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KALLIKREINS AND INVESTIGATION OF PEA3 RELATED KLKs REGULATION

BAYRAM KAŞ

A THESIS SUBMITTED FOR THE DEGREE OF

MASTER OF SCIENCE

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GEBZE TEKNİK ÜNİVERSİTESİ

FEN BİLİMLERİ ENSTİTÜSÜ

KALLİKREİNLER VE PEA3 İLE ALAKALI KLK REGÜLASYONLARININ İNCELENMESİ

BAYRAM KAŞ

YÜKSEK LİSANS TEZİ

MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI

PROF. DR. IŞIL AKSAN KURNAZ

GEBZE

2020

GEBZE TEKNİK ÜNİVERSİTESİ	**YÜKSEK LİSANS JÜRİ ONAY FORMU**

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...../...../..... tarih ve ...../..... sayılı kararı.

SUMMARY

Previous transcriptomic studies in our laboratory have identified new Pea3 target genes in the neuronal system, including Ephrin and ephrin receptors, semaphorines, cell adhesion molecules, metalloproteases, and kallikreins. This regulation can be either indirect, or by direct binding of Pea3 to the promoter and regulating gene expression. To understand the mechanistic details of this control, In this study we have aimed to validate the changes in kallikrein gene expression upon Pea3 overexpression that has been observed in microarray analysis, and clone the target promoters into the luciferase reporter vector, and thus we aimed to confirm the role of Pea3 in axonal growth throughby regulationng of ECM-reducing kallikrein enzymes in the nervous system.

Key Words: Transcription Factors, ETS Family, Pea3, Kallikreins.

ÖZET

Laboratuvarımızda yapılan önceki transkriptomik çalışmalar, nöronal sistemde Ephrin ve ephrin reseptörleri, semaforinler, hücre adezyon molekülleri, metaloproteazlar ve kallikreinler dahil olmak üzere yeni Pea3 hedef genlerini tanımladı. Bu düzenleme dolaylı olabileceği gibi Pea3'ün promotere doğrudan bağlanması ve gen ekspresyonunun düzenlenmesi yoluyla da olabilir. Bu kontrolün mekanik detaylarını anlamak için, bu çalışmada mikroarray analizinde gözlemlenen Pea3 aşırı ifadesi üzerine kallikrein gen ekspresyonundaki değişiklikleri doğrulamayı ve hedef promotörleri lusiferaz raportör vektörüne klonlamayı amaçladık. Böylece sinir sisteminde ECM azaltıcı kallikrein enzimlerinin düzenlenmesi yoluyla Pea3'ün aksonal büyümedeki rolünü doğrulamayı amaçlanmıştır.

Anahtar Kelimeler: ETS Ailesi, Kallikreinler Transkripsiyon Faktörleri, Pea3.

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TABLE of CONTENTS

	Page
SUMMARY	v
ÖZET	vi
ACKNOWLEDMENTS	vii
TABLE of CONTENTS	viii
LIST of ABBREVIATIONS and ACRONYMS	x
LIST of FIGURES	xi
LIST of TABLES	xii
1. INTRODUCTION	1
1.1 ETS Transcription Factor Family	1
1.2 PEA3 Family Subfamily	2
1.3 Kallikreins	3
2. MATERIALS and METHODS	6
2.1. Materials	6
2.1.1. Bioinformatic Tools	6
2.1.2. Plasmids	7
2.1.3. Cell Lines and Bacterial Strains	7
2.1.4. Cell Culture Solutions	7
2.1.5. Bacterial Media	7
2.1.6. Commercial Kits and Reagents	8
2.1.7. Gel Electrophoresis Solutions	8
2.1.8. Material and Devices Which is Used	8
2.2. Methods	9
2.2.1. Promoter Analysis	9
2.2.2. Primer Design	10
2.2.3. Cloning for Promoter of Selected KLK Targets	10
2.2.4. Bacterial Growth Medias Preparation	12
2.2.5. Competent Cell Preparation	12
2.2.6. Transformation of Plasmids	13
2.2.7. Plasmid isolation	14

2.2.8. Cell Culture 14
2.2.9. RNA isolation 15
2.2.10. Genomic DNA Isolation 15
2.2.11. Colony Verification PCR 16
2.2.12. Testing RNA Quality 16
2.2.13. cDNA Synthesis 17
2.2.14. Quantitative Real Time PCR 17
3. RESULTS 19
3.1. Promoter Analysis Results 19
3.2. Designed Primers 22
3.3. Gradient PCRs for Designed Primers 23
3.4. Promoter Cloning 23
3.5. Human U87 Glioblastoma Cell Culture and Transfection 29
3.6. Total RNA Isolation and Checking RNA Integrity 30
3.7. Quantitative Real Time PCR Results 32
4. DISCUSSION and CONCLUSION 36

REFERENCES 37
BIOGRAPHY 38

LIST of ABBREVIATIONS and ACRONYMS

Abbreviations and Acronyms		**Explanations**
APS	:	Ammonium Persulfate
APP	:	Amyloid precursor protein
DMEM	:	Dulbecco's Modified Eagle Medium
DMSO	:	Dimethyl Sulfoxide
DH5α	:	*Escherichia coli* bacterial strains
ETS	:	E-twenty-Six / E-26 Transformation Specific
FGF	:	Fibroblast Growth Factor
GAPDH	:	Glyceraldehyde-3-phosphate Dehydrogenase
GDNF	:	Glial Cell-Derived Neurotrophic Factor
KLK	:	Kallikrein
L1CAM	:	L1 cell adhesion molecule
MAPK	:	Mitogen Activated Protein Kinases
MOPS	:	3-(N-morpholino) propane-sulfonic acid
NF-L	:	Human Neurofilament Light Chain
NGF	:	Nerve Growth Factor
PEA3	:	Polyomavirus Enhancer Activator 3
PEI	:	Polyethylenimine
PLB	:	Passive Lysis Buffer
PNT	:	Pointed Domain
SDM	:	Site Directed Mutagenesis
SH-SY5Y	:	Human Neuroblastoma Cell Line
TEMED	:	Tetramethylethylenediamine
U87 Cells	:	Glioblastoma Cell Line

LIST of FIGURES

Figure No:		Page
1.1:	The ETS Sub families.	2
2.1:	pGL3-Basic Vector.	6
3.1:	KLK6_Human binding site from ALGGEN for PEA3 Sequence.	19
3.2:	Putative Pea3 binding motifs for hKLK6.	20
3.3:	KLK6 Mouse binding site from ALGGEN for PEA3 Sequence.	20
3.4:	Putative Pea3 binding motifs for mKLK6.	21
3.5:	KLK8 Mouse binding site from ALGGEN for PEA3 Sequence.	21
3.6:	Putative Pea3 binding motifs for mKLK8.	22
3.7:	Gradient PCR fot mKLK6.	23
3.8:	Template NSC-34 cells p23 PCR High-Fidelity enzymes.	24
3.9:	Digestion of plasmid pGL3 by restiriction enzymes.	24
3.10:	Digestion of plasmid KLK6 by restiriction enzymes.	25
3.11:	Colony PCR mKLK6-pGL3 plasmid. Ligation rate 3:1.	26
3.12:	GFP optimization for U87 human glioblastoma cells 75K 100K 125K 150K 1:3 PEI/Plasmid.	27
3.13:	GFP optimization for U87 human glioblastoma cells 75K 100K 125K 150K 1:4 PEI/Plasmid.	30
3.14:	GFP optimization for U87 human glioblastoma cells 75K 100K 125K 150K 1:5 PEI/Plasmid.	31
3.15:	U87 Glioblastoma cell line passage 23 total RNA in formaldehyde agarose gel electrophoresis.	31
3.16:	U87 Glioblastoma cell p24 total RNA in formaldehyde agarose gel electrophoresis.	31
3.17:	Relative expression of PEA3 in human U87 glioblastoma cells.	32
3.18:	Relative expression of KLK6 in human U87 glioblastoma cells.	33
3.19:	Relative expression of KLK6 in human U87 glioblastoma cells.	33
3.20:	Relative expression of KLK8 in human U87 glioblastoma cells.	33

LIST OF TABLES

Table No:		Page
1.1:	All Classified Human Tissue Kallikreins.	5
2.1:	Bioinformatic Tools which used in experiments.	6
2.2:	Gradient PCR for KLK primers.	10
2.3:	PCR program for optimizing annealing temperature.	10
2.4:	Phusion DNA polymerase reaction setup for insert amplification.	11
2.5:	Preparation of mix for Real Time PCR.	18
2.6:	96 wells design for Real Time PCR Reaction.	18
3.1:	Designed Primers for promoter cloning.	22
3.2:	Primers with Restriction enzymes.	23

1. INTRODUCTION

Transcription factor is a protein that can bind to a sequence on DNA to regulate transcription of genes [1]. These are also referred to as sequence-specific DNA binding proteins [2]. Transcription factors, alone or in combination with other proteins contained in a complex, control the regulation of gene expression. These transcription factors can activate or repress transcription through binding to *cis*-regulatory elements [3]. in Figure 1 adapted from Rojano et al.

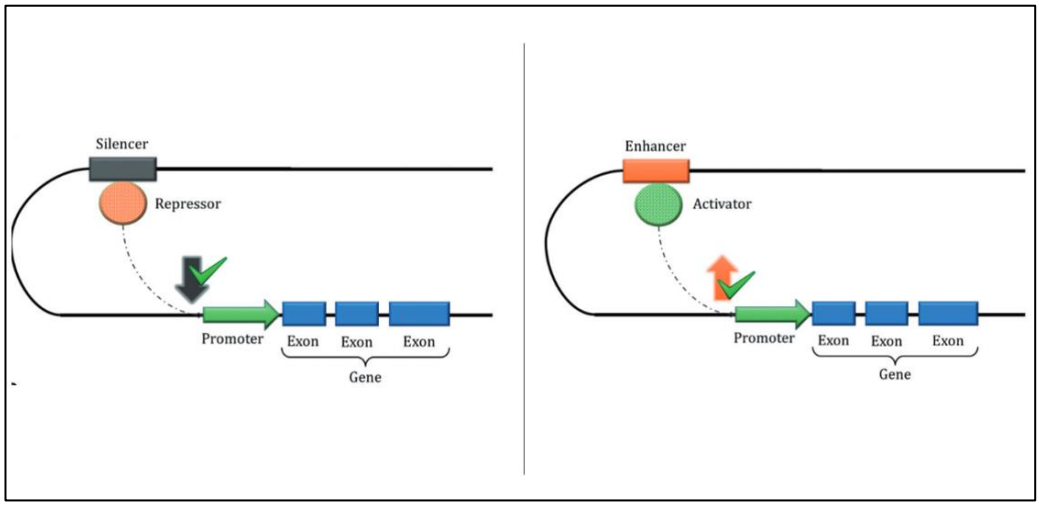


Figure 1.1: Representation of the effects of cis-regulatory elements: enhancers (Right), silencers (Left).

1.1. ETS Transcription Factor Family

The first member of ETS transcription factor gene family, *v-ets* oncogene, which was discovered in the avian transforming retrovirus of chicken, called "E-twenty-six (E26). This oncogene was found to cause erythroblastic and myeloblastic leukemias After *v-ets*, many other members of the ETS family have been also identified in different organisms, including *Caenorhabditis elegans*, *Drosophila melanogaster* and human [4].

Interestingly, the ETS domain transcription factor superfamily is represented only in the metazoan line: it consists of 12 subfamilies and approximately 30 members. [5]. Of these, 28 were found in humans, 26 were in mice, 10 were in *C. elegans*, and

9 were in *D. melanogaster*. The hallmark of the ETS family is a highly evolutionary conserved 85-amino acid DNA binding domain (ETS-domain) which binds 5-GGAA/T-3 sequence (called the ets motif) to regulate transcriptional processes. The members of the ETS family have been implicated in the development of different type of tissues especially as well as cancer progression [6].

Subfamily	Mammalian family members
ELF	ELF1, ELF2 (NERF), ELF4 (MEF)
ELG	GABPα
ERG	ERG, FLI1, FEV
ERF	ERF (PE2), ETV3 (PE1)
ESE	ELF3 (ESE1/ESX), ELF5 (ESE2), ESE3 (EHF)
ETS	ETS1, ETS2
PDEF	SPDEF (PDEF/PSE)
PEA3	ETV4 (PEA3/E1AF), ETV5 (ERM), ETV1 (ER81)
ER71	ETV2 (ER71)
SPI	SPI1 (PU.1), SPIB, SPIC
TCF	ELK1, ELK4 (SAP1), ELK3 (NET/SAP2)
TEL	ETV6 (TEL), ETV7 (TEL2)

Figure 1.2: The ETS Sub families.

1.2. PEA3 Subfamily – Structure and Function

Polyoma enhancer activator 3 (PEA3) / ETS translocation variant 4 (ETV4) which is the founding a member of the PEA3 subfamily of ETS transcription factors. They are also known as polyoma enhancer activator 3 (PEA3). The PEA3 subfamily contains three related transcription factors: Pea3/ETV4, Erm/ETV5, and Er81/ETV1 PEA3 family transcription factors have regulatory domains that are AD; acidic domain, CIDD; central inhibitory DNA-binding domain, ETS; DNA-binding domain, and CT; carboxy-terminal [7].

Pea3 is a nuclear phosphoprotein which is implicated in developmental procedures as well as brain tissue development. Throughout the neurogenesis new born neurons migrate. Then several molecular mechanisms results in dendritic branching

and synapse formation. PEA3/ETV4 knock-out mice were shown that not able to form synapse and dendritic branching within the motor neurons of the spinal cord [8].

Our previous lab members Kandemir B. *et.al.* identified novel neuronal targets of Pea3 in a combinatorial approach, through curation, computational analysis and microarray studies in a neuronal model system, SH-SY5Y neuroblastoma cells. Among the potential target promoters in neuronal systems, novel targets such as ephrins and ephrin receptors, semaphorins, cell adhesion molecules, metalloproteases and kallikreins have been identified [9].

1.3. Kallikreins

Proteolytic enzymes are classified according to their catalytic mechanisms. Proteases are a class of proteolytic enzymes. They are both involved in extracellular matrix structure and in the synthesis and secretion of proteins involved in important cellular functions such as apoptosis, cell growth and activation, protein secretion and phagocytosis [10]. Proteases are involved in shaping extracellular matrix structure. Proteases may be extracellular, or cell membrane localized. They may also be involved in cytoplasm or organelles such as lysosomes [11].

The human genome is thought to have about 400-500 proteases and about 32% of these are serine proteases. The serine protease family includes digestive enzymes (trypsin, chymotrypsin), growth factors (tissue plasminogen activator), some clotting factors and kallikrein. Therefore, serine proteases are involved in important functions such as digestion, coagulation, fibrinolysis, tissue remodeling, hormone activation, and extracellular matrix destruction [11].

The term kallikrein was first used in 1930s to describe proteolytic enzymes that release small vasoactive peptides from high molecular weight precursors [12]. Werle *et al.* determined the proteolytic activity in the pancreatic extracts they obtained and expressed this activity with the Greek term kallikreas meaning “pancreas”. Kallikrein enzymes are a subgroup of the serine protease family and fall into two major categories; (i) plasma kallikrein (PK) and (ii) kallikrein-related peptidases (KLKs).

Plasma kallikrein is encoded by a single gene on chromosome 4. The gene contains 15 exons and 14 intron [12]. In healthy humans, plasma is also present in complex with approximately 50 micrograms/ml of PPK high molecular weight

quinones. 638 amino acids are released in the form of pre-kallikrein [12]. The signal peptide consists of 19 amino acids at the N-terminal end [13].

Tissue Kallikreins; the human kallikrein gene family has an important role in the discipline of clinical biochemistry since it contains the best tumor marker, prostate specific antigen (PSA) [14]. Only three human kallikrein genes were known in reports published in the late 1990s. These are pancreatic / renal kallikrein (KLK1), human glandular kallikrein 2 (KLK2) and PSA (KLK3) genes [17].

Table 1.1: All Classified Human Tissue Kallikreins.

Gene Name	**Protein Name**	**Other Names / Symbols**
KLK1	KLK1	Pancreatic/renal kallikrein
KLK2	KLK2	Human glandular kallikrein1
KLK3	KLK3	PSA, APS
KLK4	KLK4	Prostase, KLK-L1, EMSP1, PRSS17, ARM1
KLK5	KLK5	KLK-L2, HSCTE
KLK6	KLK6	Zyme, Protease M, Neurosin, PRSS9
KLK7	KLK7	HSCCE, PRSS6
KLK8	KLK8	Neuropsin, ovasin, TADG-14, PRSS19,HNP
KLK9	KLK9	KLK-L3
KLK10	KLK10	NES1, PSSSL1
KLK11	KLK11	TLSP/Hippostatin, PRSS20
KLK12	KLK12	KLK-L5
KLK13	KLK13	KLK-L4
KLK14	KLK14	KLK-L6
KLK15	KLK15	Prostinogen, HSRNASPH

In the 2000s, various kallikrein-like genes were found alongside the classic kallikrein gene family. These genes encode serine protease and are located in the same chromosome locus as the classic kallikrein [18]. The Human Genome Organization (HUGO) was classified kallikreins up to 1-15.

All KLK genes encode single chain preproenzymes and that are between 244-293 amino acids in length [10]. In the nervous system, especially KLK6 and KLK8 have been found to modify critical neuronal proteins such as alpha synuclein,

glutamate receptor, and amyloid precursor protein (APP) [21]. When we look at the literature, we can see that some critical neuron-related proteins such as alpha synuclein, glutamate receptor, APP have been modified by KLKs [22]. [24].

The highest expression of KLK6 in humans is observed in the central nervous system [25]. KLK6 is strongly expressed in the particularly in the grey matter of the brain and in peripheral nerves. KLK6 is predominantly expressed in oligodendrocytes, pyramidal cells and astrocytes. In Table 1 All Classified Human Tissue Kallikreins shown [24].

So, in this thesis we aim to understand the mechanistic details of Pea3 and Kallikreins regulation, confirm the role of Pea3 on axonal outgrowth through regulation of ECM degrading kallikreins in the nervous system. In addition to this, we are intented to understand the mechanistic details of this control and to validate the changes in kallikrein gene expression upon Pea3 overexpression that has been observed in microarray analysis. Also, by cloning the target promoters into the luciferase reporter vector, we aimed to confirm the role of Pea3 in axonal growth throughby regulationng of ECM-reducing kallikrein enzymes in the nervous system.

2. MATERIAL and METHODS

2.1. Materials

2.1.1. Bioinformatic Tools

Table 2.1: Tools which used in experiments.

Tool	Web Site
Pubmed	http://www.ncbi.nlm.nih.gov/pubmed
IDT Oligo Analyzer	https://www.idtdna.com/pages/tools/oligoanalyz
Primer BLAST	https://www.ncbi.nlm.nih.gov/tools/primer-blast
TRED*	https://cb.utdallas.edu/cgi-bin/TRED/tred.cg
ALGGEN Promo3.0	http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo
Eukaryotic Promoter Database	https://epd.epfl.ch//index.php
Multiple Sequence Alignment	https://www.ebi.ac.uk/Tools/msa/
Pairwase Sequence Alignment	https://www.ebi.ac.uk/Tools/psa/

2.1.2. Plasmid

Pea3-VP16 Constitutively active expression vector and pCDNA3-mPea3 is originally a gift from Prof. Dr. A.D.

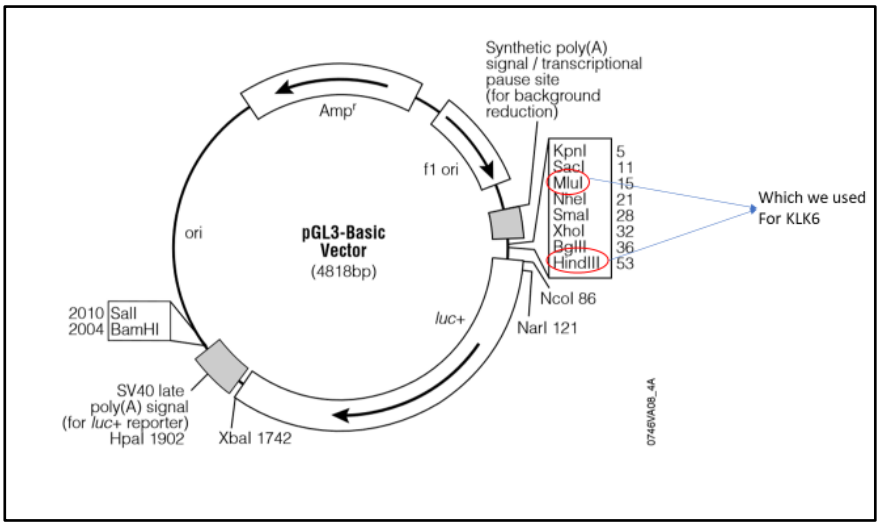


Figure 2.1: pGL3 Basic Vector.

Sharrocks. Empty pcDNA3 vector and pGL3 (shown figure 2.1. above) luciferase reporter vector were purchased from commercial companies, Promega and ThermoFisher, with the following sequence.

2.1.3. Cell Lines and Bacterial Strains

In the cell culture studies, experiments were performed with SH-SY5Y human neuroblastoma cell line (ATCC® CRL-2266™) and NSC-34 mouse motor neuron-like hybrid cell line (CLU140) and U87- glioblastoma cells (ATCC number: HTB-14). In the cloning studies, DH5α *Escherichia coli* bacterial strains was used.

2.1.4. Cell Culture Solutions

Penicilin/Streptomycin 10000U-ml Penicillin/10mg/ml Streptomycin (PAN Biotech), Dulbecco's Modified Eagle Medium (DMEM) (1X) with 4,5 g/L Glucose Dulbecco's Phosphate Buffered Saline (1X) (DPBS) (Gibco, 14190-94), 0.05% Trypsin-EDTA Solution (1X) (Gibco, 25300-054), L-glutamine and pyruvate (Gibco, 41966-029), Fetal Bovine Serum (FBS) (Gibco, 10270), PEI transfection reagent (Polysciences), Dimethylsulfoxide (DMSO), Phosphate Buffered Saline (PBS) (Gibco).

2.1.5. Bacterial Media

LB Broth (Caisson Labs, LBP01-500GM), LB Agar (Caisson Labs, LBP02500GM), Ampicillin (Biomatik), 87% Glycerol (Sigma, 15524), CaCl2 (Acros Organics).Petri Plates (Isolab), 100 mMCaCl2 solution.

2.1.6. Commercial Kits and Reagents

DNA-midi SV, Plasmid DNA Purification Kit Intron Biotechnology Catalog/Lot Number 17252, DNA-spin, Plasmid DNA Purification Kit Intron Biotechnology Catalog/Lot Number 17097, PureLink RNA MiniKit Invitrogen Catalog/Lot Number

12183018A, iScript cDNASynthesis Kit BioRad Lot Number 1708891, SsoAdvanced Universal SYBR Green Supermix BioRad Catalog/Lot Number 1725274

2.1.7. Gel Electrophoresis Solutions

GeneRuler 1kb Plus DNA Ladder (Thermo Scientific, #SM1331), 6X DNA Loading Dye (Fermentas, R0611), RedSafe Nucleic Acid Staining Solution (Intron Biotechnology, 21141), Agarose (Sigma), Tris-Acetate-EDTA (TAE) MOPS 10X (Fisher Chemical) 1132-61-2 37% Formaldehyde Sigma 25249 Sodium acetate.

2.1.8. Material and Devices Used

Rotator (JeioTech), Varioskan Flash (Thermo Scientific), Spectrophotometer – NanoDrop (Schimadzu), Thermo Scientific™ NanoDrop™ Lite Spectrophotometer 100 mm, 150 mm tissue culture plate (TRP), 0.22 µm syringe filter (Merck), 15 ml and 50 ml Falcon (CAPP), 24-well culture plate (TRP), 5 ml, 10 ml ,25 ml, 50 ml serological pipettes (CAPP) 80°C freezer (VWR) 96-well plate (NUNC) Agarose Gel Electrophoresis System (Bio-Rad) Criterion Blotter (Bio-Rad) Laminar Flow Cabinet –Safe 2020 (Thermo Scientific) Micro pipettes (Eppendorf) Microcentrifuge tubes (0,2 ml, 0,5 ml, 1,5 ml, 2ml) MJ Mini Personal Thermal Cycler (Bio-Rad) Petri dish (100 mm) (Lamtek) Power Supply – Power Pac Basic (Bio-Rad) Spin (Sprout) Syringe (1,5 ml, 10 ml, 20 ml, 50 ml) T25, T75, T150 tissue culture flasks (TRP) UV illuminator (Vilber Lourmat), Vortex (JeioTech), Water Bath (JeioTech)Hemocytometer (Marienfeld)Autoclave (Hirayama HV-110L) Microfuge 22R Centrifuge (Beckman Coulter)

2.2. Methods

2.2.1. Promoter Analysis

An online database (Transcriptional Regulatory Element Database, TRED; URL: http://https://cb.utdallas.edu/cgibin/TRED/tred.) was used in the initial analyses and the access of this site is no longer available. To obtain selected promoter sequence, the 'retrieve promoters' option was used. The organism was specified, the gene name or accession number from NCBI database was written and database browsed. Among the list of found promoters, the one with the highest quality was selected and the sequence between -1000 to +100 base pair relative to transcription starts site (TSS). Promoters were retrieved from PROMO v.3. analyzed presence of a transcription factor (TF) binding motif using a web-based tool PROMO v.3. I have performed the following steps to identify putative TF binding sites on promoter sequences.

- The regions and factors in the analysis of human promoters were limited to human.
- All factors and regions were also performed for the analysis of promoter sequences of mice.
- The promoter sequence obtained from TRED and ı choose the maximum matrix dissimilarity rate was set to 15% . Submitted to the tool in .fasta format
- Finally found sequences within the promoter specific to PEA3 were extracted in electronic file format.

2.2.2. Primer Design

Oligo Analyzer 3.1 software were used which is developed by Integrated DNA Technology to design NCBI Primer Blast tools. For the design of the primers to be used in expression analyses or cloning promoter sides sequences, obtained from National Center for Biotechnology and EPD The Eukaryotic Promoter Database. The following were carefully considered during primer design.

- the primers being 15-25 nucleotides long,
- Tm values between 55°C and 65°C,
- G/C content between 40% and 60%,
- three G/C bases within the last five bases at the 3' end,
- and giving the Gibbs Free Energy G (ΔG) value more than -5 kcal/mol for both self- and cross-dimer were chosen.

For the purpose of determining the specificity of the designed primer pairs; Primer Blast software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used. After that the sequence information of the designed primers were submitted. Database of Refseq. mRNA or Genome (reference assembly from selected organism) was selected. The name of organism was specified, and the analysis was initiated. The specificity of the templates was checked. In case of non-specific template, the primary design was repeated.

2.2.3. Molecular Cloning for Promoter of Selected KLK Targets

Firs step was Gradient PCR to determine optimization primer conditions. Gradient PCR was performed for KLK primers with MyTaq ™ DNA Polymerase and Bioline program which were used in the gradient PCR tool.

Table 2.2: Gradient PCR for KLK primers.

Reaction Component	Volume per each reaction
5X Buffer	5 µl
Template	2 µl (300 ng)
F Primer	1,25 µl
R Primer	1,25 µl
Enzyme	0,5 µl
dH2O	15 µl (up to 25 ul)

For human KLK primers SH-SY5Y: Human Neuroblastoma Cell Line genomic DNA as template were used and for mouse KLK primers Mouse Motor Neuron-Like Hybrid Cell Line (NSC-34) used as template DNA. Four tube reactions were performed for 4 different temperatures, 60°C, 62°C, 65°C.

Table 2.3: PCR program for optimizing annealing temperature.

Program for Gradient PCR		
Pre-denaturation	95°C	2 min.
Denaturation	95°C	30 sec.
Annealing	TM 35	30 sec.
Extension	72°C	30 sec.
Last extension	72°C	5 sec.

These procedures were applied for each cloning primer. (35 cycle) like showed in Table 2.3.

The pGL3 Basic Vector was used to clone the KLK promoters. Amplification made by High fidelity Phusion DNA polymerase due to comprised proofreading polymerase activity and low error rate.

Table 2.4: Phusion DNA polymerase reaction setup for insert amplification.

Reaction Component	Volume per each reaction
5X Phusion GC Buffer	10 µl
10 mM dNTP	1µl
Forward Primer	1,6 µl
Reverse Primer	1,6 µl
Template DNA	3 µl (300 ng)
Enzyme	0,5 µl
dH_2O	Up to 50 µl

In the run protocol: Initial Denaturation at 95°C 2 minutes, [Denaturation: at 95°C and 30 second, Annealing TM 65°C and 30 second, Extension 72°C and 2 minutes] “this part 38 times” and Final Extension at 72°C 10 minutes.

After agarose gel electrophoresis was performed. And checked the amplification of product. The gel was % 1,5 agarose and that have red safe to visualization. The resulting In the run protocol: Initial Denaturation at 95°C 2 minutes, [Denaturation: at 95°C and 30 second, Annealing TM 65°C and 30 second, Extension 72°C and 2 minutes] “this part 38 times” and Final Extension at 72°C 10 minutes.After agarose gel electrophoresis was performed. And checked the amplification of product. The gel was % 1,5 agarose and that have red safe to visualization.

2.2.4. Bacterial Growth Medias Preparation

Luria-Bertani (LB) broth is the most extensively used medium for the growth of bacteria and in my experiment, it was used. Preparation of LB broth is so simple 25 g powder LB Broth is added into 1L of purified water (autoclaved). Then 100µl ampicillin is added for each 100ml LB Broth. For LB Agar, 40 g powder agar is added into 1L of purified water and it is autoclaved. 100 µl ampicillin is added for each 100 ml LB Agar before the autoclaved agar gelation. Then the agar is decanted into 10 cm petri dish. Stored +4°C.

2.2.5. Competent Cell Preparation

E. coli cells are more likely to incorporate foreign DNA if cell walls are changed. DNA can pass more easily. And these cells called “competent”. The cells are competent by a process using calcium chloride and heat shock. The process which I have used is that

- These operations should be done next to the fire. Firstly 10 ml LB Broth added to 15 ml falcon tubes.
- After than from stock where in – 80°C. DH5α strain of bacteria were inoculated in 5 ml of LB Broth without any antibiotics overnight at 37°C with shaking at 180

- 200 rpm.

- After 16 hours of incubation, 1ml of inoculum from 10ml of falcon is taken and placed in 50ml of falcon containing 49 ml of LB broth.
- The mouth of the 50ml falcon is covered with paraffin. And incubated at 37°C 3 hour and 200 rpm.
- After 3-hour incubation centrifuge for 10 minutes at 5500 rpm and poured supernatant
- Dissolve the pellet with 1 ml $CaCl_2$ and additional 4 ml is added. Incubate on ice for 1 hour.
- Centrifuge for 5 min at 3500 rpm. The supernatant can be thawed with 1ml of pellet and stored at +4 °C

2.2.6. Transformation of Plasmids

Usual protocol was performed for plasmid transformation in our laboratory. First DH5α competent bacterial cells thawed in the icebox. And tubes labelled for each plasmid. 2 µl plasmid, 50µl competent cell (thawed) varied in the labelled tube and incubated 15 minutes in the ice. After then all tubes taken from ice and putted 42°C heater block for 90 seconds. Then tubes are incubated in ice 2 minutes again.

The continuation 200 µl LB Broth without ampicillin is added to tubes and they were incubated 37°C in shaker for 1 hour. For each sample used 50µl competent. To check if there was any contamination, prepared negative control also which just 50µl competent without plasmid. After that mixture within the tubes are spread 10cm ampicillin positive petri agar plate by using loop next to fire. And incubated 37°C 16 hour (overnight). After incubation day single colony were picked from ach sample plates and inoculated to 200 ml LB Broth with ampicillin. Then each sample are incubated 37°C over-night in shaker.

In summary Incubate on ice for 30 min, 90 sec at 42 ° C, re-ice for 2 min, add 200ul of LB Broth, shaker at 37 ° C in warm room for 1 hr, Spread on LB Agar plate. Incubate at 30 degrees for 16 hours to obtain transformed colonies.

2.2.7. Plasmid isolation

For plasmids used two types of kits which is MiniPrep (Intron Biotechnology, 17252). Single colonies were picked from LB Agar plate, which was grown overnight after transformation and inoculated in 5 ml of LB Broth with ampicillin at 37°C with overnight shaking. Pellets were resuspended with 250 µl of resuspension buffer containing RNase A with vortex. Than 250 µl of lysis buffer was added onto cells, and tubes were inverted ten times and incubated for 3 min at room temperature. 350 µl of neutralization buffer was added and tubes were inverted ten times and incubated on ice for 5 min. Lysate was centrifuged at 13000 rpm for 10 min at 4°C. Supernatant was transferred into columns and they were centrifuged at 13000 rpm for 1 min. Flow-through that was precipitated in collection tube was discarded and 500 µl of first washing buffer was added into filter columns. Tubes were centrifuged at 13000 rpm for 1 min. Flowthrough was discarded, again. 700 µl second washing buffer was added into filter columns and they were centrifuged at 13000 rpm for 1 min. Flow-through was discarded from collection tubes. Collection tubes with filter column were dried with centrifugation at 13000 rpm for 1 min. For elution of plasmid DNA from filter column new clean microfuge tubes was changed with collection tubes and 30 µl of dH2O was added into columns. Lastly, they were centrifuged at 13000 rpm for 1 min. Columns are incubated at RT for 1 minute. DNA concentration of plasmid DNA was measured using spectrophotometric measurement

In the other hand ZymoPURE ™ Plasmid Miniprep Kit was used according to the protocol. The first centrifugation was modified for 10 minutes at 8000rpm. Plasmid isolation from the cloning was performed successfully. Plasmids are stored -20°C

2.2.8. Cell Culture

Standard cell culture procedure of our AxanLab was used. For SH-SY5Y human neuroblastoma cell line and NSC34 mouse motor neuron cell line also U87 glioblastoma cell line, standard culture medium DMEM with 4,5g/L glucose, L-glutamine, pyruvate addition of 10 % FBS and 1 % penicillin/streptomycin antibiotics was used.

First step is washing by PBS (phosphate buffered saline) to remove cell

wreckage that are not bound or dead cells. Then 0.05% 1X Trypsin-EDTA (Gibco™) is added into flask. and incubate the cells for 2-3 minutes at 37 °C. The complete medium is added into flask to inhibit the trypsin and cells are collected from flask or plate to falcon tube. Then the cells are centrifuged at 1500 rpm for 5 minutes. And supernatant is discarded. Suspended pellet cells counted with hemocytometer. Wish for amounts of cells moved into new flask or plate with required quantity of complete medium. The cultured cells are incubated 37°C and 5% CO_2 placed. In the cell culture we used T75 or T250 flask to place. If cells reach 80% confluency in the place, they should be subculture.

For the storage of cells in -80°C or liquid nitrogen, cryotubes and DMSO are used to

2.2.9. RNA isolation

Pure Link RNA Mini Kit (Thermo Fisher Scientific) kit was used for RNA isolation. Initially Medium of cultured cells are discarded. Than next step is scraping 200 µl PBS is added on plate to collect cell with scraper into tube. Tubes are centrifuged at 2000g for 5 minutes and supernatant are discarded. Then 600 µl lysis buffer with 6 µl 2-mercaptoethanol added to sample and tubes are vortexed. In order to homogenization the mix is then passed from syringe 15 times. After that 600 µl ethanol are added and vortex. Up to 700 µl sample transferred to column. Columns are centrifuged at 12000g for 15 seconds. Repeat two times. 700 wash I was added to the column and centrifuged at 12000g for 15 seconds. 500 wash II was added to the column and centrifuged at 12000g for 15 seconds again. Repeat two times. Prepared new collection tube. To remove ethanol, columns are centrifuged at 12000g for 2 minutes without addition any solution and columns are transferred into clean collection tubes. This step is dry centrifugation. And finally, elution step for RNA isolation. 30 µl RNase-free water are added into column than incubated for 1 minute and columns are centrifuged at maximum speed for 2 minutes. RNA samples are stored at -80°C.

2.2.10. Genomic DNA Isolation

Genomic DNA isolations used in the experiments were performed using PureLink ™ Genomic DNA Mini Kit (Invitrogen ™). First, cells from -80 degrees are

thawed using non-sterile 200 microliters of PBS. Add 20 µl proteinase K into 1.5 mL or 2 mL microtubes. Transfer the dissolved cells with PBS into the prepared tube. 20 µl RNase A is added into the tube. Incubate at room temperature for 2 minutes. Add 200 µl of PureLink Genomic Lysis / Binding Buffer and vortex. Incubate for 10 minutes at 55 degrees in the next step to promote protein digestion. Add 200 µl of lysate to 96-100% ethanol and vortex. proceed to the purification stage. The sample is taken to the column. Centrifuge for 1 min at 1000g. The supernatant is poured. After washing process with buffer by 2 min maximum speed dry process completed. DNA is dissolved with 50 microliters of water and made ready for use.

2.2.11. Colony Verification PCR

MyTaq DNA polymerase (Bioline) kit was used for colony PCR. Firstly, white colonies with ampicillin resistance from LB agar plates were carefully selected. The concentrations of plasmids obtained from isolation from selected colonies were measured and diluted to 100 nanograms in microliter. pcr reaction total volume 25 microliters 5X buffer 10µl enzyme polymerase 0.5µl 10mM dNTP mix 1µl forward primer 1,25 µl reverse primer 1,25µl dH2O was performed in PCR tubes for each colony in quantities of 15µL (up to 25µL). PCR amplicons were run in 1% agarose gel. after that visualized with Bio-Rad ChemiDoc Imaging System. Bacterial colonies that gave positive results were picked to further use in cloning experiments.

2.2.12. Testing RNA Quality

To testing RNA integrity, 1 % MOPS (morpholinopropanesulfonic acid) agarose gel electrophoresis protocol was used. The reagents used in the experiment are: Agarose (electrophoresis grade), Formaldehyde (37%; ACS grade), Formamide, MOPS (morpholinopropanesulfonic acid) buffer (10×), RNA sample of interest, diluted in H2O to the appropriate concentration, size marker (0.5-to10-kbRNA marker ladder Invitrogen), Stain to detect RNA markers, Tracking dye.

Firstly, Formaldehyde-Agarose Gel is prepared: 1.5%, 15×15-cm gel (150-mL volume) as follows Mix 2.25 g of agarose and 109.5 mL of H_2O. Melted the agarose in a microwave and then cooled it to 65˚C. This step has to be in a hood! Added 15

mL of 10×MOPS buffer and 25.5 mL of 37%formaldehyde; mix well. MOPS is sensitive to high temperatures formaldehyde is dangerous. Therefore, they should not add to the gel mixture until it cools slightly. Then, in a chemical fume hood, the agarose mixture is poured into a 15 × 15 cm gel tray. Allow the gel to solidify while placing the comb. Next step is dilution of 10x MOPS. Enough 1 × MOPS buffer was prepared for the gel tank reservoirs by diluting with 10 × stock H2O. Place the gel in the tank and allow 1 × MOPS buffer before the gel could dry in the hood. Next step is Preparation of RNA Samples for Electrophoresis. Add 4.7 µL of RNA sample to a 1.5 mL microcentrifuge tube. A new sample buffer stock containing formamide, 10 × MOPS and formaldehyde was prepared as follows. Ultrapure formamide 660 µL, MOPS buffer (10×) 200 µL, Formaldehyde (37%) 270 µL Total 1.13 mL. Than 11.3 µL of sample buffer was added to the reaction tube containing 4.7 µL of RNA. The tube was heated at 60 ° C for 5 minutes and cooled on ice. 4 µL of monitoring paint added. The total volume was adjusted to 20µL.

Final step is Electrophoresis and Visualization of RNA. The gel was loaded with RNA samples and size markers and operated at room temperature at low voltage (40-50 V) until it came down to the gel. Electronic imaging was done in UV light and data was saved.

2.2.13. cDNA Synthesis

For cDNA synthesis, RNA concentrations obtained primarily from the u87 cell line are checked. then 50 nanograms are diluted to 10 microliters. The reagents used in the experiment are: iScript cDNA synthesis kit (BioRad). 4µl reaction mix, 1µl reaction enzyme, 500np/µl RNA template up to 20µl dH_2O.

25°C for 5 minutes 42°C for 30 minutes 85°C for 5 minutes Forever 4°C Synthesis is performed by adjusting the reaction protocol. cDNAs can be stored at -20°C for short periods and at -80°C for long periods.

2.2.14. Quantitative Real Time PCR

Expression analysis was performed with qPCR by using SsoAdvanced™ Universal SYBR® Green Supermix (Bio Rad). TPP Tıssue Culture Plates, 96 wells

were used. The plate was sealed with optically transparent film, tightly. Then in order to get rid of any remaining air bubbles and to collect the reaction mixture in the well bottom and PCR plate was centrifuged at 1,000 rpm for one minute.

Mix was prepared which is contain SYBR Green, F primer, R primer and cDNA as blow table. 5 ng cDNA was used.

Table 2.5: Preparation of mix for Real Time PCR.

MIX	PEA3	hKLK6	hKLK8	hKLK10	ACTNB	GAPDH
Sybr Green	80	80	80	80	80	80
F primer	8	8	8	8	8	8
R primer	8	8	8	8	8	8
Water	32	32	32	32	32	32

Volume per reaction component was used for SYBR Green 5µl Forward Primer 0.3-0.4µl Reverse Primer 0.3-0.4µl Template 2.5ng/µl Water Variable Total 10µl. In the experiment I used 2 biological replicate which is passage 24 and passage 25 and each biological replicate repeated two times (Technical Repeat) with U87 cells. Biological group was PC-DNA3, Pea3 and Pea3Vp16. And used non template control.

Table2.6: Wells 96 design for Real Time PCR Reaction.

	PEA3		hKLK6		hKLK8		hKLK10	
PC	PC	PC	PC	PC	PC	PC	PC	PC
PEA3	PEA3	PEA3	PEA3	PEA3	PEA3	PEA3	PEA3	PEA3
PEA3V P16	PEA3V P16	PEA3V P16	PEA3V P16	PEA3V P16	PEA3V P16	PEA3V P16	PEA3V P16	PEA3V P16
PC	PC	PC	PC	PC	PC	PC	PC	PC
PEA3	PEA3	PEA3	PEA3	PEA3	PEA3	PEA3	PEA3	PEA3
PEA3V P16	PEA3V P16	PEA3V P16	PEA3V P16	PEA3V P16	PEA3V P16	PEA3V P16	PEA3V P16	PEA3V P16

3. RESULTS

3.1. Promoter Analysis Results

In promoter analysis to determination of the possible PEA3 / ETV4 targets as a first step of the in silico analysis the genes were retrieved according to their function. Transcription Regulatory Element Database (TRED) was used to access promoter sequences from databases in initial studies. After the gene is determined, it is put into the search bar and the tool lists the promoter sequences and ranks them according to reliability as known curated, known and predicted sequences. So that this database gives to user chance to select start and end sites of the promoter sequence as flanking around the transcription start site (TSS).

In order to analyze all putative binding sites for the transcription factor of interest, Than PROMO3.0 tool was used (URL: http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo).

```
AGACCCAGGAGTCCAGGCCCTCACTGCACTCAGGGACCAGTGCTCCCTTCCCTGGAGGCCTGGTCAGG
TCACCAAGAGCAGAGCGTGGGGGCGGGAGGAATGTGTGTGGGAGGCCTGGGTAAGGAGGAAAAGGGTG
GCCAGTCTCCTGGCTCAGGGACCTGAGAGACAGGGGTTAAAAGGACGTTCCAGAAGCATCTGGGGACA
ACCAGCCTCTTCCAGGGAGGCCTGGGAGCTGGGGGTGTGTGTCTGGCAGTCCCTGCAGCCCTGGGCTC
CGGCCCCTGCGTCCTCCGCTTGGCTCTGCCACTGCATCTGAGTGTCTTCTCTCCTCACGGCTCCCCGC
TTCTAACTCTTTCTGCCTCCTCGTCTCAAAGCTGTTCCTTCCCCCGACTCAAGAATCCCCGGAGGCCC
AGGCCTGCAGCAGGAGCGGCCATGAAGAAGCTGATGGTGGTGCTGAGTCTGATTGCTGCAGGAATCTT
GGTCTTCCTGGGGAAGCATAACCTTCGGCAAAGGGAGAGTTCCCAGGAGCAGAGTTCTGTTGTCCGGG
GTGATCCACCCTGACTATGATGCCGCCAGCCATGACCAGGACATCATGCTGTTGCGCCTGGCACGCCC
CCAAACTCTCTGAACTCATCCAGCCCCTTCCCCTGGAGAGGGACTGCTCAGCCAACACCACCAGCTGC
CATCCTGGGCTGGGGCAAGACAGCAGATGGTGATTTCCCTGACACCATCCAGTGTGCATACATCCACC
GTGTCCCGTGAGGAGTGTGAGCATGCCTACCCTGGCCAGATCACCCAGAACATGTTGTGTGCTGGGGA
AGAAGTACGGGAAGGATTCCTGCCAGGGTGATTCTGGGGGTCCGCTGGTATGTGGAGACCACCTCCGA
CCTTGTGTCATGGGGTAACATCCCCTGTGGATCAAAGGAGAAGCCAGGAGTCTACACCAACGTCTGCA
TACACGAACTGGATCCAAAAAACCATTCAGGCCAAGTGACCCTGACATGTGACATCTACCTCCCGACC
CCACCCCACTGGCTGGTTCCAGAACGTCTCTCACCTAGACCTTGCCTCCCCTCCTCTCCTGCCCAGCT
GACCCTGATGCTTAATAAACGCAGCGACGTGAGGGTCCTGATTCTCCCTGGTTTTACCCCAGCTCCAT
TTGCATCACTGGGGAGGACGTGATGAGTGAGGACTTGGGTCCTCGGTCTTACCCCCACCACTAAGAGA
ACAGGAAAATCCCTTCTAGGCATCTCCTCTCCCCAACCCTTCCACACGTTTGATTTCTTCCTGCAGAG
CCAGCCACGTGTCTGGAATCCCAGCTCCGCTGCTTACTGTCGGTGTCCCCTTGGGATGTACCTTTCTT
CTGCAGATTTCTCACCTGTAAGATGAAGATAAGGATGATACAGTCTCCATAAGGCAGTGGCTGTTGGA
GATTTAAGGTTTCACACCTATGACATACATGGAATAGCACCTGGGCCACCATGCACTCAATAAAGAAT
                        ATTTTATTATGAAAAAAAAAAAAAAAA
```

Figure 3.1: KLK6_Human binding site from ALGGEN for PEA3 Sequence.

This tool analyzes the promoter regions for selected transcription factor and displays results as dissimilarity score. Dissimilarity rate basically suggests the variance between the consensus binding motif for the selected transcription factor and the

putative binding site on the target promoter as percentage So smaller number is a pointer of higher possibility for PEA3/ETV4 binding. When promoters of these genes were analyzed, they were choose predicted to contain one or more putative Pea3 binding motifs that exhibit % 0– %8 dissimilarity rate was shown Figure 3.2. Figure 3.3. ad Figure 3.4. Green highlight show that PEA3 bindig site and Yellows highlight show primers in the Figure 3.1.

PEA3 [T00685] was predicted in:

Sequence	ACTCATCCA	CCACATCCT	CACCATCCA	ATACATCCA	GGGATGAGA	TAACATCCC	CTCCATCCT	GGGATGTAC	AGGATGATA
	644	652 698	706 743	751 758	766 835	843 926	934 1183	1191 1383	1391 1432 1440
Dissimilarity	7.42%	1.19%	10.54%	3.71%	9.94%	6.82%	6.82%	6.82%	3.71%
RE equally	0.26899	0.05380	0.21519	0.07173	0.14346	0.17933	0.17933	0.17933	0.07173
RE query	0.30070	0.04953	0.25117	0.05599	0.14197	0.16996	0.16996	0.16996	0.05599

Consensus sequence and matrix:

A	0	0	2	0	3	0	0	0	1
C	0	0	0	3	0	0	3	3	0
G	0	0	0	0	0	0	0	0	0
T	1	1	1	0	0	3	0	0	2
	T	T	A	C	A	T	C	C	T

Figure 3.2: Putative Pea3 binding motifs for hKLK6.

sequence **mKlk6** **from** -1000 **to** 100 bp relative to TSS

```
TCCGGGGAACAGACAATGACCGATTGTTGGAAGTCTCTTTCCCCATATTGCTCATGCCGC
TTCCCAGCCTGGGTTAGGGGCGGGGGACAGTTTAGTGAAACATCCTTTCTCAATAGTTGG
GTGTCAGCTCCCACTCTCCTTGGGACACGGAGGTCTTCAGCACCCACAGAGGGACTTACA
GGTAGGTGAGTCAGCCCTGACCCCACTGAACCTGGGCAGCTGGTTCCAGGGCCTCAGAGA
TACAAACCCAGGAGAAGAAGCTGCAGGATCCCCAGAGGATATAGGTGGGGTACCTGTGGA
GGCCGGGGCACCACTGGCTAACCTGGGAACGGGGCATTTAGTCCTCAGTCTTATTATGTC
ACAAATCCTACAAATCCAAAAGCAAGAATGAGCTCCCTAAAGGGATCTGCAATCCTATAG
GTGGAACAACAATATGAACTAACCAGTACCCCGGAGCTCTTGACTCTAGCTGCATATGTA
TCAAAAGATGGCCTAGTCGGCCATCACTGGGAAGAGAGGCCCATTGGACTTGCAAACTTT
ATATGCCCCAGTACAGGGGAATGTCAGGGCCAAAAAGTGGGAGTGGGTGGGTAGGGGAGG
GGGGGAGGGTATGGGGGACTTTTGGGATAGCATTGGAAATGTAATTGAGAAAAATATGTA
ATAAAAAAATATATATAAAAGAATGAGCTCCAATCTCTCTCTACCTCAGTTCTAAAGGTT
TCAGCCCAACCCTCCTATTTATGTCCAGCCTTTCTCCTTCAGATCCAGGCGAACAGACCC
ATTTCTTCCCTCAAAATTCAGAGATCTGGGGTCACTGTGTTCCAGAGTGGGTCACTGGCT
TCCCTGTAGCTATCTGGTCAAGGGTCACCAGAAGCAGAGCTGTGGGGCAGAAGGAATGCA
TGTGGGCGGCTTAGGTGAAGAAAAGGGCGTGACTGGCTTCCTCCTGGCTCAGAGACTAGA
AGAGAGAGGGGTTAAAAAGGAGCTGGGAGACTCTGAGAACAGAGTCCGCCTCTCTCTGGG
AGGCCACAGAGCCAGGAGGGTTTCTGGAGACAGTCTGCTACTTGGGACCCAGCATCCAAG
                    TGCCCCATATTTCATGCAGGT
```

Figure 3.3: KLK6 Mouse binding site from ALGGEN for PEA3 Sequence.

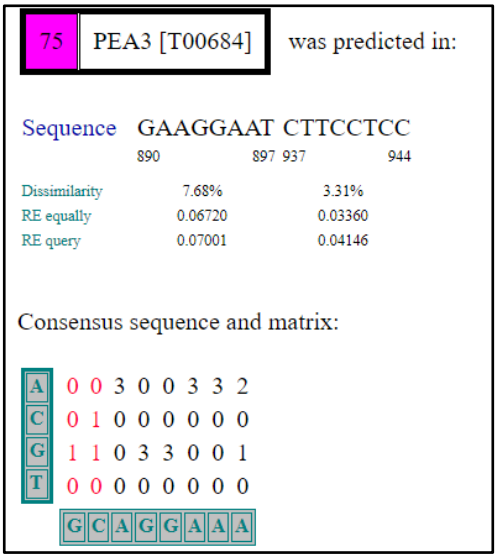


Figure 3.4: Putative Pea3 binding motifs for mKLK6.

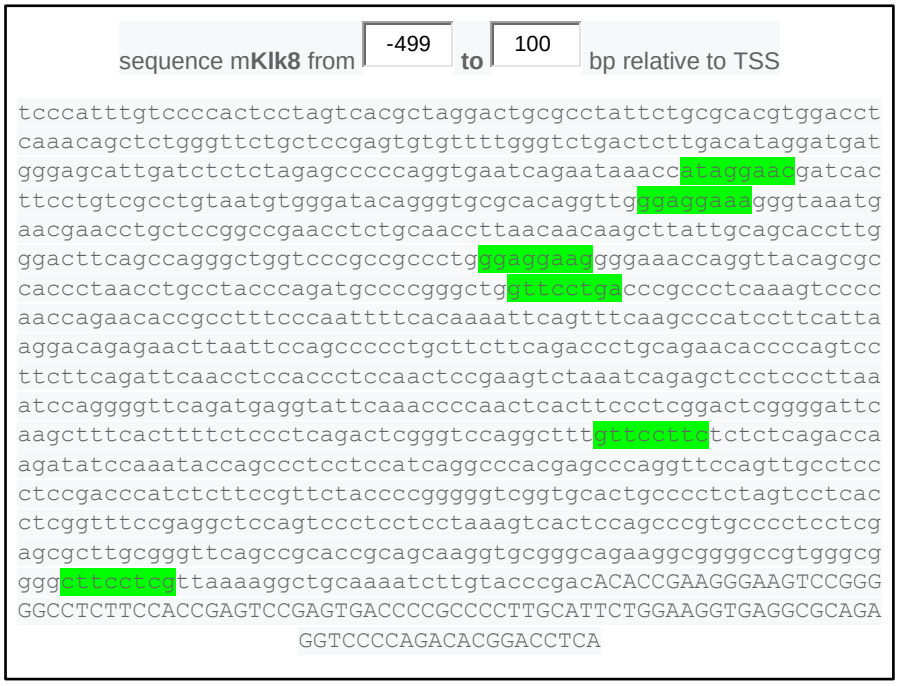


Figure 3.5: KLK8 Mouse binding site from Promo 3.0 for PEA3 Sequence.

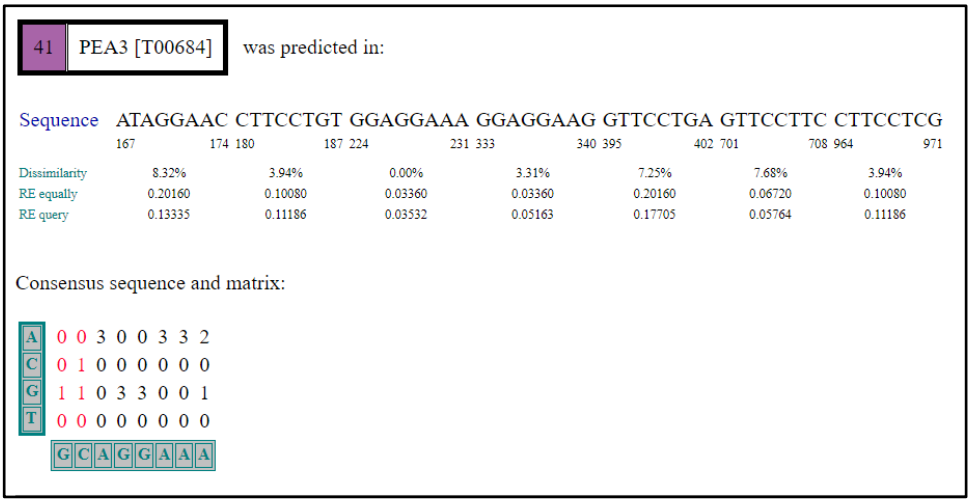


Figure 3.6: Putative Pea3 binding motifs for mKLK8.

3.2. Primer Design

In order to amplify promoter regions from the genomic DNA, specific primers were designed. Promoter sequences were taken from TRED and sites appropriate for primer binding were determined. Then their parameters from the point of, GC content, melting temperature and secondary structure potentials were screened by Oligo Analyzer 3.0 (IDT)[29]. Their binding potentials through the genomic DNA were determined by Primer Blast (NCBI). A flanking region and restriction enzyme recognition sites were added to the terminal site of the primers.

Table 3.1: Primers were Designed.

	Sequence	GC	TM	R.E.
hKLK6_pGL3_HindIII_F	CAC AGA GAG TGC TGG GGG	66.70%	58 °C	ctcgag
hKLK6_pGL3_XhoI_R	CGG GGA TTC TTG AGT CGG	61.10%	55.3 °C	aagctt
hKLK8_pGL3_MluI_F	CTG GCG GAG ACC TGG GGG	77.80%	63.5 °C	ACGCGT
hKLK8_pGL3_BglII_R	CCA GAA TCC AGG GGC GGG GTC	71.40%	64.8 °C	AGATCT
mKLK6_pGL3_HindIII_F	GGG GAA CAG ACA ATG ACC	55.60%	53 °C	ctcgag
mKLK6_pGL3_XhoI_R	AAT ATG GGG CAC TTG GAT G	47.40%	52.5 °C	aagctt
mKLK8_pGL3_MluI_F	CCC ATT TGT CCC CAC TCC	61.10%	55.8 °C	ACGCGT
mKLK8_pGL3_BglII_R	GGT CAC TCG GAC TCG GTG	66.70%	57.7 °C	AGATCT

Table 3.2: Primers with Restriction enzymes.

Sequence	GC	TM	R.E.
GAGA ctcgag CACAGAGAGTGCTGGGGG	57.10%	66.6 °C	XhoI
GAGA aagctt CGGGGATTCTTGAGTCG G	53.60%	62.3 °C	hindIII
GAGA acgcgt CTG GCG GAG ACC TGG GGG	71.40%	70.5 °C	mlu
GAGA agatct CCA GAA TCC AGG GGC GGG GTC	61.30%	67 °C	bgl
GTGT ctcgag GGG GAA CAG ACA ATG ACC	57.10%	63.7 °C	XhoI
GTGT aagctt AAT ATG GGG CAC TTG GAT G	44.80%	59.3 °C	hindIII
CTCT acgcgt CCC ATT TGT CCC CAC TCC	60.70%	65.5 °C	mlu
CTCT agatct GGT CAC TCG GAC TCG GTG	57.10%	62.4 °C	bgl

3.3. Gradient PCR for Designed Primers

First, to clone the promoter region of KLK the annealing temperature was determined by gradient PCR. For this purpose, regular PCR reaction as described in the section of methods was followed and annealing temperature was selected within the range of 55°C to °65C (Figure 3.7.).

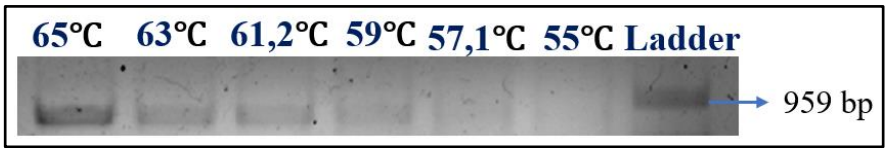


Figure 3.7: Gradient PCR for mKLK6.

65°C was selected as annealing temperature and the next reaction was setup according to this gradient PCR result.

3.4. Promoter Cloning

According to selected as annealing temperature as described in the method section mKLK6 was amplificated by Phusion DNA polymerase. After this step, DNA was purified, both the insert and pGL3 plasmid were digested by the restriction enzymes HindIII and MluI (Figure 3.8.) This process was followed by ligation procedure through T4 ligase, transformation of bacteria and spreading them on the

agar plate containing a type of antibiotic (ampicillin). The transformed bacterial cells were grown on Ampicillin containing (Amp+) plates growth agar. As mentioned in Methods (page 13), colonies were observed at a ratio of 1: 3 vector insert for mKLK6. In the next stage plasmid isolations were made from the colonies taken. Next step was colony PCR to verification. Experiments with MyTaq DNA polymerase (Bioline) kit gave positive results and samples were prepared for sequencing.

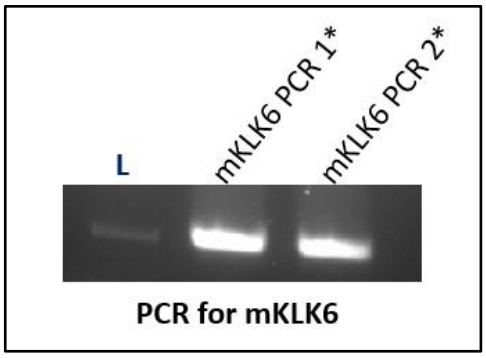


Figure 3.8: PCR for mKLK6 Template was NSC-34 ThermoSicentic High- Fidelity.

The PCR reaction prepared at 50 µl microliters total volume for each tube was loaded equally into two wells of agarose gel.

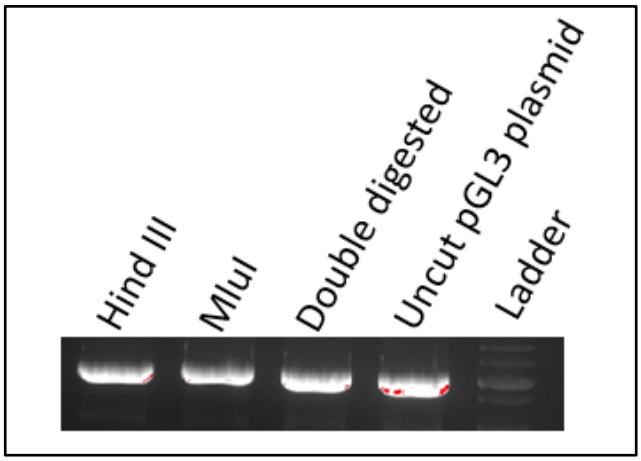


Figure 3.9: Digestion of plasmid empty pGL3 by Restriction Enzymes Hind III (3 Hour) Mlu I (2 Hour) at 37°C to optimize.

There was problem about digestion of enzymes. Therefore, optimization was performed for temperatures and durations. The experiment was continued with the

selected parameters. For Hind III restriction enzymes 3 hour for Mlu I enzymes 2 hour at 37°C in water bath.

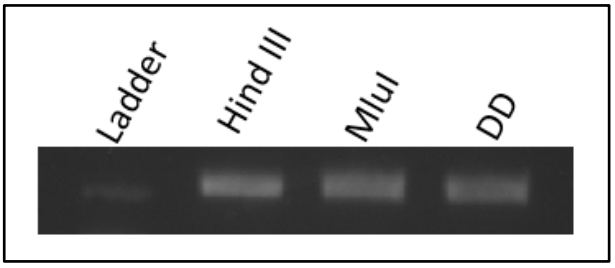


Figure 3.10: Digestion for mKLK6 by Restriction Enzymes Hind III (3 Hour) Mlu I (2 Hour) at 37°C *DD : Double Digested plasmid.

Digestion was performed for the mKLK6 promoter to be cloned into the pGL3 vector at 37°C in Figure 3.10.

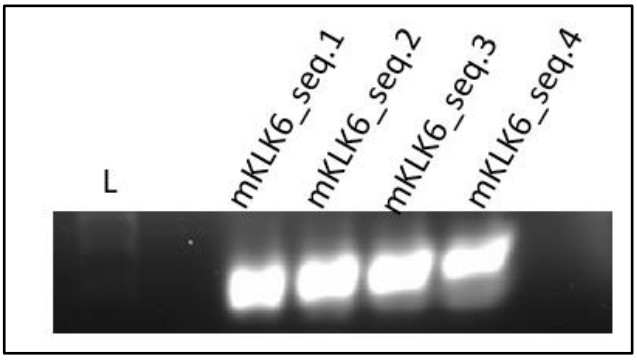


Figure 3.11: Colony PCR mKLK6-pGL3 plasmid. Ligation rate 3:1.

DNA concentrations after plasmid isolation were calculated respectively as 302,6 ng/µl; 411,0 ng/µl; 354,5ng/µl; 319,2 ng/µ in colony PCR Figure 3.11.

After promoters were amplified by PCR with the appropriate target primers and cloned into vector plasmids, they were sent forsequencing (MedSanTek Ankara) for confirmation.

3.4.1. Sequences and Alligment Result for Cloned Plasmids

An example of sequencing data of one of recombinant plasmid. DNA sequencing is done by MedSanTek Ltd Şti. Ankara. Results of DNA sequencing is provided electronically as AB1 files and FinchTV free software from Geospiza Inc was used to

process these files in figure 3.12. Sequences were obtained by doing the same with other samples for the same method. In the conversion of sequences to fasta format, Chromas | Technelysium Pty Ltd application was used.

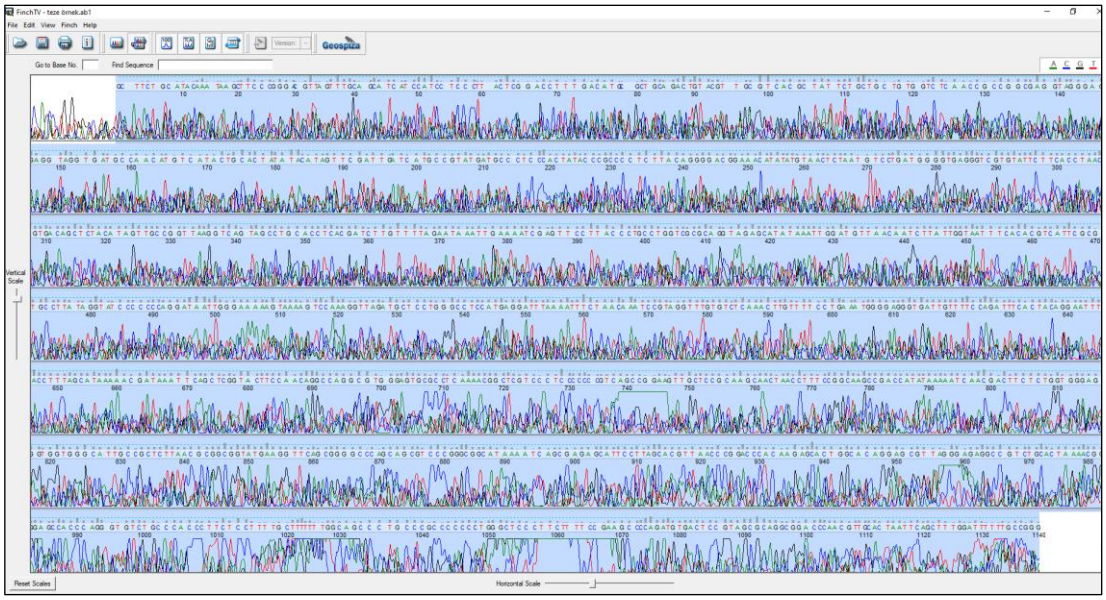
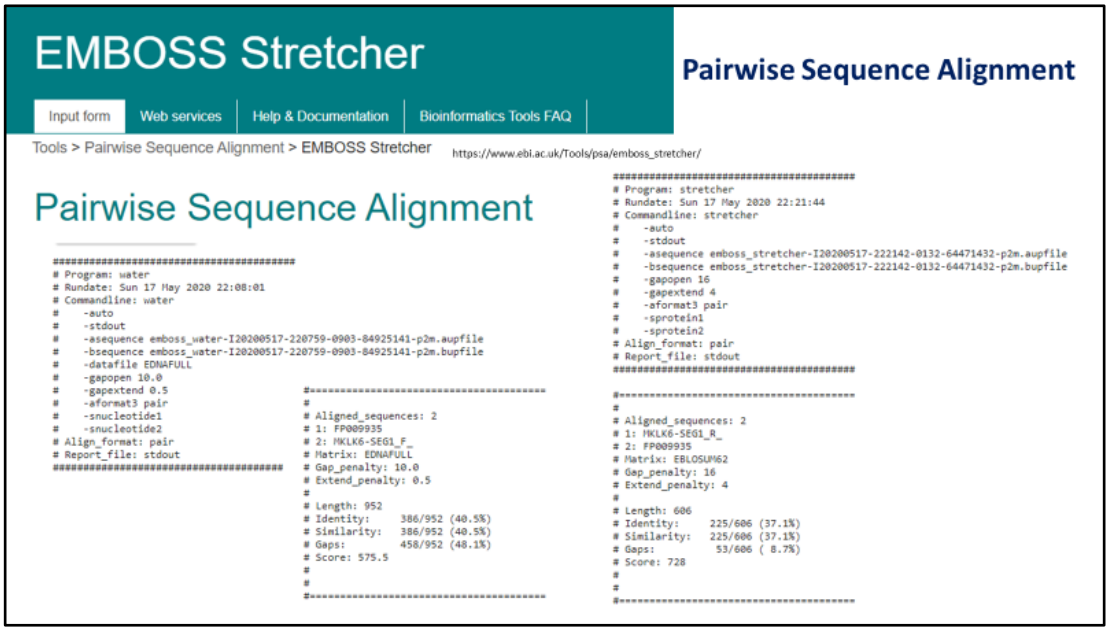


Figure 3.12: Chromotogram for mKLK6 promoter inserted plasmid sequence.

I use Multiple Sequence Alignment by CLUSTALW which is a general purpose DNA or protein multiple sequence alignment program for three or more sequences.I comperad obtained fasta sequence from promoter coloned plasmid with promoter database sequence by CLUSTALW

Alingment with ClusterW was also performed for other colonies sent to sequencing. And similar results have been obtained. Sequence studies show that there are problems with cloning. Alingnment not as expected. Then ı use another alignment tool wich is EMBOSS to Pairwase Sequence alignment and Multiple sequence alignment in Figure 3.13. Figure 3.14. Figure 3.15 and figure 3.16.



EMBOSS Stretcher

Pairwise Sequence Alignment

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Tools > Pairwise Sequence Alignment > EMBOSS Stretcher https://www.ebi.ac.uk/Tools/psa/emboss_stretcher/

Pairwise Sequence Alignment

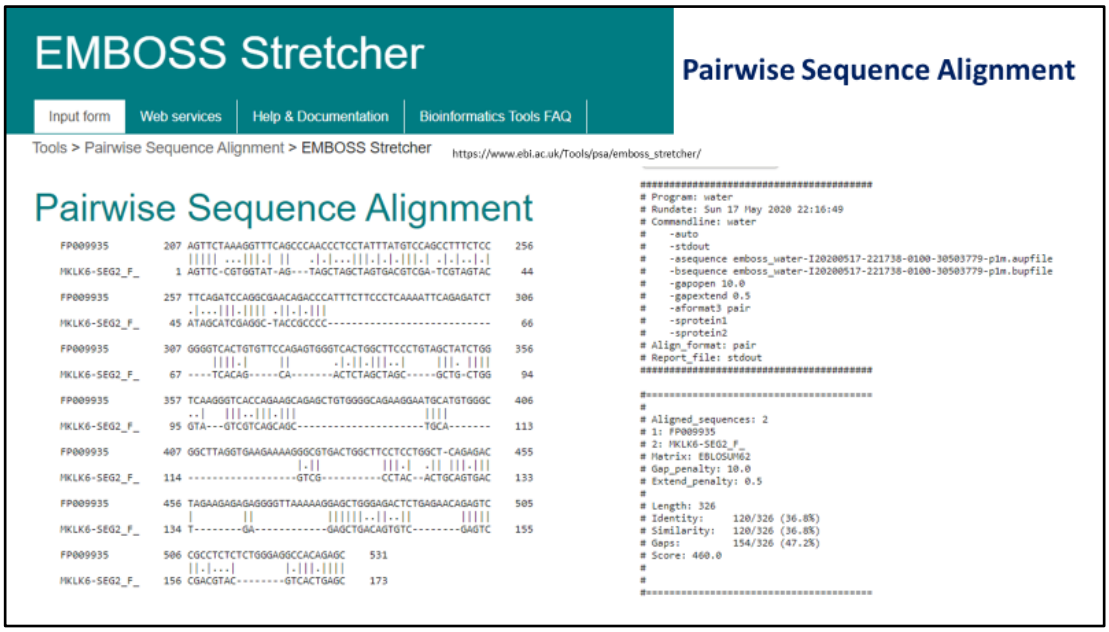
```
#######################################
# Program: water
# Rundate: Sun 17 May 2020 22:08:01
# Commandline: water
#    -auto
#    -stdout
#    -asequence emboss_water-I20200517-220759-0903-84925141-p2m.aupfile
#    -bsequence emboss_water-I20200517-220759-0903-84925141-p2m.bupfile
#    -datafile EDNAFULL
#    -gapopen 10.0
#    -gapextend 0.5
#    -aformat3 pair
#    -snucleotide1
#    -snucleotide2
# Align_format: pair
# Report_file: stdout
#######################################

#=======================================
#
# Aligned_sequences: 2
# 1: FP009935
# 2: MKLK6-SEG1_F_
# Matrix: EDNAFULL
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 952
# Identity:     386/952 (40.5%)
# Similarity:   386/952 (40.5%)
# Gaps:         458/952 (48.1%)
# Score: 575.5
#
#
#=======================================
```

```
#######################################
# Program: stretcher
# Rundate: Sun 17 May 2020 22:21:44
# Commandline: stretcher
#    -auto
#    -stdout
#    -asequence emboss_stretcher-I20200517-222142-0132-64471432-p2m.aupfile
#    -bsequence emboss_stretcher-I20200517-222142-0132-64471432-p2m.bupfile
#    -gapopen 16
#    -gapextend 4
#    -aformat3 pair
#    -sprotein1
#    -sprotein2
# Align_format: pair
# Report_file: stdout
#######################################

#=======================================
#
# Aligned_sequences: 2
# 1: MKLK6-SEG1_R_
# 2: FP009935
# Matrix: EBLOSUM62
# Gap_penalty: 16
# Extend_penalty: 4
#
# Length: 606
# Identity:     225/606 (37.1%)
# Similarity:   225/606 (37.1%)
# Gaps:          53/606 ( 8.7%)
# Score: 728
#
#
#=======================================
```

Figure 3.13: Pairwise sequence alignment for mKLK6 sample 1 result.



EMBOSS Stretcher

Pairwise Sequence Alignment

Input form | Web services | Help & Documentation | Bioinformatics Tools FAQ

Tools > Pairwise Sequence Alignment > EMBOSS Stretcher https://www.ebi.ac.uk/Tools/psa/emboss_stretcher/

Pairwise Sequence Alignment

```
FP009935        207 AGTTCTAAAGGTTTCAGCCCAACCCTCCTATTTATGTCCAGCCTTTCTCC    256
                    ||||| ...|||.| ||   .|.|...|||.|.|.|||.| .|.|..|.|
MKLK6-SEG2_F_     1 AGTTC-CGTGGTAT-AG---TAGCTAGCTAGTGACGTCGA-TCGTAGTAC     44

FP009935        257 TTCAGATCCAGGCGAACAGACCCATTTCTTCCCTCAAAATTCAGAGATCT    306
                    .|...|||.|||| .||.|.|||
MKLK6-SEG2_F_    45 ATAGCATCGAGGC-TACCGCCCC---------------------------     66

FP009935        307 GGGGTCACTGTGTTCCAGAGTGGGTCACTGGCTTCCCTGTAGCTATCTGG    356
                        ||||.|     ||      .|.||.|||..|    |||. ||||
MKLK6-SEG2_F_    67 ----TCACAG-----CA-------ACTCTAGCTAGC-----GCTG-CTGG     94

FP009935        357 TCAAGGGTCACCAGAAGCAGAGCTGTGGGGCAGAAGGAATGCATGTGGGC    406
                    ..|   |||..|||.|||                    ||||
MKLK6-SEG2_F_    95 GTA---GTCGTCAGCAGC---------------------TGCA-------    113

FP009935        407 GGCTTAGGTGAAGAAAAGGGCGTGACTGGCTTCCTCCTGGCT-CAGAGAC    455
                                    |.||         |||.|  .|| |||.|||
MKLK6-SEG2_F_   114 ------------------GTCG----------CCTAC--ACTGCAGTGAC    133

FP009935        456 TAGAAGAGAGAGGGGTTAAAAAGGAGCTGGGAGACTCTGAGAACAGAGTC    505
                    |          ||            ||||||..||..||        |||||
MKLK6-SEG2_F_   134 T--------GA------------GAGCTGACAGTGTC--------GAGTC    155

FP009935        506 CGCCTCTCTCTGGGAGGCCACAGAGC    531
                    ||.|...|        |.|||.||||
MKLK6-SEG2_F_   156 CGACGTAC--------GTCACTGAGC    173
```

```
#######################################
# Program: water
# Rundate: Sun 17 May 2020 22:16:49
# Commandline: water
#    -auto
#    -stdout
#    -asequence emboss_water-I20200517-221738-0100-30503779-p1m.aupfile
#    -bsequence emboss_water-I20200517-221738-0100-30503779-p1m.bupfile
#    -gapopen 10.0
#    -gapextend 0.5
#    -aformat3 pair
#    -sprotein1
#    -sprotein2
# Align_format: pair
# Report_file: stdout
#######################################

#=======================================
#
# Aligned_sequences: 2
# 1: FP009935
# 2: MKLK6-SEG2_F_
# Matrix: EBLOSUM62
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 326
# Identity:     120/326 (36.8%)
# Similarity:   120/326 (36.8%)
# Gaps:         154/326 (47.2%)
# Score: 460.0
#
#
#=======================================
```

Figure 3.14: Pairwise sequence alignment for mKLK6 sample 2 result and match sites shown in left.

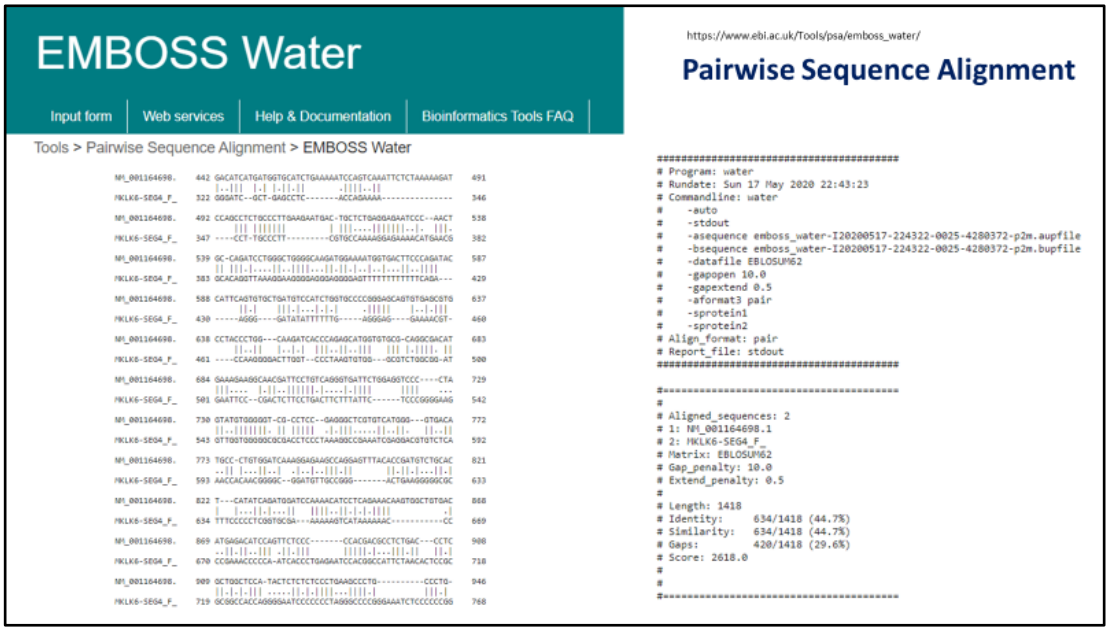


Figure 3.15: Pairwise sequence alignment for mKLK6 sample sequence.

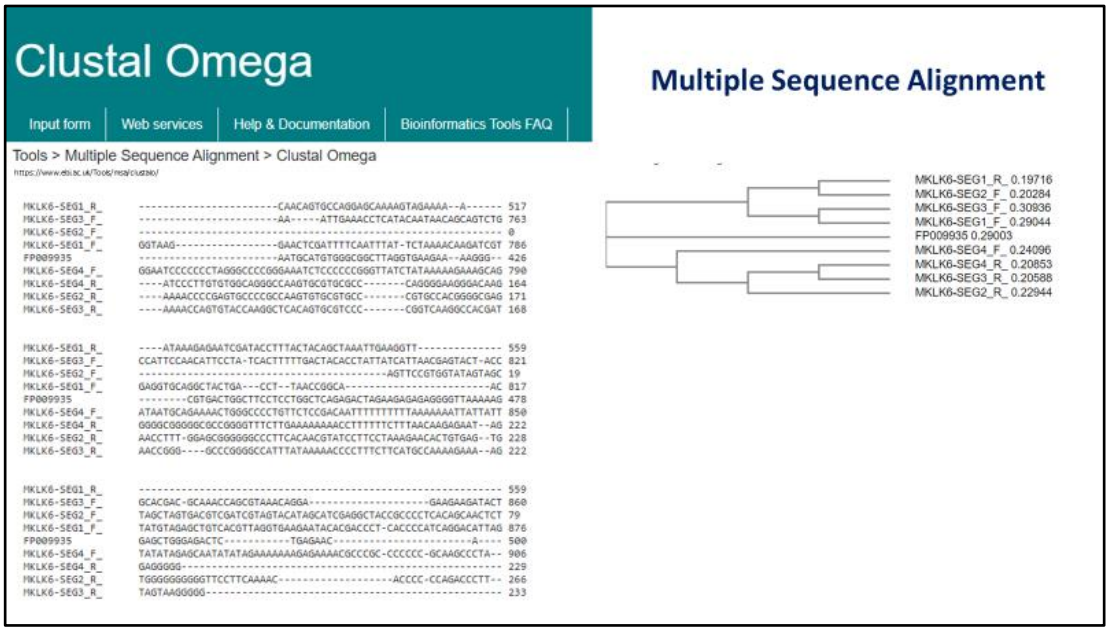


Figure 3.16: Multiple sequence alignment for all mKLK6 sample which sequenced similarity tree shown right.

This promoter sent for sequencing (MedSanTek Ankara) to confirmation Alingnment not as expected. The desired promoter region was not 100 % correctly cloned into the plasmid. They have avarege %40 idientity with template promoter sequence. remarkably this idiential sequence were like ETS family Pea3 conserved

sequece Even In this case, the next step, luciferas assayit won't be very reassuring they can try.

3.5. U87 Glioblastoma Cell Culture Transfection Optimization

To determine the amounts of components especially PEI ratio GFP optimization experiments was performed. Optimization shown below figures. The images were taken with ZOE Fluorescent Cell Imager (BioRad).

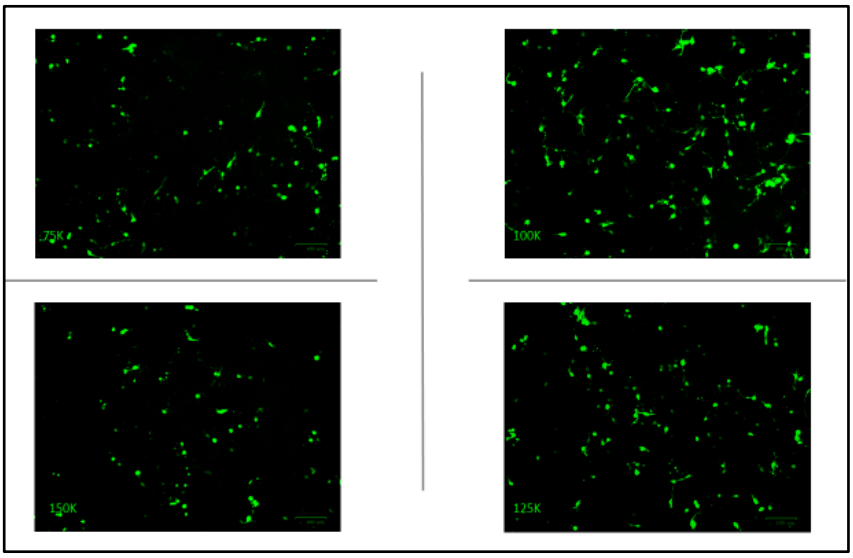


Figure 3.17: GFP optimization for U87 human glioblastoma cells 75K 100K 125K 150K 1:3 PEI/Plasmid.

In this experiments, both the white light and GFP images were taken and the appropriate GFP images were selected and shown in Figure 3.12. Figure 3.13. and Figure 3.14.

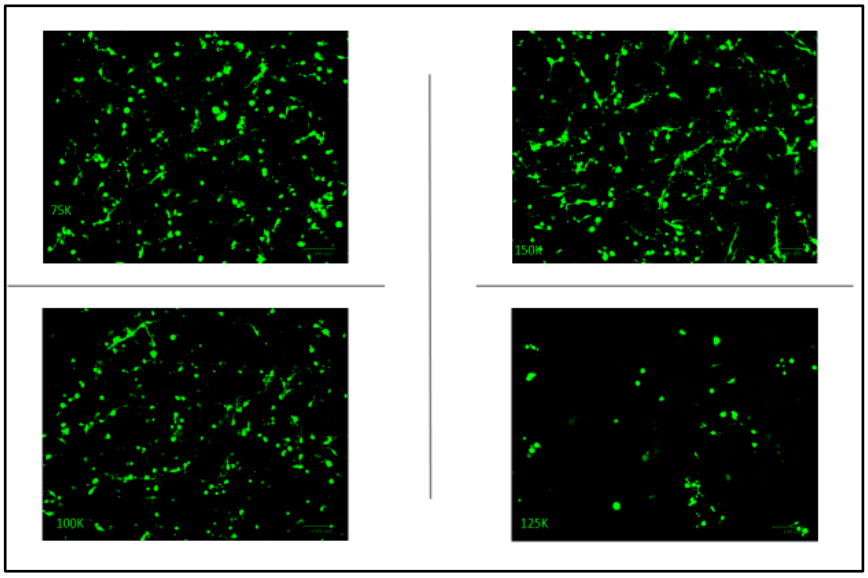


Figure 3.18: GFP optimization for U87 human glioblastoma cells 75K 100K 125K 150K 1:4 PEI/Plasmid.

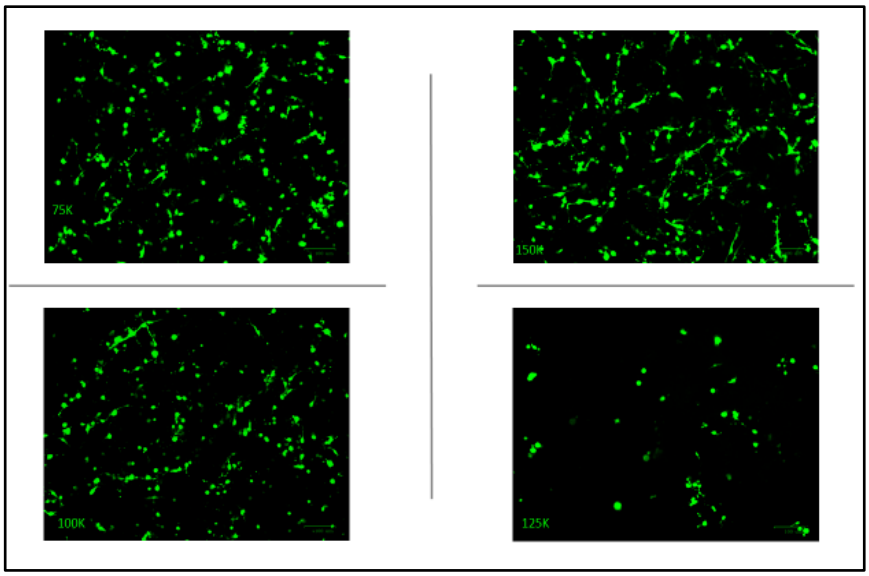


Figure 3.19: GFP optimization for U87 human glioblastoma cells 75K 100K 125K 150K 1:5 PEI/Plasmid.

As a result of experiments with GFP for transfection optimization, parameters with the best transfection at 1: 4 PEI / Plasmid ratio and 150K U87 cell concentration were determined.

3.6. Total RNA Isolation and Checking RNA Integrity

After the Transfection optimisation in the selected parameters process is performed overnight total RNA isolation were performed as shown in the method section. For elution RNase free water were used and their concentration were measured by UV spectroscopy. It was diluted and used for cDNA synthesis. The quality of RNAs had to be measured for the experiments. Hence a common method MOPS (morpholinopropanesulfonic acid) formaldehyde agarose gel electrophoresis method were performed according to the protocol of Donald C. Rio Denaturation and Electrophoresis of RNA with Formaldehyde[30]. imaging was performed with BioRad Image Lab 6.0.

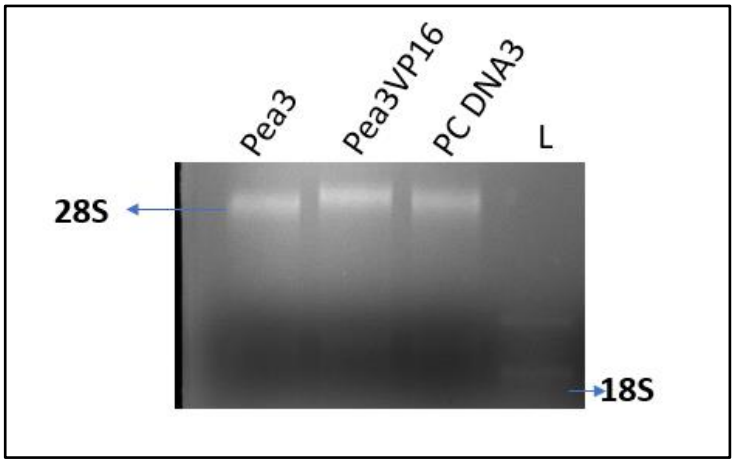


Figure 3.20: U87 Glioblastoma cell line passage 23 total RNA in formaldehyde agarose gel.

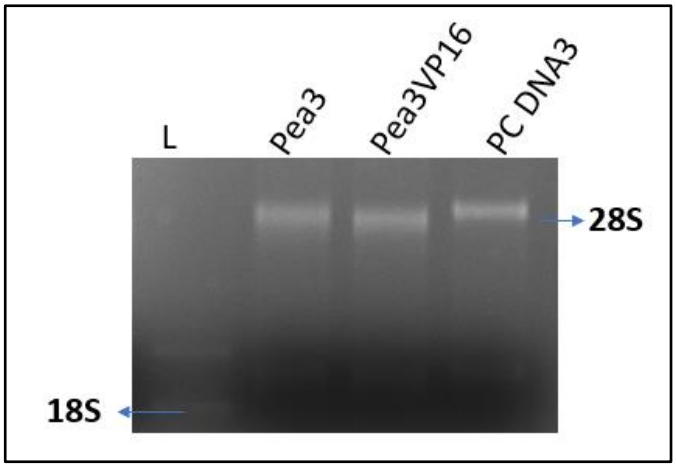


Figure 3.21: U87 Glioblastoma cell p24 total RNA in formaldehyde agarose gel.

3.7. Quantitative Real Time PCR Results

In the Q PCR experiments P24 and P25 U87 Glioblastoma cells were used as biological replicate. Biological groups were pcDNA3, Pea3 and Pea3Vp16 which is constructively active. Pea3, pCDNA3 and Pea3VP16 transfected cells performed qPCR to quantify relative expression of hKLK6, hKLK8, hKLK10. qPCR data was normalized by Gapdh and b-Actin as a housekeeping genes.

The difference of gene expression level between transfected cells and control was evaluated according C_T, ΔC_T, $\Delta\Delta C_T$ values. The endpoint of real-time PCR analysis is C_T, the threshold cycle. The C_T is determined from a log–linear plot of the PCT signal versus the cycle number. $2^{-\Delta\Delta C_T}$ were gave a liner form representing the factor change in the gene expression.

According to analysis result, relative expression of Pea3, KLK6, KLK8 and KLK10 increased. But no significant results were found in the statistical analysis. The results which relative expression fold change in U87 Glioblastoma cells shown in Figure 3.18. Figure 3.19. Figure 3.20. and Figure 3.21.

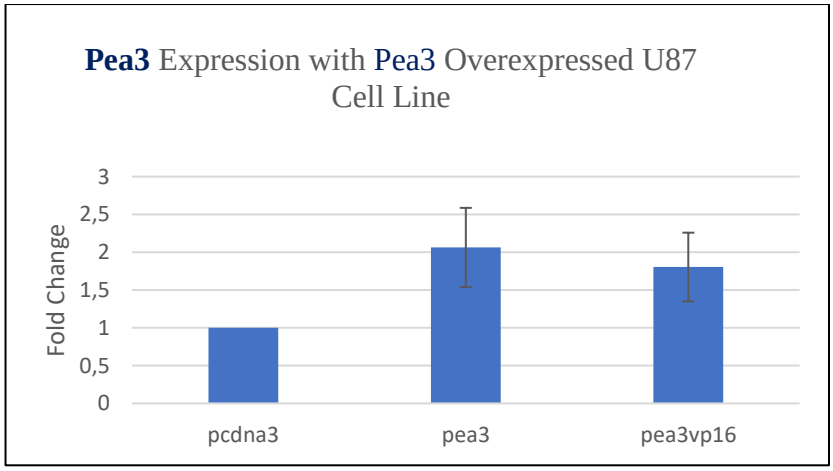


Figure 3.22: Relative expression of PEA3 in human U87 glioblastoma cells.

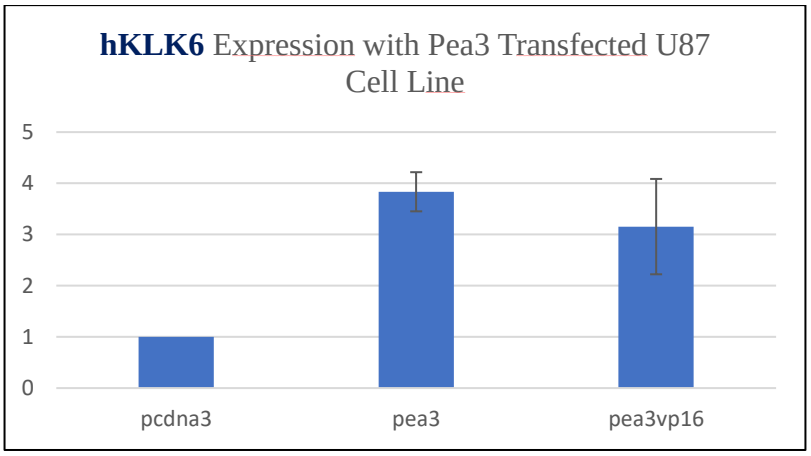


Figure 3.23: Relative expression of KLK6 in human U87 glioblastoma cells

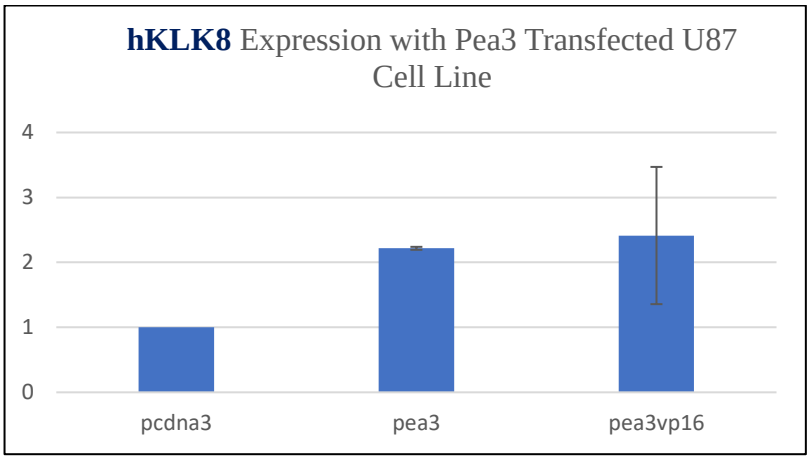


Figure 3.24: Relative expression of KLK8 in human U87 glioblastoma cells

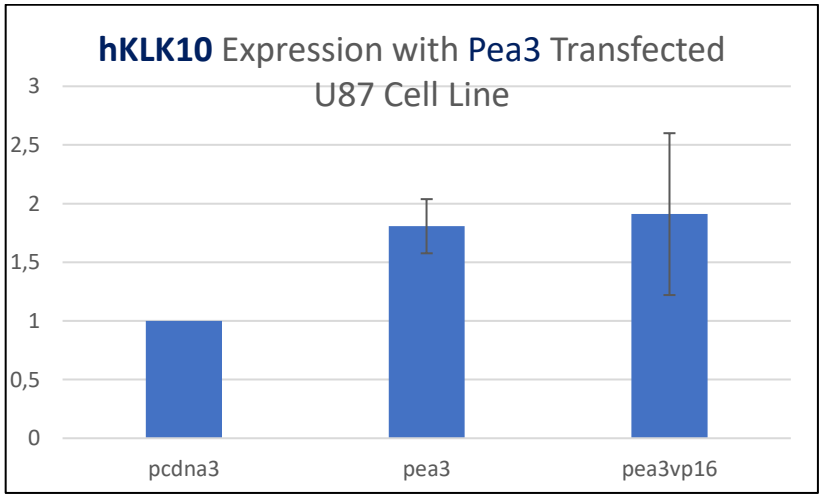


Figure 3.25: Relative expression of KLK10 in human U87 glioblastoma cells.

4. DISCUSSION and CONCLUSION

PEA3 Family members specifically know each other. If the cell expresses ER81, it is in the circuit with other ER81 expressing interneurons or motor neurons. Otherwise If PEA3 is expressed, it is only in the circuit with motor neurons that express PEA3. In other words, lookin at it by PEA3, it provides circuit selectivity. But this mechanism could not know clearly. PEA3 have a lot of phosphorylation motifs and It can go to different transcription factors by different phosphorylation. Studies are underway in our laboratory to understand these mechanisms. In the "Novel transcriptional targets of Pea3" article Kandemir B. et al. Identified potential new target promoters for regulation with transcription factors [9].

In this study we were focused transcription factor part and PEA3 regulation. According to the obtained data, the expression of kallikrein increases when the expression of Pea3 increases to. There is positive regulation between them, correlation can either be through direct regulation by Pea3 can do this directly. It can, by directly binding the promoter and regulating gene expression, or bind to the promoter of KLK or indirectly. Pea3 may indirect control KLKs by upregulation or downregulation. Besides, as mentioned in previous chapters, kallikreins are common targets both in cancer studies and axonal growth. In my thesis, I tried to clone the promoter regions of these targets. Samples were first sent to the sequence to understand that the promoter regions were not cloned correctly. Sequence results were evaluated with various bioinformatics tools.The results revealed an average of 40% similarity despite of this rate would be expected to be higher. If everything went well, luciferase assay would be done in the next stage and the effect would be examined.

On the other hand, in Real Time PCR works Relative expression of KLKs and PEA3 in human U87 glioblastoma cells were performed. In experiments with Three Biological groups (pcDNA3, Pea3 and Pea3Vp16) and two technical repeats although the expected change is observed statistically significant results could not be achieved.

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BIOGRAPHY

Bayram Kaş was born in Niksar / Tokat, 1992. He achieved her bachelor's degree from Department of Molecular Biology and Genetics, Erzurum Technical Universtiy in 2016. He has continued master degree program in the Department of Molecular Biology and Genetics at Molecular Neurobiology Laboratory, Graduate School of Natural and Applied Sciences, Gebze Technical University, since 2017. He presented posters in GTU Graduate Studies Symposium and Introductory Days 2018 namely Kallikreins and Investigation of Pea3 Related KLKs Regulation' during master degree program. He would like to study on neuroscience especially on synaptic plasticity, learning and AI.