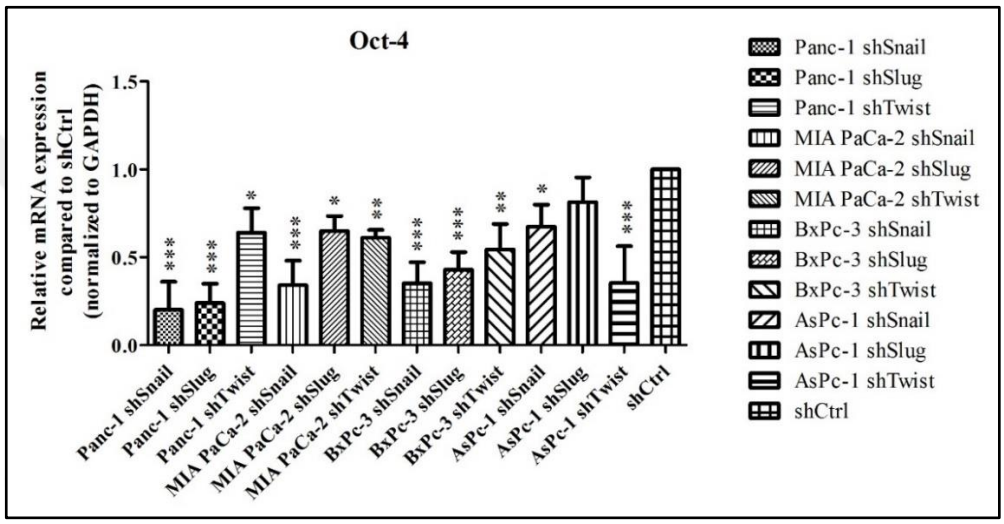


Figure 3.91 Graphical representation of Oct3/4 expression .One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$)

As stated in the figure 3.91, down regulation of EMT transcription factors Snail, Slug and Twist has significantly decreased the expression of Oct3/4 in PC cell lines. EMT silencing has decreased the amount of Oct3/4 to 20per cent in Panc-1 shSnail cells, 24 per cent in Panc-1 shSlug cells, 64 per cent in Panc-1 shTwist cells, 34 per cent in MIA PaCa-2 shSnail cells, 65 per cent in MIA PaCa-2 shSlug cells, 61 per cent in MIA PaCa-2 shTwist cells, 35 per cent in BxPc-3 shSnail cells 42 per cent in BxPc-3 shSlug cells, 54 per cent in BxPc-3 shTwist cells and, 67 per cent in AsPc-1 shSnail cells, 81 per cent in AsPc-1 shSlug cells and 35 per cent in AsPc-1 shTwist cells when their negative controls were normalized to 100 per cent. The expression values were analyzed by $\Delta\Delta$Ct method and the statistical evaluation of the differences between groups were analyzed with One-way ANOVA with Tukey's

posttest. These changes indicate that EMT silencing (Snail, Slug and Twist downregulation) also decreases the expressions of pluripotency marker Oct3/4.

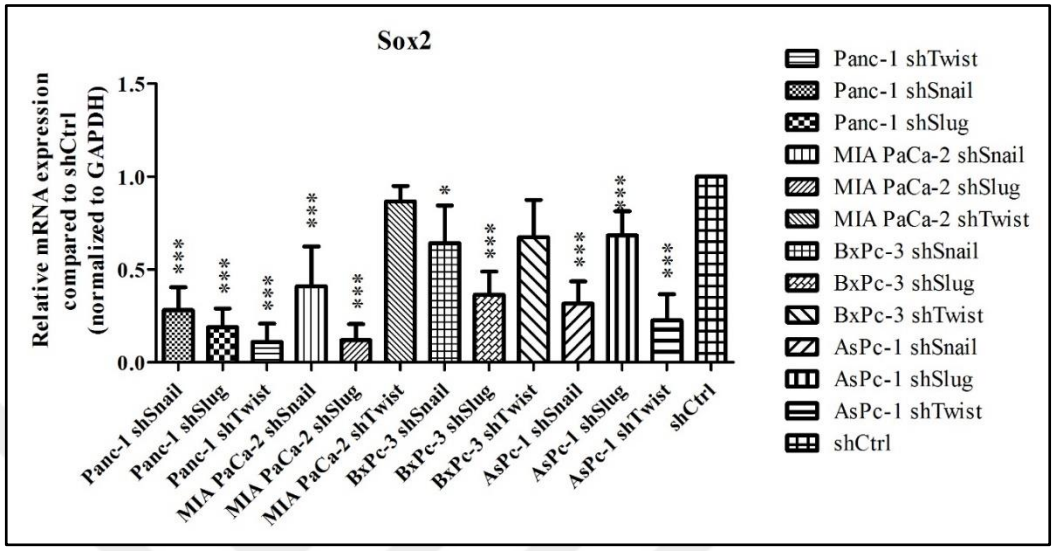


Figure 3.92 Graphical representation of Sox 2 expression.One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$)

As stated in Figure 3.92, downregulation of Snail, Slug and Twist has decreased the expression of embryonic stem cell marker Sox2 in all cell lines and in all silencing conditions except MIA PaCa-2 shTwist.

EMT silencing has decreased the amount of Sox2 to 28per cent in Panc-1 shSnail cells, 19 per cent in Panc-1 shSlug cells, 10 per cent in Panc-1 shTwist cells, 41 per cent in MIA PaCa-2 shSnail cells, 12 per cent in MIA PaCa-2 shSlug cells, 86 per cent in MIA PaCa-2 shTwist cells, 64 per cent in BxPc-3 shSnail cells 36 per cent in BxPc-3 shSlug cells, 67 per cent in BxPc-3 shTwist cells and, 31 per cent in AsPc-1 shSnail cells, 68 per cent in AsPc-1 shSlug cells and 22 per cent in AsPc-1 shTwist cells when their negative controls were normalized to 100 per cent. The expression values were analyzed by ΔΔCt method and the statistical evaluation of the differences between groups were analyzed with One-way ANOVA with Tukey's posttest. These changes indicate that EMT silencing (Snail, Slug and Twist downregulation) also decreases the expressions of pluripotency marker Sox2.

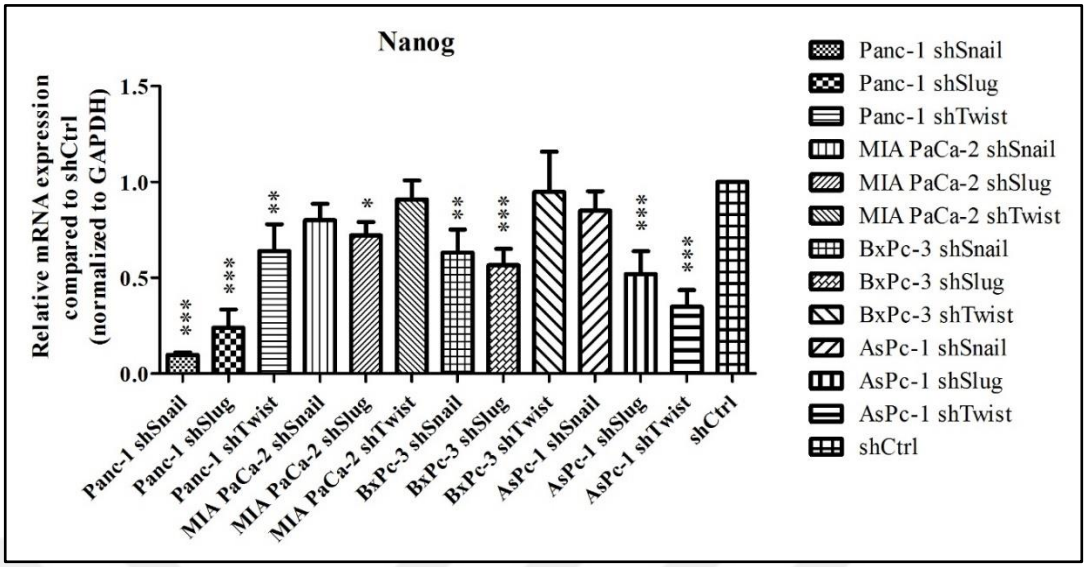


Figure 3.93 Graphical representation of Nanog expression.One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$)

As stated in Figure 3.93, downregulation of Snail, Slug and Twist has decreased the expression of embryonic stem cell marker Nanog in all cell lines and in all silencing conditions except MIA PaCa-2 shTwist, BxPc-3 shTwist and AsPc-1 shSnail.

EMT silencing has decreased the amount of Nanog to 24per cent in Panc-1 shSnail cells, 64 per cent in Panc-1 shSlug cells, 80 per cent in Panc-1 shTwist cells, 80 per cent in MIA PaCa-2 shSnail cells, 72 per cent in MIA PaCa-2 shSlug cells, 90 per cent in MIA PaCa-2 shTwist cells, 63 per cent in BxPc-3 shSnail cells 56 per cent in BxPc-3 shSlug cells, 94 per cent in BxPc-3 shTwist cells and, 85 per cent in AsPc-1 shSnail cells, 52 per cent in AsPc-1 shSlug cells and 35 per cent in AsPc-1 shTwist cells when their negative controls were normalized to 100 per cent. The expression values were analyzed by ΔΔCt method and the statistical evaluation of the differences between groups were analyzed with One-way ANOVA with Tukey's posttest. These changes indicate that EMT silencing (Snail, Slug and Twist downregulation) also decreases the expressions of pluripotency marker Nanog.

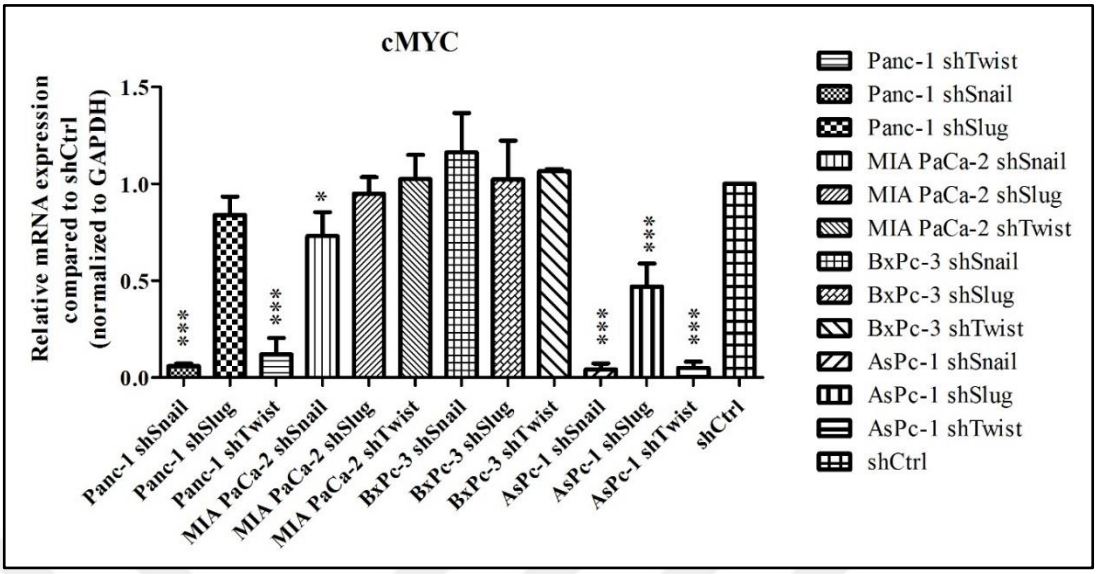


Figure 3.94 Graphical representation of cMYC expression.One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$)

As indicated in the Figure 3.94, downregulation of Snail, Slug and Twist has significantly decreased the expression of embryonic stem cell marker cMYC in Panc-1 shSnail and shTwist cells and in AsPc-1 shSnail, shSlug and shTwist cells.

EMT silencing has decreased the amount of cMYC to six per cent in Panc-1 shSnail cells, 84 per cent in Panc-1 shSlug cells, 12 per cent in Panc-1 shTwist cells, 73 per cent in MIA PaCa-2 shSnail cells, 95 per cent in MIA PaCa-2 shSlug cells, 100 per cent in MIA PaCa-2 shTwist cells, change the expression to 115 per cent BxPc-3 shSnail cells 100 per cent in BxPc-3 shSlug cells, 100 per cent in BxPc-3 shTwist cells and, four per cent in AsPc-1 shSnail cells, 47 per cent in AsPc-1 shSlug cells and 5 per cent in AsPc-1 shTwist cells when their negative controls were normalized to 100 per cent. The expression values were analyzed by $\Delta\Delta$Ct method and the statistical evaluation of the differences between groups were analyzed with One-way ANOVA with Tukey's posttest. These changes indicate that EMT silencing (Snail, Slug and Twist downregulation) also decreases the expressions of pluripotency marker cMYC in some of the groups.

3.12. EXPRESSION OF EMT RELATED MARKERS

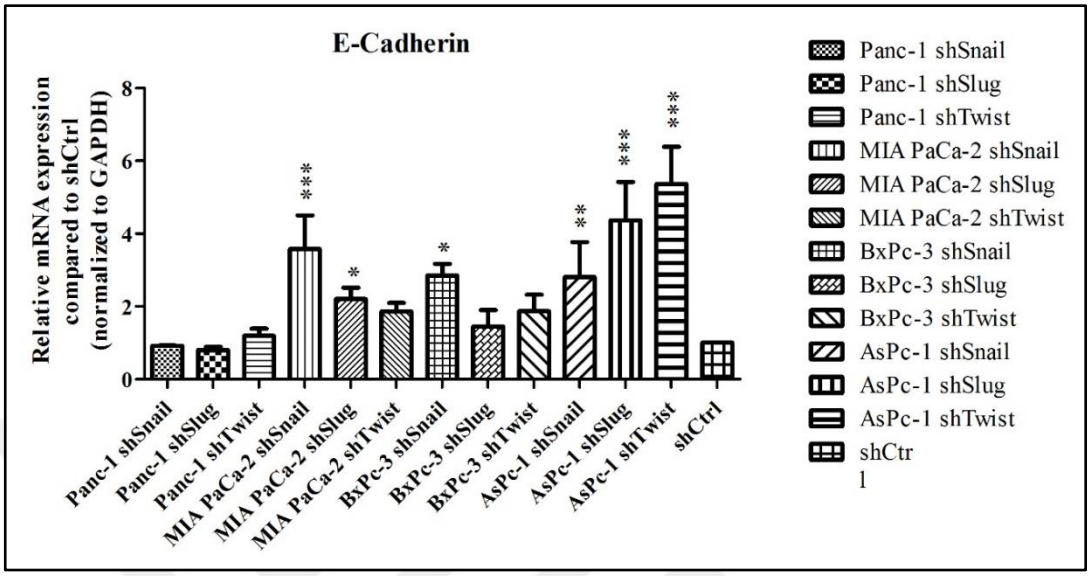


Figure 3.95 Graphical representation of E-cadherin expression change.One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$, ** indicates that $p<0.005$ and *** indicates that $p<0.001$)

As indicated in Figure 3.95, it has found that, gene silencing of Snail, Slug and Twist in MIA PaCa-2, BxPc-3 shSnail and AsPC-1 has significantly increased the expression of the most dominant epithelial marker E-cadherin.

EMT silencing changed the expression values of E-cadherin to 92 per cent in Panc-1 shSnail cells, 80 per cent in Panc-1 shSlug cells, 119 per cent in Panc-1 shTwist cells, 358 per cent in MIA PaCa-2 shSnail cells, 220 per cent in MIA PaCa-2 shSlug cells, 160 per cent in MIA PaCa-2 shTwist cells, change the expression to 285 per cent BxPc-3 shSnail cells 145 per cent in BxPc-3 shSlug cells, 187 per cent in BxPc-3 shTwist cells and, 280 per cent in AsPc-1 shSnail cells, 436 per cent in AsPc-1 shSlug cells and 537 per cent in AsPc-1 shTwist cells when their negative controls were normalized to 100 percent. The expression values were analyzed by ΔΔCt method and the statistical evaluation of the differences between groups were analyzed with One-way ANOVA with Tukey's posttest.

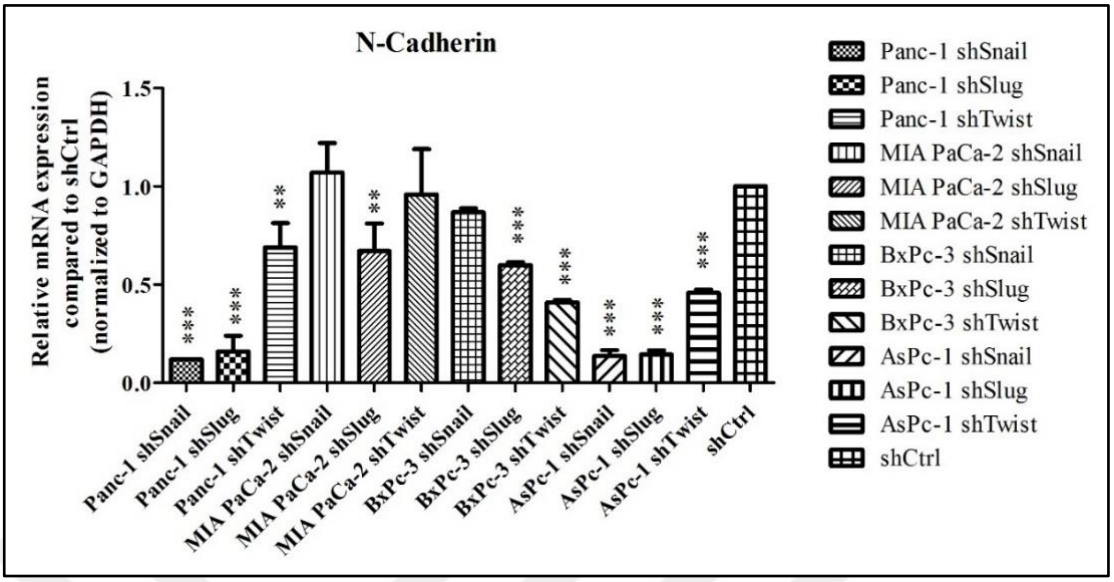


Figure 3.96 Graphical representation of expression change of N-cadherin.One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$, ** indicates that $p<0.005$ and *** indicates that $p<0.001$)

It was shown in Figure 3.96, downregulation of EMT inducer transcription factors Snail, Slug and Twist has downregulated n-cadherin, the most dominant mesenchymal marker, in nearly all of the cells.

EMT silencing changed the expression values of N-cadherin to 12 per cent in Panc-1 shSnail cells, 16 per cent in Panc-1 shSlug cells, 69 per cent in Panc-1 shTwist cells, 100 per cent in MIA PaCa-2 shSnail cells, 67 per cent in MIA PaCa-2 shSlug cells, 96 per cent in MIA PaCa-2 shTwist cells, change the expression to 86 per cent BxPc-3 shSnail cells 59 per cent in BxPc-3 shSlug cells, 40 per cent in BxPc-3 shTwist cells and, 13 per cent in AsPc-1 shSnail cells, 14 per cent in AsPc-1 shSlug cells and 46 per cent in AsPc-1 shTwist cells when their negative controls were normalized to 100 percent. The expression values were analyzed by ΔΔCt method and the statistical evaluation of the differences between groups were analyzed with One-way ANOVA with Tukey's posttest.

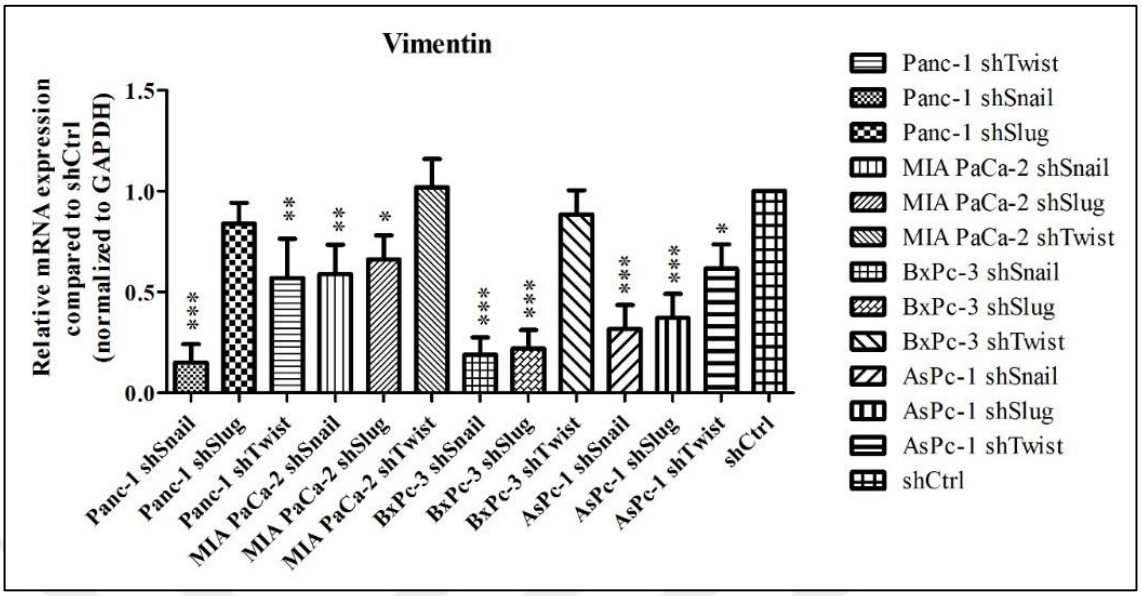


Figure 3.97 Graphical representation of expression change of Vimantin.One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$, ** indicates that $p<0.005$ and *** indicates that $p<0.001$)

As indicated in the Figure 3.97, it was found that, EMT silencing has significantly downregulated Vimentin expression in nearly the most of the cells and silencing options.

EMT silencing changed the expression values of vimentin to 15 per cent in Panc-1 shSnail cells, 84 per cent in Panc-1 shSlug cells, 57 per cent in Panc-1 shTwist cells, 56 per cent in MIA PaCa-2 shSnail cells, 66 per cent in MIA PaCa-2 shSlug cells, 100 per cent in MIA PaCa-2 shTwist cells, to 19 per cent BxPc-3 shSnail cells 22 per cent in BxPc-3 shSlug cells, 88 per cent in BxPc-3 shTwist cells and, 31 per cent in AsPc-1 shSnail cells, 37 per cent in AsPc-1 shSlug cells and 61 per cent in AsPc-1 shTwist cells when their negative controls were normalized to 100 percent. The expression values were analyzed by ΔΔCt method and the statistical evaluation of the differences between groups were analyzed with One-way ANOVA with Tukey's posttest.

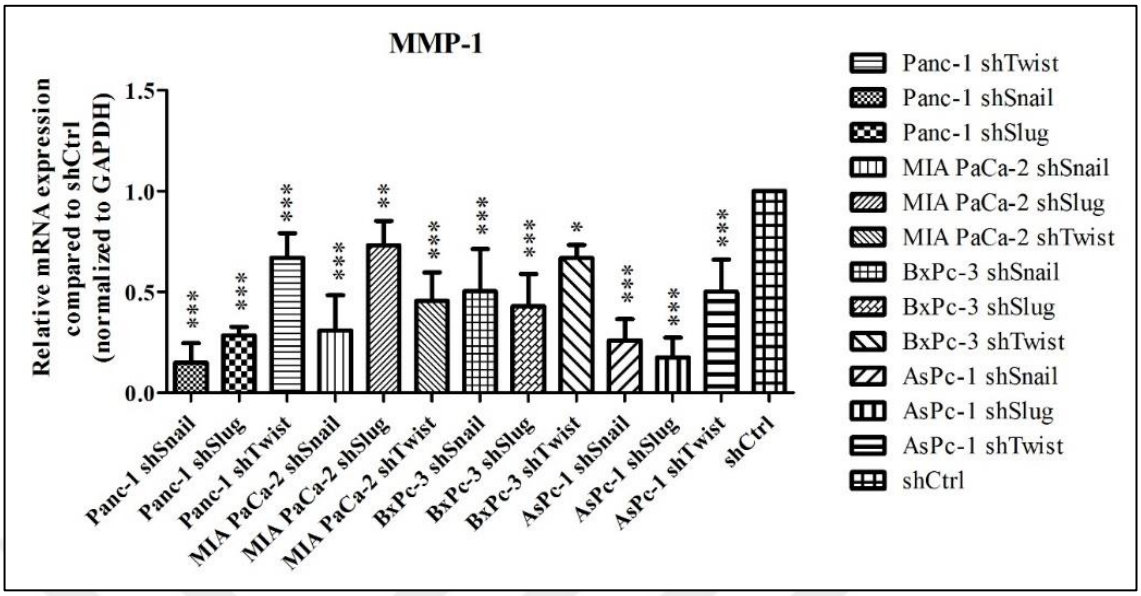


Figure 3.98 Graphical representation of expression change of MMP1.One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$, ** indicates that $p<0.005$ and *** indicates that $p<0.001$)

As shown in the Figure 3.98, it was found that, downregulation of Snail, Slug and Twist also downregulated the expression of one of the major element of metastasis, MMP1 significantly.

EMT silencing changed the expression values of MMP1 to 15 per cent in Panc-1 shSnail cells, 28 per cent in Panc-1 shSlug cells, 55 per cent in Panc-1 shTwist cells, 31 per cent in MIA PaCa-2 shSnail cells, 73 per cent in MIA PaCa-2 shSlug cells, 46 per cent in MIA PaCa-2 shTwist cells, to 50 per cent BxPc-3 shSnail cells 42 per cent in BxPc-3 shSlug cells, 66 per cent in BxPc-3 shTwist cells and, 26 per cent in AsPc-1 shSnail cells, 17 per cent in AsPc-1 shSlug cells and 50 per cent in AsPc-1 shTwist cells when their negative controls were normalized to 100 percent. The expression values were analyzed by $\Delta\Delta$Ct method and the statistical evaluation of the differences between groups were analyzed with One-way ANOVA with Tukey's posttest.

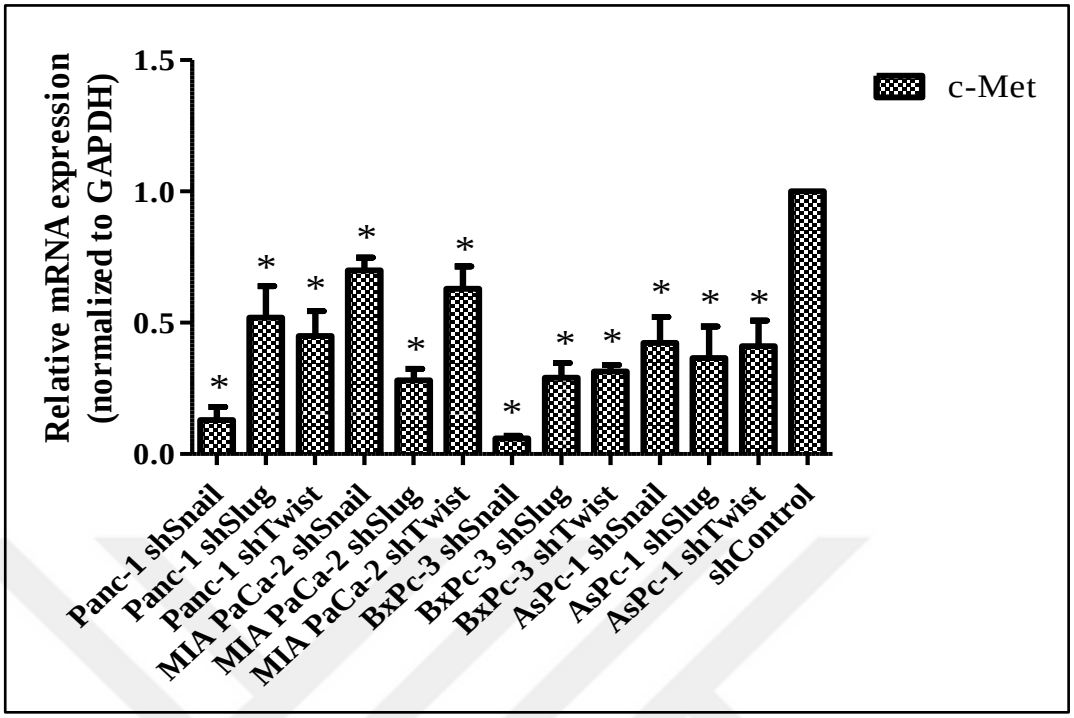


Figure 3.99 Graphical representation of expression change of c-met .One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$)

As shown in Figure 3.99, it was found that, gene silencing decreases c-met expression significantly.

EMT silencing changed the expression values of c-met to 13 per cent in Panc-1 shSnail cells, 52 per cent in Panc-1 shSlug cells, 45 per cent in Panc-1 shTwist cells, 70 per cent in MIA PaCa-2 shSnail cells, 28 per cent in MIA PaCa-2 shSlug cells, 63 per cent in MIA PaCa-2 shTwist cells, to 6 per cent BxPc-3 shSnail cells 29 per cent in BxPc-3 shSlug cells, 31 per cent in BxPc-3 shTwist cells and, 42 per cent in AsPc-1 shSnail cells, 36 per cent in AsPc-1 shSlug cells and 41 per cent in AsPc-1 shTwist cells when their negative controls were normalized to 100 percent. The expression values were analyzed by ΔΔCt method and the statistical evaluation of the differences between groups were analyzed with One-way ANOVA with Tukey's posttest.

3.13. DRUG RESISTANCE

3.13.1. Determination of the Toxic Dose of Chosen Chemicals in Healthy Pancreas Cell Line

In order to determine the toxic dose of the chosen chemotherapeutics, healthy human pancreas cell line (hTert-HPNE) was treated with various concentrations of 5-Fluorouracil (200µM, 100µM, 50µM and 25µM), Docetaxel (10nM, 5nM, 2.5nM and 1.25nM), Mitomycin C (0.8µM, 0.4µM, 0.2µM and 0.1µM) for at 48 hours.

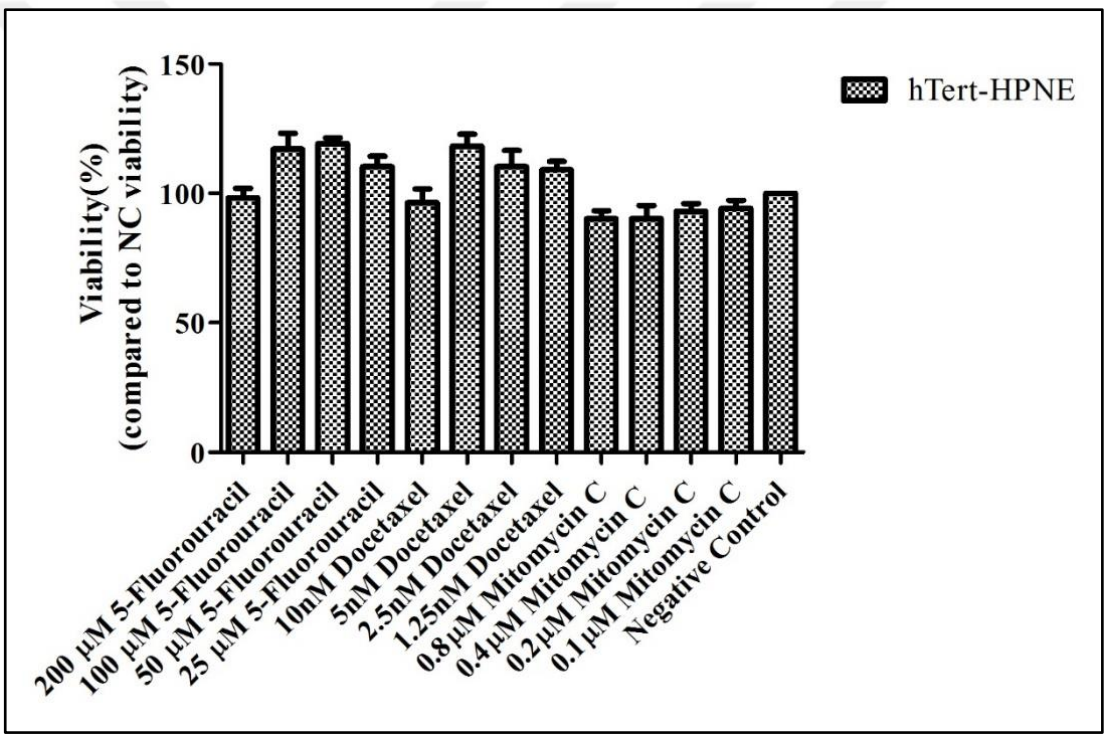


Figure 3.100 Graphical representation of cellular viability of hTert-HPNE cells after chemotherapy

The results shown in Figure 3.100 indicate that chosen concentrations for chemotherapeutics were not toxic to healthy pancreas cells and parallel to the literature. The viabilities for each group, after 48 hours of treatment, was not less than 100 per cent. So the chosen concentrations were not toxic to the healthy control, hTert-HPNE. The chemicals mentioned above were used in the given concentrations to treat AsPc-1 cells after gene therapy.

3.13.2. Evaluation of Drug Resistance in AsPc-1 Cell Line after Gene Therapy

EMT is known to increase the drug resistance. In order to evaluate how silencing changes drug resistance, AsPc-1 cell line was treated with the chosen chemicals in pre-determined concentrations. The time of effect was chosen as 48 hours because of the short doubling time of AsPc-1 cell line.

5-Fluorouracil was chosen as the first FDA approved pancreatic cancer chemotherapeutic. 5-Fluorouracil is a common chemotherapeutic, used for carcinomas.

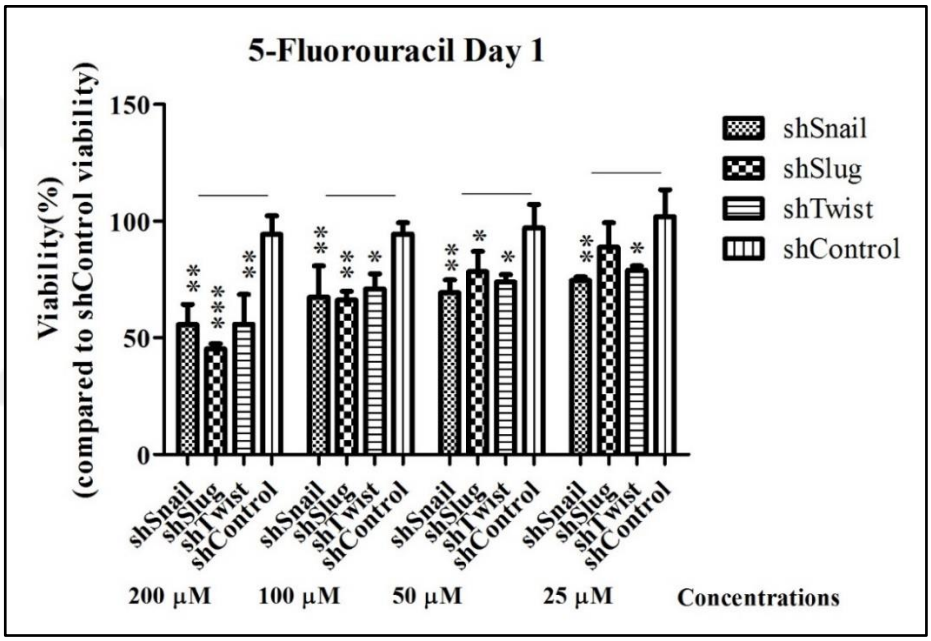


Figure 3.101 Graphical representation of cellular viability after 5-Fluorouracil treatment for 24 hours .One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$, ** indicates that $p<0.005$ and *** indicates that $p<0.001$)

As given in the Table 3.37, after 24 hours of treatment with 200 µM 5-Fluorouracil, the viabilities of AsPc-1 cells were decreased to 57.67 percent in shSnail, 45.24 per cent, in shSlug, 55.79 per cent in shTwist when the shControl viability was 94.35. Treatment with 100 µM 5-Fluorouracil decreased the viabilities of shSnail to 67.50 per cent, shSlug to 66.19 per cent, shTwist to 70.89 per cent while the viability of shControl was 94.40 per cent. Treatment with 50 µM 5-Fluorouracil decreased the viability of shSnail to 69.25 per cent, shSlug to 78.29 per cent, shTwist to73.95 per cent while shControl viability was 97.17. 24

hour treatment with 25 μM 5-Fluorouracil decreased the viability of shSnail to 74 per cent, shSlug to 88.76 per cent and shTwis to 78.91 per cent while the viability of shControl was 100 per cent

Table 3.37 The average viability percentages of AsPc-1 cells after 24 hours of 5-Fluorouracil treatment

Average Viabilty per cent (24 Hours)	**shSnail**	**shSlug**	**shTwist**	**shControl**
5-Fluorouracil (200μM)	55.67	45.24	55.79	94.35
5-Fluorouracil (100μM)	67.50	66.19	70.89	94.40
5-Fluorouracil (50μM)	69.25	78.29	73.95	97.17
5-Fluorouracil (25μM)	74.55	88.76	78.91	101.91

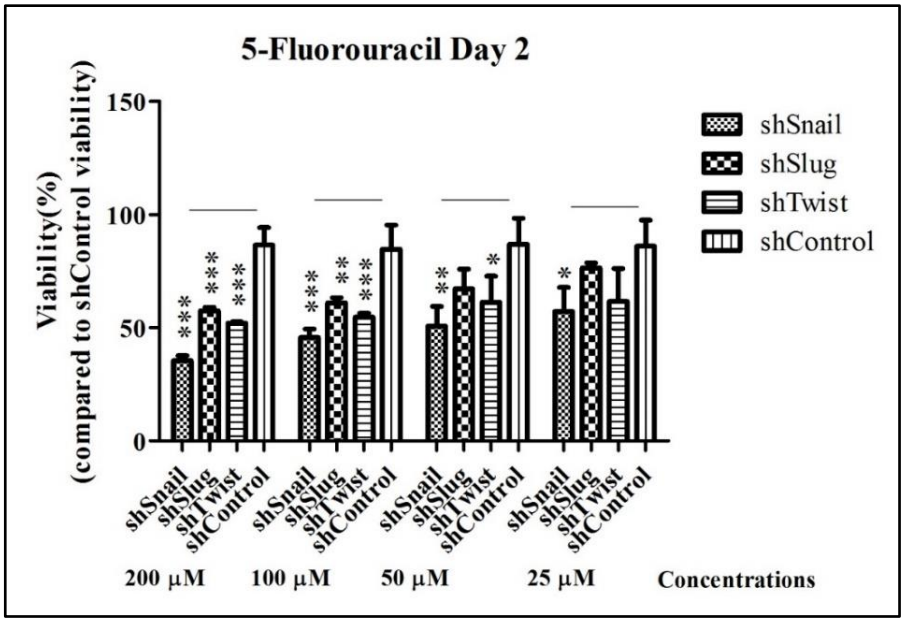


Figure 3.102 Graphical representation of cellular viability after 5-Fluorouracil treatment for 48 hours .One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$, ** indicates that $p<0.005$ and *** indicates that $p<0.001$)

As shown in the Figure 3.102, the results of two days of 5-Fluorouracil treatment indicate that, downregulation of Snail, Slug and Twist has significantly decreased the resistance towards 5-Fluorouracil.

As shown in the Table 3.38, 48 hours of treatment with 200 μM 5-Fluorouracil decreased the viability of shSnail to 35 percent, shSlug to 58 per cent and shTwist to 52 percent while the viability of shControl remained 86.52 per cent. 48 hours of treatment with 100 μM 5-Fluorouracil decreased the viabilities of shSnail to 46 per cent, shSlug to 61 per cent and shTwist to 54 per cent while the viability for shControl vas 84 per cent. 48 hours of treatment with 50 μM 5-Fluorouracil decreased the viability of shSnail to 50 per cent, shSlug to 61 per cent, shTwist to 54 per cent while the viability for shControl was 87 per cent. Treatment with 25 μM 5-Fluorouracil for 48 hours decreeased the viabilities of shSnail, shSlug and shTwist to 57, 76 and 61 per cent while the viability of shControl was 86 per cent.

Table 3.38 The average viability percentages of AsPc-1 cells after 48 hours of 5-Fluorouracil treatment

Average Viabilty per cent (48 Hours)	**shSnail**	**shSlug**	**shTwist**	**shControl**
5-Fluorouracil (200μM)	35.48	57.61	52.11	86.52
5-Fluorouracil (100μM)	45.70	61.01	54.68	84.66
5-Fluorouracil (50μM)	50.69	61.19	61.26	86.902
5-Fluorouracil (25μM)	57.203	76.42	61.73	86.11

The second chosen chemotherapeutic, Docetaxel, is another FDA approved drug for pancreas cancer. It is generally used in advanced pancreatic cancer patients.

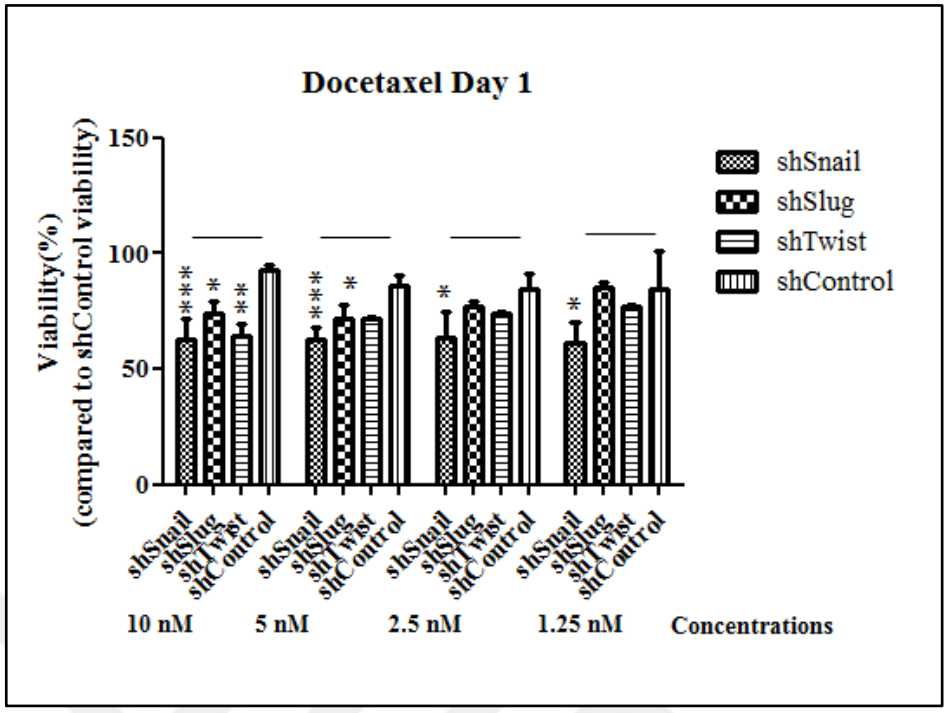


Figure 3.103 Graphical representation of cellular viability after Docetaxel treatment for 24 hours .One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$, ** indicates that $p<0.005$ and *** indicates that $p<0.001$)

As indicated in the figure 3.103, even in the first day of treatment with different contentrations of Docetaxel, the viability values for EMT silenced cels were statistically less, compared to shControl's viability.

As given in Table 3.39, after 24 hours of treatment with 10nM Docetaxel, the viability value of shSnail was decreased to 62 per cent, shSlug to 74 per cent, shTwist to 64 per cent while the viability of shControl was 92.5 per cent. 24 hour treatment with 5nM Docetaxel decreased the viability of shSnail to 62.5 per cent, shSlug to 71.61 per cent, shTwist to 71.67 per cent while the viability of shControl was 86 per cent. The treatment with 2.5 nM Docetaxel decreased the viability of shSnail to 63 per cent, shSlug to 76 per cent and shTwist to 74 per cent while the viability was 84 per cent in shControl cells. The treatment with 1.25 nM Docetaxel decreased the viability of shSnail cell to 61 per cent, shSlug to 85 per cent, shTwist to 77 per cent while the viability value for shControl was 87 per cent. By this study it was shown that, the viabilities of EMT silenced cells decreased more than shControl cells even after 24 hours of Docetaxel treatment.

Table 3.39 The average viability percentages of AsPc-1 cells after 24 hours of Docetaxel treatment

Average Viabilty per cent (24 Hours)	shSnail	shSlug	shTwist	shControl
Docetaxel (10nM)	62.74	73.72	64.45	92.52
Docetaxel (5nM)	62.56	71.61	71.67	85.71
Docetaxel (2.5nM)	63.50	76.71	74.34	84.26
Docetaxel (1.25 nm)	61.28	85.54	77.02	84.74

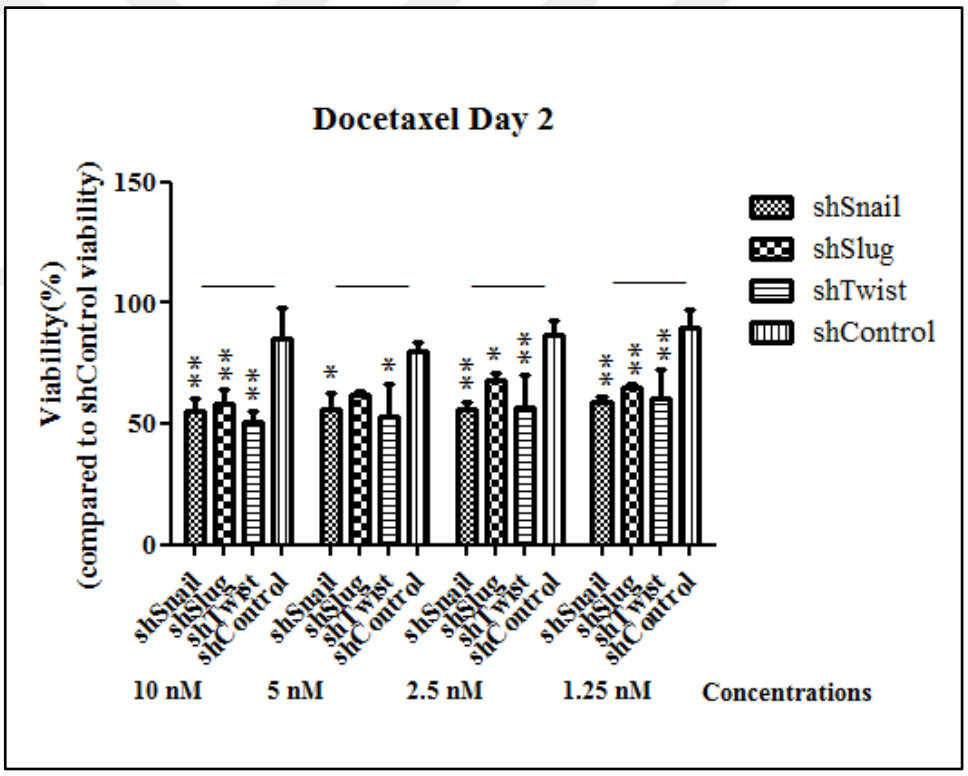


Figure 3.104 Graphical representation of cellular viability after Docetaxel treatment for 48 hours .One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$, ** indicates that $p<0.005$ and *** indicates that $p<0.001$)

As given in the Figure 3.104, the results of two days of Docetaxel treatment indicate that, downregulation of Snail, Slug and Twist has significantly decreased the resistance towards Docetaxel.

As mentioned in the Table 3.40, 48 hours of treatment with various concentrations of Docetaxel yielded with different results between treatment groups. After 48 hours of 10nM Docetaxel treatment, the viability of shSnail cells were 55 per cent, shSlug cells were 58 per cent, shTwist cells were 64 per cent while shControl viability was 85 per cent. After 48 hours of 5nM Docetaxel treatment, it was observed that viability values were decreased to 56 per cent in shSnail cells, 62 per cent in shSlug cells, 71 per cent in shTwist cells while shControl remained at 80 per cent. 48 hours of 2.5 nM docetaxel treatment decreased the viabilities of shSnail cells to 56 per cent, shSlug to 68 per cent, shTwist to 74 per cent while the viability of shControl group was 86 per cent. Treatment for 48 hours with 1.25 nM docetaxel decreased the viability of shSnail cells to 58 per cent, shSlug to 64 per cent, shTwist to 77 per cent while shControl viability was 89 per cent.

Table 3.40 The average viability percentages of AsPc-1 cells after 48 hours of Docetaxel treatment

Average Viabilty per cent (48 Hours)	**shSnail**	**shSlug**	**shTwist**	**shControl**
Docetaxel (10nM)	55.35	58.52	64.45	85.11
Docetaxel (5nM)	56.11	61.99	71.67	80.26
Docetaxel (2.5nM)	55.95	67.74	74.34	86.54
Docetaxel (1.25 nm)	58.97	64.63	77.02	89.67

The last chosen chemotherapeutic for drug resistance analysis is Mitomycin C. Mitomycin C is another FDA approved drug for treatment of pancreas cancer.

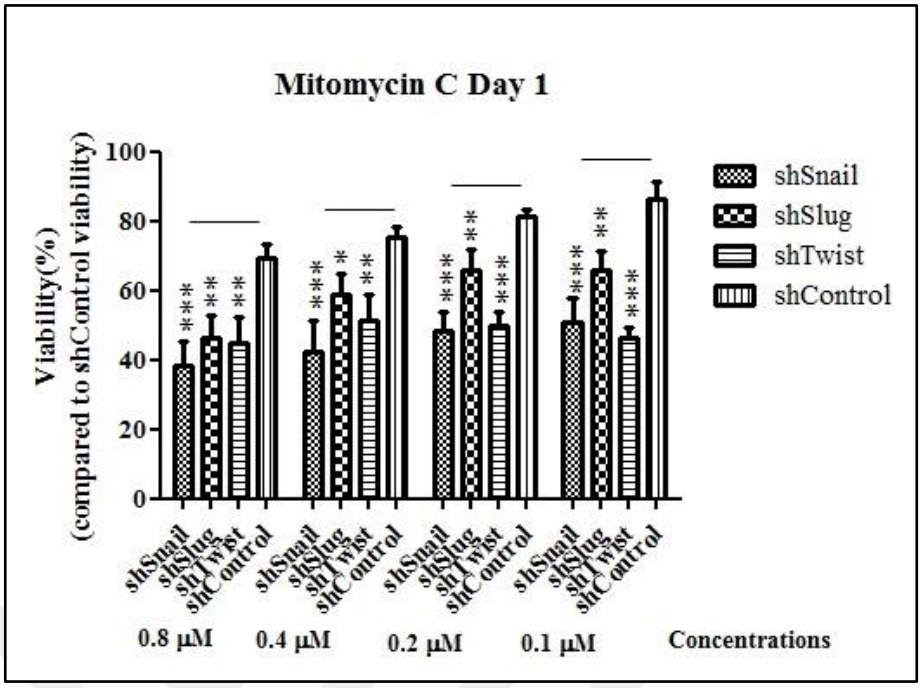


Figure 3.105 Graphical representation of cellular viability after Mitomycin C treatment for 24 hours .One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$, ** indicates that $p<0.005$ and *** indicates that $p<0.001$)

As shown in the Figure 3.105, the viabilities of EMT downregulated cells decreased more with various concentrations of Mitomycin C treatment compared to their corresponding negative control groups.

As mentioned in Table 3.41, the viabilities of 0.8 µM Mitomycin C treated cells were decreased to, 38.5 per cent in shSnail cells, 46 per cent in shSlug cells, 44.74 in shTwist cells while the viability of shControl was 69.57 per cent. The viabilities after 24 hours of 0.4 µM Mitomycin C treatment were decreased to 42 per cent in shSnail, 58.87 percent in shSlug, 51 per cent in shTwist when the viability of shControl was 75 per cent. 24 hour of 0.2 µM Mitomycin C treatment decreased the viability of shSnail cells to 48 per cent, shSlug cells to 66 per cent, shTwist cells to 50 per cent while the 81 per cent of shControl cells were alive. 0.1 µM Mitomycin C treatment decreased the viability values of shSnail, shSlug and shTwist cells to 51, 66, 46 per cent respectively while the viability for shControl cell was 86 per cent.

Table 3.41 The average viability percentages of AsPc-1 cells after 24 hours of Mitomycin C treatment

Average Viabilty per cent (24 Hours)	shSnail	shSlug	shTwist	shControl
Mitomycin C (0.8µM)	38.50	46.16	44.74	69.57
Mitomycin C (0.4µM)	42.33	58.87	51.31	75.17
Mitomycin C (0.2µM)	48.25	65.98	50.00	81.21
Mitomycin C (0.1µM)	51.03	65.88	46.49	86.18

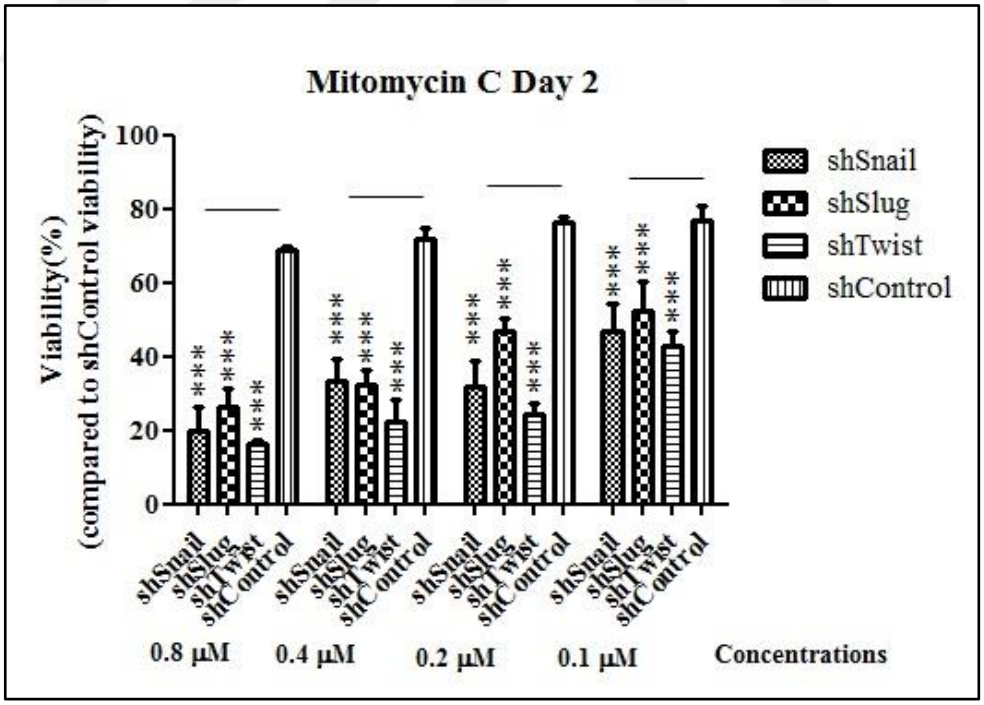


Figure 3.106 Graphical representation of cellular viability after Mitomycin C treatment for 48 hours .One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$, ** indicates that $p<0.005$ and *** indicates that $p<0.001$)

As indicated in the Table 3.42, 48 hours of 0.8 μM Mitomycin C treatment decreased the viability of shSnail cells to 20 per cent, shSlug cells to 26 per cent, shTwist to 16 per cent while the viability of their control cell line remained 69 per cent. 48 hours of 0.4 μM Mitomycin C decreased the viabilities of shSnail, shSlug and shTwist cells to 33.5 per cent, 32 per cent and 22 per cent respectively while the viability of shControl cells were 71 per cent. The treatment with 0.2 μM Mitomycin C decreased the viability of shSnail, shSlug and shTwist cells to 31 per cent, 46.7 per cent and 24 per cent respectively when 76 per cent of shControl cells were alive. Treatment with 0.1 μM Mitomycin C decreased the viability of shSnail cells to 47 per cent, shSlug cells to 52 per cent and shTwist to 43 per cent while the viability of shControl cells were 77 per cent.

Table 3.42 The average viability percentages of AsPc-1 cells after 48 hours of Mitomycin C treatment

Average Viabilty per cent (48 Hours)	**shSnail**	**shSlug**	**shTwist**	**shControl**
Mitomycin C (0.8μM)	20.07	26.15	16.25	68.95
Mitomycin C (0.4μM)	33.52	32.53	22.35	71.95
Mitomycin C (0.2μM)	31.74	46.70	24.19	76.23
Mitomycin C (0.1μM)	46.94	52.18	42.83	77.04

The results of two days of Mitomycin C treatment indicate that, downregulation of Snail, Slug and Twist has significantly decreased the resistance towards Mitomycin C.

3.13.3. Changes in Gene Expression Profile towards Drug Resistance with EMT Silencing

In order to understand how gene therapy changed the expression of selected genes (Survivin, ABCG2 and MUC1), gene expression analysis was done to untreated group.

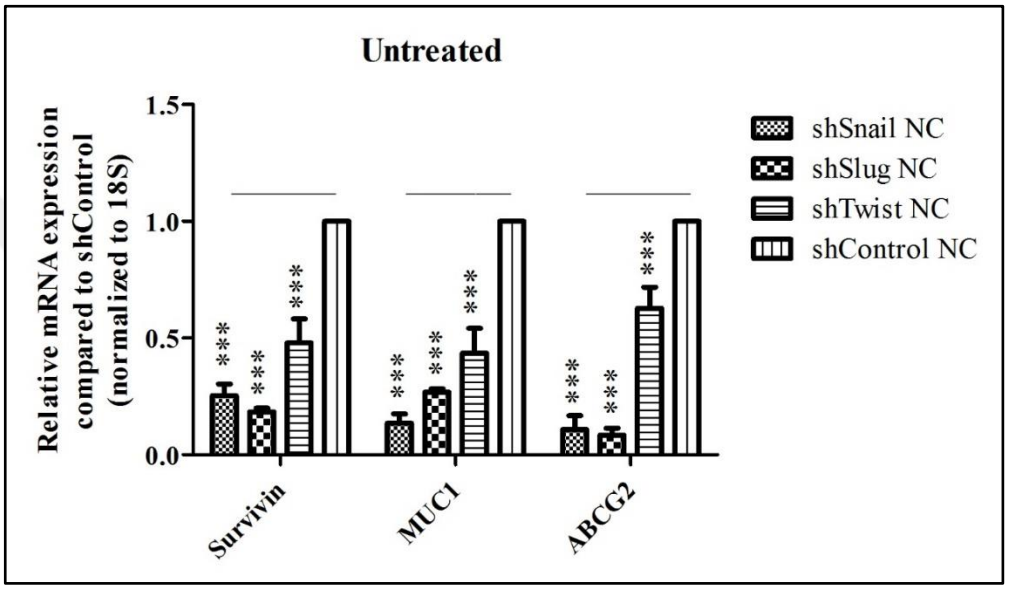


Figure 3.107 Relative expression values of selected genes in untreated shSnail, shSlug, shTwist and shControl cells .One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$, ** indicates that $p<0.005$ and *** indicates that $p<0.001$)

As it is shown in the figure 3.107 the expression of survival and drug resistance genes have significantly decreased by downregulation of Snail, Slug and Twist genes. The results are correlated with the decreased viabilities of the cells with gene silencing.

As given in the table 3.43, gene expression of Survivin was decreased to 0.25, 0.18 and 0.45 in shSnail, shSlug and shTwist cells when the expression in shControl cells were normalized to one. The expression of MUC-1 was decreased to 0.13, 0.27 and 0.44 for shSnail, shSlug and shTwist cells respectively when the expression in shControl cells were normalized to one. The expression of ABCG2 was decreased to 0.1, 0.08 and 0.63 in shSnail, shSlug and shTwist cells respectively when the expression in shControl cells were normalized to one.

Table 3.43 Relative expressions of selected genes in untreated shSnail, shSlug, shTwist and shControl cells

	shSnail	**shSlug**	**shTwist**
Survivin	0.25	0.18	0.45
MUC1	0.13	0.27	0.44
ABCG2	0.1	0.08	0.63

The values given in the Table 3.43 are calculated with ΔΔCt method, normalized to shControl cells (shControl is fixed to one) and 18S used as housekeeping gene.

The gene expression values for the cells treated with selected drugs were also analyzed.

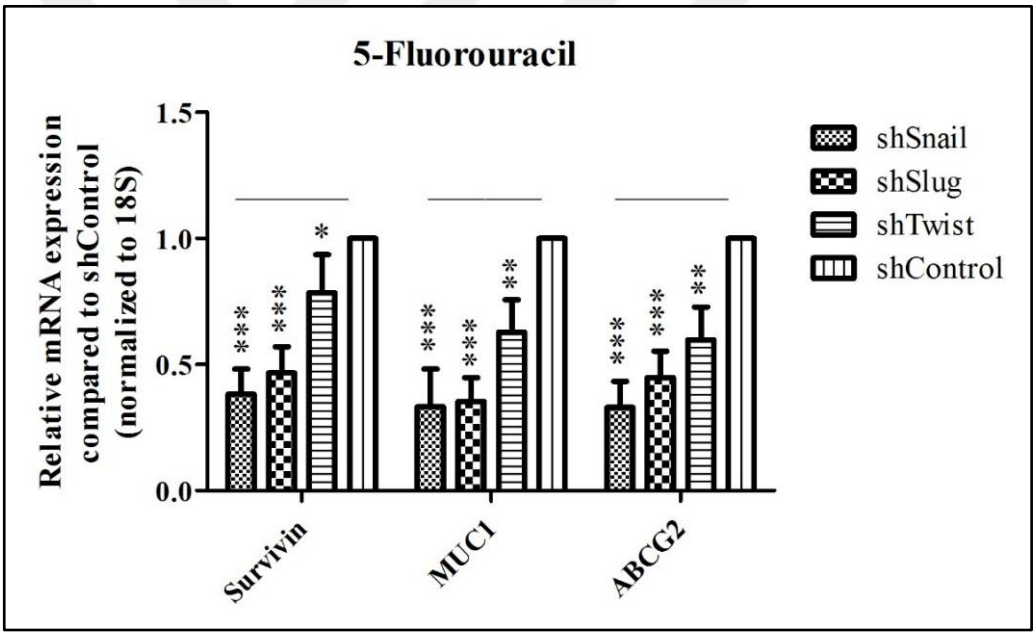


Figure 3.108 Relative mRNA expression amounts of selected genes in 5-Fluorouracil treated shSnail, shSlug, shTwist and shControl cells .One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$, ** indicates that $p<0.005$ and *** indicates that $p<0.001$)

Figure 3.108 shows the significant decrease of selected genes in shSnail, shSlug and shTwist cells compared to shControl treated with the same amount of drug. The chosen 5-Fluorouracil concentration for this experiment is 200μM and the treatment period is 48 hours.

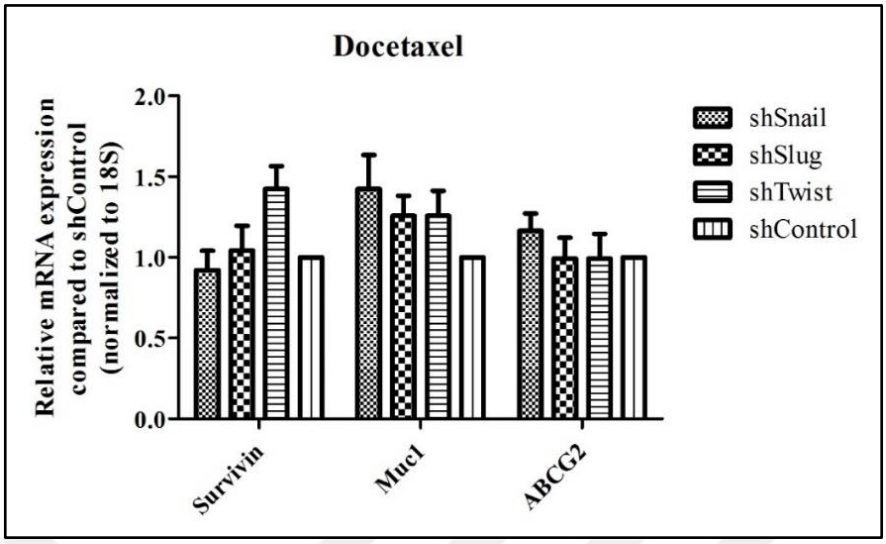


Figure 3.109 Relative mRNA expression amounts of selected genes in docetaxel treated shSnail, shSlug, shTwist and shControl cells .One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$, ** indicates that $p<0.005$ and *** indicates that $p<0.001$)

As shown in the Figure 3.109, no significant decreases were observed in selected genes after docetaxel Treatment. The chosen docetaxel concentration for this experiment is 10nM and the treatment period is 48 hours.

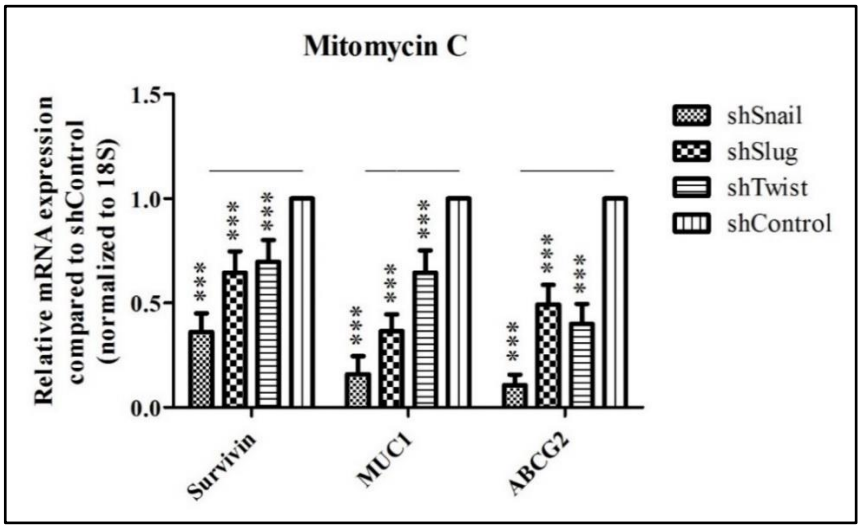


Figure 3.110 Relative mRNA expression amounts of selected genes in Mitomycin C treated shSnail, shSlug, shTwist and shControl cells .One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$, ** indicates that $p<0.005$ and *** indicates that $p<0.001$)

The Figure 3.110 shows the significant decrease of selected genes in shSnail, shSlug and shTwist cells compared to shControl treated with the same amount of drug. The chosen Mitomycin C concentration for this experiment is 0.8μM and the treatment period is 48 hours.

As given in the table 3.44, after 48 hour 5-Fluorouracil treatment, the expression of Survivin, Muc-1 and ABCG2 was changed to 0.38, 0.33 and 0.33 in shSnail cells. The expressions of Survivin, Muc-1 and ABCG2 was changed to 0.46, 0.35 and 0.45 in shSlug cells. The expressions of Survivin, Muc-1 and ABCG2 was changed to 0.78, 0.62 and 0.59 in shTwist cells. The expression for these genes in negative control were normalized to one.

After 48 hours Docetaxel treatment the expression of Survivin, Muc-1 and ABCG2 was changed to 0.92, 1.42 and 1.16 in shSnail cells, 1.04, 1.25 and 0.99 in shSlug cells and 1.42, 1.25 and 0.99 in shTwist cells. The expression for these genes in negative control were normalized to one.

After 48 hours of Mitomycin C treatment the expression of Survivin, Muc-1 and ABCG2 wsa changed to 0.36, 0.16 and 0.11 in shSnail cells, 0.65, 0.36 and 0.09 in shSlug cells and 0.69, 0.65 and 0.4 in Twist cells. The expression for these genes in negative control were normalized to one.

With respect to the results given in Table 3.44, it was observed that, the gene expressions of survival genes and drug resistance genes with EMT silencing. Also instead of getting increased after chemotherapy, the expressions of selected genes (Survivin, Muc-1 and ABCG2) were further decreased in the groups of cell therapy except docetaxel treatment. The expressions in docetaxel treatment were not decreased or increased but stayed constant when compared to the expression values of their control groups.

The expression of the selected genes in docetaxel treated shSnail, shSlug and shTwist AsPc-1 cells were not decreased. This may be because of the mechanism of action of the docetaxel chemotherapeutic. It is generally used in combination with other chemotherapeutics in order to increase their effectiveness. When used alone it may be expected for a cell to keep its Survivin, Muc-1 and ABCG2 amounts stable.

Table 3.44 Relative gene expression values of selected genes of cells treated with chemoterapeutics

	shSnail	**shSlug**	**shTwist**
	5-Fluorouracil Treatment		
Survivin	0.38	0.46	0.78
MUC1	0.33	0.35	0.62
ABCG2	0.33	0.45	0.59
	Docetaxel Treatment		
Survivin	0.92	1.04	1.42
MUC1	1.42	1.25	1.25
ABCG2	1.16	0.99	0.99
	Mitomycin C Treatment		
Survivin	0.36	0.65	0.69
MUC1	0.16	0.36	0.65
ABCG2	0.11	0.09	0.40

The table above indicates the gene expression values of shSnail, shSlug and shTwist cells for selected drug resistance and survival genes. The values are calculated with ΔΔCt method, normalized to shControl cells (shControl is fixed to one) and 18S used as housekeeping gene.

3.14. COMBINATION THERAPY

3.14.1. Changes in Gene Expression Profiles after Combination Therapy with SD208 and CX4945 and Their Combination

Gene expression values of selected EMT markers were evaluated after 48 hours of incubation with selected small molecule EMT inhibitors.

Table 3.45 Gene expression values of AsPc-1 shSnail cells after SD208 treatment

	SD208	
	shSnail	**shControl**
Snail	0.131	0.08
Slug*	0.158	0.302
Twist*	0.012	0.25
E-cadherin	2.18	3.19
N-cadherin*	0.12	0.248
Vimentin*	0.09	0.118
CD133	0.476	0.182
CXCR4*	0.302	0.88
CD24*	0.186	0.316
CD44*	0.196	0.363
EpCAM*	0.236	0.414
Nanog*	0.221	0.414
Klf4*	0.21	0.302
c-Myc*	0.239	0.93
MMP1*	0.084	0.476
MMP9	0.053	0.056
c-Met*	0.248	0.669
MTA1*	0.012	0.305
MTA2*	0.109	0.188

Table 3.46 Gene expression values of AsPc-1 shSnail cells after CX-4945 treatment

	CX-4945	
	shSnail	**shControl**
Snail*	0.327	0.07
Slug*	0.284	0.223
Twist*	0.329	0.449
E-cadherin*	2.706	4
N-cadherin*	0.012	0.18
Vimentin	0.201	0.389
CD133*	0.547	0.277
CXCR4*	0.12	0.607
CD24*	0.44	0.399
CD44*	0.201	0.261
EpCAM*	0.12	0.669
Nanog*	0.203	0.423
Klf4*	0.18	0.34
c-Myc*	0.263	0.67
MMP1*	0.18	0.432
MMP9*	0.071	0.49
c-Met*	0.567	0.846
MTA1	0.32	0.447
MTA2	0.21	0.237

Table 3.47 Gene expression values of AsPc-1 shSnail cells after SD208 and CX-4945 treatment

	SD208 and CX-4945	
	shSnail	**shControl**
Snail	0.261	0.06
Slug	0.264	0.2
Twist*	0.152	0.49
E-cadherin*	4.18	3
N-cadherin*	0.1	0.169
Vimentin*	0.23	0.603
CD133	0.489	0.124
CXCR4*	0.402	0.683
CD24*	0.374	0.473
CD44*	0.102	0.272
EpCAM*	0.603	0.727
Nanog	0.19	0.23
Klf4*	0.562	0.752
c-Myc*	0.42	0.59
MMP1*	0.245	0.582
MMP9*	0.037	0.11
c-Met*	0.418	0.759
MTA1*	0.36	0.401
MTA2*	0.21	0.257

Table 3.48 Gene expression values of AsPc-1 shSlug cells after SD208 treatment

	SD208	
	shSlug	**shControl**
Snail*	0.0125	0.08
Slug*	0.075	0.302
Twist*	0.111	0.25
E-cadherin*	5.38	3.19
N-cadherin	0.225	0.248
Vimentin	0.305	0.118
CD133*	0.054	0.182
CXCR4*	0.2	0.88
CD24*	0.279	0.316
CD44*	0.26	0.363
EpCAM*	0.279	0.414
Nanog*	0.245	0.414
Klf4*	0.103	0.302
c-Myc*	0.22	0.93
MMP1*	0.235	0.476
MMP9	0.055	0.056
c-Met*	0.222	0.669
MTA1*	0.077	0.305
MTA2	0.139	0.188

Table 3.49 Gene expression values of AsPc-1 shSlug cells after CX-4945 treatment

	CX-4945	
	shSlug	**shControl**
Snail*	0.015	0.07
Slug*	0.132	0.223
Twist*	0.04	0.449
E-cadherin*	5.06	4
N-cadherin*	0.061	0.18
Vimentin	0.543	0.389
CD133*	0.129	0.277
CXCR4*	0.205	0.607
CD24*	0.253	0.399
CD44*	0.126	0.261
EpCAM*	0.253	0.669
Nanog*	0.221	0.423
Klf4*	0.132	0.34
c-Myc*	0.43	0.67
MMP1*	0.26	0.432
MMP9*	0.157	0.49
c-Met*	0.195	0.846
MTA1	0.403	0.447
MTA2	0.295	0.237

Table 3.50 Gene expression values of AsPc-1 shSlug cells after SD208 and CX-4945 treatment

	SD208 and CX-4945	
	shSlug	**shControl**
Snail*	0.0198	0.06
Slug*	0.128	0.2
Twist*	0.013	0.49
E-cadherin*	4.013	3
N-cadherin	0.152	0.169
Vimentin*	0.395	0.603
CD133	0.107	0.124
CXCR4*	0.17	0.683
CD24*	0.147	0.473
CD44*	0.207	0.272
EpCAM*	0.146	0.727
Nanog	0.223	0.23
Klf4*	0.316	0.752
c-Myc*	0.26	0.59
MMP1*	0.248	0.582
MMP9*	0.047	0.11
c-Met*	0.324	0.759
MTA1*	0.344	0.401
MTA2*	0.188	0.257

Table 3.51 Gene expression values of AsPc-1 shTwist cells after SD208 treatment

	SD208	
	shTwist	**shControl**
Snail*	0.010	0.08
Slug*	0.063	0.302
Twist*	0.026	0.25
E-cadherin*	6.10	3.19
N-cadherin*	0.206	0.248
Vimentin	0.205	0.118
CD133*	0.045	0.182
CXCR4*	0.302	0.88
CD24*	0.106	0.316
CD44*	0.210	0.363
EpCAM*	0.306	0.414
Nanog*	0.302	0.414
Klf4*	0.096	0.302
c-Myc*	0.186	0.93
MMP1*	0.302	0.476
MMP9*	0.015	0.056
c-Met*	0.302	0.669
MTA1*	0.095	0.305
MTA2	0.185	0.188

Table 3.52 Gene expression values of AsPc-1 shTwist cells after CX-4945 treatment

	CX-4945	
	shTwist	**shControl**
Snail	0.083	0.07
Slug*	0.126	0.223
Twist*	0.09	0.449
E-cadherin*	4,96	4
N-cadherin*	0.079	0.18
Vimentin*	0.301	0.389
CD133*	0.204	0.277
CXCR4*	0.402	0.607
CD24*	0.306	0.399
CD44*	0.206	0.261
EpCAM*	0.323	0.669
Nanog*	0.400	0.423
Klf4*	0.220	0.34
c-Myc*	0.503	0.67
MMP1*	0.102	0.432
MMP9*	0.185	0.49
c-Met*	0.302	0.846
MTA1*	0.342	0.447
MTA2*	0.203	0.237

Table 3.53 Gene expression values of AsPc-1 shTwist cells after SD208 and CX-4945 treatment

	SD208 and CX-4945	
	shTwist	**shControl**
Snail	0.03	0.06
Slug	0.216	0.2
Twist*	0.103	0.49
E-cadherin*	6.01	3
N-cadherin	0.140	0.169
Vimentin*	0.296	0.603
CD133*	0.106	0.124
CXCR4*	0.312	0.683
CD24*	0.260	0.473
CD44*	0.203	0.272
EpCAM*	0.410	0.727
Nanog*	0.196	0.23
Klf4*	0.503	0.752
c-Myc	0.506	0.59
MMP1*	0.341	0.582
MMP9*	0.16	0.11
c-Met*	0.475	0.759
MTA1*	0.260	0.401
MTA2*	0.128	0.257

After gene expression values were analyzed, shSnail and shSlug groups have showed better potential. The shSnail and shSlug groups were used in further experiments. The combination of SD208 and CX4945 has not shown a cumulative result so the chemicals were used one by one.

3.14.2. Changes in Migration after Combination Therapy with SD208 and CX4945

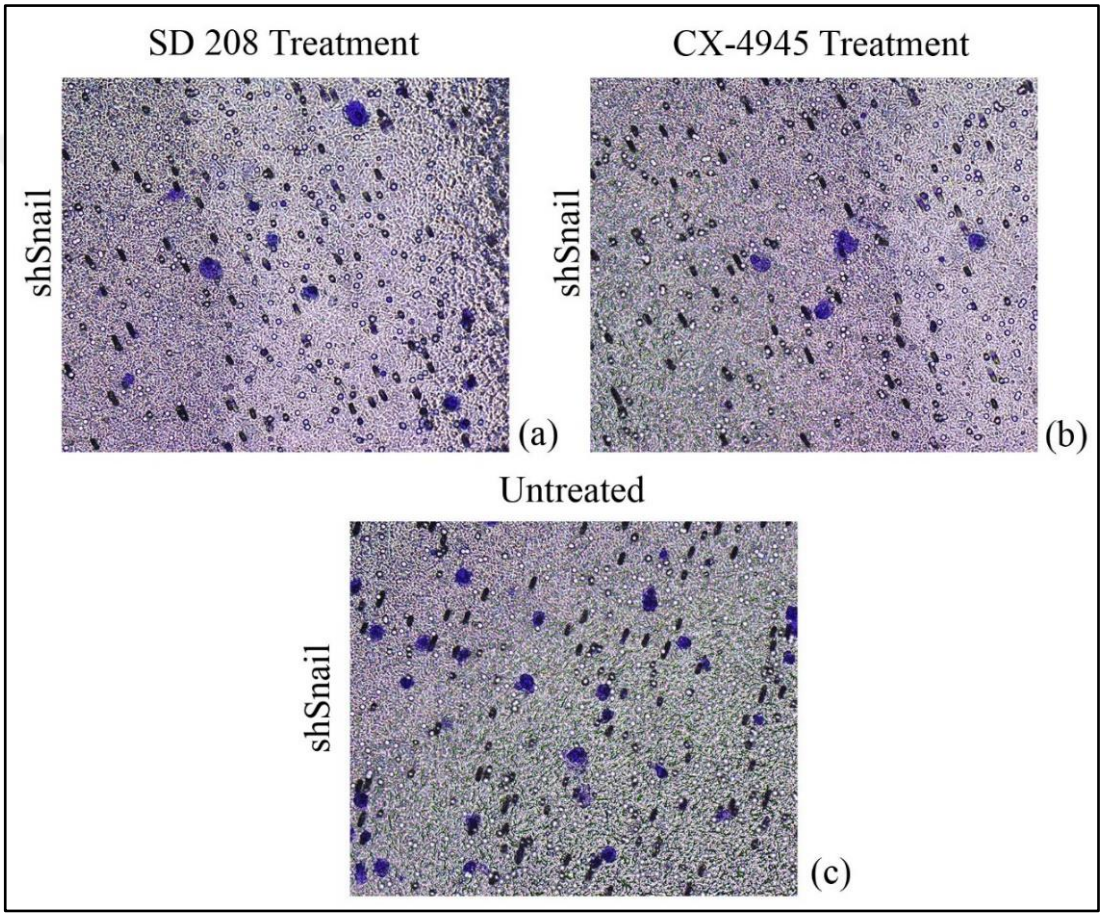


Figure 3.111 Cell images of shSnail after SD208, CX4945 treatment and untreated shSnailwith (a) SD-208, (b) CX4945 treatments and (c) untreated

Figure 3.11 contains the microscope images of untreated shSnail and shSnail after SD208 and CX-4959 treatment. Decreased number of migrated cells shows the decreased migration of shSnail cells after inhibitor treatment.

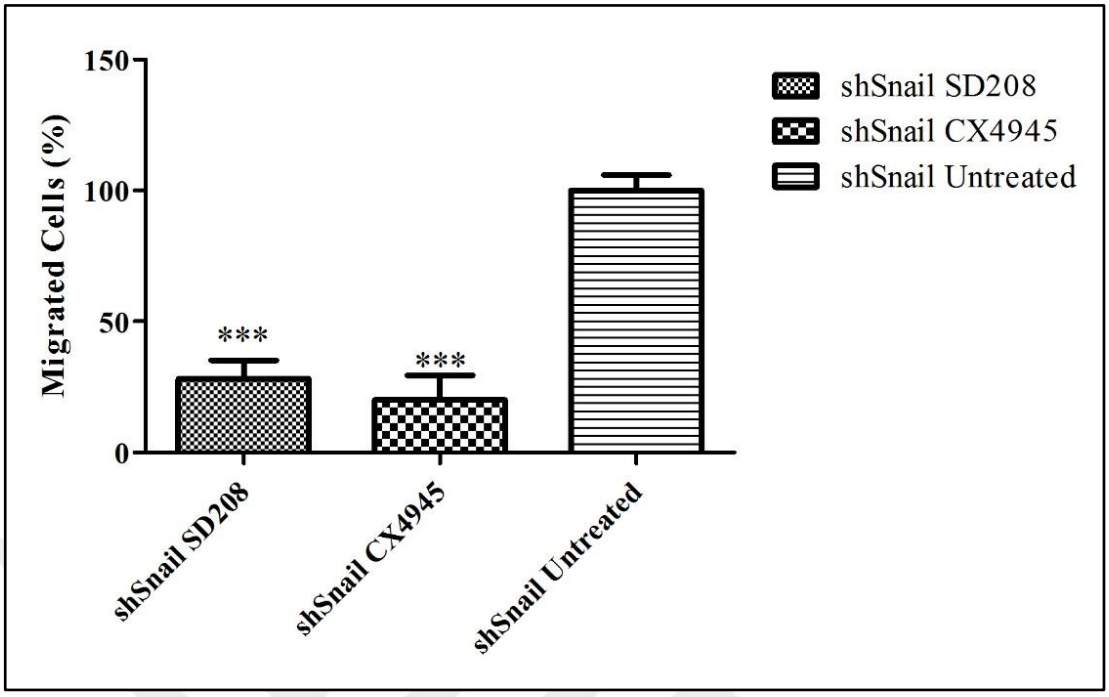


Figure 3.112 Graphical representation of migrated cell per cents of shSnail cells in SD208, CX4945 and Untreated groups.One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$, ** indicates that $p<0.005$ and *** indicates that $p<0.001$)

It was seen that both SD208 and CX4945 treated shSnail cells migrated significantly in less amounts compared to untreated group. As given in the table 3.54, number of migrated cells were decreased to 28 and 20 per cent in shSnail SD208 and shSnail CX49454 groups compared to shSnail untreated cells.

Table 3.54 Migrated cell percentages of shSnail Cells compared to untreated shSnail cells

Migrated Cells (per cent)		
shSnail SD208	**shSnail CX4945**	**shSnail Untreated**
28	20	100

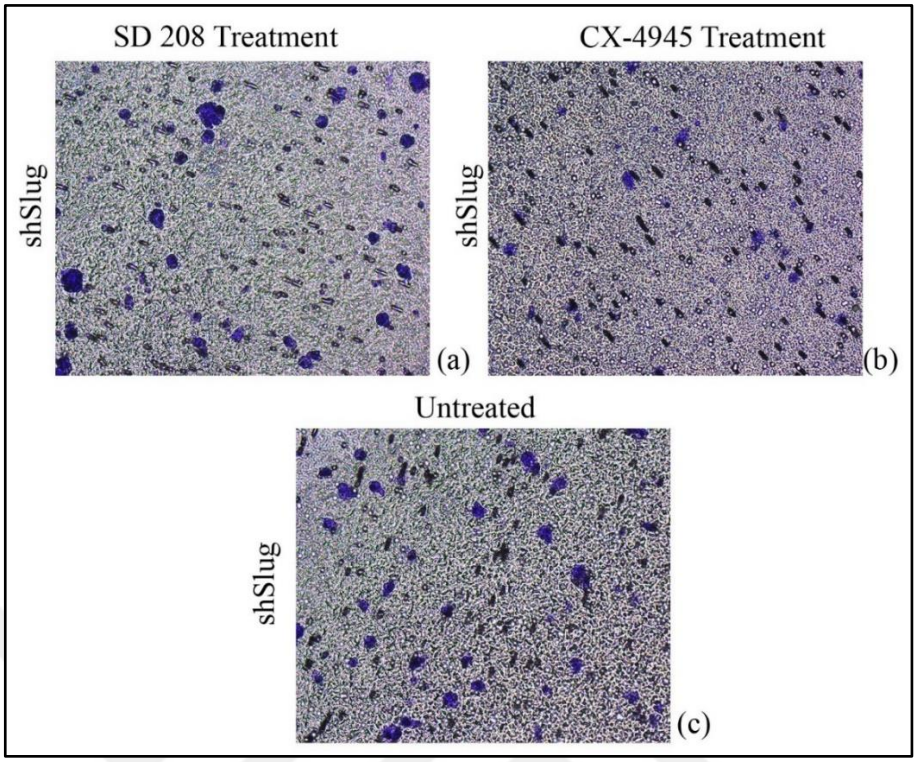


Figure 3.113 Cell images of shSlug after SD208, CX4945 treatment and untreated shSlug with (a) SD-208, (b) CX4945 treatments and (c) untreated

As shown in Figure 3.113 images of untreated shSlug and shSlug after SD208 and CX-4959 treatment. Decreased number of migrated cells shows the decreased migration of shSlug cells after inhibitor treatment.

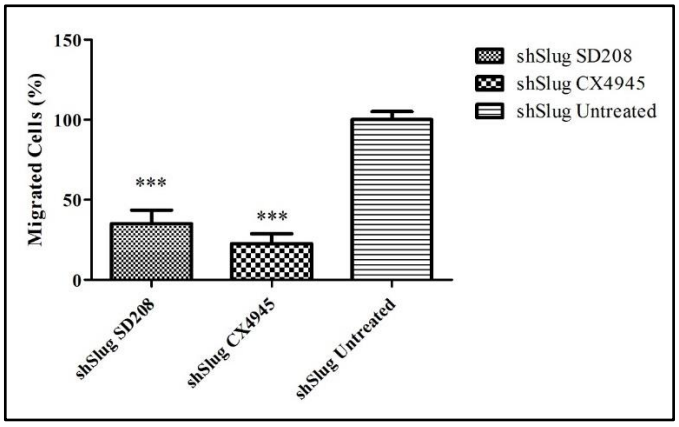


Figure 3.114 Graphical representation of migrated cell per cents of shSnail cells in SD208, CX4945 and Untreated groups.One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$, ** indicates that $p<0.005$ and *** indicates that $p<0.001$)

It was seen that both SD208 and CX4945 treated shSlug cells migrated in less amounts compared to untreated group. These treatment shows that small molecule inhibitors significantly decrease migration.

As indicated in the Table 3.55, the amount of migrated cells were decreased to 26.9 per cent in shSlug SD208, 38.5 in shSlug CX4945 when the amount of migrated cells were accepted to 100 per cent

Table 3.55 Migrated cell percentages of shSlug cells compared to untreated shSlug cells

Migrated Cells (per cent)		
shSlug SD208	**shSlug CX4945**	**shSlug Untreated**
26.9	38.5	100

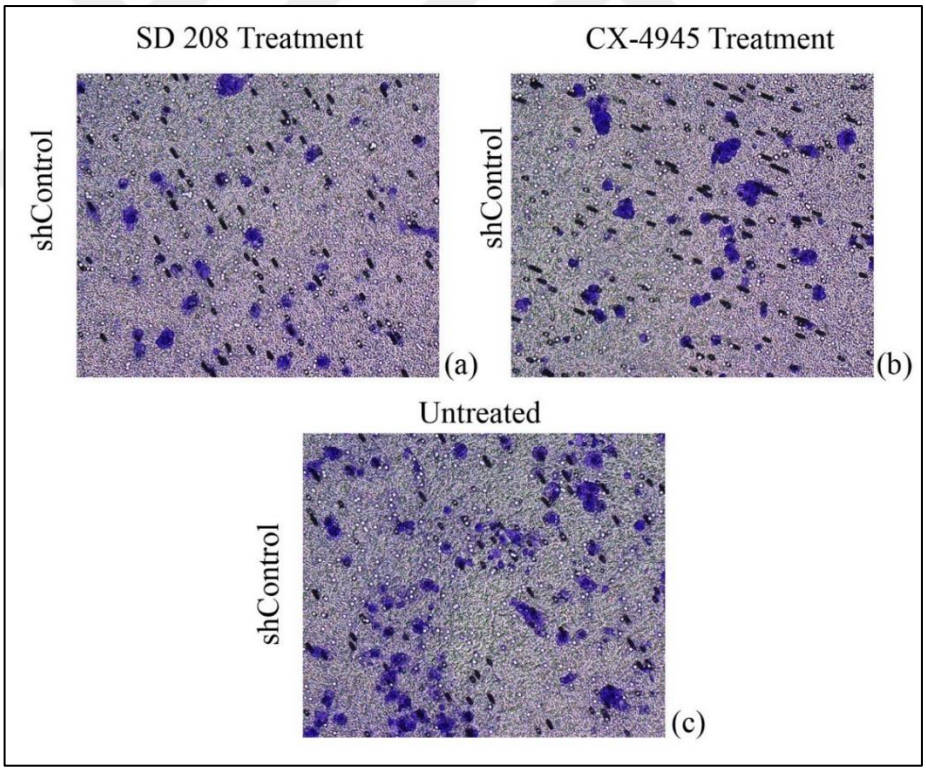


Figure 3.115 Cell images of shControl after SD208, CX4945 treatment and untreated shControlwith (a) SD-208, (b) CX4945 treatments and (c) untreated

Images of untreated shControl and shControl after SD208 and CX-4959 treatment. Decreased number of migrated cells shows the decreased migration of shControl cells after inhibitor treatment.

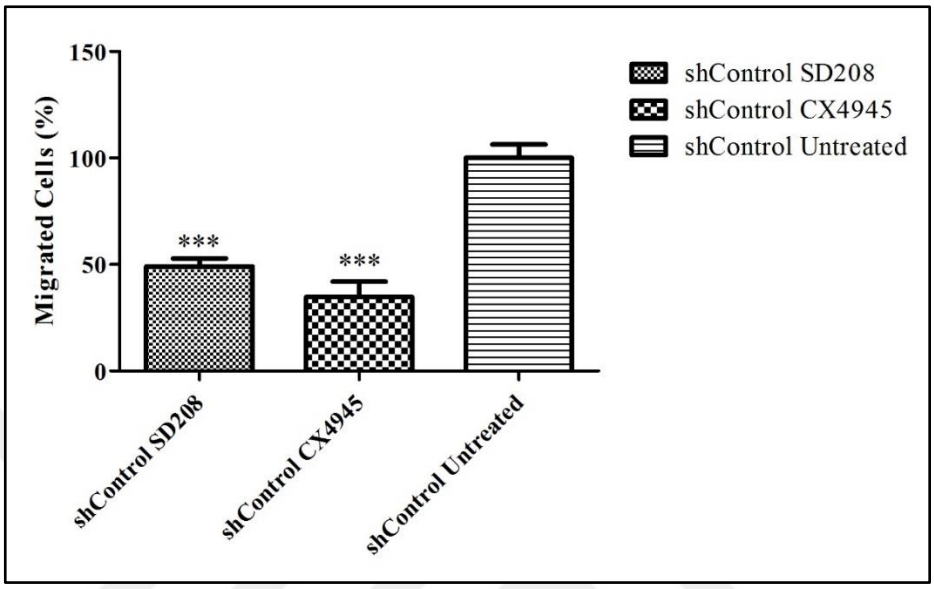


Figure 3.116 Graphical representation of migrated cell per cents of shControl cells in SD208, CX4945 and Untreated groups.One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$, ** indicates that $p<0.005$ and *** indicates that $p<0.001$)

It seen in Figure 3.116 both SD208 and CX4945 treated shControl cells migrated in less amounts compared to untreated group.

As given in Table 3.56 with small molecule treatment the amount of migrated cells were decreased to 26.9 per cent in SD208 group and to 38.5 percent in CX4945 group when it is compared to shControl untreated group which is accepted as 100 per cent.

Table 3.56 Migrated cell percentages of shSlug cells compared to untreated shControl cells

Migrated Cells (per cent)		
shControl SD208	**shControl CX4945**	**shControl Untreated**
26.9	38.5	100

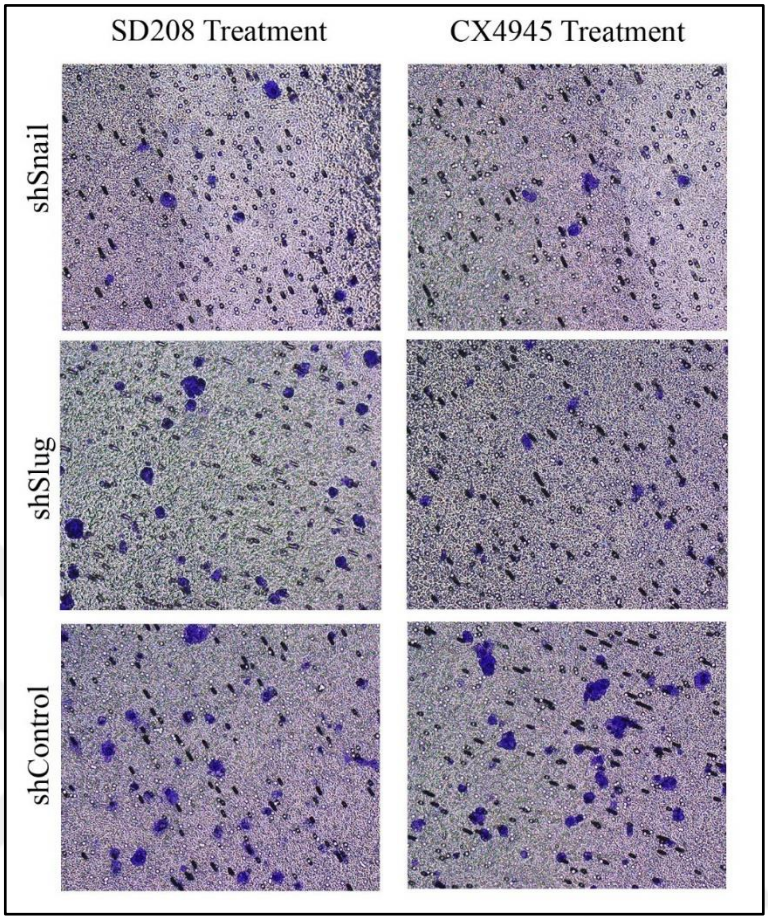


Figure 3.117 Compared images of shSnail, shSlug and shControl cells with SD208 and CX4945 treatments

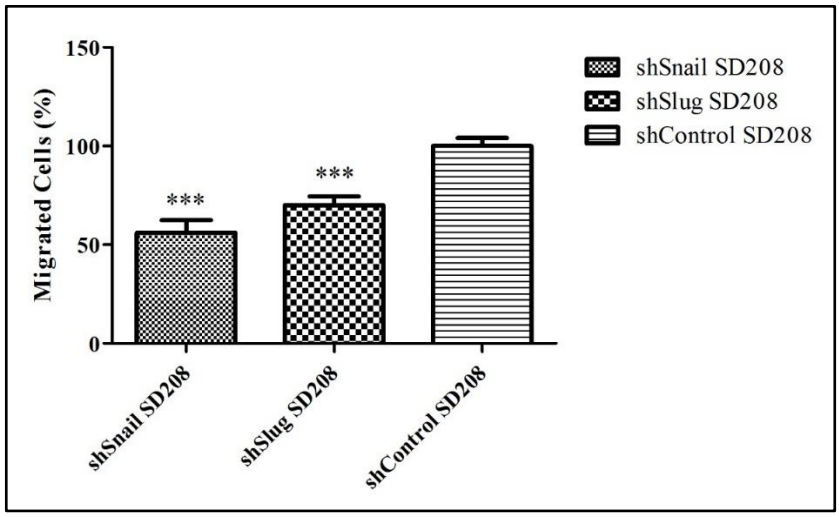


Figure 3.118 Graphical representation of migrated cell per cents treated with SD208.One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$, ** indicates that $p<0.005$ and *** indicates that $p<0.001$)

It was shown that, SD208 treatment decreased migrated cell amounts of shSnail, shSlug cells compared to SD208 treated shControl, when shControl is accepted as 100 per cent.

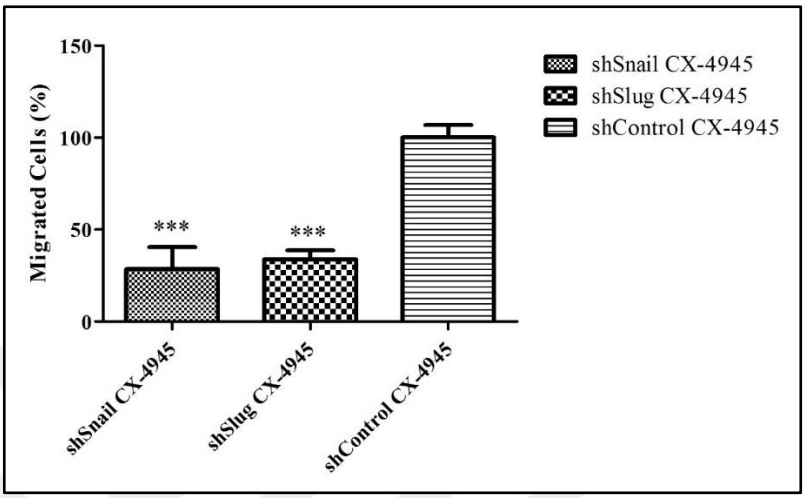


Figure 3.119 Graphical representation of migrated cell per cents treated with CX-4945 It was shown that, CX-4945 treatment has significantly decreased migration abilities of shSnail and shSlug cells compared to shControl cells, when shControl is accepted as 100 per cent..One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$, ** indicates that $p<0.005$ and *** indicates that $p<0.001$)

Table 3.57 Migrated cell percentages compared to shControl cells

Migrated Cells (per cent)		
shSnail SD208	**shSlug SD208**	**shControl SD208**
56	70	100
shSnail CX4945	**shSlug CX4945**	**shControl CX4945**
18	33	100
shSnail NC	**shSlug NC**	**shControl NC**
48	68	100

Table3.54 shows the invaded cell per cents compared to the shControl groups. It was found that, both SD208 and CX4945 treatment significantly decreased the migrated cell amounts.

3.14.3. Changes in Invasiveness after Combination Therapy with SD208 and CX4945

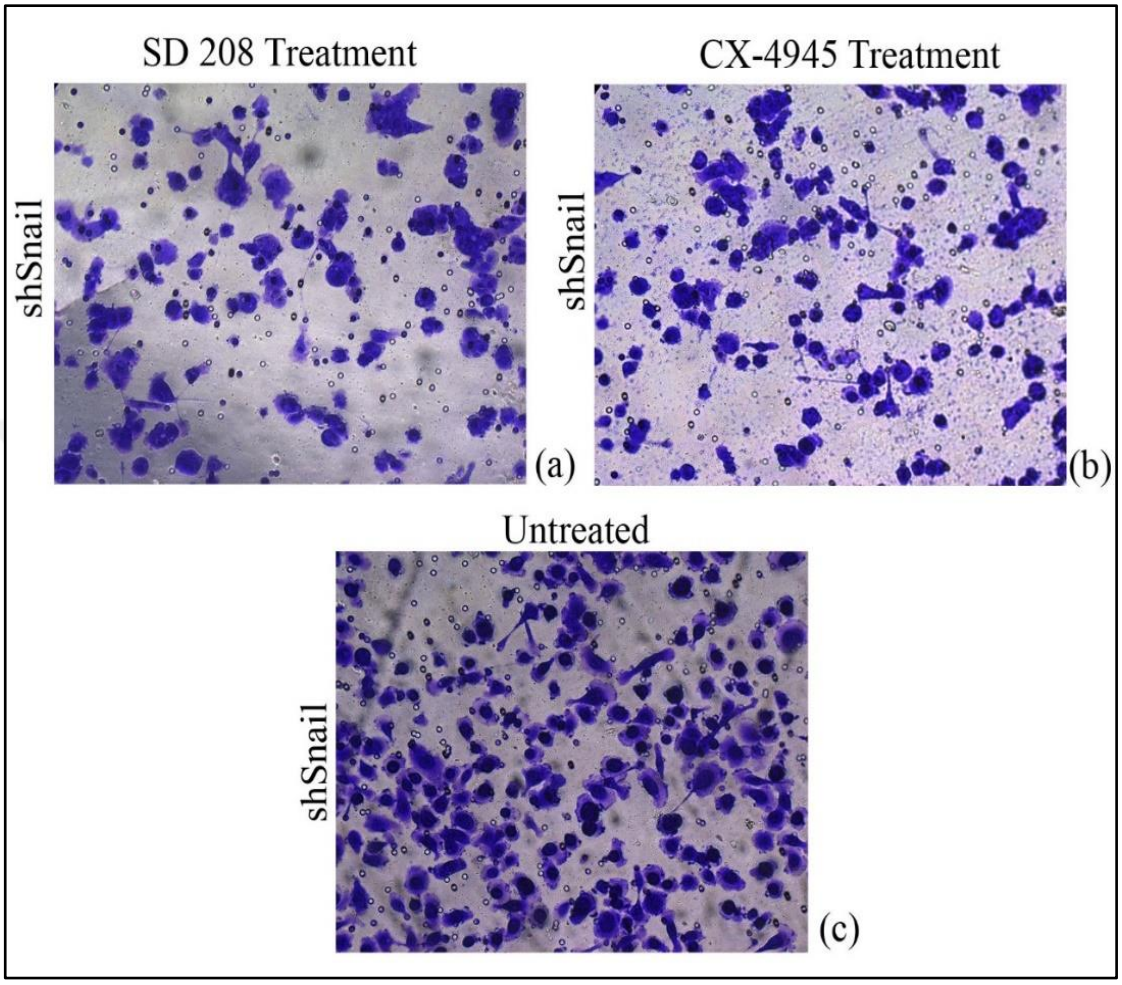


Figure 3.120 Cell images of shSnail after SD208, CX4945 treatment and untreated shSnailwith (a)SD-208, (b) CX4945 treatment and (c) Untreated

Images of untreated shSnail and shSnail after SD208 and CX-4959 treatment. Decreased number of invaded cells shows the decreased invasiveness of shSnail cells after inhibitor treatment.

Number of invasive cells (purple) were less in SD208 treated shSnail AsPc-1 cells and in CX4945 treated shSnail AsPc-1 cells compared to untreated shSnail AsPc-1 cells.

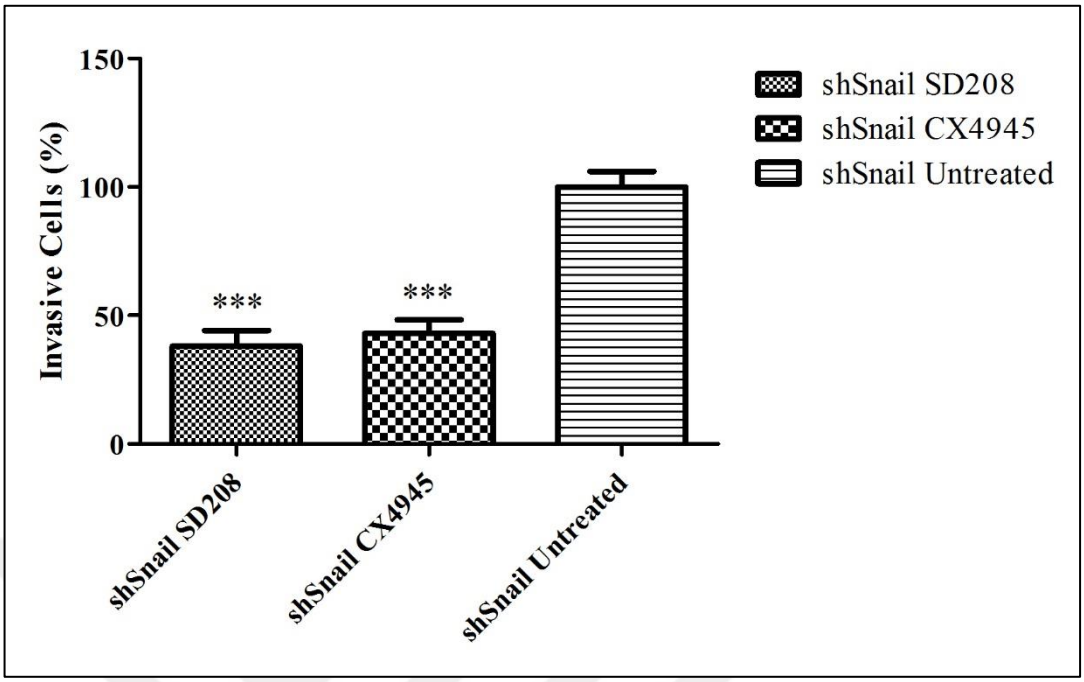


Figure 3.121 Graphical representation of invaded cell per cents of shSnail cells in SD208, CX4945 and Untreated groups.One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$, ** indicates that $p<0.005$ and *** indicates that $p<0.001$)

As seen in figure 3.121 it was observed that both SD208 and CX4945 treated shSnail cells invaded in less amounts compared to untreated group.

Table 3.58 indicates the invaded cell numbers were decreased to 38 per cent in shSnail SD208 group, 43 per cent in shSnail CX4945 group compared to Untreated shSnail cells which is accepted as 100 per cent

Table 3.58 Invaded cell percentages of shSnail Cells compared to untreated shSnail cells

Invaded Cells (per cent)		
shSnail SD208	**shSnail CX4945**	**shSnail Untreated**
38	43	100

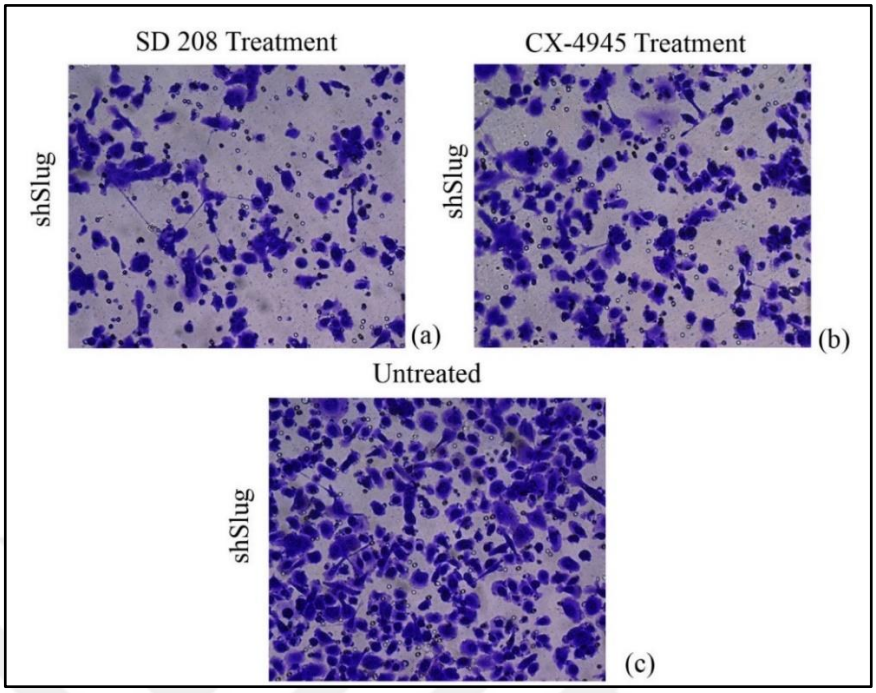


Figure 3.122 Cell images of shControl after SD208, CX4945 treatment and untreated shSlugwith (a) SD-208, (b) CX4945 treatments and (c) untreated

Figure 3.122 contains the images of untreated shSlug and shSlug after SD208 and CX-4959 treatment. Decreased number of invaded cells shows the decreased invasiveness of shSlug cells after inhibitor treatment.

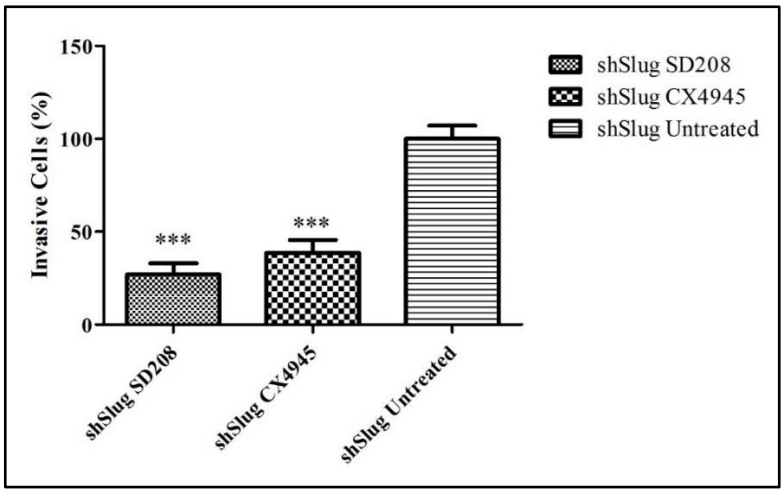


Figure 3.123 Graphical representation of invaded cell per cents of shSlug cells in SD208, CX4945 and Untreated groups.One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$, ** indicates that $p<0.005$ and *** indicates that $p<0.001$)

As shown in the Figure 3.123 both SD208 and CX4945 treated shSlug cells invaded in less amounts compared to untreated group.

As given in Table 3.59, invaded cell percentages of shSlug SD208 was decreased to 26.9 per cent, shSlug CX4945 was decreased to 38.5 per cent compared to shSlug Untreated cells which are accepted as 100 per cent.

Table 3.59 Invaded cell percentages of shSlug cells compared to untreated shSlug cells

Invaded Cells (per cent)		
shSlug SD208	**shSlug CX4945**	**shSlug Untreated**
26.9	38.5	100

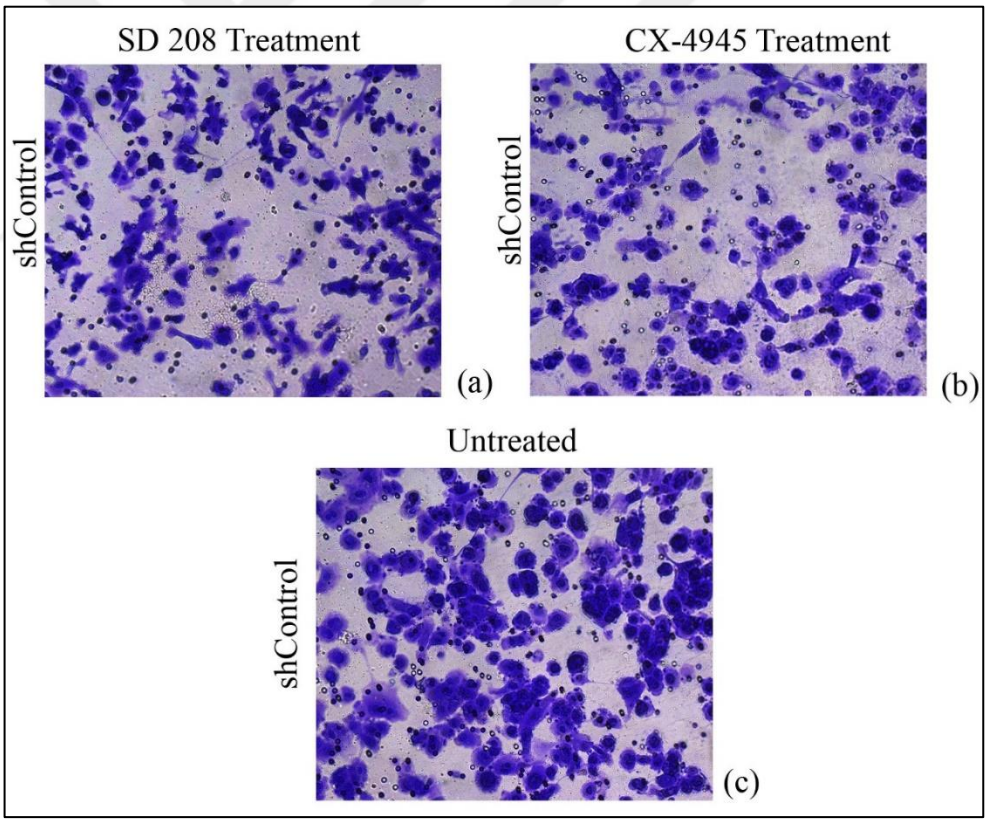


Figure 3.124 Cell images of shControl after SD208, CX4945 treatment and untreated shControlwith (a) SD-208, (b) CX4945 treatments and (c) untreated

Figure 3. 124 contains the images of untreated shControl and shControl after SD208 and CX-4959 treatment. Decreased number of invaded cells shows the decreased invasiveness of shControl cells after inhibitor treatment.

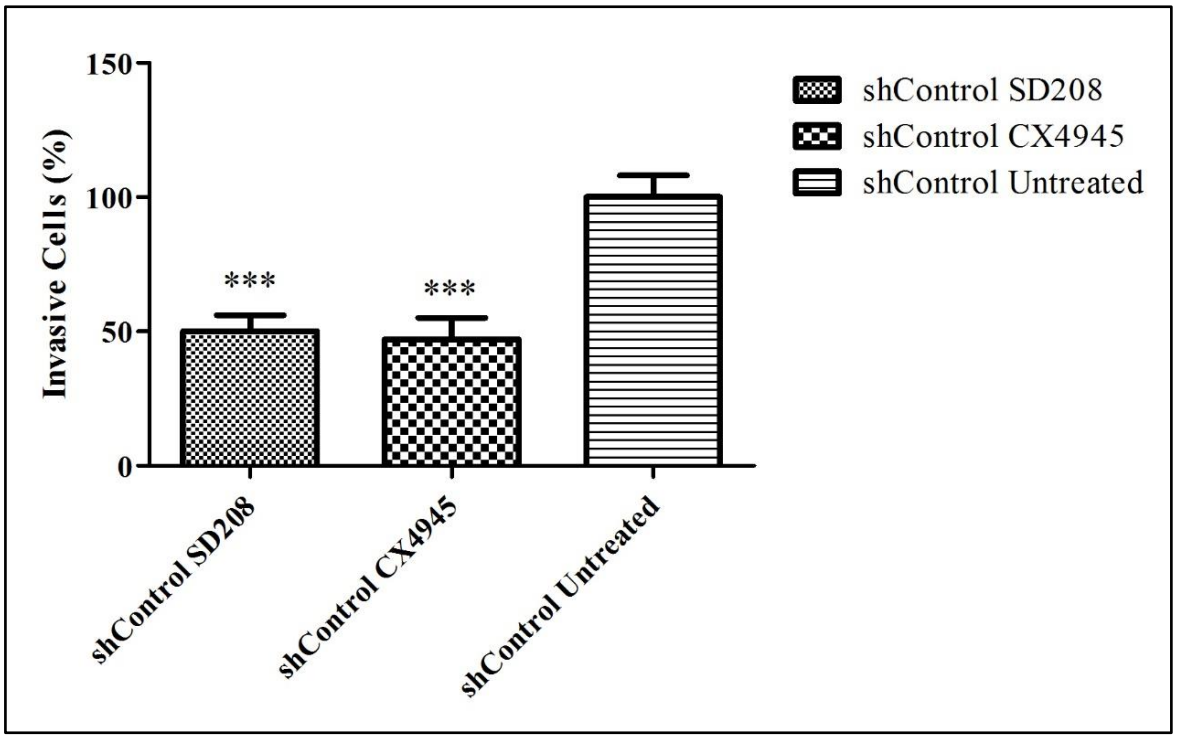


Figure 3.125 Graphical representation of invaded cell per cents of shControl cells in SD208, CX4945 and Untreated groups.One-way ANOVA with Tukey's Post Test was used to statistically analyze the differences between groups (* indicates that $p<0.05$, ** indicates that $p<0.005$ and *** indicates that $p<0.001$)

It was seen in figure 3.125, both SD208 and CX4945 treated shControl cells invaded in less amounts compared to untreated group.

Table 3.60 indicates the changes in invasion in shControl group. According to Table 3.60, with SD208 treatment invasiveness of shControl cells were decreased to 50 per cent and with CX4945 it was decreased to 47.1 per cent when compared to untreated shcontrol cells which was accepted as 100 per cent.

Table 3.60 Invaded cell percentages of shControl Cells compared to Untreated shControl

Invaded Cells (per cent)		
shControl SD208	**shControl CX4945**	**shControl Untreated**
50.6	47.1	100

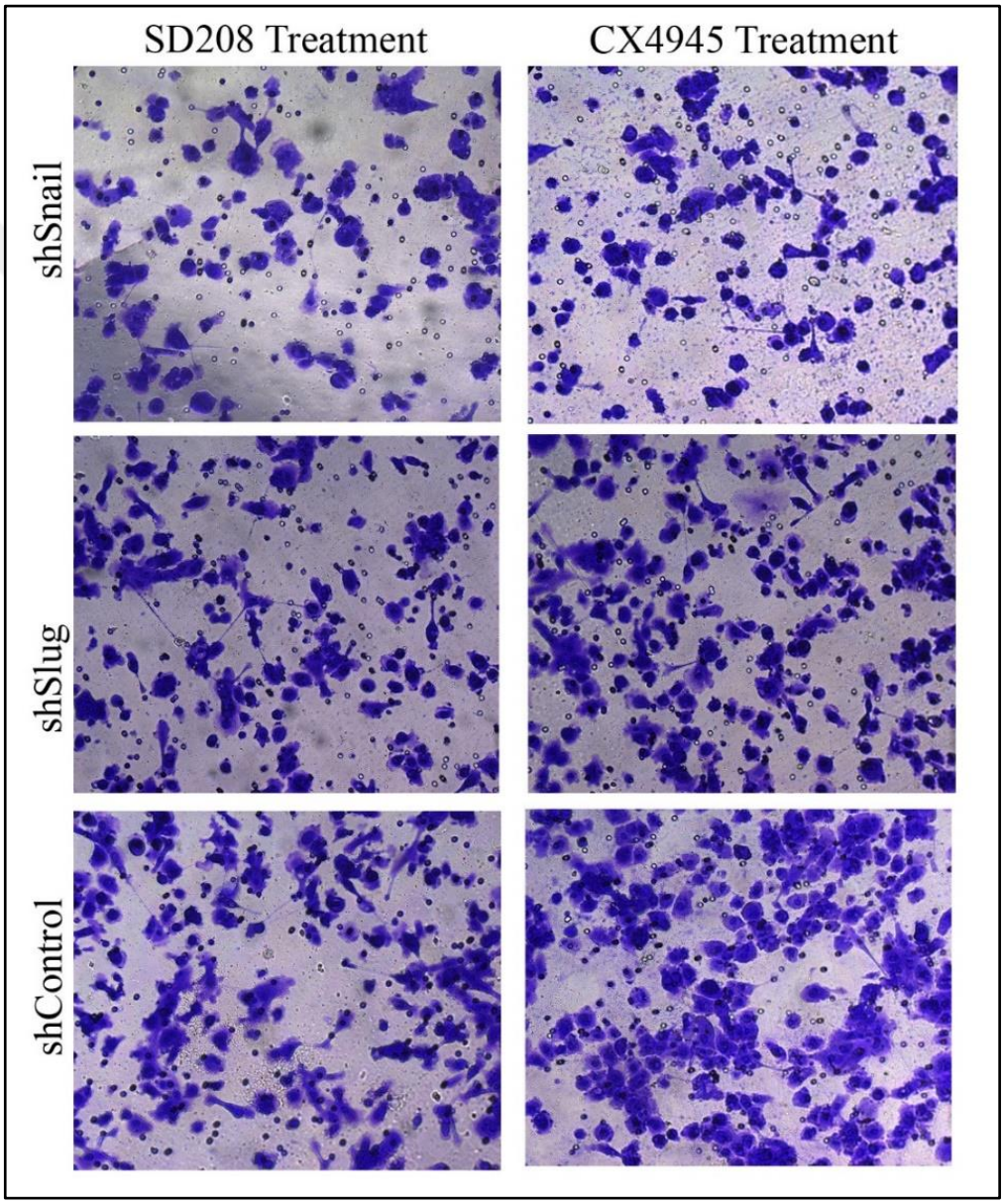


Figure 3.126 Compared images of shSnail, shSlug and shControl cells with SD208 and CX4945 treatments

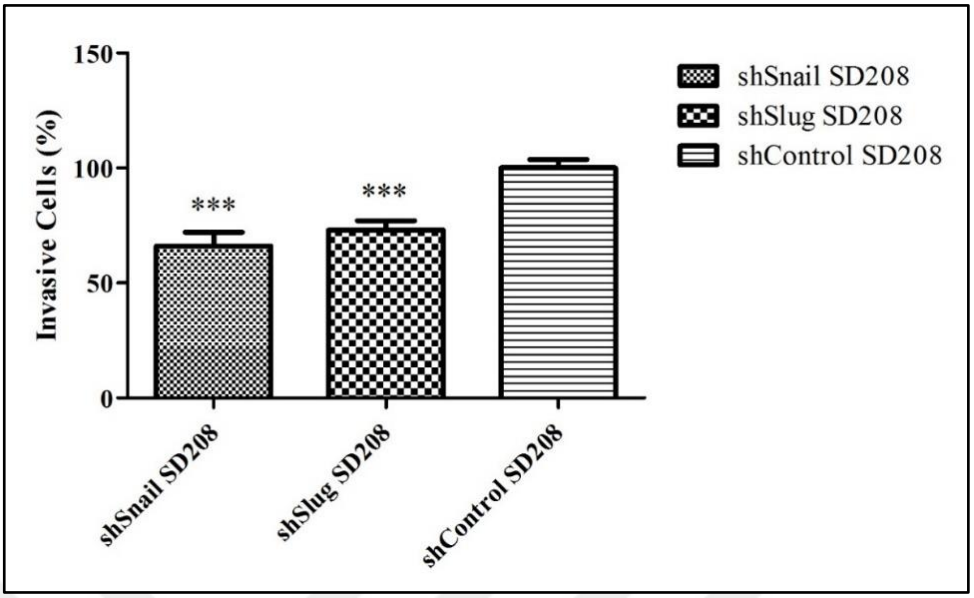


Figure 3.127 Graphical representation of invaded cell per cents treated with SD208

It was shown that, SD208 treatment decreased invaded cell amounts of shSnail, shSlug cells compared to SD208 treated shControl, when shControl is accepted as 100 per cent.

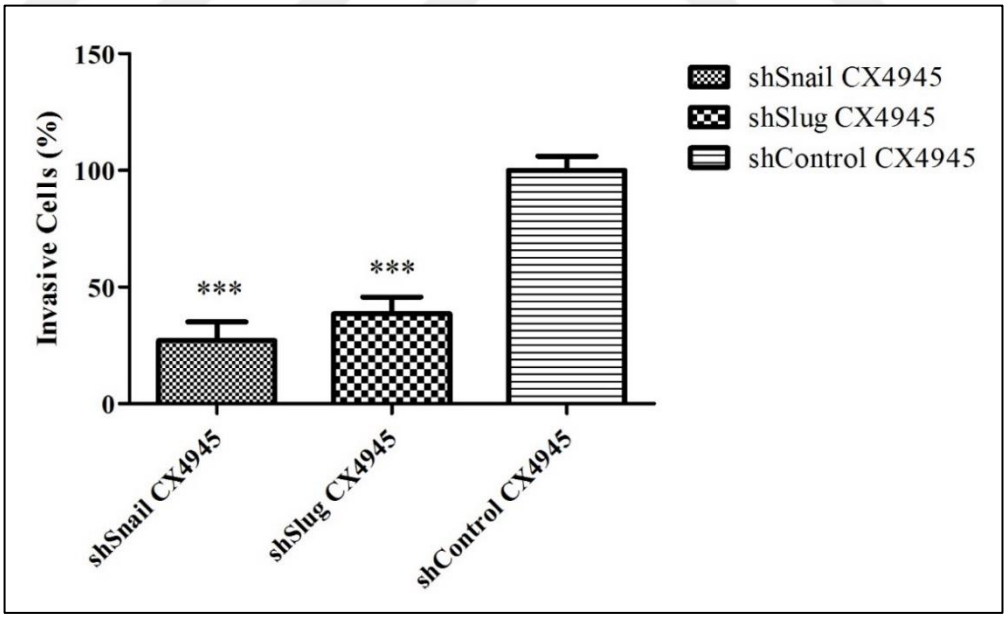


Figure 3.128 Graphical representation of invaded cell per cents treated with CX4945

It was shown that, CX4945 treatment decreased invaded cell amounts of shSnail, shSlug cells compared to CX4945 treated shControl when shControl is accepted as 100 per cent.

Table 3.61 Invaded cell percentages compared to shControl cells

Invaded Cells (per cent)		
shSnail SD208	**shSlug SD208**	**shControl SD208**
66	73	100
shSnail CX4945	**shSlug CX4945**	**shControl CX4945**
27	38.6	100
shSnail NC	**shSlug NC**	**shControl NC**
50.6	47.1	100

Table above shows the invaded cell per cents compared to the shControl groups. It was found that, both SD208 and CX4945 treatment significantly decreased the invasive cell amounts.

4. DISCUSSION

Despite the improvements in surgical techniques, diagnostic tools and chemotherapeutic diversity, PC still remains to be a major problem of medicine with a high mortality rate. Metastasis is the leading cause of PC related deaths. Metastasis is controlled by a series of events where cells spread from the initial tumor site, migrate through circulatory system and form new secondary tumors in distant organs. One of the key regulator of metastasis is epithelial to mesenchymal transition (EMT) in which cells lose their epithelial phenotype and turn into cells with mesenchymal properties. Contradictory to some studies suggesting that EMT is dispensable for metastasis [205], many other studies indicate the importance of EMT mechanism in metastasis initiation [206, 207]. EMT is controlled by several pathways and transcription factors; Snail, Slug and Twist are being the most prominent ones [208]. Expression of these transcription factors induce the EMT program, which may have roles in stem cell traits, drug resistance, senescence, immunosuppression and metastasis through controlling the expression of their associated genes [209].

During metastasis cells lose their epithelial phenotype and invade to the local tissues. Invasive tumors are characterized with an increased expression of Snail, Slug and Twist [210, 211]. To understand the effect of Snail, Slug and Twist downregulation, invasion assay was done. As indicated by other studies on different cancer cell types [212-216] we have observed a reduction in invasion after gene therapy. Snail was found to be the most effective gene on invasion among all cell lines of interest.

Decrease of the invasive profile may be due to the link of these transcription factors to matrix metalloproteinases (MMPs). Particularly MMP-1 is known to contribute to invasion [217]. To support this hypothesis, MMP-1 mRNA expression level was measured. After gene silencing, MMP-1 expression was found to be decreased significantly, in all cells. Additionally this decrease may be hinged upon the decrease in c-met, one of the primary genes that controls both invasion and migration thus metastasis [218-221], which is known to be found in increased amounts in pancreatic adenocarcinoma (PDAC) [222], and counted as a marker of pancreas cancer stem cells [29]. Together with the following contributors on the decrease in invasiveness, the lessening of vimentin with EMT silencing may also be the reason of decreased invasiveness. Vimentin is known to cooperate with actin and

microtubules for elongation of invadopodia therefore orchestrating invasion [223]. The decreased expression of N-cadherin in nearly all cells and the increased expression of E-cadherin after gene therapy are also connected to the reduced invasiveness. Cadherins are transmembrane proteins that control cell-cell adhesion [224, 225] and invasiveness. One of the major changes of EMT process is the "cadherin switch" which is basically the degradation of E-cadherin and the synthesis of N-cadherin in its place [226]. Cadherin switch is an important cause of tumor aggressiveness in epithelial carcinomas [227]. By silencing EMT transcription factors and hence reversing the cadherin switch, may have contributed to decrease the invasive potential.

An invading cell must interacts with the basal membrane (BM) between the primary tissue and secondary site via extending specialized parts from its membrane, named as invadopodia [228]. The basal membrane is dense and consists of greatly cross-linked sheets of extracellular matrix proteins, including several proteoglycans, collagen, elastin, fibronectin and laminin [229, 230]. During invasion, invasive cells attach, penetrate and digest these extracellular proteins. Additionally in various cancer cell lines metastatic ability is found to be directly proportional to the level of laminin receptors on the cell surface [231, 232] and when laminin adherent cells were selected in vitro, they showed more malignancy in *in vivo* conditions [233]. The incapability to demonstrate the complex interactions between the ECM and the surface receptors of the invasive cells both *in vivo* and *in vitro*, made researchers to be content with simplified experiments, for instance attachment to laminin assay in order to evaluate the capability of the cell to connect ECM. In our study, attachment to laminin assay was done to PC cells subjected to Snail, Slug and Twist silencing. All cells in all silencing conditions has shown less tendency to attach to laminin as expected since Snail [234], Slug [235] and Twist [236] is known to control the expression of integrins, including the receptor for laminin. Together with effect of the EMT silencing on integrins, the decrease of CD44 may also contribute to the decrease in laminin attachment. Besides being a cancer stem cell marker for PDAC [237], CD44 is known to interact with various ECM proteins including laminin [238]. Decreased invasion and laminin attachment reveals that Snail, Slug or Twist silencing are good candidates for overcoming invasiveness of PC cells.

Being motile is a precondition and necessity for invasion and metastasis [239]. The contributions of Snail [240, 241], Slug [241] and Twist [242] on migration was previously

stated by various studies. With the purpose of understanding the influence of EMT silencing on PC cells, we have made scratch assay and trans-well migration assay and observed reduced wound healing and decreased transmembrane migration as expected. In addition to its effect on invasion, cadherin switch also controls cellular migration. Predominantly the loss of E-cadherin and the gain of N-cadherin, promote cellular motility [243, 244]. E-cadherin expression level is controlled by E-box elements in the promoter region and numerous Zn-finger transcription factors, including Snail [245, 246] and Slug [247] are able to directly bind to these elements. Removal of Snail and Slug by gene silencing resulted the increase of E-cadherin. Also Snail and Slug activate the expression of N-cadherin and vimentin by an indirect mechanism still not clearly understood [248]. The decrease of N-cadherin in the majority of Snail and Slug silenced treatment groups has proved that, the decrease in migration ability may be due to the direct and indirect link between N-cadherin and Zn-finger transcription factors. Additionally the decreased expression of other Zn-finger transcription factors, Zeb-1 and Zeb-2 (data not shown) may also be linked to the decrease in migration. Like Snail and Slug, Twist is also a regulator of cadherins. It is found that the downregulation of Twist suppresses N-cadherin accompanied with an increase in E-cadherin in various carcinomas [211, 249]. The outcomes of EMT silencing such as the reduction of vimentin and c-met in our study may also contribute the decreased migratory ability. The decrease in migration with Snail, Slug or Twist silencing, together with the decreased invasiveness, prove that it is possible to overwhelm the highly metastatic character of PDACs.

It is known that, cellular attachment and movement ability is highly connected to proliferation [250]. Thus EMT also controls the proliferation of cancer cells. To evaluate this hypothesis, we have made proliferation assay. We have shown that, in all gene edited cells (except Panc-1 shSlug and shControl group) the proliferation rate was significantly decreased. The result of this decrease might be due to the tightened cell-cell attachment and decreased cancer stemness. Proliferation pathways and cell cycle controllers were not evaluated in detail. EMT's effect on proliferation may be worked extensively in further researches.

Due to the decrease in expression of c-met and CD44 we decided to control the levels of PC specific cancer stem like cell markers including, CD24 [28], CD133 [28], EpCAM [251], CXCR4 [251]. According to literature, CD44 is known to contribute cancer stemness [252]

and together with CD133, higher levels of these proteins is associated with tumor sphere formation [253]. A cancer stem cell does not always have to be CD133+ to be called as a cancer stem cell [254] but CD133 is counted as one of the most important surface marker for cancer stem cells. Also the contributions of CD24 and CXCR4 to pancreas cancer stemness are in remarkable amounts [255-257]. Additionally we wanted to investigate the expression levels of embryonic stem cell markers that are known to contribute cancer stemness and pancreatic carcinogenesis including Oct4 [258], Nanog [258, 259], Sox2 [260], and cMYC[251]. We have found that the expression levels of, the cancer stem cell markers, CD24, CD44, CD133, CXCR4 was significantly decreased with gene therapy. Moreover we have observed that the expression values of embryonic stem cell markers have also decreased.

Cancer stem cells are responsible for self-renewal and the maintenance of tumor growth and migration [261] and pluripotent stem cell markers are found to have important roles in carcinogenesis and prognosis of pancreas cancer [259]. Alike in previous studies [262], EMT is known to contribute cancer stemness [260]. To investigate the effect of the decrease in cancer/embryonic stem cell associated genes on tumorigenesis, tumor sphere formation assay was done. The formation of tumor spheres, self-renewal capacity while still being in undifferentiated state, is a cancer stem cell hallmark [261]. Snail, Slug and Twist silenced PC cells has shown reduced potential to create tumor spheres. Along being less numerically, in EMT silenced groups tumor sphere sizes were smaller.

The decrease in number of tumor spheres with the downregulation of EMT transcription factors was expected. Snail downregulation is previously associated with decreased number of spheres in Panc-1 cells together with the decreased expression of Oct4 [263]. Slug was associated with tumor sphere formation and with metastasis in human lung carcinoma [264] and Twist is previously found to decrease the size and density of tumorspheres in T47D and MCF7 breast cancer cell lines [242]. The decrease in expression levels of cancer/embryonic stemness related genes additionally contributed the fewer numbers of tumor spheres and sphere sizes. The experiments above is not enough to tell that Snail, Slug and Twist decrease cancer stemness but it was found that, this treatment has potential to decrease tumorigenesis and agressiveness of pancreas cancer.

Interestingly, in a cell nearly every pathway is connected to each other. It is known that some embryonic stem cell markers influence invasion and migration. Sox2 is a perfect example

for this fact. It is found that Sox2 overexpression promotes migration and invasion by controlling EMT via Wnt/β-catenin pathway [265] and targets fibronectin to increase migration and invasion [266], controls Src kinase and enhance migration [267] in many different cancer types. It is also known that it is possible to control EMT, MET and even metastasis with genetic and epigenic modifications on Sox2 [268, 269]. Like Sox2 other embryonic stem cell markers also have influence in metastasis. It was found that Oct4 and Nanog is related to the relapse and metastasis of breast cancer [270]. These studies are proof that, EMT system controls even the pluripotency markers and these markers can also contribute the cancer's aggressiveness. In our study, it was found that, expression of embryonic stem cell markers were also decreased. This decrease may also contribute the decreased metastatic potential in our treatment groups.

In several studies CD133+/CXCR4+ cells are found to have shown more invasive abilities in esophageal squamous cell carcinoma [271] and pancreatic ductal tumors [272]. In our study chosen PC cell lines were found to be highly CD133 positive and all of the cells were positive for CXCR4. While the decrease in these surface markers are probably the cause of decreased tumor sphere numbers, this can also contribute the decreased invasiveness. In further studies, double positive cells can be investigated extensively to see the further effects of EMT silencing on these cells.

EMT is known to highly contribute drug resistance [36, 205]. To this respect, we have decided to investigate this how EMT contributed the drug resistance of AsPc-1. Up to date, this cell line was never investigated in detail about EMT's effects and it was the best possible target we had for EMT silencing because of its metastatic characteristic. The experiments for invasion, migration, laminin attachment has shown the best result in this cell line and it was used in the further experiments.

To investigate how Snail, Slug and Twist downregulation effects the drug resistance, we have treated AsPc-1 cells with three different chemotherapeutics. The chosen chemotherapeutics for this purpose are 5-Fluoruracil, Docetaxel and Mitomycin C, all being FDA approved drugs for treatment of pancreas cancer. All of the treatment groups have shown less resistance to these drugs, compared to their control groups. In order to understand how drug resistance and survival genes response to chemotherapeutics, the expression levels of ABCG2, MUC1 and Survivin was investigated after treatment.

ABCG2 is a member of ABC transporter super family and it is selected for being a multidrug resistance gene and it is famous for its interesting role in protecting cancer stem cells [273].
MUC-1

Muc-1 is a membrane bound surface glycoprotein and is selected because it is known to contribute chemoresistance and increase metastasis in pancreas cancer [274]. Survivin on the other hand is defined as an apoptosis inhibitor. It is found to inhibit caspases that are activated during intrinsic and extrinsic apoptotic pathways[275].

In normal conditions ABCG2 is expressed highly in AsPc-1 cell line [276] and even in ATCC, AsPc-1 is characterized by being MUC1+. Survivin is also positive in pancreatic adenocarcinoma cell lines and used as a prognostic marker in pancreas cancer patients [277].

To start with we have compared the expression levels of these three genes before drug treatment, we have seen that all three were decreased significantly compared to the control group. The connection between the levels of EMT transcription factors were previously studied in several studies. Snail, Slug and Twist silencing was found to actively control the expression levels of Survivin due to the connection of EMT to HIF-1α pathway [278-280]. MUC-1 was found to be controlled and control EMT even it is found to contribute the metastatic character and poor prognosis in pancreas, gall bladder and colon cancer [274]. Additionally ABCG2 gene expression is known to be connected to EMT inducer genes, especially Snail, Twist and Zeb-1 [281].

Afterwards, we have treated AsPc-1 cells with the selected drugs; 5-Fluorouracil, a DNA synthesis blocker [282], Docetaxel, a blocker of bcl-2 thus apoptosis initiator [283], Mitomycin C, an alkylating agent and DNA synthesis blocker[284]. After treatment we have again investigated the increase of the three selected genes, which must normally occur when a resistant cancer cell is treated with a chemotherapeutic. In our 5-fluorouracil and Mitomycin C treated shSnail, shSlug and shTwist cells, we have observed that expression of Survivin, Muc1 and ABCG2 genes were only increased slightly compared to the shControl group. This effect was parallel to viability assay since cells were found to be less resistant to 5-fluorouracil and Mitomycin C. Cells treated with Docetaxel on the other hand has shown similar increase in gene expression of Survivin, Muc-1 and ABCG2 with their control group. It was also expected since this drug is a bcl-2 blocker and it is generally recommended to use this drug in combination with other chemotherapeutics such as Cisplatin [285],

Gemcitabine [286] or 5-Fluorouracil [287]. Even though the drug resistance and survival genes were increased as much as the control group, it was found that, cellular viability was decreased around 50 per cent. Additional pathways may have contributed the decreased resistance against docetaxel. With respect to the study above we can state that, downregulation of Snail, Slug and Twist significantly decrease drug resistance against various pancreas cancer chemotherapeutics.

When gene therapy is not enough or when an alternative for gene therapy is sought, it is possible to use combination therapy. In this part of our study, we have investigated the effects of two small molecules, SD208; a TGFβR1 blocker [179] and CX4945; a Casein Kinase-2 inhibitor [186] on EMT associated gene expressions. As previously mentioned, TGFβ pathway is the major controller of EMT [288] and CK2 is found to control TGFβ1 induced cadherin switch [289] and these two chemicals are found to actively control EMT by inhibiting TGFβ pathway.

First we have investigated the gene expression levels of EMT related genes (Snail, Slug, Twist, E-cadherin, N-cadherin and Vimentin), PC stem cell specific markers (CD133, CXCR4, CD24, CD44 and EpCAM), pluripotency markers (Nanog, Klf4 and c-MYC), metastasis related markers (MMP1, MMP9, c-Met, MTA1 and MTA2) in AsPc-1 shSnail, shSlug, shTwist and shControl cells after SD208, CX4945, SD208+CX4945 treatment. The aim of this experiment was to evaluate how these chemicals act alone or in combination and to see if the shControl group's expression values will be as low as the shSnail, shSlug and shTwist group. It turned out that these two chemicals successfully decreased all the mentioned gene levels (except E-cadherin, level of E-cadherin was increased as expected), but not as much as the groups with gene therapy. It was found that, combination therapy (gene therapy plus small molecule inhibitor treatment) was superior compared to gene therapy alone or small molecule inhibitor therapy alone. Additionally, we observed that, combination of these two small molecule inhibitors did not show better gene expression values when the results were compared to SD208 alone or CX4945 alone. As a result of the experiments above from three treatment groups, being shSnail, shSlug and shTwist, we have decided to work with shSnail and shSlug groups due to the better changes in gene expression values and we have also decided not to use SD208 and CX-4945 in combination.

We have investigated how the treatment with SD208 and CX4945 effects migration ability. It was found that, small molecule inhibitors have a positive effect for blocking migration. It

was also recognized that when shSnail and shSlug cells were treated with small molecules, the amount of migrated cells were less compared to shControl treated with SD208 or CX-4945. Additionally it was seen that, CX-4945 has a greater potential for overcoming migration compared to SD-208.

Afterwards we have made invasion assay to observe the changes of invasiveness of AsPc-1 cells. Compared to shControl SD208 and CX4945 treated group, the invaded cells were significantly lower in shSnail and shSlug (SD208 and CX4945) cells. It was found that, small molecule inhibitors addition to short hairpin silencing can be used to overcome invasiveness of metastatic pancreas cancer cell line AsPc-1. After invasion assay, it is possible to say that CX-4945 has more potential for overcoming invasiveness.

Even though the gene expressional changes after SD-208 and CX-4945 treatments were close to each other, we can say that for treating metastasis, it is more suitable to use CX-4945 rather than SD-208. It is recognized also that, gene therapy cannot be replaced by using small molecule inhibitors of EMT.

We have also recognized that, the invasiveness of AsPc-1 cells (untreated) were slightly increased compared to earlier passages of the cells. The reason of the decrease may be because the used AsPc-1 cells were aliquots of the original ones. They were thawed once and it was observed by our group and by other researchers around the world that freeze-thaw cycles highly decrease the effect of short hairpin silencing. The small molecule inhibition may be used to overcome this situation. Another reason can be the serum starvation prior to the invasion assay. The rush though the serum may be the reason of increased invasion amounts.

5. CONCLUSIONS

In this study, the effect on epithelial to mesenchymal treatment on several pancreas cancer cell lines was investigated. EMT mechanism is known to contribute many different events in a cell's life. It coordinates migration, invasion, proliferation, drug resistance, it supports a cancer cell when it decides to be aggressive. It is not possible to understand the EMT mechanism completely in a study that is only done in couple of years but with this research, we have shed light on its abilities on pancreas cancer.

We have found that, when EMT related transcription factors have downregulated; the proliferation rate was decreased, cells became less motile, less invasive then before. The laminin attachment ability of the cells was decreased.

The gene treatment has also decreased the cancer stem cell properties and decreased cells' ability on tumorigenesis.

We have also investigated how EMT silencing changes drug resistance on one of the cell lines. It was found that, as expected, the drug resistance has also decreased due to the inability of drug efflux and inhibition of apoptotic blockers.

The results above are the prove that, EMT is probably the best possible route for overcoming this highly metastatic, aggressive, drug resistance cancer type.

Additionally, we have selected two small molecule inhibitors in order to use them in combination with gene therapy. We have observed that it is possible to use small molecule inhibitors to enhance gene therapy towards EMT.

One day it may be possible to use multiple small molecules, targeting different pathways that act on cancer progression and put an end to the sufferings of cancer patients. We believe that our study has brought this dream one step closer to the reality.

For future studies, it is possible to study the potential pathways that connect EMT to cellular proliferation and how it controls the cell cycle checkpoints. Cancer stem cells can also be selected for being double positive for CD133 and CXCR4 to understand the effects of EMT silencing on these cells. Cells treated with combination therapy can be used for extensive genomic research. Arrays for expression and protein levels can be investigated in detail.

Additionally it is important to observe this study in *in vivo* conditions. The most accurate results can be taken by using the same strategy in animal studies.

REFERENCES

1. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. 2000;100(1):57-70.,

2. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144(5):646-74.

3. Gallucci BB. Selected concepts of cancer as a disease: from the Greeks to 1900. *Oncology nursing forum*. 1985;12(4):67-71.

4. Bjerkvig R, Tysnes BB, Aboody KS, Najbauer J, Terzis AJ. Opinion: the origin of the cancer stem cell: current controversies and new insights. *Nature reviews Cancer.* 2005;5(11):899-904.

5. Siddiqui IA, Sanna V, Ahmad N, Sechi M, Mukhtar H. Resveratrol nanoformulation for cancer prevention and therapy. *Annals of the New York Academy of Sciences.* 2015;1348(1):20-31.

6. Rodriguez-Brenes IA, Wodarz D, Komarova NL. Minimizing the risk of cancer: tissue architecture and cellular replication limits. *Journal of the Royal Society, Interface / the Royal Society*. 2013;10(86):20130410.

7. Ip C, Ganther HE. Combination of blocking agents and suppressing agents in cancer prevention. *Carcinogenesis.* 1991;12(2):365-7.

8. Stoner GD, Morse MA, Kelloff GJ. Perspectives in cancer chemoprevention. *Environmental health perspectives.* 1997;105 Suppl 4:945-54.

9. Troll W, Wiesner R. The role of oxygen radicals as a possible mechanism of tumor promotion. *Annual review of pharmacology and toxicology*. 1985;25:509-28.

10. Pitot HC, Goldsworthy T, Moran S. The natural history of carcinogenesis: implications of experimental carcinogenesis in the genesis of human cancer. *Journal of supramolecular structure and cellular biochemistry*. 1981;17(2):133-46.

11. Lodish H BA, Zipursky SL. Proto-Oncogenes and Tumor-Suppressor Genes. In: Freeman WH, editor. Molecular Cell Biology. New York: W. H. Freeman and Company.; 2000.

12. Burck KB, Liu ET, Larrick JW. Proto-Oncogenes. *Oncogenes.* New York: Springer; 1988. p. 78-94.

13. Heisterkamp N, Stam K, Groffen J, de Klein A, Grosveld G. Structural organization of the bcr gene and its role in the Ph' translocation. *Nature.* 1985;315(6022):758-61.

14. Goodrich DW. The retinoblastoma tumor-suppressor gene, the exception that proves the rule. *Oncogene.* 2006;25(38):5233-43.

15. Nielsen LL, Maneval DC. P53 tumor suppressor gene therapy for cancer. *Cancer gene therapy*. 1998;5(1):52-63.

16. Cooper GM. Tumor Suppressor Genes. In: Hausman RE, editor. T*he Cell: A Molecular Approach*. 7th edition. USA: Sinauer Associates; 2000. p. 741-8.

17. Dell' Antone P. Energy metabolism in cancer cells: how to explain the Warburg and Crabtree effects? *Medical hypotheses.* 2012;79(3):388-92.

18. Warburg O. On respiratory impairment in cancer cells. *Science*. 1956;124(3215):269-70.

19. Lunt SY, Vander Heiden MG. Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. *Annual review of cell and developmental biology*. 2011;27:441-64.

20. Agani F, Jiang BH. Oxygen-independent regulation of HIF-1: novel involvement of PI3K/AKT/mTOR pathway in cancer. *Current cancer drug targets*. 2013;13(3):245-51.

21. DeBerardinis RJ, Lum JJ, Hatzivassiliou G, Thompson CB. The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. *Cell metabolism.* 2008;7(1):11-20.

22. Maschek G, Savaraj N, Priebe W, et al. 2-deoxy-D-glucose increases the efficacy of adriamycin and paclitaxel in human osteosarcoma and non-small cell lung cancers in vivo. *Cancer research*. 2004;64(1):31-4.

23. Lu W, Hu Y, Chen G, et al. Novel role of NOX in supporting aerobic glycolysis in cancer cells with mitochondrial dysfunction and as a potential target for cancer therapy. *PLoS biology*. 2012;10(5):e1001326.

24. Sun Q, Chen X, Ma J, et al. Mammalian target of rapamycin up-regulation of pyruvate kinase isoenzyme type M2 is critical for aerobic glycolysis and tumor growth. *Proceedings of the National Academy of Sciences of the United States of America*. 2011;108(10):4129-34.

25. Zheng S, Xin L, Liang A, Fu Y. Cancer stem cell hypothesis: a brief summary and two proposals. *Cytotechnology*. 2013;65(4):505-12.

26. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature medicine*. 1997;3(7):730-7.

27. Dean M. ABC transporters, drug resistance, and cancer stem cells. *Journal of mammary gland biology and neoplasia*. 2009;14(1):3-9.

28. Li C, Heidt DG, Dalerba P, et al. Identification of pancreatic cancer stem cells. *Cancer research*. 2007;67(3):1030-7.

29. Li C, Wu JJ, Hynes M, et al. c-Met is a marker of pancreatic cancer stem cells and therapeutic target. *Gastroenterology*. 2011;141(6):2218-27 e5.

30. Went PT, Lugli A, Meier S, et al. Frequent EpCam protein expression in human carcinomas. *Human pathology*. 2004;35(1):122-8.

31. Bailey JM, Alsina J, Rasheed ZA, et al. DCLK1 marks a morphologically distinct subpopulation of cells with stem cell properties in preinvasive pancreatic cancer. *Gastroenterology*. 2014;146(1):245-56.

32. Su HT, Weng CC, Hsiao PJ, et al. Stem cell marker nestin is critical for TGF-beta1-mediated tumor progression in pancreatic cancer. *Molecular cancer research : MCR.* 2013;11(7):768-79.

33. van den Hoogen C, van der Horst G, Cheung H, et al. High aldehyde dehydrogenase activity identifies tumor-initiating and metastasis-initiating cells in human prostate cancer. *Cancer research.* 2010;70(12):5163-73.

34. Duong HQ, Hwang JS, Kim HJ, Kang HJ, Seong YS, Bae I. Aldehyde dehydrogenase 1A1 confers intrinsic and acquired resistance to gemcitabine in human pancreatic adenocarcinoma MIA PaCa-2 cells. *International journal of oncology.* 2012;41(3):855-61.

35. Matsuda Y, Kure S, Ishiwata T. Nestin and other putative cancer stem cell markers in pancreatic cancer. *Medical molecular morphology*. 2012;45(2):59-65.

36. Singh A, Settleman J. EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. *Oncogene.* 2010;29(34):4741-51.

37. van der Horst G, van den Hoogen C, Buijs JT, et al. Targeting of alpha(v)-integrins in stem/progenitor cells and supportive microenvironment impairs bone metastasis in human prostate cancer. *Neoplasia.* 2011;13(6):516-25.

38. van den Brink GR, Bleuming SA, Hardwick JC, et al. Indian Hedgehog is an antagonist of Wnt signaling in colonic epithelial cell differentiation. *Nature genetics.* 2004;36(3):277-82.

39. McCoy EL, Iwanaga R, Jedlicka P, et al. Six1 expands the mouse mammary epithelial stem/progenitor cell pool and induces mammary tumors that undergo epithelial-mesenchymal transition. *The Journal of clinical investigation*. 2009;119(9):2663-77.

40. DiMeo TA, Anderson K, Phadke P, et al. A novel lung metastasis signature links Wnt signaling with cancer cell self-renewal and epithelial-mesenchymal transition in basal-like breast cancer. *Cancer research*. 2009;69(13):5364-73.

41. Feldmann G, Fendrich V, McGovern K, et al. An orally bioavailable small-molecule inhibitor of Hedgehog signaling inhibits tumor initiation and metastasis in pancreatic cancer. *Molecular cancer therapeutics.* 2008;7(9):2725-35.

42. Feldmann G, Dhara S, Fendrich V, et al. Blockade of hedgehog signaling inhibits pancreatic cancer invasion and metastases: a new paradigm for combination therapy in solid cancers. *Cancer research.* 2007;67(5):2187-96.

43. Busnardo AC, Didio LJ, Tidrick RT, Thomford NR. History of the pancreas. *American journal of surgery.* 1983;146(5):539-50.

44. Agur AMR, Lee MJ, Grant JCB. Grant's atlas of anatomy. Philadelphia: Lippincott Williams & Wilkins.; 1999.

45. Pandol SJ. The Exocrine Pancreas. Colloquium Series on Integrated Systems Physiology: *From Molecule to Function to Disease.* San Rafael (CA)2010.

46. Young B, Wheath JW. Wheater's Functional Histology: Churchill Livingstone Press; 2002.

47. Boyle P, Hsieh CC, Maisonneuve P, et al. Epidemiology of pancreas cancer (1988). *International journal of pancreatology : official journal of the International Association of Pancreatology.* 1989;5(4):327-46.

48. Are C, Dhir M, Ravipati L. History of pancreaticoduodenectomy: early misconceptions, initial milestones and the pioneers. *HPB : the official journal of the International Hepato Pancreato Biliary Association*. 2011;13(6):377-84.

49. Adsay NV, Thirabanjasak D, Altinel D. Molecular Pathology and Epidemiology. In: Andrew M. Lowy SDL, Philip A., editor. P*ancreatic Cancer*. USA: Springer US; 2008.

50. Hezel AF, Kimmelman AC, Stanger BZ, Bardeesy N, Depinho RA. Genetics and biology of pancreatic ductal adenocarcinoma. *Genes & development.* 2006;20(10):1218-49.

51. Ito T, Igarashi H, Jensen RT. Pancreatic neuroendocrine tumors: clinical features, diagnosis and medical treatment: advances. *Best practice & research Clinical gastroenterology*. 2012;26(6):737-53.

52. Wolfgang CL, Herman JM, Laheru DA, et al. *Recent progress in pancreatic cancer. CA: a cancer journal for clinicians*. 2013;63(5):318-48.

53. Borad MJ, Saadati H, Lakshmipathy A, et al. Skeletal metastases in pancreatic cancer: a retrospective study and review of the literature. *The Yale journal of biology and medicine.* 2009;82(1):1-6.

54. Lemke J, Scheele J, Kapapa T, Wirtz CR, Henne-Bruns D, Kornmann M. Brain metastasis in pancreatic cancer. *International journal of molecular sciences.* 2013;14(2):4163-73.

55. Hidalgo M. Pancreatic cancer. *The New England journal of medicine.* 2010;362(17):1605-17.

56. Cascinu S, Falconi M, Valentini V, Jelic S, Group EGW. Pancreatic cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO.* 2010;21 Suppl 5:v55-8.

57. Pleskow DK, Berger HJ, Gyves J, Allen E, McLean A, Podolsky DK. Evaluation of a serologic marker, CA19-9, in the diagnosis of pancreatic cancer. *Annals of internal medicine.* 1989;110(9):704-9.

58. Strobel O, Berens V, Hinz U, et al. Resection after neoadjuvant therapy for locally advanced, "unresectable" pancreatic cancer. *Surgery*. 2012;152(3 Suppl 1):S33-42.

59. De La Cruz MS, Young AP, Ruffin MT. Diagnosis and management of pancreatic cancer. *American family physician.* 2014;89(8):626-32.

60. Oettle H, Post S, Neuhaus P, et al. Adjuvant chemotherapy with gemcitabine vs observation in patients undergoing curative-intent resection of pancreatic cancer: a randomized controlled trial. *Jama*. 2007;297(3):267-77.

61. Regine WF, Winter KA, Abrams RA, et al. Fluorouracil vs gemcitabine chemotherapy before and after fluorouracil-based chemoradiation following resection of pancreatic adenocarcinoma: a randomized controlled trial. *Jama*. 2008;299(9):1019-26.

62. Delbaldo C, Faivre S, Dreyer C, Raymond E. Sunitinib in advanced pancreatic neuroendocrine tumors: latest evidence and clinical potential. *Therapeutic advances in medical oncolog*y. 2012;4(1):9-18.

63. Yao JC, Lombard-Bohas C, Baudin E, et al. Daily oral everolimus activity in patients with metastatic pancreatic neuroendocrine tumors after failure of cytotoxic chemotherapy: a phase II trial. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology.* 2010;28(1):69-76.

64. Weigelt B, Peterse JL, van 't Veer LJ. Breast cancer metastasis: markers and models. *Nature reviews Cancer.* 2005;5(8):591-602.

65. Fidler IJ. Cancer biology is the foundation for therapy. *Cancer biology & therapy.* 2005;4(9):1036-9.

66. Spano D, Heck C, De Antonellis P, Christofori G, Zollo M. Molecular networks that regulate cancer metastasis. *Seminars in cancer biology.* 2012;22(3):234-49.

67. Mack GS, Marshall A. Lost in migration. Nature biotechnology. 2010;28(3):214-29.

68. Klein CA. Parallel progression of primary tumours and metastases. *Nature reviews Cancer*. 2009;9(4):302-12.

69. Werb Z. ECM and cell surface proteolysis: regulating cellular ecology. *Cell.* 1997;91(4):439-42.

70. Shapiro SD, Senior RM. Matrix metalloproteinases. Matrix degradation and more. *American journal of respiratory cell and molecular biology*. 1999;20(6):1100-2.

71. Ganguly KK, Pal S, Moulik S, Chatterjee A. Integrins and metastasis. *Cell adhesion & migration.* 2013;7(3):251-61.

72. Berrier AL, Yamada KM. Cell-matrix adhesion. *Journal of cellular physiology.* 2007;213(3):565-73.

73. Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. *Cell*. 2009;139(5):871-90.

74. Yilmaz M, Christofori G. Mechanisms of motility in metastasizing cells. *Molecular cancer research : MCR*. 2010;8(5):629-42.

75. Christofori G. New signals from the invasive front. Nature. 2006;441(7092):444-50.

76. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. *Nature reviews Cancer*. 2002;2(6):442-54.

77. Huber MA, Kraut N, Beug H. Molecular requirements for epithelial-mesenchymal transition during tumor progression. *Current opinion in cell biology.* 2005;17(5):548-58.

78. van Roy F, Berx G. The cell-cell adhesion molecule E-cadherin. C*ellular and molecular life sciences : CMLS*. 2008;65(23):3756-88.

79. Gumbiner BM. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell.* 1996;84(3):345-57.

80. Barth AI, Nathke IS, Nelson WJ. Cadherins, catenins and APC protein: interplay between cytoskeletal complexes and signaling pathways. *Current opinion in cell biology*. 1997;9(5):683-90.

81. Christofori G, Semb H. The role of the cell-adhesion molecule E-cadherin as a tumour-suppressor gene. *Trends in biochemical sciences*. 1999;24(2):73-6.

82. van Zijl F, Krupitza G, Mikulits W. Initial steps of metastasis: cell invasion and endothelial transmigration. *Mutation research.* 2011;728(1-2):23-34.

83. Friedl P, Wolf K. Plasticity of cell migration: a multiscale tuning model. *The Journal of cell biology*. 2010;188(1):11-9.

84. Hegerfeldt Y, Tusch M, Brocker EB, Friedl P. Collective cell movement in primary melanoma explants: plasticity of cell-cell interaction, beta1-integrin function, and migration strategies. *Cancer research.* 2002;62(7):2125-30.

85. Vincent-Salomon A, Thiery JP. Host microenvironment in breast cancer development: epithelial-mesenchymal transition in breast cancer development. *Breast cancer research : BCR.* 2003;5(2):101-6.

86. Carragher NO, Walker SM, Scott Carragher LA, et al. Calpain 2 and Src dependence distinguishes mesenchymal and amoeboid modes of tumour cell invasion: a link to integrin function. *Oncogene*. 2006;25(42):5726-40.

87. Parri M, Taddei ML, Bianchini F, Calorini L, Chiarugi P. EphA2 reexpression prompts invasion of melanoma cells shifting from mesenchymal to amoeboid-like motility style. *Cancer research.* 2009;69(5):2072-81.

88. Gadea G, de Toledo M, Anguille C, Roux P. Loss of p53 promotes RhoA-ROCK-dependent cell migration and invasion in 3D matrices. *The Journal of cell biology*. 2007;178(1):23-30.

89. Friedl P, Wolf K. Tumour-cell invasion and migration: diversity and escape mechanisms. *Nature reviews Cancer.* 2003;3(5):362-74.

90. Pankova K, Rosel D, Novotny M, Brabek J. The molecular mechanisms of transition between mesenchymal and amoeboid invasiveness in tumor cells. *Cellular and molecular life sciences : CMLS*. 2010;67(1):63-71.

91. Sanz-Moreno V, Marshall CJ. Rho-GTPase signaling drives melanoma cell plasticity. *Cell cycle.* 2009;8(10):1484-7.

92. Berton S, Belletti B, Wolf K, et al. The tumor suppressor functions of p27(kip1) include control of the mesenchymal/amoeboid transition. *Molecular and cellular biology*. 2009;29(18):5031-45.

93. Ramis-Conde I, Chaplain MA, Anderson AR, Drasdo D. Multi-scale modelling of cancer cell intravasation: the role of cadherins in metastasis. *Physical biology.* 2009;6(1):016008.

94. Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. *Nature reviews Cancer*. 2009;9(4):239-52.

95. Kim J, Yu W, Kovalski K, Ossowski L. Requirement for specific proteases in cancer cell intravasation as revealed by a novel semiquantitative PCR-based assay. *Cell*. 1998;94(3):353-62.

96. Kessenbrock K, Plaks V, Werb Z. Matrix metalloproteinases: regulators of the tumor microenvironment. *Cell.* 2010;141(1):52-67.

97. Wyckoff JB, Wang Y, Lin EY, et al. Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. *Cancer research*. 2007;67(6):2649-56.

98. Giampieri S, Manning C, Hooper S, Jones L, Hill CS, Sahai E. Localized and reversible TGFbeta signalling switches breast cancer cells from cohesive to single cell motility. *Nature cell biology*. 2009;11(11):1287-96.

99. Xue C, Wyckoff J, Liang F, et al. Epidermal growth factor receptor overexpression results in increased tumor cell motility in vivo coordinately with enhanced intravasation and metastasis. *Cancer research*. 2006;66(1):192-7.

100. Reddy BY, Lim PK, Silverio K, Patel SA, Won BW, Rameshwar P. The Microenvironmental Effect in the Progression, Metastasis, and Dormancy of Breast Cancer: A Model System within Bone Marrow. *International journal of breast cancer.* 2012;2012:721659.

101. Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. *Nature reviews Cancer.* 2002;2(3):161-74.

102. Lynch CC, Matrisian LM. Matrix metalloproteinases in tumor-host cell communication. *Differentiation; research in biological diversity*. 2002;70(9-10):561-73.

103. Mohamed MM, Sloane BF. Cysteine cathepsins: multifunctional enzymes in cancer. *Nature reviews Cancer.* 2006;6(10):764-75.

104. Gocheva V, Joyce JA. Cysteine cathepsins and the cutting edge of cancer invasion. *Cell cycle.* 2007;6(1):60-4.

105. Nagy JA, Benjamin L, Zeng H, Dvorak AM, Dvorak HF. Vascular permeability, vascular hyperpermeability and angiogenesis. *Angiogenesis.* 2008;11(2):109-19.

106. Conn EM, Botkjaer KA, Kupriyanova TA, Andreasen PA, Deryugina EI, Quigley JP. Comparative analysis of metastasis variants derived from human prostate carcinoma cells: roles in intravasation of VEGF-mediated angiogenesis and uPA-mediated invasion. *The American journal of pathology*. 2009;175(4):1638-52.

107. Presta M, Dell'Era P, Mitola S, Moroni E, Ronca R, Rusnati M. Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. *Cytokine & growth factor reviews*. 2005;16(2):159-78.

108. Zhang L, Kharbanda S, McLeskey SW, Kern FG. Overexpression of fibroblast growth factor 1 in MCF-7 breast cancer cells facilitates tumor cell dissemination but does not support the development of macrometastases in the lungs or lymph nodes. *Cancer research.* 1999;59(19):5023-9.

109. Montesano R, Vassalli JD, Baird A, Guillemin R, Orci L. Basic fibroblast growth factor induces angiogenesis in vitro. *Proceedings of the National Academy of Sciences of the United States of America*. 1986;83(19):7297-301.

110. Mitchell MJ, King MR. Fluid shear stress sensitizes cancer cells to receptor-mediated apoptosis via trimeric death receptors. *New journal of physics.* 2013;15(1):015008.

111. Trousseau A. Clinique Medicale de l'Hotel-Dieu de Paris 1896;3(2):654–712.

112. Khorana AA. Malignancy, thrombosis and Trousseau: the case for an eponym. *Journal of thrombosis and haemostasis : JTH.* 2003;1(12):2463-5.

113. Sierko E, Wojtukiewicz MZ. Inhibition of platelet function: does it offer a chance of better cancer progression control? *Seminars in thrombosis and hemostasis.* 2007;33(7):712-21.

114. Prandoni P, Falanga A, Piccioli A. Cancer and venous thromboembolism. *The Lancet* Oncology. 2005;6(6):401-10.

115. Ruggeri ZM, Mendolicchio GL. Adhesion mechanisms in platelet function. *Circulation research*. 2007;100(12):1673-85.

116. Davi G, Patrono C. Platelet activation and atherothrombosis. T*he New England journal of medicine.* 2007;357(24):2482-94.

117. Palumbo JS, Talmage KE, Massari JV, et al. Platelets and fibrin(ogen) increase metastatic potential by impeding natural killer cell-mediated elimination of tumor cells. Blood. 2005;105(1):178-85.

118. Erpenbeck L, Schon MP. Deadly allies: the fatal interplay between platelets and metastasizing cancer cells. *Blood*. 2010;115(17):3427-36.

119. Gay LJ, Felding-Habermann B. Contribution of platelets to tumour metastasis. *Nature reviews Cancer*. 2011;11(2):123-34.

120. Degen JL, Palumbo JS. Hemostatic factors, innate immunity and malignancy. *Thrombosis research.* 2012;129 Suppl 1:S1-5.

121. Jurasz P, Alonso-Escolano D, Radomski MW. Platelet–cancer interactions: mechanisms and pharmacology of tumour cell-induced platelet aggregation. *British journal of pharmacology*. 2004;143(7):819-26.

122. Wood S, Jr. Pathogenesis of metastasis formation observed in vivo in the rabbit ear chamber. *AMA archives of pathology*. 1958;66(4):550-68.

123. Jones DS, Wallace AC, Fraser EE. Sequence of events in experimental metastases of Walker 256 tumor: light, immunofluorescent, and electron microscopic observations. *Journal of the National Cancer Institute*. 1971;46(3):493-504.

124. Machado EA, Gerard DA, Mitchell JR, Lozzio BB, Lozzio CB. Arrest and extravasation of neoplastic cells. An electron microscopy study of serial sections at sequential stages. *Virchows Archiv A, Pathological anatomy and histology.* 1982;396(1):73-89.

125. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. *Cell.* 2010;141(1):39-51.

126. Qian B, Deng Y, Im JH, et al. A distinct macrophage population mediates metastatic breast cancer cell extravasation, establishment and growth. *PloS one.* 2009;4(8):e6562.

127. Karachaliou N, Pilotto S, Bria E, Rosell R. Platelets and their role in cancer evolution and immune system. *Translational lung cancer research*. 2015;4(6):713-20.

128. Schaphorst KL, Chiang E, Jacobs KN, et al. Role of sphingosine-1 phosphate in the enhancement of endothelial barrier integrity by platelet-released products. American journal of physiology *Lung cellular and molecular physiology*. 2003;285(1):L258-67.

129. Yin F, Watsky MA. LPA and S1P increase corneal epithelial and endothelial cell transcellular resistance. *Investigative ophthalmology & visual science.* 2005;46(6):1927-33.

130. Leong HS, Robertson AE, Stoletov K, et al. Invadopodia are required for cancer cell extravasation and are a therapeutic target for metastasis. *Cell reports.* 2014;8(5):1558-70.

131. Kanada M, Zhang J, Yan L, Sakurai T, Terakawa S. Endothelial cell-initiated extravasation of cancer cells visualized in zebrafish. *PeerJ.* 2014;2:e688.

132. Weis S, Cui J, Barnes L, Cheresh D. Endothelial barrier disruption by VEGF-mediated Src activity potentiates tumor cell extravasation and metastasis. *The Journal of cell biology*. 2004;167(2):223-9.

133. Ribatti D, Mangialardi G, Vacca A. Stephen Paget and the 'seed and soil' theory of metastatic dissemination. *Clinical and experimental medicine*. 2006;6(4):145-9.

134. Proctor JW, Auclair BG, Rudenstam CM. The distribution and fate of blood-borne 125IUdR-labelled tumour cells in immune syngeneic rats. *International journal of cancer*. 1976;18(2):255-62.

135. Kalluri R, Neilson EG. Epithelial-mesenchymal transition and its implications for fibrosis. *The Journal of clinical investigation*. 2003;112(12):1776-84.

136. Kalluri R. EMT: when epithelial cells decide to become mesenchymal-like cells. The *Journal of clinical investigation*. 2009;119(6):1417.

137. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. *The Journal of clinical investigation*. 2009;119(6):1420.

138. Vicovac L, Aplin JD. Epithelial-mesenchymal transition during trophoblast differentiation. *Acta anatomica*. 1996;156(3):202-16.

139. Aplin JD, Haigh T, Vicovac L, Church HJ, Jones CJ. Anchorage in the developing placenta: an overlooked determinant of pregnancy outcome? *Human fertility*. 1998;1(1):75-9.

140. Acloque H, Adams MS, Fishwick K, Bronner-Fraser M, Nieto MA. Epithelial-mesenchymal transitions: the importance of changing cell state in development and disease. *The Journal of clinical investigation*. 2009;119(6):1438.

141. Hay ED. Role of cell-matrix contacts in cell migration and epithelial-mesenchymal transformation. Cell differentiation and development : the official journal of the *International Society of Developmental Biologists*. 1990;32(3):367-75.

142. Skromne I, Stern CD. Interactions between Wnt and Vg1 signalling pathways initiate primitive streak formation in the chick embryo. *Development*. 2001;128(15):2915-27.

143. Popperl H, Schmidt C, Wilson V, et al. Misexpression of Cwnt8C in the mouse induces an ectopic embryonic axis and causes a truncation of the anterior neuroectoderm. *Development*. 1997;124(15):2997-3005.

144. Skromne I, Stern CD. A hierarchy of gene expression accompanying induction of the primitive streak by Vg1 in the chick embryo. *Mechanisms of development.* 2002;114(1-2):115-8.

145. Barrallo-Gimeno A, Nieto MA. The Snail genes as inducers of cell movement and survival: implications in development and cancer. *Development.* 2005;132(14):3151-61.

146. Ciruna B, Rossant J. FGF signaling regulates mesoderm cell fate specification and morphogenetic movement at the primitive streak. *Developmental cell.* 2001;1(1):37-49.

147. Burns WC, Thomas MC. The molecular mediators of type 2 epithelial to mesenchymal transition (EMT) and their role in renal pathophysiology. *Expert reviews in molecular medicine.* 2010;12:e17.

148. Acloque H, Adams MS, Fishwick K, Bronner-Fraser M, Nieto MA. Epithelial-mesenchymal transitions: the importance of changing cell state in development and disease. *The Journal of clinical investigation*. 2009;119(6):1438-49.

149. Strutz F, Muller GA, Neilson EG. Transdifferentiation: a new angle on renal fibrosis. *Experimental nephrology.* 1996;4(5):267-70.

150. Patterson BK, Tjernlund A, Andersson J. Endogenous inhibitors of HIV: potent anti-HIV activity of leukemia inhibitory factor. *Current molecular medicine.* 2002;2(8):713-22.

151. Yang J, Weinberg RA. Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. *Developmental cell.* 2008;14(6):818-29.

152. Zeisberg M, Shah AA, Kalluri R. Bone morphogenic protein-7 induces mesenchymal to epithelial transition in adult renal fibroblasts and facilitates regeneration of injured kidney. *The Journal of biological chemistry*. 2005;280(9):8094-100.

153. Thiery JP, Sleeman JP. Complex networks orchestrate epithelial-mesenchymal transitions. *Nature reviews Molecular cell biology*. 2006;7(2):131-42.

154. Peinado H, Olmeda D, Cano A. Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nature reviews Cancer*. 2007;7(6):415-28.

155. Heldin CH, Landstrom M, Moustakas A. Mechanism of TGF-beta signaling to growth arrest, apoptosis, and epithelial-mesenchymal transition. *Current opinion in cell biology*. 2009;21(2):166-76.

156. Ellenrieder V, Hendler SF, Boeck W, et al. Transforming growth factor beta1 treatment leads to an epithelial-mesenchymal transdifferentiation of pancreatic cancer cells requiring extracellular signal-regulated kinase 2 activation. *Cancer research.* 2001;61(10):4222-8.

157. Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature. 2003;425(6958):577-84.

158. Maier HJ, Schmidt-Strassburger U, Huber MA, Wiedemann EM, Beug H, Wirth T. NF-kappaB promotes epithelial-mesenchymal transition, migration and invasion of pancreatic carcinoma cells. *Cancer letters.* 2010;295(2):214-28.

159. Jungert K, Buck A, von Wichert G, et al. Sp1 is required for transforming growth factor-beta-induced mesenchymal transition and migration in pancreatic cancer cells. *Cancer research.* 2007;67(4):1563-70.

160. Zeisberg M, Hanai J-i, Sugimoto H, et al. BMP-7 counteracts TGF-β1–induced epithelial-to-mesenchymal transition and reverses chronic renal injury. *Nature medicine.* 2003;9(7):964-8.

161. Yu M-A, Shin K-S, Kim JH, et al. HGF and BMP-7 ameliorate high glucose–induced epithelial-to-mesenchymal transition of peritoneal mesothelium. *Journal of the American Society of Nephrology.* 2009;20(3):567-81.

162. Wu Y-d, Zhou B. TNF-α/NF-κB/Snail pathway in cancer cell migration and invasion. *British journal of cancer.* 2010;102(4):639-44.

163. Janda E, Lehmann K, Killisch I, et al. Ras and TGF[beta] cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways. The *Journal of cell biology*. 2002;156(2):299-313.

164. Burk U, Schubert J, Wellner U, et al. A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. *EMBO reports.* 2008;9(6):582-9.

165. Huber MA, Beug H, Wirth T. Epithelial-mesenchymal transition: NF-kappaB takes center stage. *Cell cycle*. 2004;3(12):1477-80.

166. Hidalgo M, Maitra A. The hedgehog pathway and pancreatic cancer. *The New England journal of medicine*. 2009;361(21):2094-6.

167. Heuberger J, Birchmeier W. Interplay of cadherin-mediated cell adhesion and canonical Wnt signaling. *Cold Spring Harbor perspectives in biology.* 2010;2(2):a002915.

168. Seidel B, Braeg S, Adler G, Wedlich D, Menke A. E- and N-cadherin differ with respect to their associated p120ctn isoforms and their ability to suppress invasive growth in pancreatic cancer cells. *Oncogene.* 2004;23(32):5532-42.

169. Chua K-N, Sim W-J, Racine V, Lee S-Y, Goh BC, Thiery JP. A cell-based small molecule screening method for identifying inhibitors of epithelial-mesenchymal transition in carcinoma. *PloS one*. 2012;7(3):e33183.

170. Huber MA, Kraut N, Beug H. Molecular requirements for epithelial–mesenchymal transition during tumor progression. *Current opinion in cell biology.* 2005;17(5):548-58.

171. Mishra VK, Johnsen SA. Targeted therapy of epigenomic regulatory mechanisms controlling the epithelial to mesenchymal transition during tumor progression. C*ell and tissue research*. 2014;356(3):617-30.

172. Wolffe AP, Matzke MA. Epigenetics: regulation through repression. *Science.* 1999;286(5439):481-6.

173. Tojo M, Hamashima Y, Hanyu A, et al. The ALK-5 inhibitor A-83-01 inhibits Smad signaling and epithelial-to-mesenchymal transition by transforming growth factor-beta. *Cancer science*. 2005;96(11):791-800.

174. de Gouville AC, Boullay V, Krysa G, et al. Inhibition of TGF-beta signaling by an ALK5 inhibitor protects rats from dimethylnitrosamine-induced liver fibrosis. *British journal of pharmacology*. 2005;145(2):166-77.

175. Ehata S, Hanyu A, Fujime M, et al. Ki26894, a novel transforming growth factor-beta type I receptor kinase inhibitor, inhibits in vitro invasion and in vivo bone metastasis of a human breast cancer cell line. *Cancer science*. 2007;98(1):127-33.

176. Kapoun AM, Gaspar NJ, Wang Y, et al. Transforming growth factor-beta receptor type 1 (TGFbetaRI) kinase activity but not p38 activation is required for TGFbetaRI-induced myofibroblast differentiation and profibrotic gene expression. *Molecular pharmacology*. 2006;70(2):518-31.

177. Uhl M, Aulwurm S, Wischhusen J, et al. SD-208, a novel transforming growth factor beta receptor I kinase inhibitor, inhibits growth and invasiveness and enhances immunogenicity of murine and human glioma cells in vitro and in vivo. *Cancer research.* 2004;64(21):7954-61.

178. Leung SY, Niimi A, Noble A, et al. Effect of transforming growth factor-beta receptor I kinase inhibitor 2,4-disubstituted pteridine (SD-208) in chronic allergic airway inflammation and remodeling. *The Journal of pharmacology and experimental therapeutics*. 2006;319(2):586-94.

179. Manaenko A, Lekic T, Barnhart M, Hartman R, Zhang JH. Inhibition of transforming growth factor-beta attenuates brain injury and neurological deficits in a rat model of germinal matrix hemorrhage. *Stroke.* 2014;45(3):828-34.

180. Derynck R, Akhurst RJ, Balmain A. TGF-beta signaling in tumor suppression and cancer progression. *Nature genetics*. 2001;29(2):117-29.

181. Thorne CA, Hanson AJ, Schneider J, et al. Small-molecule inhibition of Wnt signaling through activation of casein kinase 1alpha. *Nature chemical biology*. 2010;6(11):829-36.

182. Cheng X, Merz KH, Vatter S, Christ J, Wolfl S, Eisenbrand G. 7,7'-Diazaindirubin--a small molecule inhibitor of casein kinase 2 in vitro and in cells. *Bioorganic & medicinal chemistry*. 2014;22(1):247-55.

183. Rena G, Bain J, Elliott M, Cohen P. D4476, a cell-permeant inhibitor of CK1, suppresses the site-specific phosphorylation and nuclear exclusion of FOXO1a. *EMBO report*s. 2004;5(1):60-5.

184. Lee JW, Hirota T, Peters EC, et al. A small molecule modulates circadian rhythms through phosphorylation of the period protein. *Angewandte Chemie*. 2011;50(45):10608-11.

185. Maslyk M, Janeczko M, Martyna A, Kubinski K. CX-4945: the protein kinase CK2 inhibitor and anti-cancer drug shows anti-fungal activity. *Molecular and cellular biochemistry*. 2017;435(1-2):193-6.

186. Siddiqui-Jain A, Drygin D, Streiner N, et al. CX-4945, an orally bioavailable selective inhibitor of protein kinase CK2, inhibits prosurvival and angiogenic signaling and exhibits antitumor efficacy. *Cancer research*. 2010;70(24):10288-98.

187. Hermann PC, Huber SL, Herrler T, et al. Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. *Cell stem cell*. 2007;1(3):313-23.

188. Javle MM, Gibbs JF, Iwata KK, et al. Epithelial-mesenchymal transition (EMT) and activated extracellular signal-regulated kinase (p-Erk) in surgically resected pancreatic cancer. *Annals of surgical oncology*. 2007;14(12):3527-33.

189. Onder TT, Gupta PB, Mani SA, Yang J, Lander ES, Weinberg RA. Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. C*ancer research*. 2008;68(10):3645-54.

190. Tomita K, van Bokhoven A, van Leenders GJ, et al. Cadherin switching in human prostate cancer progression. *Cancer research*. 2000;60(13):3650-4.

191. Hotz B, Arndt M, Dullat S, Bhargava S, Buhr HJ, Hotz HG. Epithelial to mesenchymal transition: expression of the regulators snail, slug, and twist in pancreatic cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research.* 2007;13(16):4769-76.

192. Wellner U, Brabletz T, Keck T. ZEB1 in Pancreatic Cancer. *Cancers.* 2010;2(3):1617-28.

193. Maitra A, Hruban RH. Pancreatic cancer. *Annual review of pathology*. 2008;3:157-88.

194. Friess H, Yamanaka Y, Buchler M, et al. Enhanced expression of transforming growth factor beta isoforms in pancreatic cancer correlates with decreased survival. *Gastroenterology*. 1993;105(6):1846-56.

195. McConkey DJ, Choi W, Marquis L, et al. Role of epithelial-to-mesenchymal transition (EMT) in drug sensitivity and metastasis in bladder cancer. *Cancer and Metastasis Review*s. 2009;28(3-4):335-44.

196. Yilmaz M, Christofori G. EMT, the cytoskeleton, and cancer cell invasion. C*ancer and Metastasis Reviews.* 2009;28(1-2):15-33.

197. Wellner U, Schubert J, Burk UC, et al. The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs. *Nature cell biology.* 2009;11(12):1487-95.

198. Nguyen PT, Tsunematsu T, Yanagisawa S, et al. The FGFR1 inhibitor PD173074 induces mesenchymal-epithelial transition through the transcription factor AP-1. *British journal of cancer.* 2013;109(8):2248-58.

199. Muthusamy BP, Budi EH, Katsuno Y, et al. ShcA Protects against Epithelial-Mesenchymal Transition through Compartmentalized Inhibition of TGF-beta-Induced Smad Activation. *PLoS biology.* 2015;13(12):e1002325.

200. Park HR, Jung WW, Kim HS, Bacchini P, Bertoni F, Park YK. Overexpression of metastatic tumor antigen in osteosarcoma: comparison between conventional high-grade and central low-grade osteosarcoma. *Cancer research and treatment : official journal of Korean Cancer Association*. 2005;37(6):360-4.

201. Yamada T, Tsuda M, Wagatsuma T, et al. Receptor activator of NF-kappaB ligand induces cell adhesion and integrin alpha2 expression via NF-kappaB in head and neck cancers. *Scientific report*s. 2016;6:23545.

202. Idetsu A, Suehiro T, Okada K, Shimura T, Kuwano H. Hyperbaric oxygenation promotes regeneration of biliary cells and improves cholestasis in rats. *World journal of gastroenterology*. 2011;17(17):2229-35.

203. Li CW, Wang Q, Li J, et al. Silver nanoparticles/chitosan oligosaccharide/poly(vinyl alcohol) nanofiber promotes wound healing by activating TGFbeta1/Smad signaling pathway. *International journal of nanomedicine*. 2016;11:373-86.

204. Pelosof L, Yerram SR, Ahuja N, et al. CHFR silencing or microsatellite instability is associated with increased antitumor activity of docetaxel or gemcitabine in colorectal cancer. *International journal of cancer*. 2014;134(3):596-605.

205. Zheng X, Carstens JL, Kim J, et al. Epithelial-to-mesenchymal transition is dispensable for metastasis but induces chemoresistance in pancreatic cancer. *Nature.* 2015;527(7579):525-30.

206. Korpal M, Ell BJ, Buffa FM, et al. Direct targeting of Sec23a by miR-200s influences cancer cell secretome and promotes metastatic colonization. *Nature medicine.* 2011;17(9):1101-8.

207. Chaffer CL, Brennan JP, Slavin JL, Blick T, Thompson EW, Williams ED. Mesenchymal-to-epithelial transition facilitates bladder cancer metastasis: role of fibroblast growth factor receptor-2. *Cancer research.* 2006;66(23):11271-8.

208. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. *The Journal of clinical investigation*. 2009;119(6):1420-8.

209. Wang Y, Zhou BP. Epithelial-mesenchymal transition in breast cancer progression and metastasis. *Chinese journal of cancer*. 2011;30(9):603-11.

210. Elloul S, Elstrand MB, Nesland JM, et al. Snail, Slug, and Smad-interacting protein 1 as novel parameters of disease aggressiveness in metastatic ovarian and breast carcinoma. *Cancer.* 2005;103(8):1631-43.

211. Yang Z, Zhang X, Gang H, et al. Up-regulation of gastric cancer cell invasion by Twist is accompanied by N-cadherin and fibronectin expression. *Biochemical and biophysical research communications*. 2007;358(3):925-30.

212. Zheng M, Jiang YP, Chen W, et al. Snail and Slug collaborate on EMT and tumor metastasis through miR-101-mediated EZH2 axis in oral tongue squamous cell carcinoma. *Oncotarget*. 2015;6(9):6797-810.

213. Yang X, Han M, Han H, et al. Silencing Snail suppresses tumor cell proliferation and invasion by reversing epithelial-to-mesenchymal transition and arresting G2/M phase in non-small cell lung cancer. *International journal of oncology*. 2017;50(4):1251-60.

214. Olmeda D, Jorda M, Peinado H, Fabra A, Cano A. Snail silencing effectively suppresses tumour growth and invasiveness. *Oncogene*. 2007;26(13):1862-74.

215. Yue B, Ren QX, Su T, Wang LN, Zhang L. ERK5 silencing inhibits invasion of human osteosarcoma cell via modulating the Slug/MMP-9 pathway. *European review for medical and pharmacological sciences*. 2014;18(18):2640-7.

216. Matsuo N, Shiraha H, Fujikawa T, et al. Twist expression promotes migration and invasion in hepatocellular carcinoma. *BMC cancer*. 2009;9:240.

217. Pulukuri SM, Rao JS. Matrix metalloproteinase-1 promotes prostate tumor growth and metastasis. *International journal of oncology*. 2008;32(4):757-65.

218. Bradley CA, Dunne PD, Bingham V, et al. Transcriptional upregulation of c-MET is associated with invasion and tumor budding in colorectal cancer. *Oncotarget*. 2016;7(48):78932-45.

219. Tsou HK, Chen HT, Hung YH, et al. HGF and c-Met interaction promotes migration in human chondrosarcoma cells. *PloS one*. 2013;8(1):e53974.

220. Mai A, Muharram G, Barrow-McGee R, et al. Distinct c-Met activation mechanisms induce cell rounding or invasion through pathways involving integrins, RhoA and HIP1. J*ournal of cell science*. 2014;127(Pt 9):1938-52.

221. Sierra JR, Tsao MS. c-MET as a potential therapeutic target and biomarker in cancer. *Therapeutic advances in medical oncology*. 2011;3(1 Suppl):S21-35.

222. Di Renzo MF, Poulsom R, Olivero M, Comoglio PM, Lemoine NR. Expression of the Met/hepatocyte growth factor receptor in human pancreatic cancer. *Cancer research.* 1995;55(5):1129-38.

223. Schoumacher M, Goldman RD, Louvard D, Vignjevic DM. Actin, microtubules, and vimentin intermediate filaments cooperate for elongation of invadopodia. *The Journal of cell biology*. 2010;189(3):541-56.

224. Martin AC, Gelbart M, Fernandez-Gonzalez R, Kaschube M, Wieschaus EF. Integration of contractile forces during tissue invagination. T*he Journal of cell biology*. 2010;188(5):735-49.

225. Hayashi T, Carthew RW. Surface mechanics mediate pattern formation in the developing retina. *Nature*. 2004;431(7009):647-52.

226. Araki K, Shimura T, Suzuki H, et al. E/N-cadherin switch mediates cancer progression via TGF-beta-induced epithelial-to-mesenchymal transition in extrahepatic cholangiocarcinoma. *British journal of cancer*. 2011;105(12):1885-93.

227. Hazan RB, Qiao R, Keren R, Badano I, Suyama K. Cadherin switch in tumor progression. *Annals of the New York Academy of Sciences.* 2004;1014:155-63.

228. Linder S. The matrix corroded: podosomes and invadopodia in extracellular matrix degradation. *Trends in cell biology.* 2007;17(3):107-17.

229. Kalluri R. Basement membranes: structure, assembly and role in tumour angiogenesis. *Nature reviews Cancer*. 2003;3(6):422-33.

230. Daley WP, Peters SB, Larsen M. Extracellular matrix dynamics in development and regenerative medicine. *Journal of cell science.* 2008;121(Pt 3):255-64.

231. Albini A, Aukerman SL, Ogle RC, et al. The in vitro invasiveness and interactions with laminin of K-1735 melanoma cells. Evidence for different laminin-binding affinities in high and low metastatic variants. *Clinical & experimental metastasis.* 1989;7(4):437-51.

232. Varani J, Lovett EJ, 3rd, McCoy JP, Jr., et al. Differential expression of a lamininlike substance by high- and low-metastatic tumor cells. *The American journal of pathology*. 1983;111(1):27-34.

233. Barsky SH, Rao CN, Williams JE, Liotta LA. Laminin molecular domains which alter metastasis in a murine model. *The Journal of clinical investigation*. 1984;74(3):843-8.

234. Haraguchi M, Okubo T, Miyashita Y, et al. Snail regulates cell-matrix adhesion by regulation of the expression of integrins and basement membrane proteins. The *Journal of biological chemistr*y. 2008;283(35):23514-23.

235. Turner FE, Broad S, Khanim FL, et al. Slug regulates integrin expression and cell proliferation in human epidermal keratinocytes. *The Journal of biological chemistry.* 2006;281(30):21321-31.

236. Terauchi M, Kajiyama H, Yamashita M, et al. Possible involvement of TWIST in enhanced peritoneal metastasis of epithelial ovarian carcinoma. C*linical & experimental metastasis.* 2007;24(5):329-39.

237. Immervoll H, Hoem D, Steffensen OJ, Miletic H, Molven A. Visualization of CD44 and CD133 in normal pancreas and pancreatic ductal adenocarcinomas: non-overlapping membrane expression in cell populations positive for both markers. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society*. 2011;59(4):441-55.

238. Ponta H, Sherman L, Herrlich PA. CD44: from adhesion molecules to signalling regulators. *Nature reviews Molecular cell biology*. 2003;4(1):33-45.

239. Entschladen F, Drell TLt, Lang K, Joseph J, Zaenker KS. Tumour-cell migration, invasion, and metastasis: navigation by neurotransmitters. *The Lancet Oncology*. 2004;5(4):254-8.

240. Henderson V, Smith B, Burton LJ, Randle D, Morris M, Odero-Marah VA. Snail promotes cell migration through PI3K/AKT-dependent Rac1 activation as well as PI3K/AKT-independent pathways during prostate cancer progression. *Cell adhesion & migration*. 2015;9(4):255-64.

241. Medici D, Hay ED, Olsen BR. Snail and Slug promote epithelial-mesenchymal transition through beta-catenin-T-cell factor-4-dependent expression of transforming growth factor-beta3. *Molecular biology of the cell*. 2008;19(11):4875-87.

242. Wang Y, Liu J, Ying X, Lin PC, Zhou BP. Twist-mediated Epithelial-mesenchymal Transition Promotes Breast Tumor Cell Invasion via Inhibition of Hippo Pathway. *Scientific report*s. 2016;6:24606.

243. Nieman MT, Prudoff RS, Johnson KR, Wheelock MJ. N-cadherin promotes motility in human breast cancer cells regardless of their E-cadherin expression. *The Journal of cell biology*. 1999;147(3):631-44.

244. Islam S, Carey TE, Wolf GT, Wheelock MJ, Johnson KR. Expression of N-cadherin by human squamous carcinoma cells induces a scattered fibroblastic phenotype with disrupted cell-cell adhesion. *The Journal of cell biology*. 1996;135(6 Pt 1):1643-54.

245. Batlle E, Sancho E, Francí C, et al. The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nature cell biology*. 2000;2(2):84-9.

246. Cano A, Pérez-Moreno MA, Rodrigo I, et al. The transcription factor snail controls epithelial–mesenchymal transitions by repressing E-cadherin expression. *Nature cell biology*. 2000;2(2):76-83.

247. Hajra KM, Chen DY, Fearon ER. The SLUG zinc-finger protein represses E-cadherin in breast cancer. *Cancer research.* 2002;62(6):1613-8.

248. Stanisavljevic J, Porta-de-la-Riva M, Batlle R, de Herreros AG, Baulida J. The p65 subunit of NF-kappaB and PARP1 assist Snail1 in activating fibronectin transcription. *Journal of cell science*. 2011;124(Pt 24):4161-71.

249. Yu L, Li HZ, Lu SM, et al. Down-regulation of TWIST decreases migration and invasion of laryngeal carcinoma Hep-2 cells by regulating the E-cadherin, N-cadherin expression. *Journal of cancer research and clinical oncology*. 2011;137(10):1487-93.

250. Rojas-Puentes L, Cardona AF, Carranza H, et al. Epithelial-mesenchymal transition, proliferation, and angiogenesis in locally advanced cervical cancer treated with chemoradiotherapy. *Cancer medicine.* 2016;5(8):1989-99.

251. Heiler S, Wang Z, Zoller M. Pancreatic cancer stem cell markers and exosomes - the incentive push. *World journal of gastroenterology*. 2016;22(26):5971-6007.

252. Yan Y, Zuo X, Wei D. Concise Review: Emerging Role of CD44 in Cancer Stem Cells: A Promising Biomarker and Therapeutic Target. *Stem cells translational medicine*. 2015;4(9):1033-43.

253. Zhou X, Wang G, Sun Y. A reliable parameter to standardize the scoring of stem cell spheres. PloS one. 2015;10(5):e0127348.

254. Li Z. CD133: a stem cell biomarker and beyond. *Experimental hematology & oncology*. 2013;2(1):17.

255. Chatterjee S, Behnam Azad B, Nimmagadda S. The intricate role of CXCR4 in cancer. *Advances in cancer research*. 2014;124:31-82.

256. Jaggupilli A, Elkord E. Significance of CD44 and CD24 as cancer stem cell markers: an enduring ambiguity. *Clinical & developmental immunology*. 2012;2012:708036.

257. Salaria S, Means A, Revetta F, Idrees K, Liu E, Shi C. Expression of CD24, a Stem Cell Marker, in Pancreatic and Small Intestinal Neuroendocrine Tumors. *American journal of clinical pathology*. 2015;144(4):642-8.

258. Wen J, Park JY, Park KH, et al. Oct4 and Nanog expression is associated with early stages of pancreatic carcinogenesis. *Pancreas*. 2010;39(5):622-6.

259. Gao S, Pan Y, Song L, et al. Nanog Predicts Poor Prognosis in Human Pancreatic Cancer and Is Downregulated by QingyihuaJi Formula in Pancreatic Cancer Stem Cells. *Evidence-based complementary and alternative medicine : eCAM.* 2016;2016:7028289.

260. Herreros-Villanueva M, Zhang JS, Koenig A, et al. SOX2 promotes dedifferentiation and imparts stem cell-like features to pancreatic cancer cells. *Oncogenesis.* 2013;2:e61.

261. Guo W, Lasky JL, Wu H. Cancer stem cells. *Pediatric research.* 2006;59:59R-64R.

262. Mani SA, Guo W, Liao MJ, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell*. 2008;133(4):704-15.

263. Zhou W, Lv R, Qi W, et al. Snail contributes to the maintenance of stem cell-like phenotype cells in human pancreatic cancer. *PloS one.* 2014;9(1):e87409.

264. Luanpitpong S, Li J, Manke A, et al. SLUG is required for SOX9 stabilization and functions to promote cancer stem cells and metastasis in human lung carcinoma. *Oncogene*. 2016;35(22):2824-33.

265. Yang N, Hui L, Wang Y, Yang H, Jiang X. Overexpression of SOX2 promotes migration, invasion, and epithelial-mesenchymal transition through the Wnt/beta-catenin pathway in laryngeal cancer Hep-2 cells. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine*. 2014;35(8):7965-73.

266. Lou X, Han X, Jin C, et al. SOX2 targets fibronectin 1 to promote cell migration and invasion in ovarian cancer: new molecular leads for therapeutic intervention. *Omics : a journal of integrative biology*. 2013;17(10):510-8.

267. Wang X, Ji X, Chen J, et al. SOX2 enhances the migration and invasion of ovarian cancer cells via Src kinase. *PloS one*. 2014;9(6):e99594.

268. Alonso MM, Diez-Valle R, Manterola L, et al. Genetic and epigenetic modifications of Sox2 contribute to the invasive phenotype of malignant gliomas. *PloS one.* 2011;6(11):e26740.

269. Han X, Fang X, Lou X, et al. Silencing SOX2 induced mesenchymal-epithelial transition and its expression predicts liver and lymph node metastasis of CRC patients. *PloS one*. 2012;7(8):e41335.

270. Wang D, Lu P, Zhang H, et al. Oct-4 and Nanog promote the epithelial-mesenchymal transition of breast cancer stem cells and are associated with poor prognosis in breast cancer patients. *Oncotarget.* 2014;5(21):10803-15.

271. Lu C, Xu F, Gu J, et al. Clinical and biological significance of stem-like CD133(+)CXCR4(+) cells in esophageal squamous cell carcinoma. *The Journal of thoracic and cardiovascular surgery*. 2015;150(2):386-95.

272. Bao B, Ahmad A, Azmi AS, Ali S, Sarkar FH. Overview of cancer stem cells (CSCs) and mechanisms of their regulation: implications for cancer therapy. *Current protocols in pharmacology*. 2013;Chapter 14:Unit 14 25.

273. Mo W, Zhang J-T. Human ABCG2: structure, function, and its role in multidrug resistance. *International journal of biochemistry and molecular biology.* 2012;3(1):1.

274. Nath S, Mukherjee P. MUC1: a multifaceted oncoprotein with a key role in cancer progression. *Trends in molecular medicine*. 2014;20(6):332-42.

275. Akhtar M, Gallagher L, Rohan S. Survivin: role in diagnosis, prognosis, and treatment of bladder cancer. *Advances in anatomic pathology.* 2006;13(3):122-6.

276. Roh YJ, Kim JH, Kim IW, Na K, Park JM, Choi MG. Photodynamic Therapy Using Photosensitizer-Encapsulated Polymeric Nanoparticle to Overcome ATP-Binding Cassette Transporter Subfamily G2 Function in Pancreatic Cancer. *Molecular cancer therapeutics*. 2017;16(8):1487-96.

277. Kami K, Doi R, Koizumi M, et al. Survivin expression is a prognostic marker in pancreatic cancer patients. *Surgery*. 2004;136(2):443-8.

278. Wan Z, Pan H, Liu S, et al. Downregulation of SNAIL sensitizes hepatocellular carcinoma cells to TRAIL-induced apoptosis by regulating the NF-kappaB pathway. *Oncology reports*. 2015;33(3):1560-6.

279. Liang H, Zhang L, Xu R, Ju X. Silencing of survivin using YM155 induces apoptosis and chemosensitization in neuroblastomas cells. *European review for medical and pharmacological sciences.* 2013;17(21):2909-15.

280. Sanhueza C, Wehinger S, Castillo Bennett J, Valenzuela M, Owen GI, Quest AF. The twisted survivin connection to angiogenesis. *Molecular cancer.* 2015;14:198.

281. Mato E, Gonzalez C, Moral A, et al. ABCG2/BCRP gene expression is related to epithelial-mesenchymal transition inducer genes in a papillary thyroid carcinoma cell line (TPC-1). *Journal of molecular endocrinology*. 2014;52(3):289-300.

282. Longley DB, Harkin DP, Johnston PG. 5-fluorouracil: mechanisms of action and clinical strategies. *Nature Reviews Cancer.* 2003;3(5):330-8.

283. Kraus LA, Samuel SK, Schmid SM, Dykes DJ, Waud WR, Bissery MC. The mechanism of action of docetaxel (Taxotere®) in xenograft models is not limited to bcl-2 phosphorylation. *Investigational new drugs*. 2003;21(3):259-68.

284. Verweij J, Pinedo HM. Mitomycin C: mechanism of action, usefulness and limitations. *Anti-cancer drugs.* 1990;1(1):5-14.

285. Mattson K, Saarinen A, Jekunen A, editors. Combination treatment with docetaxel (Taxotere) and platinum compounds for non-small cell lung cancer. *Seminars in oncology*; 1997.

286. Bay JO, Ray-Coquard I, Fayette J, et al. Docetaxel and gemcitabine combination in 133 advanced soft-tissue sarcomas: A retrospective analysis. *International journal of cancer*. 2006;119(3):706-11.

287. Burris III HA. Docetaxel in combination with fluorouracil for advanced solid tumors. breast cancer. 1997;2:3.

288. Xu J, Lamouille S, Derynck R. TGF-beta-induced epithelial to mesenchymal transition. *Cell research*. 2009;19(2):156-72.

289. Kim J, Hwan Kim S. CK2 inhibitor CX-4945 blocks TGF-beta1-induced epithelial-to-mesenchymal transition in A549 human lung adenocarcinoma cells. *PloS one.* 2013;8(9):e74342.