

T.R.

GEBZE TECHNICAL UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

**THE ROLE OF PEA3 AND ELK1 IN NEURONAL CIRCUITS AND
METASTASIS**

MUSTAFA DOGUKAN METINER

A THESIS SUBMITTED FOR THE DEGREE OF

MASTER OF SCIENCE

DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

GEBZE

2020

T.R.

GEBZE TECHNICAL UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

**The Role of Pea3 and Elk1 in Neuronal Circuits
and Metastasis**

MUSTAFA DOGUKAN METINER

A THESIS SUBMITTED FOR THE DEGREE OF

MASTER OF SCIENCE

DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

THESIS SUPERVISOR

PROF. DR. ISIL KURNAZ

GEBZE

2020

T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

NÖRONAL DEVRE OLUŞUMU VE METASTAZDA
PEA3 VE ELK1'İN ROLÜ

MUSTAFA DOĞUKAN METİNER
YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
PROF. DR. IŞIL KURNAZ

GEBZE
2020



YÜKSEK LİSANS JÜRİ ONAY FORMU

GTÜ Fen Bilimleri Enstitüsü Yönetim Kurulu'nun 17/07/2020 tarih ve 2020/34 sayılı kararıyla oluşturulan jüri tarafından 10/08/2020 tarihinde tez savunma sınavı yapılan Mustafa Doğukan METİNER'in tez çalışması Moleküler Biyoloji ve Genetik Anabilim Dalında YÜKSEK LİSANS tezi olarak kabul edilmiştir.

JÜRİ

ÜYE

(TEZ DANIŞMANI) : Prof. Dr. Işıl KURNAZ

ÜYE

: Prof. Dr. Damla ARISAN

ÜYE

: Dr. Öğr. Üyesi Başak KANDEMİR

ONAY

Gebze Teknik Üniversitesi Fen Bilimleri Enstitüsü Yönetim Kurulu'nun

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

SUMMARY

Pea3 proteins are a subfamily of the ETS transcription factor superfamily, consisting of Pea3, Erm and Er81. These proteins, which are expressed in different tissues exhibiting branching, play a role in a variety of events such as formation of motor neuronal circuits in the nervous system, retinal differentiation, axon prolongation. These proteins, which have been studied extensively for many years in our laboratory, were recently found to be important determinants of axon elongation through novel transcriptional targets [Kandemir et. al., 2014; Kandemir et. al., 2017]. The next step has been to identify the specific target genes that play a role in the selectivity of motor neuron circuits. In this thesis, a microfluidic-based system has been established in our laboratory in order to provide an experimental system to mimic neuronal circuit formation. In this system, wildtype and phosphomutant versions of Pea3 have been transfected to NSC-34 motor neuron cell lines, axon elongation has been monitored and quantified by NeuronJ program. The findings of this successfully implemented project are expected to be used in studying motor neuron circuitry with different Pea3 family members and different cell types. In addition to this study, Elk-1, a member of the triple complex factor (TCF) which is a subfamily of ETS transcription factors, was determined as SOX2, NANOG and POU5F1 target genes in our laboratory and correlations of Elk-1 and these target genes in neuroblastoma cell line were investigated. In this study, it is aimed to investigate the correlation of ELK-1 and target genes in glioblastoma cell lines with qPCR method. According to the results of this study, it is thought that it will be helpful in developing Glioblastoma treatment methods.

Keywords: ETS Family, Pea3, Neural Circuit, Microfluidic, Elk-1, Glioblastoma.

ÖZET

Pea3 proteinleri, ETS bölgesi transkripsiyon faktörü süper ailesine ait bir alt grup olup, Pea3, Erm ve Er81'den oluşmaktadır. Branşlaşma gösteren farklı dokularda anlatımı görülen bu proteinler, sinir sisteminde motor nöron devrelerin oluşturulması, retina farklılaşması, akson uzaması gibi çok çeşitli olaylarda rol oynamaktadırlar. Laboratuvarımızda uzun yıllar çalışılmakta olan bu proteinlerin yakın zamanda grubumuzca akson uzama mekanizmaları deşifre edilmeye ve bu olayı hangi genler üzerinden kontrol ettikleri tanımlanmaya başlanmıştır [Kandemir vd., 2014; Kandemir vd., 2017]. Bundan sonraki aşamada bu tanımlanan genlerden hangisi veya hangilerinin, özellikle motor nöron – duyu nöronu devrelerindeki seçiciliğinde ve rol oynadığını aydınlatmak kalmıştır. Bu amaca yönelik olarak yürütülen bu tezde, nöronal devre oluşumunu taklit edecek mikroakışkan çip sistemi laboratuvarımızda kullanılmaya başlanmıştır. Bu sistemde, yabancı tür ve fosfo-mutant Pea3 versiyonları transfekte edilmiş olan NSC-34 hücrelerindeki akson uzamaları takip edilmiş ve NeuronJ programı ile kantifiye edilmiştir. Başarıyla yürütülerek sonuçlanmış olan bu projenin bulguları baz alınarak, bundan sonraki aşamalarda farklı Pea3 aile üyeleri ve değişik hücre tipleri ile motor nöron devre oluşumunu çalışmak mümkün olabilecektir. Bu çalışmaya ek olarak, ETS transkripsiyon faktörlerinin bir alt familyası olan üçlü kompleks faktörün (TCF) bir üyesi olan Elk-1'in, laboratuvarımızda yapılan çalışmalarda SOX2, NANOG ve POU5F1 hedef genleri belirlenmiş ve Elk-1 ve bu hedef genlerin nöroblastom hücre dizisindeki korelasyonları araştırılmıştır. Bu çalışmada glioblastom hücre dizilerinde ELK-1 ve hedef genlerin qPCR yöntemi ile korelasyonunun araştırılması amaçlanmıştır. Bu çalışmanın sonuçlarına göre Glioblastoma tedavi yöntemlerinin geliştirilmesinde yardımcı olacağı düşünülmektedir.

Anahtar Kelimeler: ETS Ailesi, Pea3, Nöral Devre, Mikroakışkan Sistem, Elk-1, Glioblastoma.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my most sincere gratitude to Prof. Isil AKSAN KURNAZ for her supervision, guidance and advice during the period of my research.

I would like to thank all the AxanLab members, my dear lab mates: Basak KANDEMİR, Melis SAVASAN SOGUT, Oya ARI UYAR, Ekin SONMEZ, Yigit Koray BABAL, Merve USTUN, Büsra NUR CICEK, Betul ERTURAL, Esranur YIGIT and Sedef YUSUFOGULLARI for all their supports, friendship and all lovely memories.

The last but not the least, I specially thank to my parents Zehra METINER and Ozcan METINER and my little brother Mehmet Yusuf METINER for always being with me. I am really grateful for all their supports, patience and their endless love.

TABLE of CONTENTS

	<u>Page</u>
SUMMARY	v
ÖZET	vi
ACKNOWLEDGEMENTS	vii
TABLE of CONTENTS	vii
LIST of ABBREVIATIONS and ACRONYMS	x
LIST of FIGURES	xi
LIST of TABLES	xiii
1.INTRODUCTION	1
1.1.PEA3 Subfamily	2
1.2. Elk-1, a member of the TCF subfamily	7
1.3. Aim of Thesis	10
2. MATERIALS AND METHODS	11
2.1. Materials	11
2.1.1. Plasmids	11
2.1.2. Primers	11
2.1.3. Kits	12
2.1.4. Cell Culture Materials	12
2.1.5. Bacterial Materials	14
2.1.6. Material, Equipments and Devices	14
2.2. Methods	15
2.2.1. Competent Cell Preparation	15
2.2.2. Transformation of Plasmids	15
2.2.3. Plasmid Isolation	16
2.2.4. Cell Culture	17
2.2.4.1. Cell Culture for Axon Elongation Experiments	17
2.2.4.2. Cell Culture for Glioblastoma Experiments	17
2.2.5. Cell Transfection	18

2.2.5.1. Cell Transfection for Axon Elongation Experiments	18
2.2.5.2. Cell Transfection for Glioblastoma Experiments	19
2.2.9. Preparing the Microfluidic System	19
2.2.10. Imaging and Axon Length Measurement	20
2.2.6. RNA Isolation	21
2.2.7. cDNA Synthesis	21
2.2.8. Quantitative Real-Time PCR	22
3. RESULTS	24
3.1. The Role of Pea3 in Neuronal Differentiation and Axon Elongation	24
3.1.1. NSC-34 Cells Adhesion Optimization	24
3.1.2. Microfluidic Chip Optimization	25
3.1.3. Axon Elongation Between Cells Containing the Constitutively Active Pea3-VP16 Expression Plasmid	26
3.1.4. Axon elongation between cells containing wild-type (mPea3-pCDNA3) plasmid and cells containing other Pea3 variants	29
3.2. Elk-1's Role in Proliferation and Self-Renewal	33
3.2.1. The Effect of siElk-1 on Stemness Gene	33
4. DISCUSSION AND CONCLUSION	35
REFERENCES	38
BIOGRAPHY	42

LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations and Acronyms</u>	<u>Explanations</u>
ETS	: E26 Transformation Specific
TCF	: Ternary Complex Factor
NSC-34	: Mouse Motor Neuron Cell Line
MAPK	: Mitogen Activated Protein Kinase
SRF	: Serum Response Factor
PEA3	: Polyoma Enhancer Activator 3
SRE	: Serum Response Element
SH-SY5Y	: Human Neuroblastoma Cell Line
DH5 α	: <i>Escherichia coli</i> bacterial strains
FBS	: Fetal Bovine Serum
DMEM	: Dulbecco's Modified Eagle Medium
DMSO	: Dimethyl Sulfoxide
PEI	: Polyethylenimine
CNS	: Central Nervous System
GBM	: Glioblastoma Multiforme
GSC	: Glioma Stem Cell
PSC	: Pluripotent Stem Cell
NPC	: Neuronal Precursor Cell
ESC	: Embryonic Stem Cell
GFP	: Green Flourescent Protein
U87-MG	: Glioblastoma Cell Line
DPBS	: Dulbecco's Phosphate-Buffered Saline
FITC	: Flourescein Isothiocyanate
TRITC	: Tetramethlyrhodamine
PCR	: Polymerase Chain Reaction

LIST of FIGURES

<u>Figure No:</u>	<u>Page</u>
1.1: ETS superfamily.	1
1.2: Schematic representation of PEA3 subfamily proteins.	3
1.3: Evaluation of axonal elongation capacities of Pea3 phosphomimic and phospomutant variants.	5
1.4: Microfluidic system image.	7
1.5: Schematic representation of Elk-1.	8
3.1: Cell culture dishes surface coating material optimization.	24
3.2: Microfluidic chip optimization.	25
3.3: Microfluidic system.	27
3.4: Images of axon elongation assays performed with cells transfected with the Pea3-VP16 plasmid.	28
3.5: Images of cells transfected with the mPea3 expression plasmid.	31
3.6: Analysis of axon lengths measured by NeuronJ program.	32
3.7: Transfected U87-MG cells images.	33
3.8: The expression of selected potential Elk-1 target genes after siElk-1 silencing in U-87 glioblastoma cell lines.	34

LIST of TABLES

<u>Table no</u>	<u>Page</u>
2.1: Primers design.	11
2.2: List of the kits.	12
2.3: List of the cell culture materials.	13
2.4: cDNA synthesis reaction setup.	22
2.5: Reaction protocol for cDNA synthesis.	22
2.6: qPCR reaction setup.	23
2.7: Reaction protocol of qPCR.	23

1. INTRODUCTION

One of the largest transcription factor families, the ETS (based on the founding member E26 protooncogene) domain superfamily is very important for many biological processes and is evolutionarily conserved [Oikawa and Yamada 2003]. All ETS family members are characterized by the conserved DNA binding domain of approximately 85 amino acid residues that bind to the purine-rich GGAAT/T core DNA sequence. ETS family has 28 members, divided into 12 subfamilies according to the sequence homologies, DNA binding domain similarities and structural compositions (Figure 1.1) [Sharrocks, 2001; Oikawa and Yamada 2003]. Transactivation domains (TAD) in the ETS transcription factor family allow a variation in expression between members of the subfamilies by providing control and specificity of the target genes [Brown et al., 1998].

Sub Family	Family Members
ETS	ETS1, ETS2
ERG	ERG, FLI1, FEV
ELF	ELF1, ELF2, ELF4
ESE	ELF3, ELF5 EHF
ERF	ETV3, ERF, ETV3L
TEL	ETV6, ETV7, YAN
PEA3	PEA3, ERM ER81
SPI	PU.1, SPIB, SPIC
TCF	ELK1, ELK3, ELK4
PDEF	SPDEF
ETV2	ETV2
GABPA	GAB α

Figure 1.1: ETS superfamily. Main subfamilies and their members are listed.

ETS transcription factor family members have regulatory roles such as transcriptional activator or suppressor for different genes; affect cellular processes such as proliferation, cellular differentiation, development, cell cycle control, cancer

progression and migration in different tissues [Gutierrez-Hartmann vd., 2007; Craig and Sumanas, 2016; Sizemore vd., 2017]. Another molecular task of the ETS family is that it plays a role in protein-protein interaction. Therefore, members of the ETS transcription factor family have protein-protein interaction domains that recognize proteins [Hennenberg et al., 2012]. Our laboratory is focused on two subfamilies and members thereof: Elk-1 of the TCF subfamily and Pea3, Erm and Er81 of the PEA3 subfamily.

1.1. PEA3 Subfamily

PEA3 (polyoma enhancer activator-3) is one of the members of the ETS subfamilies [Ji-Hou Xin, 1992]. PEA3 subfamilies consists of three members: Pea3, Erm and ER81 and the homology of ETS domains, which are N-terminal regions localized in acidic domain among these members, is more than 95% (Figure 1.2) [de Launoit et al., 1997]. In addition to the negative regulatory regions and the transactivation site, many post-translational modifications such as SUMOylation, phosphorylation and ubiquitylation have been identified in the PEA3 subfamilies [Oh, 2012]. PEA3 members are known to be expressed in many organs during development [Hollenhorst et al., 2004]. They are expressed in epithelial cells and participate in epithelial differentiation [Chotteau-Lelievre et al., 2003]. They also play a role in branching morphogenesis and organogenesis in organs such as kidney, lung, and are involved in diseases such as metastatic breast cancer and prostate cancer; recently they have also been shown to regulate neural circuit formation in the developing nervous system [de Launoit et al., 1997; Xin et al., 1992; Higashino et al., 1993; Laudet et al., 1999]. These members are known to play a role in the formation of motor neuron circuits and axon elongation in the nervous system [Kandemir et al., 2014; Lin et al., 1998; Fontanet et al., 2016].

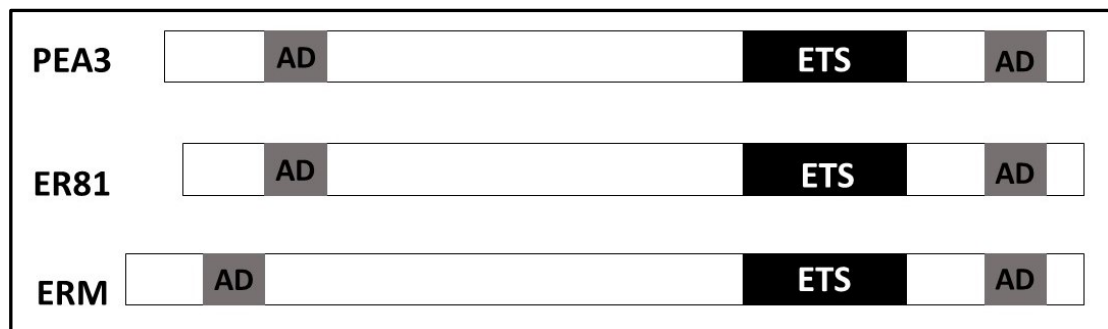


Figure 1.2: Schematic representation of PEA3 subfamily proteins. Gray box represents acidic domains at the N-terminal and C-terminal regions. Black box represents ETS-domain; gray boxes represent the activation domain (AD).

The expression of the members of the PEA3 family was determined in the development of the nervous system of mice from the E9.5 stage until the birth, expressed in high amounts in many regions such as spinal cord, hindbrain, midbrain and ventral forebrain in mouse and chick models [Lin et al., 1998; Chotteau-Lelievre et al., 2001]. In the zebrafish model, it was found to be important in nerve differentiation in the neural crown region and retina [Brown et al., 1998]. Pea3 and Erm which members of the PEA3 subfamily, have been shown to be expressed in the developing rat hippocampus during the main period of dendritic growth and synapse formation and play a key role in establishing hippocampal connections [Fontanet et al., 2016]. When neuron circuits in the spinal cord are examined, Pea3-expressing neurons specifically circuit with other Pea3-expressing neurons or Er81-expressing neurons are linked to other Er81-interacting neurons, that is the specificity and selectivity of motor neuron circuits is provided by Pea3 proteins [Lin et al., 1998]. In addition, Pea3 and Erm have been shown to mediate the formation of the vertebrae caused by the brain-derived neurotrophic factor (BDNF) in hippocampal neurons [Fontanet et al., 2016].

Our laboratory has also focused on the role of PEA3 subfamily members for some years and has identified novel targets of Pea3 transcription factor in axon elongation and neuronal differentiation. In studies in our laboratory, Pea3-VP16 constitutively active variant protein has been shown to cause axonal elongation in NSC-34 and PC12 neuronal cell models, and in this study, NF-L and NF-M (Neurofilament -L and -M) proteins are Pea3 target genes [Kandemir et al., 2014]. It was further shown that Pea3 regulated axon elongation through regulation of novel

target genes such as ephrins, ephrin receptors, semaphorins, L1CAM, and many other targets that have been determined by transcriptomic analysis [Kandemir et al., 2017]. It has previously been shown that Pea3-expressing cells almost exclusively formed neuronal circuits with other Pea3-expressing cells [Lin et al., 1998; Deppmann and Ginty, 2006]. Therefore, it is important to determine whether these novel targets of Pea3 play a role in this selectivity in motor neuron-sensory neuron circuit formation.

As with many other ETS proteins, Pea3 proteins are also subject to post-translational regulation, including phosphorylation. Although phosphorylation sites have been determined for Erm and Er81 [Janknecht, 1996], a detailed study of Pea3 phosphorylation motifs had not been performed. One focus in our laboratory was mapping of these putative phosphorylation motifs, and it has been determined using Pea3 phosphomutants that Serine 90 and Serine 458 phosphorylation sites in particular were important in axonal elongation [Kandemir et al., 2014]. The effect of other phosphomutants of Pea3 was further investigated in NSC-34 cells, and it was observed that the Serine 285 region triggered axonal elongation (Figure 1.3; Ustun et al, submitted manuscript).

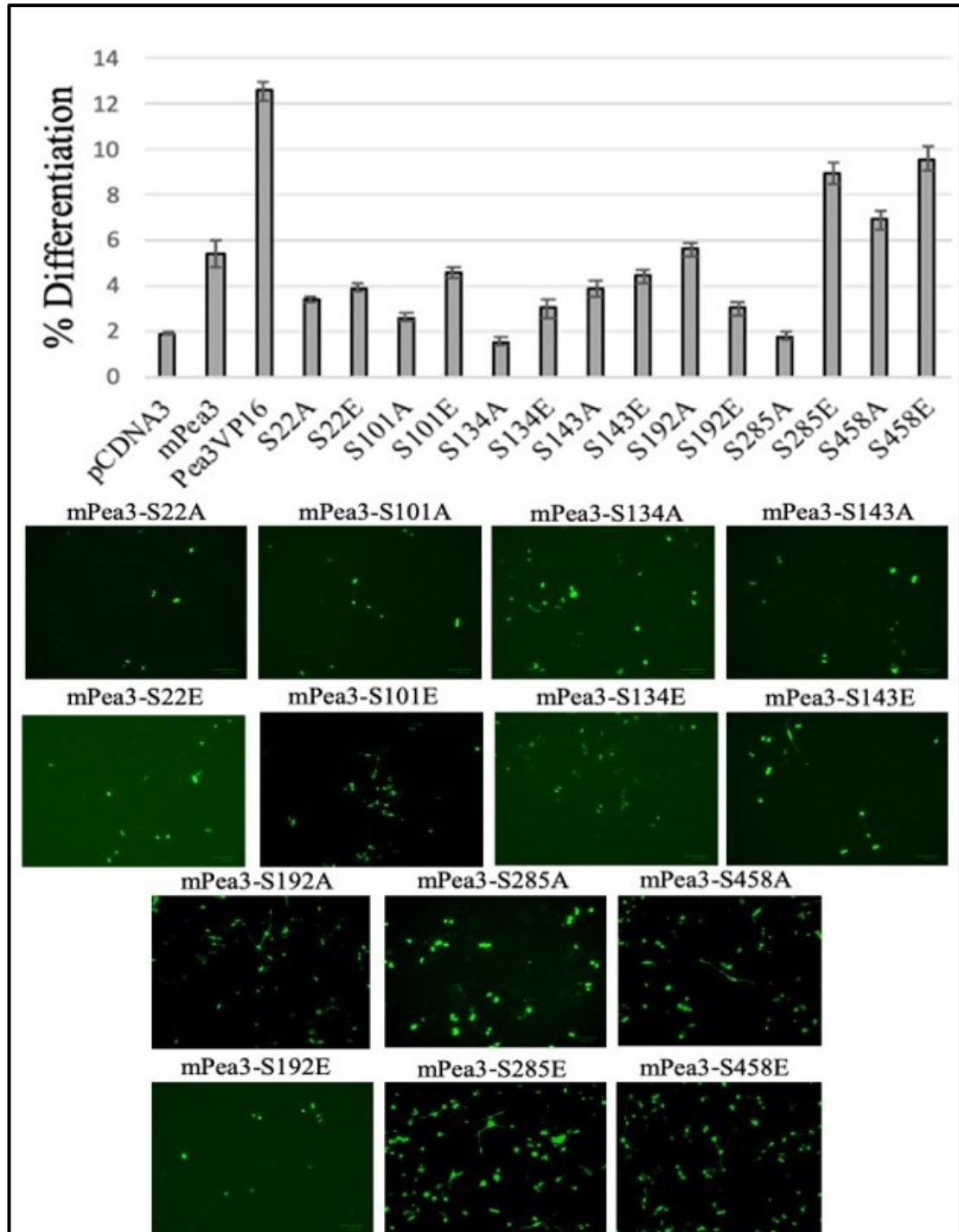


Figure 1.3: Evaluation of axonal elongation capacities of Pea3 phosphomimic and phosphomutant variants. Pea3 phospho-mutants; mPea3-S22A, mPea3-S101A, mPea3-S134A, mPea3-S143A, mPea3-S192A, mPea3-S285A, mPea3-S458A and their phosphomimic variants; images of pEGFP co-transfected with mPea3-S22E, mPea3-S101E, mPea3-S134E, mPea3-S143E, mPea3-S192E, mPea3-S285E, mPea3-S458E 3: 1 (expression plasmid: pEGFP ratio). Quantitative evaluation of the % differentiation of null pCDNA3 expression plasmid, NSC-34 cells transfected with wild-type mPea3, structurally active Pea3-VP16 and Pea3 phosphomutant expression plasmids.

In this thesis study, we aimed to investigate the role of Pea3 and its target genes in establishing functional selectivity in neural circuits, and whether Serine 90, Serine 258 and Serine 458 residues played a role in this function.

For this purpose, our model was to transfect different Pea3 mutants into motor neuron cells on a system capable of monitoring neural circuit formation. The aim of this part is to simulate the extension of the neuronal cell models expressing Pea3 towards each other by positioning the coculture system and to identify the phosphorylation motifs directly affecting in this axon prolongation. To that end, we have chosen to use a microfluidic based axon extension platform.

Microfluidic systems, which are widely used in regeneration or axon elongation studies in addition to many other applications, allow the axons to enter the microchannel to identify and examine the elongation qualitatively as well as quantitatively. It can be seen in the literature that microfluidic-based systems can also be used for spatial and temporal control of such as axons and soma for more detailed analysis upon for instance axon isolation. Although there are many different microfluidic platforms, the 4-well microfluidic system that was selected in line with the goal of this project is shown schematically in Figure 1.4. This platform, which is especially prominent for nerve cell applications, has been found suitable for observing neural circuit formation since it has previously been used in studies such as axon growth kinetics after compartmentalization and axotomy in primary neurons [Nagendran et al., 2018; Jocher et al., 2018]. The biggest advantage of this system is that analysis can be done with a relatively small number of cells.

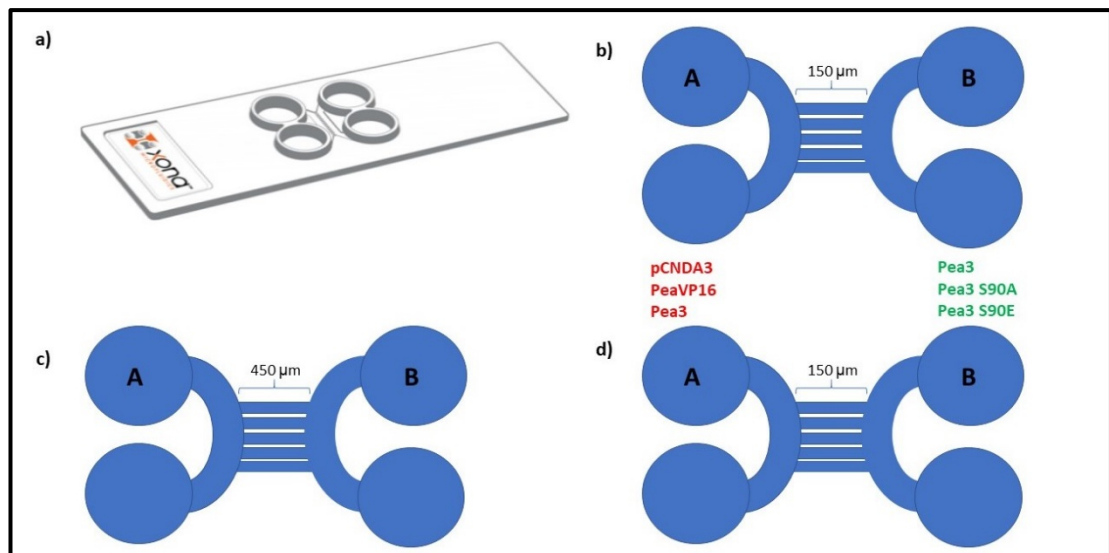


Figure 1.4: Microfluidic system image. a) Microfluidic system used in the study; b,c) Representative drawing of the system; d) Axon elongation test plan carried out within the framework of the project; Different expression plasmids and co-transfected cells with pEGFP-N2 (Clontech, USA) or pcDNA3.1 (+) / Luc2=tdT (Addgene) plasmid were added to well A and B.

1.2. Elk-1, a member of the TCF subfamily

Elk-1 is a member of the ternary complex factor (TCF) subfamily of the ETS domain superfamily of transcription factors (Figure 1.5) [Demir et al., 2012]. Elk-1 plays a dual role in neuronal functions: pro-apoptotic in the cytoplasm and pro-differentiation in the nucleus [Besnard et al., 2011]. It has been associated with mitogen-activated protein kinases (ERK / MAPK) and, which are traditionally induced by mitogen-induced immediate early gene transcription [Demir et al., 2013]. The core is targeted by three major MAP kinases, ERK, JNK and p38 pathways, as well as the CaMKII pathway. Phosphorylation of nuclear Elk-1 enhances the ability to bind DNA in serum response elements (SRE) and stimulates transcription of immediate-early genes. In this functional context, nuclear Elk-1 has been incorporated into processes such as neuronal differentiation, cellular proliferation and tumorigenesis [Sharma et al., 2010]. Elk-1 is both upregulated and phosphorylated in brain tumor cells [Demir et al., 2013]. In our laboratory, studies are performed on the localization and potential roles of Elk-1 in proliferation and survival [Sharma et al., 2010], and preliminary findings indicate a potential cross-regulation between Elk-1 and Pea3

transcription factors where Pea3 is found to repress Elk-1 transcription, which may act as a switch between stem cell renewal and proliferation (in Elk-1 presence) to differentiation (in Pea3 presence).

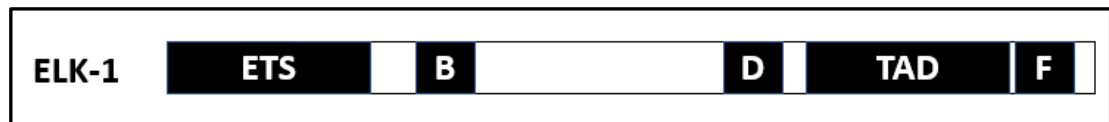


Figure 1.5: Schematic representation of Elk-1. TAD is transactivation domains. Elk-1 binds ERK/MAPK through the docking (D) domain and the FxF (F) motif, and binds Serum Response Factor (SRF) through the B domain. ETS is the consensus DNA binding domain common for all ETS superfamily members.

Glioma is a general term used to describe primary brain tumors and can be classified according to the cells from which it originates. These include astrocytic tumors (astrocytoma, anaplastic astrocytoma and glioblastoma), oligodendrogliomas, ependymomas, and different gliomas [Holland, 2000]. These are the most common tumors of the central nervous system (CNS), which makes up almost 80% of all malignant primary tumors in the brain. Glioblastoma multiforme (GBM) is the most common malignant and most common type of primary astrocytoma [Messali, 2014]. Despite the variety of modern treatments against GBM, it is still a fatal disease with an extremely poor prognosis. Patients usually show a median survival of about 14 to 15 months from diagnosis [Hanif, 2017]. It accounts for more than 60% of all brain tumors in adults [Messali, 2014]. Depending on the clinical features, GBM is divided into two as primary and secondary GBM. In primary GBMs, de novo occurs without clinical and histological evidence of the leading lesion. Unlike secondary GBMs, it progresses slowly from the pre-existing low-grade astrocytoma [Hanif, 2017].

Studies have shown that the *NANOG* gene and its isoforms play an important role in the development of a malignant phenotype in cells, along with *OCT4* and *SOX2* [Gawlik-Rzemieniewska, 2016]. The presence of cancer stem cells in cancer cells plays an important role in determining the course of the disease and the treatment process. Changes in the expression levels of stemness genes in brain cancer cells, depending on the conditions, affect the apoptosis-proliferation and survival

processes of the cells [Bradshaw, 2016]. Although not independent of GBM, Glioma Stem Cells (GSCs) can be characterized by the expression of various markers within the class of transcription factors such as NANOG, OCT4, SOX2 etc. [Guerra-Rebollo, 2019].

SOX2 is a member of the SoxB1 family of transcription factors and is an important transcriptional regulator in pluripotent stem cells (PSC). In addition, Sox2 plays an important role in somatic cell reprogramming by returning the epigenetic configuration of differentiated cells to a pluripotent embryonic state [Cui, 2014]. Binding of Sox proteins to target genes can be influenced by a variety of factors, including cofactors or transcriptional partners the interaction of Sox2 with various cofactors brings more functional diversity during developmental processes [Wegner, 2010]. Sox2 is highly expressed in proliferative neural precursor cells (NPCs) and is reduced on differentiation to post-mitotic neuronal and glial cells [Graham et al., 2003]. When Sox2 decreases in NPCs, it encourages the terminal to exit from differentiation and cell regeneration earlier, thus preventing their proliferation and self-renewal. Differentiation of NPCs to glia and neuron is inhibited by ectopic expression of Sox2 [Bylund et al., 2003].

NANOG is a critical transcription factor for maintenance and preservation of undifferentiated pluripotent cells and plays a role in the self-renewal of embryonic stem cells (ESCs) [Gawlik-Rzemieniewska et al., 2016]. In addition, Nanog overexpression ensures that ESCs maintain their pluripotency and self-regeneration in culture conditions that cause differentiation [Rodda et al., 2005]. In addition to controlling such properties, the role of NANOG in tumor formation has been noted in the adult: when the studies with Nanog are evaluated, it has been shown that Nanog is especially involved in the carcinogenicity process and can be a potential prognostic marker for malignant tumors [Siu et al., 2013]. In addition to NANOG, other stemness proteins SOX2 and OCT4 have also been shown to play a role in malignant tumor development [Gawlik-Rzemieniewska et al., 2016].

Oct-4 / POU5F1 is a member of the transcription factor family and is located within the mechanism that manages polypotency. Highly expressed Oct4 in pluripotent cells is silenced after differentiation [Shi et al.2010]. According to recent

studies, Oct4 preserves undifferentiated embryonic stem cells and has an effect on pluripotency regulator expression [Takahashi et al., 2006].

The presence of cancer stem cells plays an important role in determining the treatment process of cancer disease. Changes in brain cancer cells depending on the expression of stemness genes affect the survival, apoptosis and proliferation of cells. Studies on the relationship between the stemness genes of Elk-1, which is one of the ETS family transcription factors, with neuroblastoma-derived cell lines, has been conducted in our laboratory. Supporting the results obtained from these studies with results to be obtained from glioblastoma cancer cell line will allow for more precise comments on the effect of Elk-1 on stemness genes.

1.3. Aim of Thesis

In this thesis, we have aimed to develop a system in which neuronal model cells that have been transfected to express different mutant or wildtype forms of Pea3 protein can be monitored for neuronal circuit formation so as to determine which cells interact to that purpose. Such a system can then be used in combination with RNAi mediated knockdown of transcriptional targets of Pea3 protein, so as to identify which target gene is specifically involved in circuit formation. To that end, different Pea3 expression plasmids and pEGFP-N2 or pcDNA3.1(+)/Luc2=tdT expression plasmids were co-transfected in the mutual wells of the above system, and axon lengths in the channels were monitored at different times and the axon lengths of neurons were measured with the help of NeuronJ program.

Glioblastoma and neuroblastoma cancer stem cells were also used as a model to study the role of Elk-1 in self-renewal and proliferation of cancer stem cells, and aimed to investigate the correlation of Elk-1 and identified target genes with glioblastoma cell line.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Plasmids

pcDNA3-Pea3-VP16 and pcDNA3mPea3 mammalian expression plasmids are gifts from Prof. Dr. A.D. Sharrocks to our laboratory. The mutant variants of mPea3-S90A and mPea3-S90E plasmids were prepared in our team by B. Caglayan and M. Ustun within the scope of their Master's thesis. [Caglayan, 2010; Ustun, 2017]. pEGFP-N2 (Clontech, USA) and pcDNA3.1(+)/Luc2=tdT (Addgene) were obtained commercially.

The plasmid containing the siElk-1 sequence cloned into the psiSTRIKE hMGFP vector was prepared by Özlem DEMİR (former AxanLab member) (psiSTRIKE-sh-Elk1-GFP, referred to in text as siElk-1)). Scrambled RNA plasmid (psiSTRIKE-scrRNA-GFP, referred to in text as scrRNA) was used for control.

2.1.2. Primers

Primers which used were prepared by Melis SAVAŞAN SÖĞÜT in her PhD Thesis. Designed primers were shown in the Table 2.1.

Table 2.1: Primers design.

Gene	Site	Sequence (5'- 3')
<i>GAPDH</i>	Frw	CAT CTT CCA GGA GCG AGA TCC
	Rev	AAA TGA GCC CCA GCC TTC TCC
<i>ACTB</i>	Frw	ACG AAA CTA CCT TCA ACT CC
	Rev	GAT CTT GAT CTT CAT TGT GCT GG
<i>ELK1</i>	Frw	GCT TCC TAC GCA TAC ATT GAC C
	Rev	ACT GGA TGG AAA CTG GAA GG

Table 2.1: Continue.

SOX2	Frw	GGA AAA TGG GAG GGG TGC AAA AGA GG
	Rev	TTG CGT GAG TGT GGA TGG GAT TGG TG
POU5F1	Frw	AAG GAT GTG GTC CGA GTG TGG
	Rev	CCT GAG AAA GGA GAC CCA GCA G
NANOG	Frw	TTC AGA GAC AGA AAT ACC TCA GCC
	Rev	CCT TCT GCG TCA CAC CAT TGC

2.1.3. Kits

Table 1.2: List of the kits.

Kit	Company	Catalog/Lot Number
DNA-midi SV, Plasmid DNA Purification Kit	Intron Biotechnology	17252
iScript cDNA Synthesis Kit	Bio-Rad	1708891
SsoAdvanced Universal SYBR Green Supermix	SsoAdvanced Universal SYBR Green Supermix	172-5274
innuPREP RNA Mini Kit 2.0	Analytikjena	035-19

2.1.4. Cell Culture Materials

In this thesis, two different cell lines NSC-34 mouse motor neuron cell line and U87-MG glioblastoma cell line (gift from Assist. Prof. Tugba Bagci Onder from Koc University) are used.

Table 2.3: List of the cell culture materials.

Materials	Company	Catalog/Lot Number
Dulbecco's Modified Eagle Medium 1X (DMEM) with L-glutamine and 4,5g/L Glucose, without Pyruvate	Gibco	2007829
Dulbecco's Modified Eagle Medium 1X (DMEM) with L-glutamine, pyruvate and 4,5g/L Glucose	Gibco	41966-029
Fetal Bovine Serum (FBS)	Gibco	10270
Penicillin, Streptomycin (10000U/ml Penicillin, 10mg/ml Streptomycin)	PAN Biotech	P06-07100
Dulbecco's Phosphate Buffered Saline 1X (DPBS)	Gibco	14190-94
0.05% Trypsin-EDTA Solution 1X	Gibco	25300-054
Dimethyl sulfoxide (DMSO)	BIOFROXX	67-68-5
Poly-L-Lysine	Sigma	P8920
Poly-D-Lysine	Sigma	P7280
Collagen	Sigma	C3867

2.1.5. Bacterial Materials

DH5 α Escherichia coli bacterial strains were used for cloning studies for competent cell preparation.

LB Agar (Caisson Labs, LBP02-500GM), LB Broth (Caisson Labs, LBP01-500GM), Ampicillin (Biomatix), CaCl₂ (Acros Organics).

2.1.6. Material, Equipments and Devices

- 0.22 μ m syringe filter (Merck)
- 60 mm, 100 mm, 150 mm tissue culture plate (TRP).
- 15 ml and 50 ml Falcon (CAPP)
- 6-well culture plate (TRP)
- 5 ml, 10 ml, 25 ml serological pipettes (CAPP)
- -80°C freezer (VWR)
- CO₂ incubator - Stericycle i160 (Thermo Scientific)
- Laminar Flow Cabinet – Safe 2020 (Thermo Scientific)
- Micro pipettes (Eppendorf)
- Microcentrifuge tubes (0,2 ml, 0,5 ml, 1,5 ml, 2ml)
- Petri dish (100 mm) (Lamtek) Power Supply – Power Pac Basic (Bio-Rad)
- Spectrophotometer – NanoDrop (Schimadzu)
- Spin (Sprout)
- T25, T75, T150 tissue culture flasks (TRP)
- UV illuminator (Vilber Lourmat)
- Vortex (JeioTech)
- Leica Dmi8 Reverse Fluorescence Microscope
- Magnetic Induction Cycler (Mic) PCR Machine
- Cryotubes (1ml, 2ml)
- Heat Block (JeioTech)
- Liquid nitrogen tank (Taylor-Wharton, LS4800)
- XC150, XC450 (XONA Chip)
- Hemocytometer (Marienfeld)
- Water Bath (JeioTech)

2.2. Methods

2.2.1. Competent Cell Preparation

DH5 α colony was scraped by a sterile inoculation loop from the glycerol stock. Scraped cells were propagated it onto the LB Agar plate and incubated 37°C degree for over-night. Next day, the single colony was picked from agar plate into 5 ml of LB Broth medium and incubated shaker at 250 rpm 37°C for over-night. Next day, 1 ml of LB Broth medium containing DH5 α culture was picked into 100ml LB Broth medium. At this point, this medium was shaken at 37°C until the OD600 becomes 0.4. After this step, cells were kept at 4 °C. Therefore, all materials to be used, such as tubes and pipette tips, were pre-cooled. Media with OD600 value of 0.4 was centrifuged at 2700 g for 10 minutes at 4°C. The supernatant was removed, and 1.6 ml of cold 100 mM CaCl₂ was added on the pellet by spinning on ice gently after it was incubated on ice for 30 minutes. After 30 minutes, pellet that was resuspended with CaCl₂ was centrifuged at 2700 g for 10 minutes at 4°C. The supernatant was removed again and 16 ml of cold CaCl₂ was added to the pellet and incubated on ice for 20 minutes. Competent cells were either used directly or stored as follows: 0.5 ml cold %80 glycerol was added to cells. This mixture was solidified in fluid nitrogen as 100 μ l aliquots and stored - 80°C.

2.2.2. Transformation of Plasmids

DH5 α *Escherichia coli* bacterial competent cells were either freshly prepared or thawed on ice. 2 μ l plasmid (50-100 ng/ μ l) was added to 50 μ l thawed competent cells, and tubes were incubated for 15 minutes in ice. Negative control was performed without any plasmid using the same methods. After that, whole tubes were incubated on 42°C heat-block for 90 seconds for heat shock, and tubes were put on ice for 2 minutes. Then 200 μ l LB Broth (without antibiotic) was added to each tube, and tubes were incubated at 37°C for 1 hour with shaking at 180 rpm. After incubation, mixture within the tubes were spread onto 10 cm petri dish, which

includes LB Agar (ampicillin positive or kanamycin positive), and incubated over-night at 37°C to grow.

2.2.3. Plasmid Isolation

For midi prep, single colonies were picked from the bacterial plate and inoculated over-night at 50 ml of LB Broth at 37°C, shaking at 180 rpm. After, they were centrifuged at 4000 rpm for 10 minutes at room temperature. Supernatants were removed. Pellet was resuspended with 2ml resuspension buffer, including RNase, and they were vortexed until no clumps remain. 2 ml of lysis buffer was added onto suspended cells, they were gently inverted ten times, and cells were incubated at room temperature for 3 min. For the neutralization step, 2 ml of neutralization buffer was added into tubes, and they were gently inverted ten times. Cells were incubated in ice for 5 minutes. All lysate was transferred to pre-columns, and columns were centrifuged at 4000 rpm for 5 minutes. Flow-through was transferred into binding columns, and tubes were centrifuged at 4000 rpm for 5 minutes. Flow-through was discarded, 10 ml washing buffer was added into binding columns, and tubes were centrifuged at 4000 rpm for 5 minutes. Flow-through was discarded. The binding columns were washed with 10 ml of second washing buffer and tubes were centrifuged at 4000 rpm for 5 minutes. Then, the binding columns were centrifuged at 4000 rpm for 15 minutes, and filters were incubated 56°C for 5 minutes (dry centrifuge to remove ethanol). Then binding columns were transferred clean collection tube, 600 µl dH₂O was added into the filter of columns, and columns were incubated room temperature for 5 minutes for elution. Plasmid DNA concentration was measured with spectrophotometric measurements.

2.2.4. Cell Culture

2.2.4.1. Cell Culture for Axon Elongation Experiments

NSC-34 (Mouse motor neuron) cell line was maintained in non-pyruvate DMEM High Glucose medium (%10 FBS and %1 Penicillin Streptomycin), 37°C and %5 CO₂. After the cells were removed from the liquid nitrogen tank, they were placed at 37°C to thaw frozen cells. Cell culture dish was prepared and placed incubator (37°C and %5 CO₂) to equilibrate temperature and carbon dioxide. After the frozen cells were thawed, the cells were transferred to cell culture dishes and left for incubation. At the fourth hour of incubation, the whole medium was taken, and a fresh medium was added. After the cell density reached 80 percent, cells were trypsinized and collected, and they were passaged into two separate 100 mm cell culture dishes to proliferate. When the cell density reached 80 %, they were collected with trypsin and counted. Hemocytometer (Marienfeld) was used for cell count. Before use, the hemocytometer glass is cleaned with 70% alcohol. In the cell suspension, 10 microliters are taken with a micropipette and placed on the hemocytomer glass and covered with coverslip. This preparation is examined in 10x microscope. Cells in the 16 squares in the corners are counted, averaged, and multiplied by 10,000.

Optimization experiments were performed to determine optimum cell number and surface coating material for microfluidic system. To that end, three different surface coating material was investigated: collagen, Poly-D-Lysine and Poly-L-Lysine, and two different cell number was studied: 100.000 and 200.000 NSC-34 cells per well. These conditions were analyzed at the twentieth and twenty-fourth hour of incubation; images were taken with ZOE Fluorescent Cell Imager (Bio-Rad) and analyzed.

2.2.4.2. Cell Culture for Glioblastoma Experiments

We used the U87-MG Glioblastoma cell line that used before in our laboratory, and this cell line was grown up in DMEM High Glucose medium (%10 FBS and %1 Penicillin Streptomycin), 37°C and %5 CO₂.

The cell culture of U87-MG cell lines is that the medium used is different from the other cell culture method. Media, including pyruvate, were used in cell cultures made with U87-MG. The steps required for cell proliferation are the same as described above.

2.2.5. Cell Transfection

2.2.5.1. Cell Transfection for Axon Elongation Experiments

Pea3-VP16 and mPea3 mammalian expression plasmids were a gift from Prof. Dr. A.D. Sharrocks to our laboratory. The mutant variants of mPea3-S90A and mPea3-S90E were obtained by region-targeted mutagenesis in our team by B. Caglayan and M. Ustun within the scope of Master's thesis [Caglayan, 2010; Ustun, 2017; Ustun et al, manuscript submitted].

Unless otherwise noted, three million cells were seeded to 150 mm cell culture dishes for transfection. Plasmids were prepared according to Lipofectamine 2000 protocol. For microfluidic system, one plasmid mix included a Pea3 expression plasmid together with pEGFP (1:4 ratio, totally 12 ng) and another plasmid mix included a Pea3 expression plasmid together with pcDNA3.1 (+) / Luc2=tdT (1:3 ratio, totally 12 ng). In another falcon, 72 µl of lipofectamine reagent was added to 2.5 ml serum-free medium. Plasmid and Lipofectamine reagents are incubated for 5 minutes at room temperature separately in different Eppendorf tubes. After 5 minutes, the solution including lipofectamine reagent was added onto plasmid solutions. Plasmid – reagent solutions were incubated at 20 minutes at room temperature to form the DNA lipofectamine complex. After 20 minutes, this solution was distributed homogeneously to the cell culture dishes as indicated in text. Transfected cells were seeded into the microfluidic system 24 hours after transfection. While the cells were seeded, the mPea3-pCDNA3 and pCDNA3-Pea3-VP16 plasmids for the lower and upper wells on the left side was seeded to be 90,000 cells in 20 µl, and the cells which are transfected different plasmid, seeded to the lower and upper wells on the right was 90,000 cells in 20 µl. After waiting 5 minutes

for the cells to attach, 150 μ l of medium was added and cells were left for incubation. After the first 24 hours of incubation, axon elongations were checked for one week at 6-hour intervals. After the experiment setup was optimized, imaging was performed for two days at 12-hour intervals after the first 24 hours.

2.2.5.2. Cell Transfection for Glioblastoma Experiments

Transfection was done with polyethylenimine (PEI) transfection reagent of U87-MG cells with sh-Elk1-GFP (siElk-1) and scrambled RNA (scrRNA) plasmids according to standard PEI protocol. Before the transfection process, U87-MG human glioblastoma cells were seeded into 60 mm cell culture dishes at 750,000 cells / well. After one day, 5000 ng of each plasmid, 500 μ l serum-free medium, and 1: 4 (DNA-PEI) ratio PEI reagent was mixed into the tube and incubated for 5 minutes. After 5 minutes, 2ml complete medium was added to the mixture, the mix was vortexed for 15 seconds, and incubated at room temperature for 15 minutes. After 15 minutes, the medium of cultured cells was removed. This prepared mixture was added into the dishes and immediately afterwards 4 ml complete medium was added into the dish. Then dishes were incubated at 37°C for 2 hours. This protocol was repeated after 24 hours for a second round of siElk-1 transfection so as to effectively knockdown *Elk-1* message.

2.2.9. Preparing the Microfluidic System

XC150 microfluidic chips developed by Xona company for neuron cells were used, following manufacturer's instructions. Essentially, the microfluidic chip was placed inside a 100 mm cell culture dish in a laminar flow chamber. Firstly, the XC Pre-Coat solution was added to the upper left well (Figure 1.4) and incubated for 1 minute. After 1 minute, the XC Pre-Coat solution was added to the lower-left well for 5 minutes. After 5 minutes, the same process was repeated in the right wells. XC Pre-Coat solution in each well was removed using a vacuum. To wash the microfluidic system, 150 μ l of PBS was added to the upper left well for 1 minute. After 1 minute,

150 µl of PBS was added to the lower-left well for 5 minutes. After 5 minutes, the same process was repeated in the right wells. PBS was removed using a vacuum.

The washing step was repeated once more. The wells were coated with Poly-D-Lysine to create a surface that would allow them to attach to the cells (It has been tried in Poly-L-Lysine and Collagen in the optimization phase.). Firstly, 100 µl of Poly-D-Lysine (100 µl Poly-D-Lysin and 900 µl sterile water) prepared at the rate of 10% was placed in the upper left well and waited for 1 minute. 100 µl Poly-D-Lysine was placed in the lower left well and waited for 5 minutes. The same process was repeated in the wells on the right side and incubated for 1 hour at 37°C in 5% CO₂. After 1 hour, Poly-D-Lysine was removed using a vacuum. The washing processes were repeated. 100 µl of medium was added to the upper left well for 1 minute. 100 µl of medium was added to the bottom left well for 5 minutes. The same process was repeated in the right wells and kept in an incubator at 5% CO₂ at 37°C until the cells were ready. When the cells were prepared, the medium in the microfluidic system was removed using a vacuum, and cells were seeded. After waiting for 5 minutes for the cells to adhere to the surface, 150 µl of media was added to the wells, and system was placed to the incubator.

2.2.10. Imaging and Axon Length Measurement

Red or green fluorescence from plasmids (pcDNA3.1(+)/Luc2-tdTomato and pEGFP-N2, respectively) that were co-transfected with Pea3 expression plasmids were used to determine the length and direction of axons between groups and to understand the effect of mutant regions on axon extension. GFP luminescence was measured by fluorescence microscope's FITC filters (450-500 nm), and TdTomato fluorescence was measured with TRITC filters (500-570 nm), and the cell were analyzed.

Imaging procedures were initiated 24 hours after transfection and images were taken at 12-hour intervals. Imaging was done using a Leica Dmi8 reverse fluorescence microscope. The images of fluorescently labelled cells on each side of the microfluidic system were taken separately and then merged with Leica Application Suite (LAS) software.

After the images were taken, the lengths of axons on both sides of the channel were measured using the NeuronJ program (free access; URL: <https://imagescience.org/meijering/software/neuronj/>, Popko et al., 2009), which is the plug-in of the ImageJ program. The measured axon lengths were averaged, and graphs were created with MS Excel program, according to both the elongation values at different times and the elongation values according to the Pea3 mutants.

2.2.6. RNA Isolation

InnuPREP RNA Mini Kit 2.0 was used for RNA isolation. First, the medium in the cell culture dish was removed and washed with PBS. Then, cells were trypsinized, collected and centrifuged at 2000g for 5 minutes and supernatant was discarded. 400 µl Lysis Solution RL was added to the pellet and incubated for 2 minutes at room temperature. The pellets were re-suspended by pipetting and incubated for 3 minutes at room temperature. The lysate samples were placed in Spin Filter D and centrifuged at 11,000 g for 2 minutes. Spin Filter D was discarded. Filtrate was re-suspended by adding 70% alcohol (to the same amount as the filtrate). It was placed in the Spin Filter R and centrifuged at 11,000 g for 2 minutes. The Spin Filter R was taken into a new tube because the filter contains RNAs. 500 µl Washing Solutions HS were added to the Spin Filter R and centrifuged at 11,000 g or 1 minute. The Spin Filter R filter was again placed in a new tube and 700 µl Washing Solutions LS were added and centrifuged at 11,000 g for 1 minute. The Spin Filter R was put in a new tube and centrifuged at 11,000 g for 3 minutes without any solution to remove all ethanol. For elution, the Spin Filter R was placed in a new tube and 30 µl RNase-free water was added and incubated for 1 minute at room temperature. It was centrifuged at 11,000 g for 1 minute. RNA samples were stored at -80°C.

2.2.7. cDNA Synthesis

cDNA synthesis was done by utilizing iScript cDNA synthesis kit (Bio-Rad). For the 20µl total volume 1x reaction, 4µl reaction mixture, 1µl reaction enzyme,

500ng/ μ l RNA template and required amounts of nuclease-free water were used. cDNAs were stored at -20°C.

Table 2.4: cDNA synthesis reaction setup.

Component	Volume per reaction, μ l
5x iScript Reaction Mix	4
iScript Reverse Transcriptase	1
Nuclease Free Water	Variable
RNA Template	Variable (500ng/ μ l)
Total	20

Table 2.5: Reaction protocol for cDNA synthesis.

PCR Cycle	Temperature	Time
Priming	25°C	5 min.
Reverse Transcription	42°C	30 min.
Reverse Transcription Inactivation	85°C	5 min.

2.2.8. Quantitative Real-Time PCR

The qPCR experiments were done utilizing using ssoAdvanced Universal SYBR Green Supermix (Bio-Rad). Total amount 10 μ l 1x reaction mix, 5 μ l SYBR Green supermix, 0.7 μ l 10 μ M forward and reverse primers, 2 μ l 5 ng/ μ l cDNA template and 1.6 μ l nuclease-free water were used.

Firstly, a master mix containing primers, water, and SYBR Green Supermix were prepared (Table 2.6). Then master mixes shared to tubes and template cDNAs were added onto tubes. After that, the tubes were loaded qPCR machine, and reaction protocol was run.

Table 2.6: qPCR reaction setup.

Component	Volume per reaction
SYBR Green Supermix	5µl
Forward Primer	0.7 µl
Reverse Primer	0.7 µl
Template	5 ng/µl
Water	1.06 µl
Total	10µl

Tablo 2.7 Reaction protocol of qPCR.

Cycle	Time	Temperature	
Initial Denaturation	30 sec	95°C	
Denaturation	15 sec	95°C	39 Cycles
Annealing	60 sec	60°C	

When the measurement was finished, the fold changes in target gene expressions were determined dependent on the mean of the reference gene expression. Fold change is calculated by the formula below.

$$\Delta Ct = \Delta Ct_{targetgenes} - \Delta Ct_{housekeeping} \quad (2.1)$$

$$\Delta\Delta Ct = Exp_{\Delta Ct} - Cntrl_{\Delta Ct} \quad (2.2)$$

$$2^{-\Delta\Delta Ct} = Fold\ Change \quad (2.3)$$

3. RESULTS

3.1. The Role of Pea3 in Neuronal Differentiation and Axon Elongation

3.1.1. NSC-34 Cells Adhesion Optimization

In biological applications, poly amino acids and collagen allow cells and proteins to bind to solid surfaces more easily. Cell growth was investigated on different coatings using Poly-L-Lysine (Sigma), Poly-D-Lysine (Sigma) and Collagen to see which coating material NSC-34 grows better and elongations the extensions better (Figure 3.1). Two different cell amounts were seeded: 100,000 and 200,000 cells per well for each coating material. Poly-L-Lysine and Poly-D-Lysine were prepared in a 1:10 ratio in dH₂O. Collagen (Sigma) was prepared to contain 50 µg of collagen in 1 ml of acetic acid (20mM). Based on the results, it was observed that NSC-34 cells show better growth on Poly-D-Lysine, which was selected for further microfluidic experiments. We also observed that 100,000 cells grow better in six wells (9.6 cm²). We optimized the number of cells in future experiments accordingly.

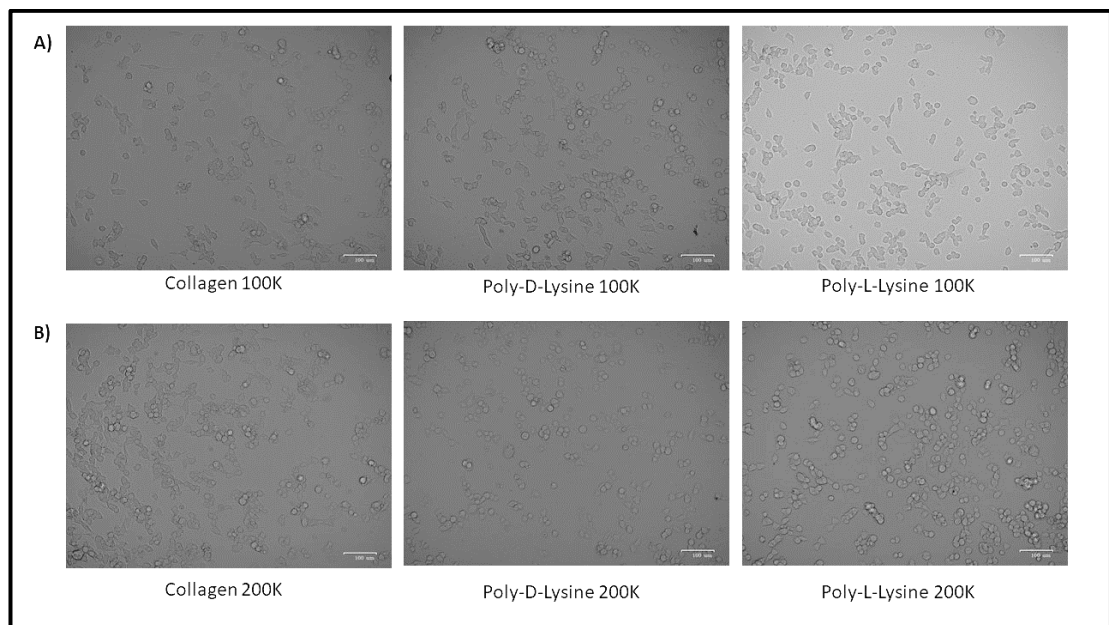


Figure 3.1: Cell culture dishes surface coating material optimization. a) 100,000 cells were seeded using three different coating materials, and axon elongation and proliferation of the cells were examined. b) 200,000 cells were seeded, and cells were observed.

3.1.2. Microfluidic Chip Optimization

We had two different microfluidic systems XC150 and XC450. XC150 had a channel length of 150 μm . XC450 had a channel length of 450 μm (Figure 1.4). Simultaneous experiments were set up to find out which channel length is ideal for axon elongation. Cells were seeded on both chips, and imaging was performed. As a result of the experiment, it was observed that the cells migrated more in the XC450 chip, but they could not extend the axons well, most probably because the channel was too long. In the XC150 chip, the cells were observed to elongate the axons better. It was therefore decided to conduct the next experiments on the XC150 chip from this point onwards (Figure 3.2).

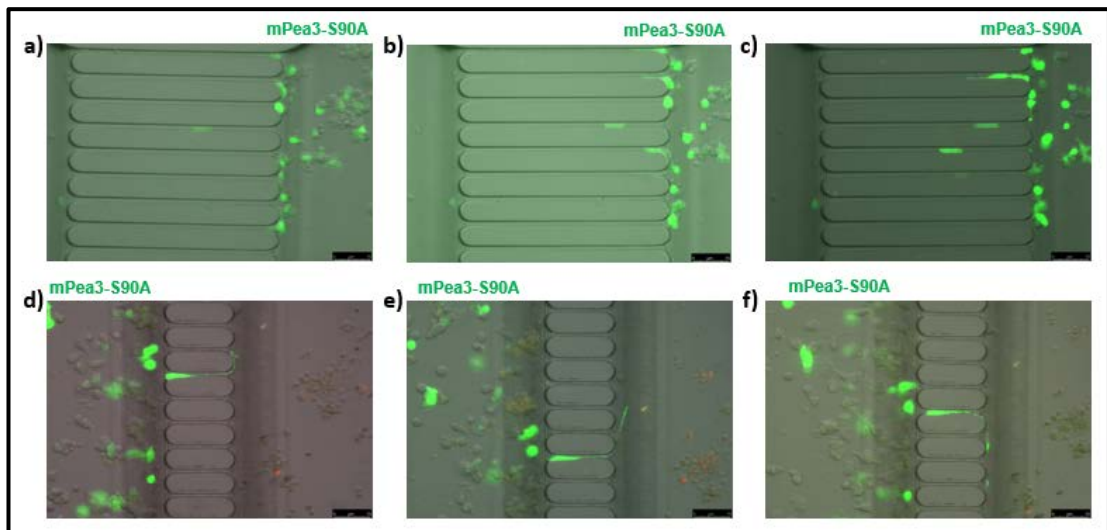


Figure 3.2: Microfluidic chip optimization. Images a,b,c belong to the XC450 chip with a channel length of 450 μm . The images belong to the same area and were taken at 24,30 and 36 hours, respectively. Because the canal was long, the cells could not extend the axon, and it was observed that they only migrated. The images d,e,f are images of the XC150 chip with a channel length of 150 μm . In addition to seeing the tendency of cells to migrate, axon extensions were also seen. Scale bar = 0-75 μm .

After this stage, an optimization experiment was done to determine the number of cells in the microfluidic chip. In the XONA microfluidic chip protocol of the manufacturer, a range of 50.000 to 90.000 cells was proposed for cell cultivation. We have initially seeded 90.000 cells to the XC150 chip to determine imaging time intervals and how long imaging analysis could be performed; to that end, images

were taken for 162 hours at 6-hour intervals. As a result, it was decided to take images at 12-hour intervals for 72 hours after the 24th hour of incubation.

3.1.3. Axon Elongation Between Cells Containing the Constitutively Active Pea3-VP16 Expression Plasmid

Before analyzing how Pea3 proteins provide specificity in neural circuit formation, a co-culture system in which this problem could be asked had to be developed. In this purpose, NSC-34 cells transfected with different Pea3 variants were used. These cells were seeded on one side of the well by co-transfected with the pEGFP-N2 plasmid and Pea3 expression vector, and cells that were seeded in the opposite well were co-transfected with pcDNA3.1(+)/Luc2=tdTomato and a different Pea3 expression vector or empty expression vector as control.

In the initial set, cells transfected with tdTomato and Pea3-VP16 plasmids were seeded on one side, and cells transfected with GFP and wildtype or S90A phosphomutant Pea3 plasmids were seeded on the other side of the microfluidic chambers (Figure 3.3). In this study, the cells were seeded in 150 mm cell culture dishes in parallel sets as 3 million cells. After 24 hours of incubation the cells were transfected in the plasmid combinations as mentioned above using Lipofectamine reagent as described in Materials and Methods. Cells that were co-transfected with the expression vector encoding Pea3-VP16 constitutively active variant and pcDNA3.1 (+) / Luc2=tdTomato plasmid were removed by trypsinization at the end of the first day of transfection and seeded to the left chamber of the microfluidic system. Cells that were co-transfected with pEGFP plasmid together with either the empty expression plasmid, or expression plasmids encoding wild-type Pea3 or Pea3-S90A were removed by trypsinization at the end of the first day of transfection and seeded to the right chamber of the microfluidic system.

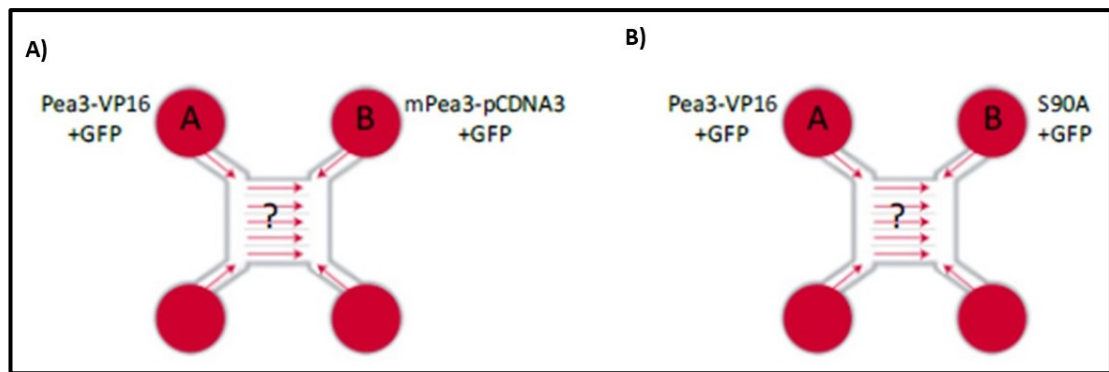


Figure 3.3: Microfluidic system. a) System optimization will be performed by detecting the direction and amount of axon elongations in the coculture system of Pea3-VP16 + GFP transfected cells in well A and in the coculture system by means of GFP radiation. b) Despite the Pea3-VP16 + GFP expression cells in well A, each mutant will be planted individually in B well and the direction and amount of axon elongation in microchannels will be examined in the same way.

Within the scope of this study, the same transfection parameters were used in co-transfections with both red and green fluorescent proteins, and the same number of cells were cultivated (however, it should be emphasized that cells transfected with plasmid encoding the TdTomato red fluorescent protein in general had lower fluorescence intensity than those transfected with pEGFP).

When cells containing Pea3-VP16 on one chamber were across from cells containing the empty pCDNA3 expression plasmid, it appeared that the cells containing pCDNA3 had axon elongation towards from the opposite side and furthermore the cells which containing pCDNA3 themselves were observed to migrate through the channels towards to Pea3-VP16 transfected cells (Figure 3.4 A:9-16). In some areas of the channel, cells did not seem to interact on either side of the channel, most likely due to low cell numbers or lack of microfluidic flow (no pump is attached to the system, the flow is obtained through intrinsic volumes of the inlet, and equilibrates upon incubation). In conditions where there are no cells on the opposite side of pCDNA3 cells, less axon elongation was observed in pCDNA3 transfected cells (Figure 3.4 A 9-12)

In the experimental sets where Pea3-VP16 transfected cells and Pea3-S90A transfected cells were seeded (Figure 3.4 A:1-8), axon elongation was very low in cells containing the phospho-mutant plasmid (green fluorescence, Figure 3.4 A:2,6), and axon elongation and cell migration were observed in cells containing the Pea3-VP16 plasmid (red fluorescence, Figure 3.4 A:3,4), although no cell migration was observed.

In the Pea3-VP16 / mPea3 experiment sets, there was no significant axon elongation and migration (Figure 3.4 B:12).

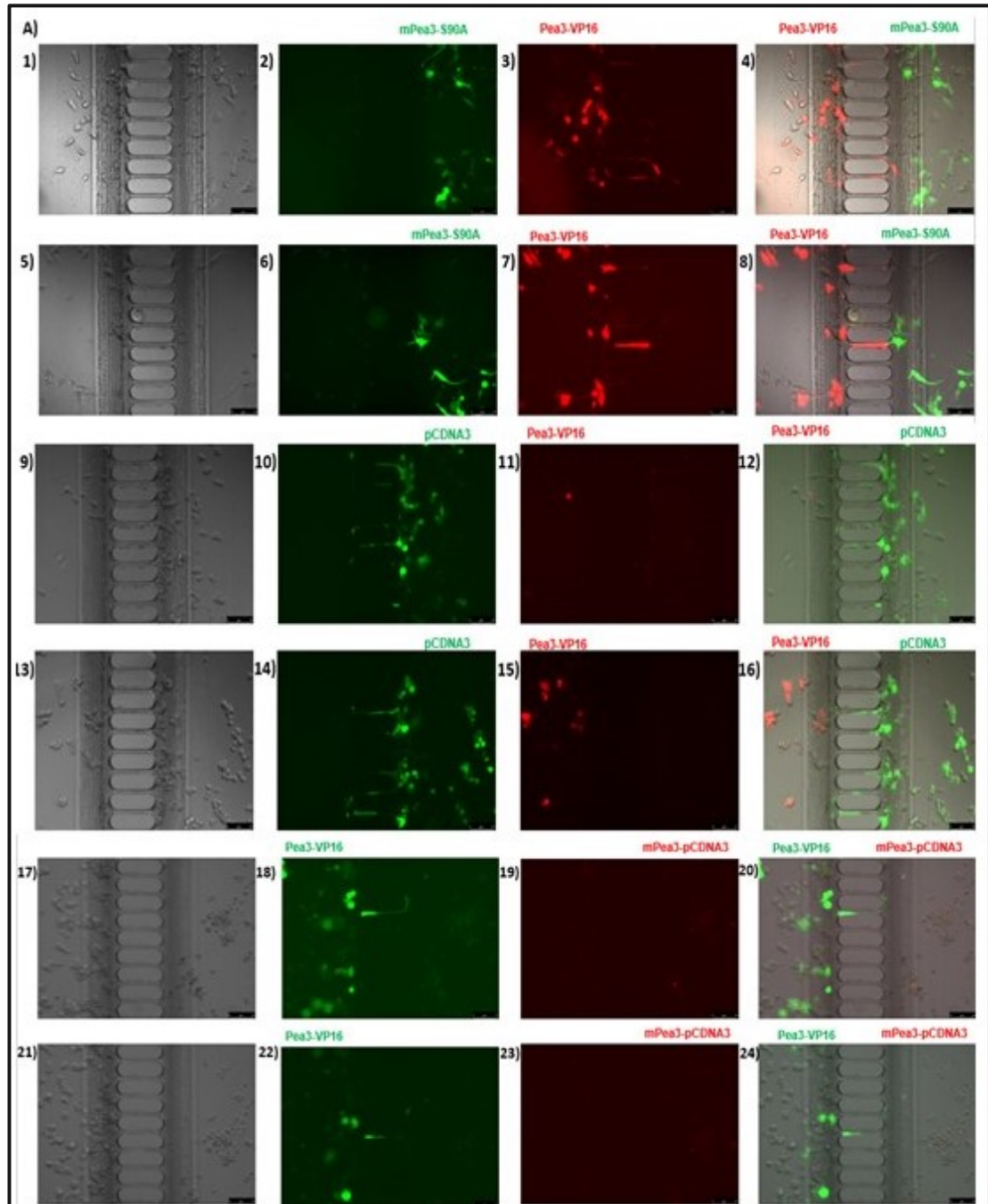


Figure 3.4: Images of axon elongation assays performed with cells transfected with the Pea3-VP16 plasmid. Red labeled cells Pea3-VP16 overexpressing cells. Transfection rates and the number of cells seeded in the microfluidic system are the same in all experimental sets. A) Separate and merged cell images in representative sets; 1-8: Pea3-VP16 / S90A-pCDNA3 images; 9-16: Pea3-VP16 / pCDNA3 images; 17-24: Pea3-VP16 / mPea3-pCDNA3 images. B) Combined images of interaction between cells expressing Pea3-VP16 and Pea3 variants. 1-6: Pea3-VP16 / pCDNA3 images; 7-11: Pea3-VP16 / S90A-pCDNA3 images; 12: Pea3-VP16 / mPea3-pCDNA3 image. Scale bar: 75 μ m.

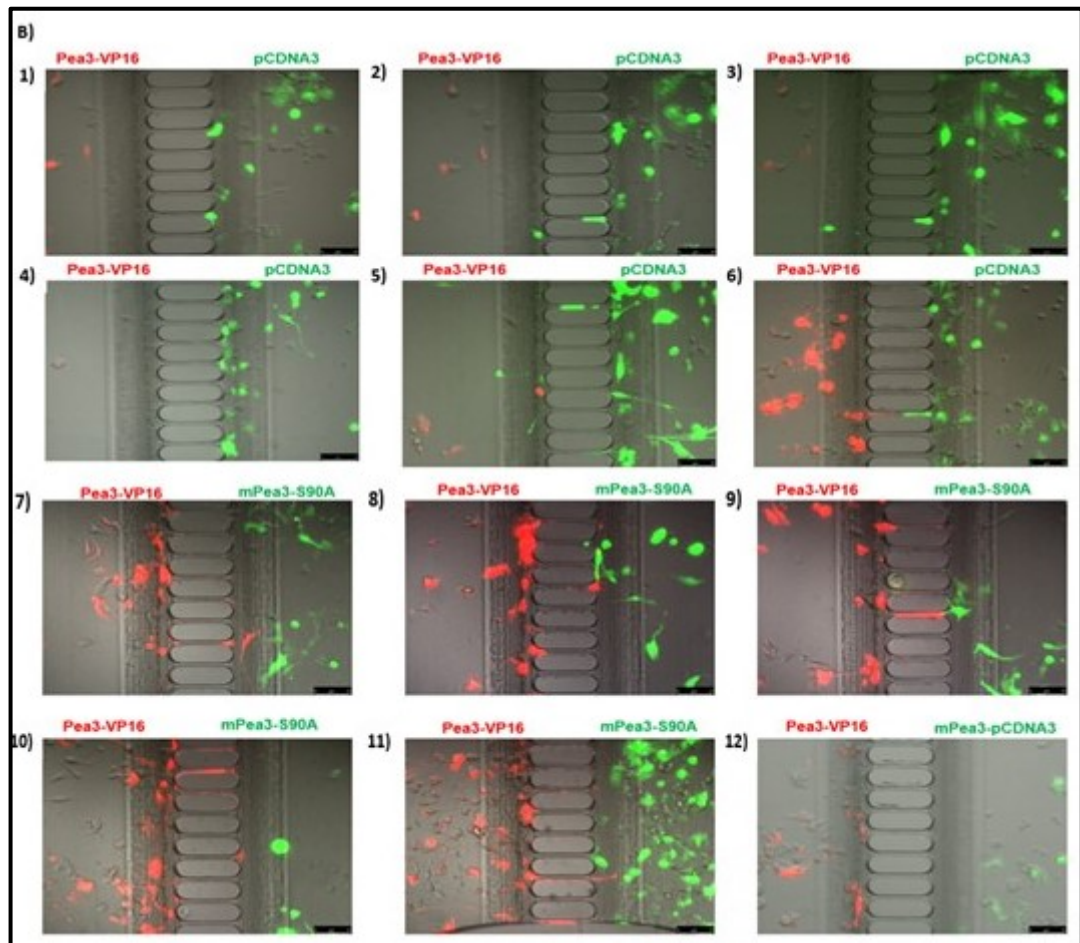


Figure 3.4: Continue.

3.1.4. Axon elongation between cells containing wild-type (mPea3-pCDNA3) plasmid and cells containing other Pea3 variants

In this set of experiments, we have investigated circuit selectivity between wildtype and phosphomutant Pea3 variants, ie mPea3, phosphomutant Pea3-S90A and phosphomimic Pea3-S90E. These expression plasmids were co-transfected with pEGFP to one set of cells, which were seeded in one chamber, while the other set of cells was co-transfected with pcDNA3.1(+)/Luc2=tdTomato and wildtype Pea3 plasmid. Although the number of cells seeded with the transfection parameters in parallel, the distribution of cells to the channel can vary between sets depending on microfluidic flow and how many cells can be carried with that flow to the channels.

When mPea3 transfected cells were seeded on both sides, we shown that axon elongation and cells were migrated. When cells expressing the wild-type Pea3 on one chamber and Pea3-S90A mutants on the other chamber of the microfluidic chip, the amount of axon elongation was relatively low. In this condition, axon elongation was observed, although it is less than other experiments (Figure 3.5). On the other hand, it was observed that the amount of axon elongation detected at the beginning of the analyzes performed at different times (54, 66 and 72 hours) decreased over time (Figure 3.6).

Contrary to the experiments with Pea3-VP16, very little if any cell body migration was observed in this set of experiments. It is possible that the constitutively active VP16 fusion of Pea3 protein is mimicking overphosphorylation of the transcription factor, and initiating a metastasis-like phenotype, overactivating cells' motility.

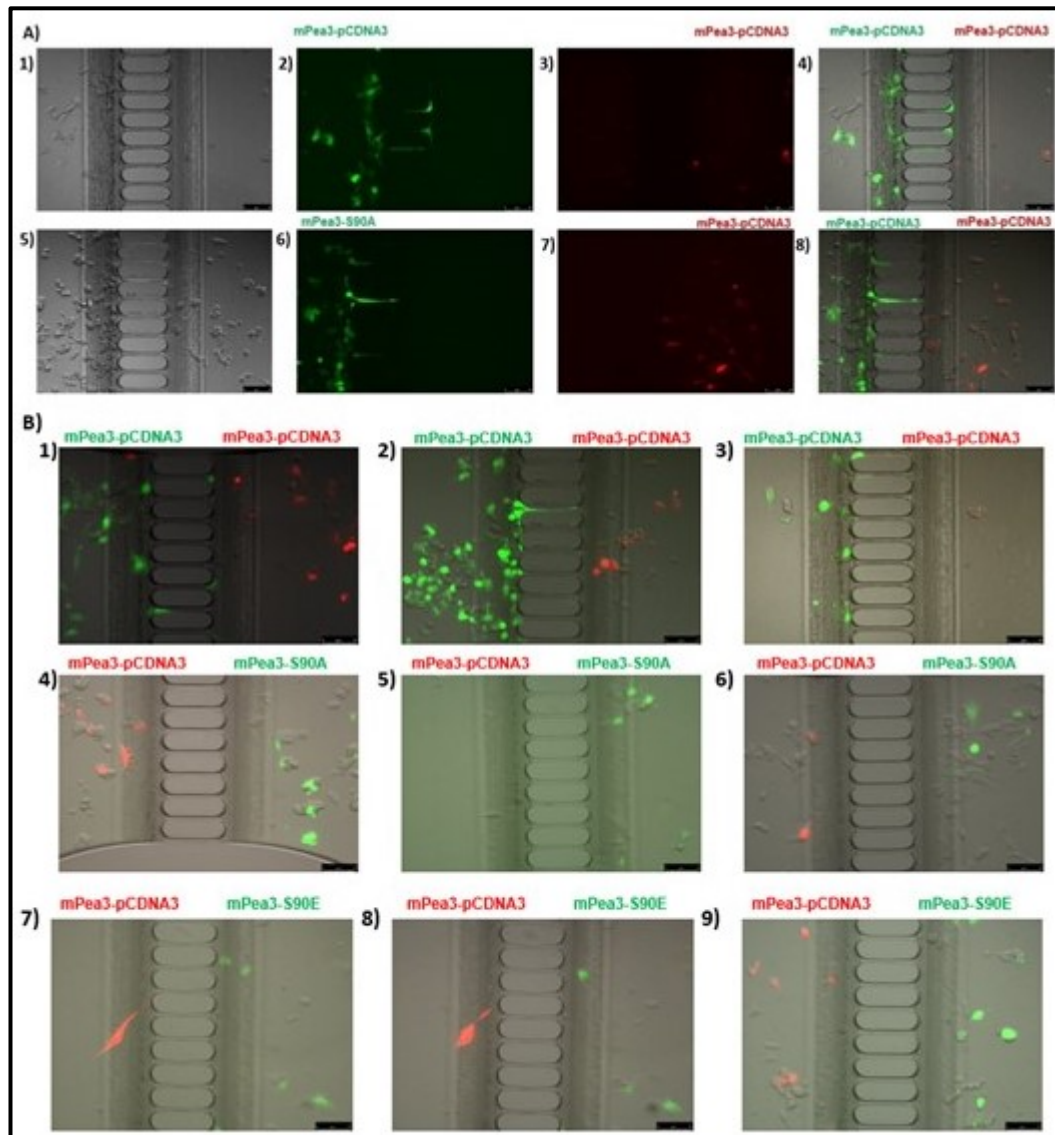


Figure 3.5: Images of cells transfected with the mPea3 expression plasmid. Red labeled cells are wild-type Pea3 group cells. A) Separate and combined images of mPea3-pCDNA3 / mPea3-pCDNA3 chips. B) Combined images of cells expressing different mPea3 variants: 1-3, mPea3-pCDNA3 / mPea3-pCDNA3 images; 4-6 mPea3-pCDNA3 / S90A-pCDNA3 images; 7-9 mPea3-pCDNA3 / S90E-pCDNA3 images. Scale bar: 75 μm.

When images were analyzed by NeuronJ program, axon elongations appeared to alter significantly from microscopic area to area in pcDNA3/Pea3VP16 and mPea3/mPea3 experimental setups, as indicated by high variability among samples (Figure 3.6). Although mPea3 / Pea3-VP16 (Figure 3.6 a) did not yield long axon elongation lengths at 54 and 66 hours, quite long axons (around 200 micrometer) were seen at 72 hours (Figure 3.6 a).

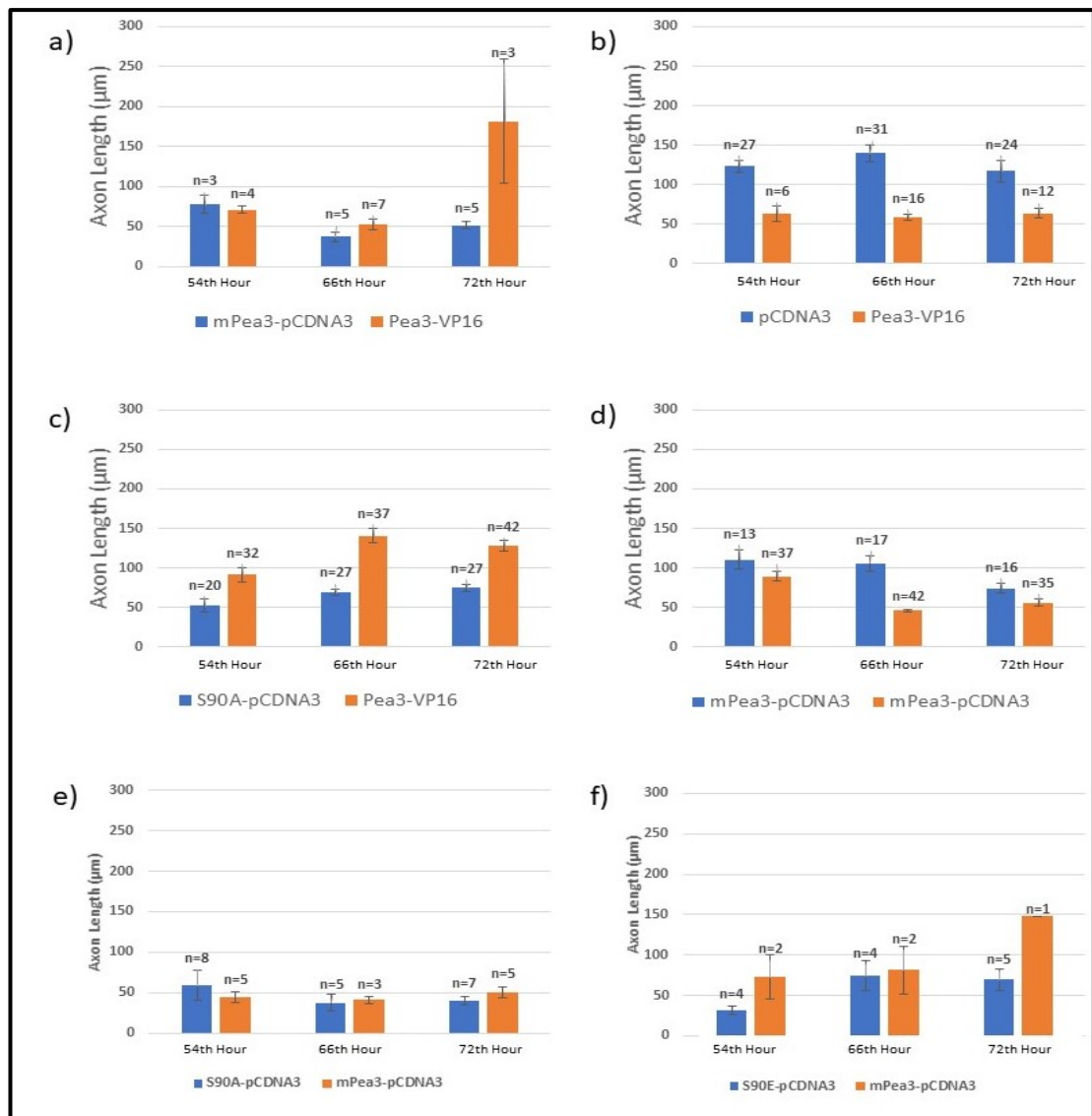


Figure 3.6: Analysis of axon lengths measured by NeuronJ program. n: number of axons measured. Error bars show the value ($\pm$ SEM). The axons of cells (n) showing axon elongation from fluorescently labeled cells were individually counted and their length was measured. (The number of transfected cells and the number of cells seeded in the microfluidic system are the same in each set of experiments set, but the number of neurons observed in each set varies in axons; these numbers are indicated on the rods).

When the combination of S90A-Pea3 phosphomutant and Pea3-VP16 were seeded on opposite chambers, there was a partial decrease in axon elongation at the 72nd hour on Pea3-VP16 side, on the other hand, the partial increase was observed in the elongation at 66th hour (Figure 3.6 c). In the combination of mPea3-S90A and mPea3-pCDNA3, an average of 50 μ m axon elongation occurred (Figure 3.6 e).

On the other hand, we showed that axon elongation in mPea3/mPea3 experimental setup shorter than a condition with Pea3-VP16/mPea3 at all time

intervals. However, it was observed that the mPea3-pCDNA3 / Pea3-S90A experiment had a shorter axon length, especially than them mPea3-pCDNA3 / Pea3-S90E experiment (Figure 3.6).

3.2. Elk-1's Role in Proliferation and Self-Renewal

3.2.1. The Effect of siElk-1 on Stemness Gene

Previous transcriptomic studies in our laboratory had identified novel targets for Elk-1 transcription factor, including stemness genes such as Sox2, Nanog and Oct-4, which have been validated to play a role in neuroblastoma cancer stem cell proliferation [Savasan Sogut et al, manuscript in preparation; Savasan Sogut PhD Thesis 2019]. In order to examine whether these results are cell type-specific and to reveal regulation in other types of cancer, we wanted to look at the effect of the Elk-1 gene on glioblastoma using the glioblastoma cell line, U87-MG (Figure 3.7).

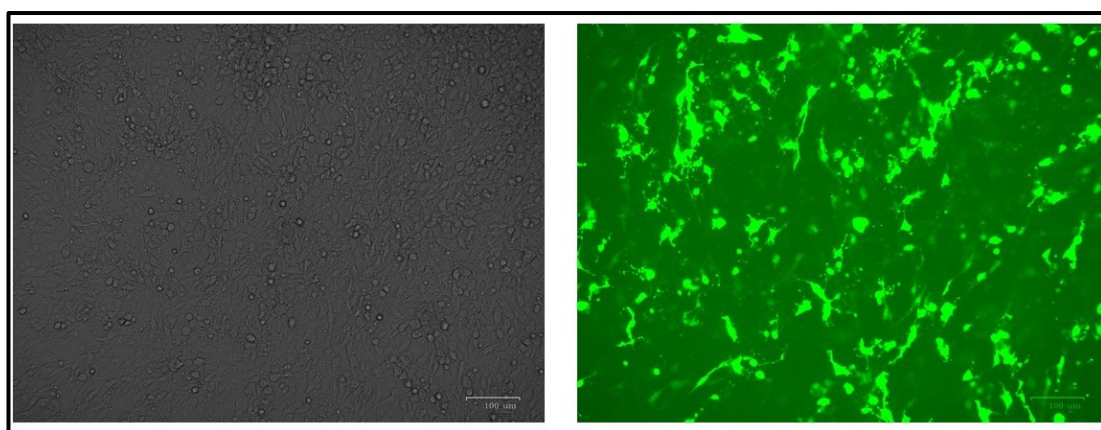


Figure 3.7: Transfected U87-MG cells images. Images taken by ZOE Fluorescent Cell Imager (Bio-Rad).

To that end, we have silenced Elk-1 message in U87-MG glioblastoma cell line using RNAi method and analyzed the expression of these target genes using qPCR method. After the cells were transfected with either siELK-1 (sh-Elk1-GFP) and scrambled RNA control plasmid (scrRNA) as described in Materials and Methods,

expression levels of Sox2, Nanog and Oct4 messages in cells were determined. *Beta-actin* and *GAPDH* genes were used for control and normalization.

When results were analyzed, it was seen that Elk-1 message was silenced about 50%. There was a nonsignificant increase in Sox2 and Pouf51 genes after silencing, contrary to microarray results that showed positive regulation of these genes by Elk-1 (Figure 3.8). Furthermore, *NANOG* gene was increased around two-fold in siElk-1 cells.

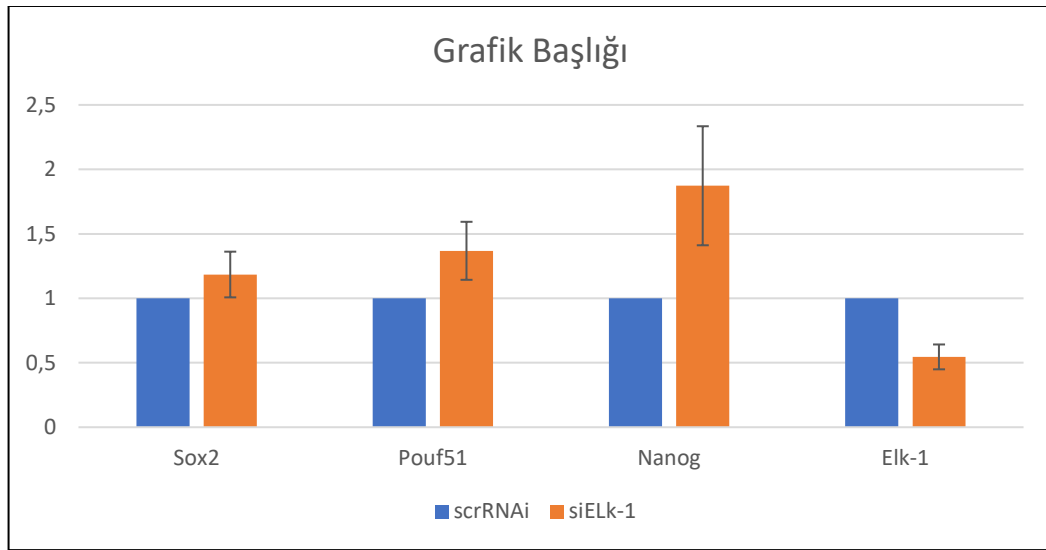


Figure 3.8: The expression of selected potential Elk-1 target genes after siElk-1 silencing in U-87 glioblastoma cell lines.

In previous studies on SK-N-SH neuroblastoma cell lines, *NANOG* and *POUF51* (*OCT4*) genes were increased and *SOX2* gene was decreased when the Elk-1 gene was silenced [Savasan Sogut, PhD Thesis 2019], indicating both that the microarray results in SH-SY5Y are not conserved in different cell lines, and that Elk-1 does not always act as a transactivator in these cells.

4. DISCUSSION AND CONCLUSION

ETS domain transcription factors are involved in various stages of development in different tissues. In this thesis we have particularly focused on the role of the Pea3 subfamily in neuronal differentiation and axon elongation [Kandemir et al., 2014; Kandemir et al., 2017], and the role of Elk-1 in regulation of novel targets involved in cancer stem cell self-renewal.

In previous studies conducted in our laboratory, there was an increase in NANOG, SOX2, POU5F1, which are the stemness genes of cells (SH-SY5Y, SKNBE (2), T98G) in which Elk-1 is over-expressed. There was no significant change in SOX2 when Elk-1 was silenced, but a decrease in NANOG and POU5F1 (Oct4) was observed [Savasan Sogut et al, manuscript in preparation; Savasan Sogut PhD Thesis 2019]. In this study conducted in the U87 cell, when the Elk-1 gene was silenced, an increase in stemness genes was observed. In this case, it shows that Elk-1 can have a suppressor effect on glioblastoma cells. When we look at the literature, some cases where the Elk-1 gene is suppressor are observed. In studies with Elk-1 expression conducted by Zhang et al., miR-326 identified in neurons has been shown to provide down regulation. In miR-326 transfected gliomas, tumor formation has been observed to be reduced [Wang et al., 2013], and it has been found to play an important role in the MAPK signaling pathway [Xu et al., 2013]. In another study, Elk-1 was observed to play a role in the suppression of megakaryoblastic leukemia (MKL) target genes [Lee et al., 2010].

Interestingly, different studies in the literature had shown that members of the Pea3 family are expressed in different motor neuron pools in a mutually exclusive manner: for example, motor neurons expressing Pea3 have been observed to selectively circuit with neurons expressing Pea3, whereas those expressing Er81 selectively circuit with Er81 expressing neurons [Lin et. Al., 1998]. In earlier microarray studies, our laboratory had defined novel transcriptional targets for Pea3-VP16 constitutively active protein, including ephrins, semaphorins and their receptors; more recent transcriptome studies performed by our laboratory, we have further investigated how such closely homologous family members (namely Pea3,

ERM and Er81) can provide such a selectivity in neuronal circuit formation [Kandemir et al., submitted].

To complement these studies, this thesis project aimed to set up and use an *in vitro* system capable of mimicking motor neuron circuit selectivity, to be used in further studying which of these transcriptional targets were responsible for circuit formation. To that end, a microfluidic co-culture system has been established in which axon elongation of neuronal model cell lines transfected with different Pea3 members can be tracked and quantified for axon elongation and selective interaction. In this thesis, we have also addressed whether specific phosphorylations of Pea3 were important in this circuit formation.

In addition, some cells in Pea3-VP16 overexpression experiment sets were seen to migrate through channels, as well as or instead of axon extension. It has been reported that Pea3 overexpression causes cell migration in different tissues and metastasis in many cancer types [Kherrouche et al., 2015; de Launiot et al., 2000; Li et al., 2013; Mesci et al., 2014]. Matrix metalloproteinases (MMP1, MMP2 and MMP9), which is involved in initiating cell migration in breast cancer, has also been described to be effective in Pea3 / ETV4 [Qin et al., 2008]. As a result of previous *in silico* analysis conducted in our laboratory, Pea3 was found to be associated with 404 human genes that affect neuronal migration (NCAM1, NRCAM, L1CAM etc.) [Kandemir et al., 2017], migrating cells were not quantified as they were not directly within the scope of this thesis study. We showed that in thesis, transfected cells with mPea-pCDNA3 and Pea3-VP16 plasmids were migrated. However, it was observed that phosphorylation of Pea3 in relation to neuronal migration during development should be examined in more detail in future studies.

We have also observed that in the experiments with Pea3-VP16, a much larger number of cells generally extended the axon (Figure 3.6). However, in sets with wildtype mPea3 variants without constitutive active VP16 fusion protein, the number of axon-prolonging neurons was also observed to be much lower (Figure 3.6). Another interesting observation noted that in the Pea3-VP16 / pCDNA3 assay sets, axons extending from pCDNA3 transfected cells to Pea3-VP16 transfected cells were also quite a lot number and long (Figure 3.6, blue bars). In this study, we showed that axon elongation appears more in cells transfected with the Pea3-VP16 plasmid. In

previous studies conducted in our laboratory on SH-SY5Y and PC12 cells, it was shown that the cells transfected with mPea3-S90E mutant had more axon elongation, and less axon extension was observed in cells transfected with mPea3-S90A [Kandemir et al., 2014]. In this study, the same situation was shown in NSC-34 cells. The aim of this study was to show the selectivity between Pea3 and Pea3. When we look at the results, we see that there is more selectivity between cells transfected with the same plasmids. But between selectivity of the cells that were transfected with different Pea3 variants is less. In future studies, we may try to increase cell experiments transfected with the same plasmids to confirm these conditions. In addition, experimental setups with other members of the PEA3 subfamily, ERM and ER81, should be established and examined and we use different cell type. In addition, a comparison can be made using different phosphomutants of Pea3. The microfluidic platform used in this study is planned to be used routinely in future neuronal circuit formation studies. It is expected to contribute to future research on the use of Pea3 proteins in neuroregeneration.

REFERENCES

- Besnard A., Galan-Rodriguez B., Vanhoutte P., Caboche J., (2011), "Elk-1 a transcription factor with multiple facets in the brain", *Frontiers in Neuroscience*, 5:35.
- Brown L. A., Amores, A., Schilling, T.F., Jowett, T., Baert, J. L., de Launoit Y., Sharrocks A. D., (1998), "Molecular characterization of the zebrafish PEA3 ETS-domain transcription factor", *Oncogene*, 17(1), 93-104.
- Bylund M., Andersson E., Novitch B. G., Muhr J., (2003), Vertebrate neurogenesis is counteracted by Sox1-3 activity. *Nat Neurosci*. 6:1162–1168.
- Caglayan B., (2010), "Identification of upstream regulators of MAPK stimulated Pea3 mediated neural differentiation of PC12 cells", Master of Science Thesis, Yeditepe University.
- Chotteau-Lelievre A., Dolle P., Peronne V., Coutte L., de Launoit Y., Desbiens X., (2001), "Expression patterns of the Ets transcription factors from the PEA3 group during early stages of mouse development", *Mechanisms of Development*, 108(1-2), 191-195.
- Chotteau-Lelievre A., Montesano R., Soriano J., Soulie P., Desbiens X., de Launoit Y., (2003), "PEA3 transcription factors are expressed in tissues undergoing branching morphogenesis and promote formation of duct-like structures by mammary epithelial cells in vitro", *Developmental Biology*, 259(2), 241-257.
- Craig M. P., Sumanas S., (2016), "ETS transcription factors in embryonic vascular development", *Angiogenesis*, 19(3), 275-285.
- Cui W., Zhang S., (2014), "Sox2, a key factor in the regulation of pluripotency and neural differentiation", *World J Stem Cells*, 305-311.
- de Launoit Y., Baert J. L., Chotteau A., Monte D., Defossez P. A., Coutte L., Pelczar H., Leenders F., (1997), "Structure-function relationships of the PEA3 group of Ets-related transcription factors", *Biochem Mol Med*, 61(2), 127-135.
- de Launoit Y., Chotteau-Lelievre A., Beaudoin C., Coutte L., Netzer S., Brenner C., Huvent I., Baert, J. L., (2000), "the PEA3 group of ETS-related transcription factors. Role in breast cancer metastasis", *Adv Exp Med Biol*, 480, 107 – 116.
- Demir O., Aksan Kurnaz I., (2013), "Phospho-Ser383-Elk-1 is localized to the mitotic spindles during cell cycle and interacts with mitotic kinase Aurora-A", *Cell Biochemistry and Function*

- Demir O., Aysit N., Önder Z., Turkel N., Öztürk G., Sharrocks A.D., Kurnaz I., (2011), "ETS-domain transcription factor Elk-1 mediates neuronal survival: SMN as a potential target", *BBA-Mol. Basis Dis.*, 1812, pp. 652-662.
- Deppmann C. D., Ginty D. D., (2006), "Retrograde control of neural circuit formation", *Cell* 127(7), 1306-1307.
- Fontanet P. A., Ríos A. S., Alsina F. C., Paratcha G., Ledda F., (2016), "Pea3 Transcription Factors, Etv4 and Etv5, Are Required for Proper Hippocampal Dendrite Development and Plasticity", *Cerebral Cortex*, 28(1), 236-249.
- Gawlik-Rzemieniewska N., Bednarek I., (2016), "The role of NANOG transcriptional factor in the development of malignant phenotype of cancer cells", *Cancer Biol Ther.* ;17(1):1–10.
- Graham V., Khudyakov J., Ellis P., Pevny L., (2003), "SOX2 functions to maintain neural progenitor identity", *Neuron*, 749–765.
- Gutierrez-Hartmann A., Duval D. L., Bradford A. P., (2007), "ETS transcription factors in endocrine systems", *Trends Endocrinol Metab*, 18(4), 150-158.
- Hanif F., Muzaffar K., Perveen K., Malhi S. M., Simjee ShU. (2017), "Glioblastoma Multiforme: A Review of its Epidemiology and Pathogenesis through Clinical Presentation and Treatment", *Asian Pac J Cancer Prev.* ;18(1):3–9.
- Hennenberg M., Strittmatter F., Beckmann C., Rutz B., Füllhase C., Waidelich R., Montorsi F., Hedlund P., Andersson K. E., Stief C. G., Gratzke C., (2012), "Silodosin inhibits noradrenaline-activated transcription factors Elk1 and SRF in human prostate smooth muscle.", *PLoS One*, 7 (11).
- Higashino F., Yoshida K., Fujinaga Y., Kamio K., Fujinaga K., (1993), "Isolation of a cDNA encoding the adenovirus E1A enhancer binding protein: a new human member of the ets oncogene family", *Nucleic Acids Res*, 21(3), 547-553.
- Holland E. C., (2000), "Glioblastoma multiforme:the terminator." *Proc Natl Acad Sci.*, 97:6242–44.
- Hollenhorst P. C., Jones D. A., Graves B. J., (2004), "Expression profiles frame the promoter specificity dilemma of the ETS family of transcription factors", *Nucleic Acids Res*, 32(18), 5693-5702.
- Jocher G., Mannschatz S. H., Offterdinger M., Schweigreiter R., (2018), "Microfluidics of small-population neurons allows for a precise quantification of the peripheral axonal growth state", *Front Cell Neurosci*, 12, 166.
- Kandemir B., Caglayan B., Hausott B., Erdogan B., Dag U., Demir O., Savasan Sogut M., Klimaschewski L., Aksan Kurnaz I., (2014), "Pea3 transcription factor promotes neurite outgrowth", *Front Mol Neurosci*, 7, 59.

Kandemir B., Dag U., Bakir Gungor B., Durasi I. M., Erdogan B., Sahin E., Sezerman U., Aksan Kurnaz, I., (2017), "In silico analyses and global transcriptional profiling reveal novel putative targets for Pea3 transcription factor related to its function in neurons", *PLoS One*, 12(2), e0170585.

Kandemir B., Gülfidan Yildiz G., Arga K. Y., Yilmaz B., Aksan Kurnaz I., (2019), "Transcriptomic profile of Pea3 family members reveal regulatory codes for axon outgrowth and neuronal connection specificity".

Kherrouche Z., Monte D., Werkmeister E., Stoven L., De Launoit Y., Cortot A. B., Tulasne D., Chotteau-Lelievre A., (2015), "PEA3 transcription factors are downstream effectors of Met signaling involved in migration and invasiveness of Met-addicted tumor cells", *Mol Oncol*, 9(9), 1852-1867.

Laudet V., Hanni C., Stehelin D., Duterrque-Coquillaud M., (1999), "Molecular phylogeny of the ETS gene family", *Oncogene*, 18(6), 1351-1359.

Lee S. M., Vasishtha M., Prywes R., (2010), "Activation and repression of cellular immediate early genes by serum response factor cofactors", *The Journal of biological chemistry*, 285(29), 22036–22049.

Li S., Huang X., Zhang D., Huang Q., Pei G., Wang L., Jiang W., Hu Q., Tan R., Hua Z. C., (2013), "Requirement of Pea3 for transcriptional activation of FAK gene in tumor metastasis", *PLoS One*, 8(11); e79336.

Lin J. H., Saito T., Anderson D. J., Lance-Jones D. J., Jessell T. M., Arber S., (1998), "Functionally Related Motor Neuron Pool and Muscle Sensory Afferent Subtypes Defined by Coordinate ETS Gene Expression", *Cell*, 95(3), 393-407.

Mesci A., Taeb S., Huang X., Jairath R., Sivaloganathan D., Liu S. K., (2014), "Pea3 expression promotes the invasive and metastatic potential of colorectal carcinoma", *World J Gastroenterol*, 20(46), 17376 – 17387.

Messali A., Villacorta R., Hay J.W., (2014), "A Review of the economic burden of Glioblastoma and the cost effectiveness of pharmacologic treatments", *Pharmacoeconomics*, 32:1201–1201.

Nagendran T., Poole V., Harris J., Taylor A. M., (2018), "Use of pre-assembled plastic microfluidic chips for compartmentalizing primary murine neurons", *J Visual Exp*, 141, e5841.

Oikawa T., Yamada T., (2003), "Molecular biology of the Ets family of transcription factors", *Gene*, 303, 11-34.

Popko J., Fernandes A., Brites D., Lanier L. M., (2009), "Automated analysis of NeuronJ tracing data", *Cytometry A*, 75(4), 371-376.

Rodda D. J., Chem J., Lim L., Loh Y., Wang B., Ng H., Robson P., (2005), "Transcriptional Regulation of Nanog by OCT4 and SOX2", *The Journal of Biological Chemistry*, 280, 24731-24737.

Sharma A., Callahan L. M., Sul J., Kim T. K., Barrett L., Kim M., Powers J. M., Federoff H., Eberwine J., (2010), "A Neurotoxic Phosphoform of Elk-1 Associates with Inclusions from Multiple Neurodegenerative Diseases" *Plos One*.

Sharrocks A. D., (2001), "The ETS-domain transcription factor family", *Nat Rev Mol Cell Biol*, 2(11), 827-837.

Shi G., Jin Y., (2010), "Role of Oct4 in maintaining and regaining stem cell pluripotency", *Stem Cell Res Ther*.

Siu M. K. Y., Wong E. S. Y., Kong D. S. H., Chan H. Y., Jiand L., Wong O. G. W., Lam E. W-F., Chan K. K. L., Ngan H. Y. S., Le X-F., Cheung A. N., (2013), "Stem cell transcription factor NANOG controls cell migration and invasion via dysregulation of E-cadherin and FoxJ1 and contributes to adverse clinical outcome in ovarian cancers", *Oncogene*, 32,3500-3509.

Sizemore G. M., Pitarresi J. R., Balakrishnan S., Ostrowski, M. C., (2017), "The ETS family of oncogenic transcription factors in solid tumours", *Nat Rev Cancer*, 17(6), 337-351.

Takahashi K., Yamanaka S., (2006), "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors", *Cell*, 126: 663-676

Ustun M., (2017), "Identifying Pea3 phosphorylation sites specifically regulated by different growth factors", *Master of Science Thesis*, Gebze Technical University.

Wang S., Lu S., Geng S., (2013), "Expression and clinical significance of microRNA-326 in human glioma miR-326 expression in glioma" *Med Oncol*, **30**, 373.

Wegner M., (2010), "All purpose Sox: The many roles of Sox proteins in gene expression", *Int J Biochem Cell Biol*, 42:381–390.

Xin J. H., Cowie A., Lachance P., Hassell J. A., (1992), "Molecular cloning and characterization of PEA3, a new member of the Ets oncogene family that is differentially expressed in mouse embryonic cells", *Genes Dev*, 6(3), 481-496.

Zhang P., Kong F., Deng X., Yu Y., Hou C., Liang T., Zhu, L., (2017), "MicroRNA-326 suppresses the proliferation, migration and invasion of cervical cancer cells by targeting ELK1", *Oncology Letters*, 13, 2949-2956.

BIOGRAPHY

Mustafa Dogukan METINER was born in Istanbul, 1994. He achieved bachelor's degree from Department of Molecular Biologyt and Genetics, Bartın University in 2016. He was a Biology teacher in a private high school for a year. He has continued master degree program in the Departmen of Molecular Biology and Genetics at Molecular Neurobiology Laboratory, Gebze Technical University, since 2018 spring.