REPUBLIC OF TURKEY
DOKUZ EYLUL UNIVERSITY
IZMIR INTERNATIONAL BIOMEDICINE AND GENOME INSTITUTE

INVESTIGATION OF THE ROLE OF WNT/β-CATENIN SIGNALING IN BRAIN REGENERATION USING ZEBRAFISH AS A MODEL SYSTEM

GÖKHAN CUCUN

DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

MASTER OF SCIENCE THESIS

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T.C.
DOKUZ EYLÜL ÜNİVERSİTESİ
İZMİR ULUSLARARASI BİYOTIP VE GENOM ENSTİTÜSÜ

ÇEVRİM İÇİ YÜKSEK LİSANS TEZ SAVUNMA SINAVI TUTANAĞI

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☒ Çevrim İçi

☒ Sınav kayıt altına alınmıştır.

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ABBREVIATIONS

CNS ..... Central Nervous System
Ctgfa ..... Connective tissue growth factor a
Dpl ..... Days post lesion
EGFP ..... Enhanced Green Fluorescent Protein
Fsta ..... Follistatin-A
GFAP ..... Glial Fibrillary Acidic Protein
GS ..... Gluthamine Sythase
GSK-3β ..... Glycogen Synthase Kinase 3 beta
Hpl ..... Hours post lesion
Inhbab ..... Inhibin, beta Ab
Klf11b ..... Kruppel-like factor 11b
LEF1 ..... Lymphoid enhancer factor 1
Neurod1 ..... Neurogenic Differentiation Factor 1
Olig2 ..... Oligodendrocyte Transcription Factor
Pappaa ..... Pregnancy-associated plasma protein A
PCNA ..... Proliferating Cell Nuclear Antigen
Prg4b ..... Proteoglycan 4b
PV ..... Parvalbumin
RG ..... Radial Glia
S100β ..... S100 Calcium-Binding Protein β
TCF ..... T-Cell Factor

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INVESTIGATION OF THE ROLE OF WNT/β-CATENIN SIGNALING IN BRAIN REGENERATION USING ZEBRAFISH AS A MODEL SYSTEM

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ABSTRACT

Regeneration ability is highly limited in the mammalian central nervous system. On the contrary, one of the teleost models zebrafish has enormous brain regeneration capacity among vertebrates. Taking the brain regeneration ability of adult zebrafish as an advantage, elucidation of molecular mechanisms of brain regeneration has a pivotal role for regenerative medicine research.

Wnt signaling is one of the tightly regulated signaling pathways that involve in organ regeneration and tissue homeostasis. Dysregulation and certain mutation in both components and downstream targets of Wnt signaling lead to improper tissue regeneration and cancer. Identification of novel Wnt modulators paves the way for the treatment of various Wnt signaling dependent diseases.

In the scope of this thesis, traumatic brain injury model was performed in the adult zebrafish telencephalon. Reporter activity was measured by q-PCR in both injured and uninjured hemispheres of the telencephalon in Wnt reporter line at two separate stages; early-stage and proliferative stage. In addition, localization of Wnt signaling was detected in the regions of telencephalon by immunohistochemistry staining. RNA Sequencing was also performed to identify Wnt modulators which involve in brain regeneration. In conclusion, novel Wnt modulators and their potential interactors have been identified at two crucial stages of brain regeneration.

Keywords: Brain injury, Wnt/β-catenin signaling, RNA-Seq, Telencephalon

WNT/β-KATENİN SİNYAL YOLAĞININ ZEBRABALIĞI MODELİ KULLANILARAK BEYİN REJENERASYONUNDAKİ ROLÜNÜN ARAŞTIRILMASI

Gökhan Cucun, Dokuz Eylül Üniversitesi, İzmir Uluslararası Biyotıp ve Genom Enstitüsü, Sağlık Kampüsü Balçova 35340- İzmir / TÜRKİYE

ÖZET

Memeli merkezi sinir sisteminde rejenerasyon yeteneği kısıtlıdır. Buna karşılık, teleost modeli olan zebrabalığı omurgalılar arasında büyük ölçüde rejenerasyon kapasitesine sahiptir. Erişkin zebrabalığının bu kapasitesiniden yararlanarak, beyin rejenerasyonunun moleküler mekanizmalarının aydınlatılması rejeneratif tıp araştırmaları için önemli bir role sahiptir.

Wnt sinyal yolağı organ rejenerasyonu ve doku homeostasisinde görev alan, sıkı bir şekilde kontrol edilen sinyal yolaklarından biridir. Wnt sinyal yolağındaki bozukluk ve bileşenlerindeki belirli mutasyonlar rejenerasyonun tam gerçekleşmemesine ve kansere neden olmaktadır. Yeni Wnt modülatörlerinin belirlenmesi çeşitli Wnt sinyal yolağı ile ilişkili olan hastalıkların tedavisi için yol açmaktadır.

Bu tez kapsamında, erişkin zebrabalığının telensefalon bölgesinde travmatik beyin hasarı modeli uygulanmıştır. Sinyal aktivitesi kantitatif PCR ile hem hasarlı hemde hasarsız hemisferde, erken ve proliferatif evrelerde ölçülmüştür. Ayrıca, immunohistokimya boyaması ile Wnt sinyali telensefalonun bölgelerinde belirlenmiştir. Beyin rejenerasyonunda görevli olan Wnt modülatörlerini belirlemek için ayrıca RNA sekanslaması gerçekleştirilmiştir. Sonuç olarak, beyin rejenerasyonunun iki önemli evresinde yeni Wnt hedefleri ve potansiyel olarak etkileşimleri belirlenmiştir.

Anahtar kelimeler: Beyin hasarı, Wnt/β-katenin sinyal yolağı, RNA sekanslama, Telensefalon

1. INTRODUCTION AND AIM

1.1 Statement and Importance of the Problem

Wnt/β-catenin signaling is one of the signaling pathways that is involved in regenerating organs including the brain. Activation of this signaling pathway in regenerating tissues emphasis the cruciality and necessity of Wnt/β-catenin signaling. Unfortunately, the capacity of brain repair is highly restricted in mammals, but zebrafish has a remarkable regenerative ability to repair various tissues via Wnt/β-catenin signaling. In zebrafish, the role of Wnt/β-catenin signaling in brain regeneration has been clarified except the telencephalon region.

1.2 Aim of Study

This study aims to broaden our deep understanding of the role of Wnt/β-catenin signaling during brain regeneration. For this aim, we performed a stab wound assay in the part of adult zebrafish telencephalon and immunohistochemistry staining to determine the activation of Wnt/β-catenin signaling. Additionally, RNA-Seq is performed to elucidate Wnt/β-catenin signaling-related genes.

1.3 Hypothesis of Study

The hypothesis of this study includes the activation of Wnt/β-catenin signaling at the early stage of brain regeneration and identifying regeneration associated genes that play essential roles in regenerating tissues.

2. GENERAL INFORMATION

2.1 Canonical Wnt signaling

Canonical Wnt signaling is one of the highly conserved signaling pathways which involves in various biological process in animals (Holstein, 2012). Activation of Canonical Wnt signaling relies on the interaction between secreted lipid-modified and glycosylated Wnt proteins and their transmembrane receptor proteins, Frizzled, and co-receptor, low-density lipoprotein receptor protein 5/6 (LRP5/6) (Nile, Mukund, Stanger, Wang, & Hannoush, 2017). Wnt proteins can follow discrete routes to reach their target cells to activate the expression of its downstream target genes (Driehuis & Clevers, 2017), (Gross, Chaudhary, Bartscherer, & Boutros, 2012), (Neumann et al., 2009).

β-catenin plays a central role in transcriptional activation of downstream targets of Canonical Wnt signaling. This protein contains 12 copies of a 42 amino acid motifs called armadillo repeat sequences that enable its nuclear transport by interacting with nucleoporins (Xing et al., 2008),(Sharma, Jamieson, Johnson, Molloy, & Henderson, 2012). In the absence of Wnt glycoprotein, cytoplasmic β-catenin stabilizing complex which is consist of Axin, Adenomatous polyposis coli (APC), Glycogen Synthase Kinase 3 beta (GSK3-β) and Casein kinase (CK1α) promotes degradation of β-catenin. Axin is a scaffold protein that has a binding region of other components of destruction complex such as APC (Zhan, Rindtorff, & Boutros, 2017). This protein functions as a tumor suppressor in colon cancer. In addition, kinases such as GSK3-β and CK1α have an inhibitory role in the regulation of Canonical Wnt signaling by phosphorylating β-catenin at Ser33/Ser37/Thr41 and at Ser45 residues respectively (Jiang, Zhang, Sun, & Yang, 2018), (Clevers & Nusse, 2012). In the presence of Wnt ligands such as Wnt1 and Wnt3a, Dishevelled (Dvl) inhibits the formation of destruction complex by destabilizing this destruction complex. This process leads to translocation of the increased number of cytoplasmic β-catenin into the nucleus, where it binds to T-cell factor/Lymphoid enhancer factor (TCF/LEF) transcription factors (Figure 1).

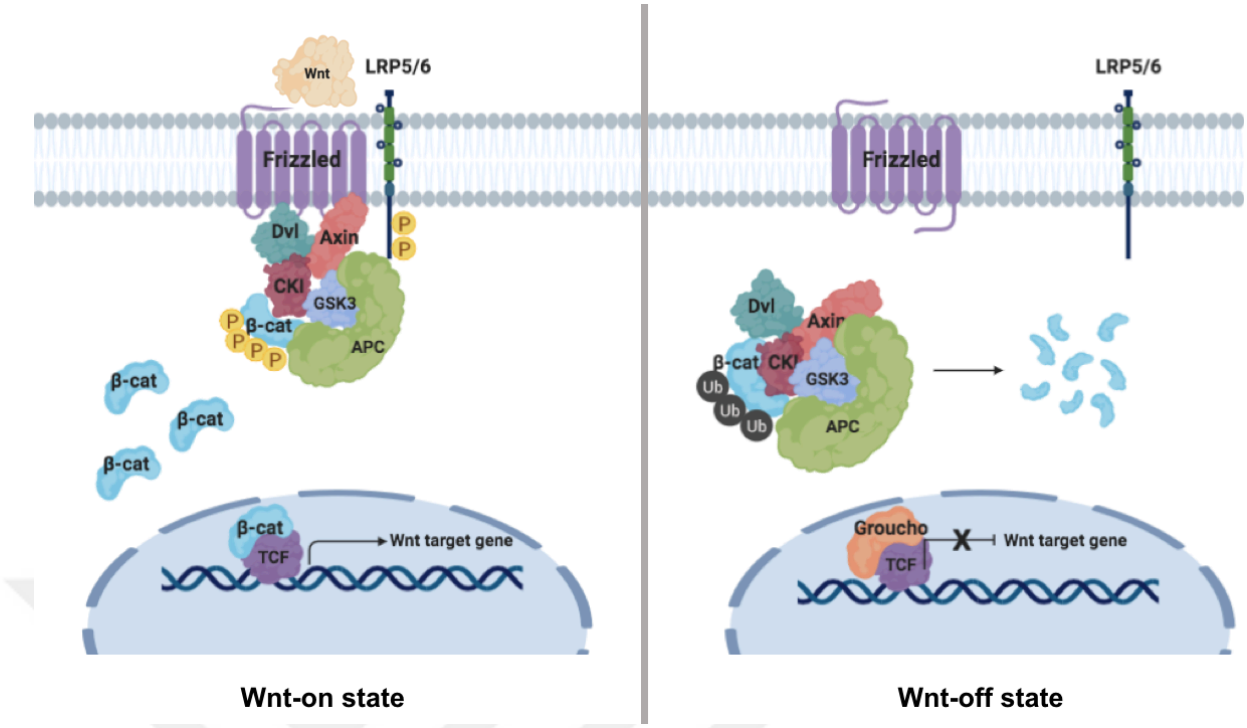


Figure 1: Canonical Wnt signaling *(adapted from Centellus et al., 2012)*

2.1.1 Wnt signaling in homeostasis

Wnt signaling has diverse roles in numerous biological processes from embryonic development to adult organ regeneration (Nüsslein-Volhard & Wieschaus, 1980), (Yamada et al., 2009),(Matsushita et al., 2020).

Functional roles of Wnt signaling have been well documented in the adult stem cell maintenance and their proliferation in different types of tissues (Polakis, 2000),(Reya & Clevers, 2005). The involvement of Wnt signaling in adult intestinal epithelial cell turnover is highlighted by a variety of studies (Fevr, Robine, Louvard, & Huelsken, 2007), (Sukhotnik et al., 2014), (Mah, Yan, & Kuo, 2016).

Activation of Wnt signaling has been associated with maintenance and regeneration of hair follicle stem cells (Mah et al., 2016), (Qiu et al., 2017). Additionally, perturbation of Wnt signaling may leads to development of cancer, neurological diseases, and developmental abnormalities during embryonic development. For instance, a certain mutation in the component of Wnt signaling disrupts bone homeostasis. (Gong et al., 2001), (Boyden et al., 2002)

2.2 Wnt signaling in Central Nervous System Regeneration

2.2.1 Spinal Cord Regeneration

Spinal cord injury is one of the prevalent injury types in the nervous system. Various signaling pathways have been shown to involve spinal cord repair (Li, Gu, & Yi, 2017), (Kamei et al., 2012), (Schellino, Boido, & Vercelli, 2019). Wnt signaling is one of the regulatory pathways in spinal cord regeneration (Yamagami, Pleasure, Lam, & Zhou, 2018).

Transient activation of β-catenin dependent Wnt signaling is required in the injured spinal cord of larval and adult zebrafish to repair (Strand et al., 2016), (Wehner et al., 2017). Negative regulation of Canonical Wnt signaling blocks the functional restoration of the transected spinal cord by inhibiting the formation of bipolar glial cells (Strand et al., 2016). In addition to the glial cell, fibroblast cells are another regeneration inducing cell types in regenerating spinal cord. (Wehner et al., 2017) demonstrated that Wnt signaling induces the expression of col12a1a/b genes in fibroblast-like cells in zebrafish. These cells are the most Wnt responding cells in the injured spinal cord indicating fibroblast-like cells have a crucial role during spinal cord regeneration by modulating extracellular matrix protein named collagen via Wnt signaling.

In addition to the zebrafish model, the beneficial role of Wnt signaling has been emphasized in the rat model. Transplantation of Wnt3a-secreting fibroblasts improves the locomotor function in the injured rat spinal cord. In addition to the beneficial role of Wnt signaling, also its inhibitory roles have been indicated in spinal cord injury. Wnt glycoproteins including Wnt5a and Wnt1 do not induce spinal cord recovery (Suh et al., 2011). On the other side, spinal cord regeneration associated genes adversely affected by inhibition of Wnt secretion indicating some of Canonical Wnt ligands such as Wnt 3 and Wnt 10 are necessary for the activation of regeneration-associated genes in regenerating spinal cord (Herman et al., 2018). In the spinal cord, not only Wnt ligands are decisive in regeneration, but also receptors of Wnt signaling such as Frizzleds play an essential role in this process. Diversified amount of frizzled receptors have been detected in different cell types including oligodendrocyte, reactive microglia/macrophage, neurons astrocytes, and glial precursors during spinal cord regeneration. Interestingly, the expression of Frizzled 5 does not show cellular specificity indicating this receptor is much more sensitive to injury (Gonzalez, Fernandez-Martos, Gonzalez-Fernandez, Arenas, & Rodriguez, 2012). Existence of overexpressed Frizzled 4 in astrocyte and Frizzled 1

in neuronal cells induces cell-type-specific transcriptional activation in the near of spinal cord injury (González-Fernández, Fernández-Martos, Shields, Arenas, & Javier Rodríguez, 2014).

2.2.2 Brain Regeneration

The varying roles of Wnt signaling have been established in neurons, microglia, and astrocytes in brain regeneration ((He, Liao, & Pan, 2018), (L'Episcopo et al., 2018). Various brain regeneration models have been developed to understand the mechanism of brain healing.

Activation of Canonical Wnt signaling results in cell survival and neuronal cell proliferation by activating its downstream transcription factors such as Cyclin D1 and NeuroD1. Additionally, inhibition of GSK3-β induces neurogenesis in cerebral ischemia via activating Akt signaling (Xu et al., 2019). (Wang et al., 2020) showed that Non-canonical Wnt signaling also has a neuroprotective role in the subventricular zone and hippocampus by reducing cell apoptosis.

In Alzheimer's disease (AD), neuronal cells are prone to programmed cell death in the inhibition of Canonical Wnt signaling indicating the importance of Wnt signaling for cell survival (Z. Zhang et al., 1998). Accumulation of amyloid peptide in the brain is one of the hallmarks of AD. (N. Zhang, Parr, Birch, Goldfinger, & Sastre, 2018) showed that interaction of both amyloid precursor protein (APP) and β-catenin may disrupt β-catenin-dependent Wnt signaling by inhibiting transcriptional activation of its downstream targets which has been known to be required for neuronal survival genes such as cyclin D1. Furthermore, targeting GSK3-β keeps neurons healthy in amyloid toxicity-induced cell death in a rat model (Inestrosa et al., 2002). Wnt signaling not only involves in amyloid or tau toxicity caused neurodegenerative disease but also involves the aggregation of Lewy bodies causes disease such as Parkinson's disease (PD). Deregulation of Axin scaffold protein which is a negative regulator of Wnt signaling protects neurons against apoptosis in the hippocampus by regulating transcriptional activation of microRNA (G. Zhang et al., 2020). Additionally, targeting GSK3-β to enhance the activation of Wnt signaling supports neuronal survival through Nurr1 in PD patients (Guttuso, 2019).

The importance of Canonical Wnt signaling has been highlighted in neuro-immune interaction by modulating microglia polarization from the pro-inflammatory phase to the anti-inflammatory phase in a stroke model. Activation of this signaling leads to the activation of anti-inflammatory microglia which has been shown to induce CNS repair(Song et al., 2019).

Intranasal administered Wnt3a attenuated ischemic stroke-induced reactive gliosis by modulating transcriptional activation of pro-inflammatory and anti-inflammatory mediators (D. Zhang et al., 2019).

Wnt signaling maintains the Blood-Brain Barrier (BBB) integrity and angiogenesis under both normal and pathological circumstances. The sufficiency of Wnt signaling has been elucidated in neurodegenerative disease models including brain injury, stroke, and seizure (Munji et al., 2019). Some of the downstream targets of Wnt signaling such as LEF1 and TCF4 have a binding region of some of the transporters and tight junctions protein families that maintain BBB integrity. It has been well established that transcriptional regulation of certain Claudin gene family members depends on Wnt signaling. Transcriptional activation of Claudin-2 is regulated by cdx gene which is known to be modulated by Canonical Wnt signaling while destabilization of β-catenin also negatively affects the expression of another claudin gene family member, Claudin-3 in endothelial cells (Mankertz et al., 2004), (Liebner et al., 2008). Transcriptional suppression of such tight junctions result in increased BBB permeability that causes the activation of inflammatory response. Various signaling pathways including Wnt also associated with endothelial transcriptional regulation of plasmalemma vesicle-associated protein (plvap) which is a specific and glycosylated protein for endothelial cells (Stan, 2004). It was shown that plvap protein is associated with vascular leakage in brain cancer and brain (Bosma, van Noorden, Schlingemann, & Klaassen, 2018), (Shue et al., 2008). Activation of Canonical Wnt signaling leads to the accumulation of β-catenin in nucleus where it inhibits the expression level of plvap to induce maturation of the blood-brain barrier and blood-retinal barrier (Liebner & Plate, 2010).

In addition to Canonical Wnt pathway, the Noncanonical Wnt pathway also involves in the proliferation and maintenance of endothelial cells. Wnt5a stimulation increases tight junction integrity by activating Wnt/Par/aPKC PCP pathway in endothelial cells (Artus et al., 2014). (Rao et al., 2007) showed that Angiopoietin 2 induced production of β-catenin-independent Wnt ligands by macrophages maintains vessel formation. In a various conditions such as pathological and developmental stage, the ligands of Noncanonical Wnt signaling play an essential role in vascular remodeling and pruning (Korn et al., 2014).

The involvement of Wnt signaling in regenerating tissue is highly conserved across species. The role of Wnt signaling in brain regeneration is not only detected in the mice model, but also emphasized in zebrafish brain regeneration models. Wnt signaling is required for

proliferation and differentiation of radial glia in the adult optic tectum upon stab wound injury model. Inhibition of Wnt signaling leads to reduced number of proliferating radial glial cells that limit the regeneration ability of zebrafish (Y. Shimizu, Ueda, & Ohshima, 2018). Another region of adult zebrafish brain, habenula is conserved and rich in stem cells. It is observed that the expression level of Wnt-2 increased at the early stage of brain regeneration indicating Canonical Wnt signaling is involved in habenular regeneration at early stage. Strikingly, enhanced expression of Canonical Wnt inhibitor, Wnt inhibitory factor 1 (WIF-1) demonstrates that Wnt signaling is tightly regulated during brain regeneration (Lim, Ogawa, Smith, & Parhar, 2017).

2.3 Telencephalon

Telencephalon region shows numerous similarities in terms of physiology and function across species. In zebrafish, the dorso-lateral region of the pallium, central zone of the pallium, and medial zone of dorsal telencephalon are equivalent to the mammalian hippocampus, cortex, and amygdala respectively (Kozol et al., 2016). As mentioned, zebrafish telencephalon shows spatial heterogeneity in terms of mammalian counterpart.

The telencephalon is identified as one of the neurogenic zones owing to have radial glia (RG) in the adult zebrafish brain. RG cells are distributed along the dorsal part of the telencephalon with a variety of radial morphologies under discrete circumstances. The abundance of RG cells in telencephalon is a crucial factor for the enhanced regeneration ability of adult telencephalon.

Anatomically, telencephalon consists of two identical hemispheres and ordinarily divided into 2 subregions; Pallium and Subpallium regions (Figure 2).

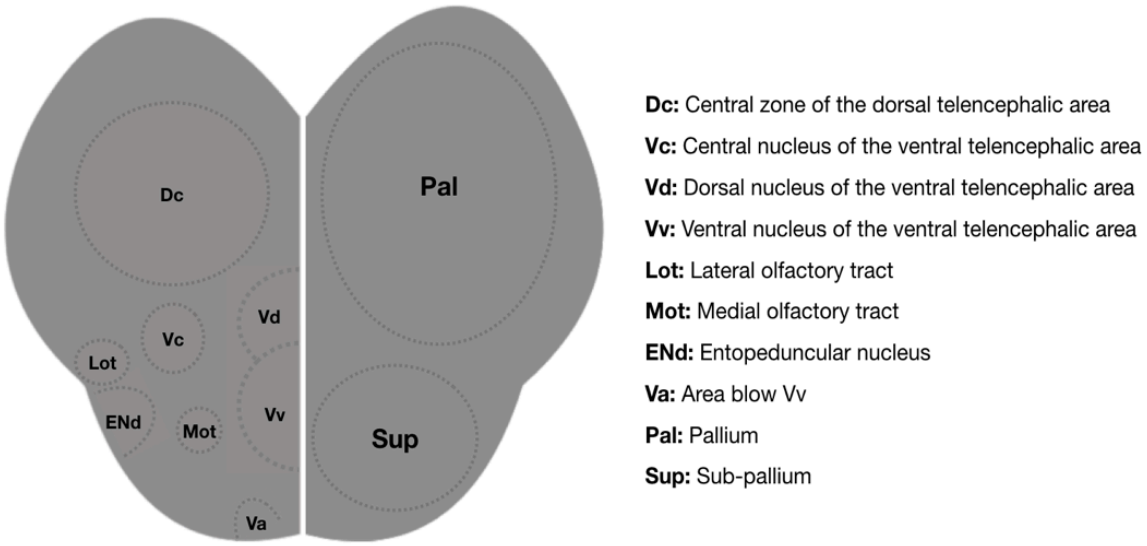


Figure 2: Parts of adult telencephalon (adapted from Schmidt et al., 2013)

Pallium region is predominantly constituted by parenchyma and cell bodies while the Sub-pallium region is constituted by migrating cells.

In addition to olfactory bulb and optic tectum, telencephalon is another regions that particularly studied in brain regeneration research in the adult zebrafish model. Several injury models have been developed to clarify the molecular mechanism of brain regeneration until now(Maheras et al., 2018), (McCutcheon et al., 2017), (Baumgart, Barbosa, Bally-Cuif, Götz, & Ninkovic, 2012). Stab wound injury model has been frequently used to generate brain injury mimicking mammalian traumatic brain injury (TBI). The regenerative response of telencephalon is well established in terms of cellular activation (Figure 3).

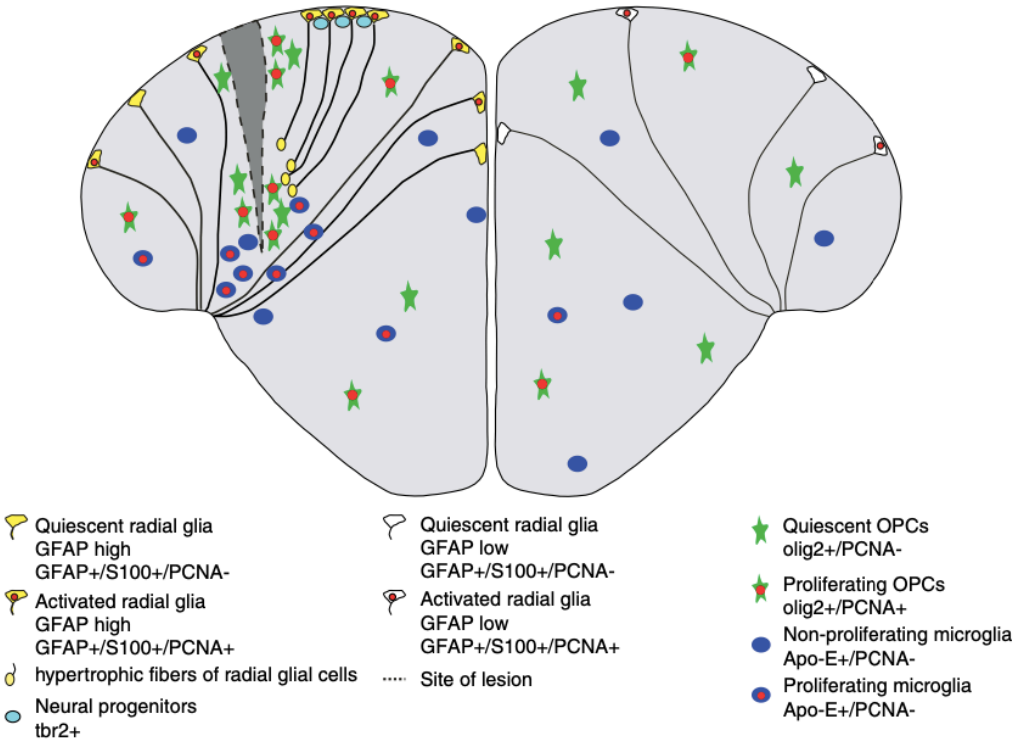


Figure 3: Scheme of regenerating brain (Schmidt et al., 2013)

2.3.1 Main Cell Types in Telencephalon

2.3.1.1 Radial Glia

Radial glia (RG) cells are able to be distinguished with small radial morphology and long processes in the different parts of the central nervous system (CNS) including telencephalon, retina, and hindbrain in the zebrafish. Radial glial cells in the retina have been named Müller glia expressing glial markers including GFAP, glutamine synthase (GS), and uniquely carbonic anhydrase (Raymond, Barthel, Bernardos, & Perkowski, 2006). GFAP-positive radial cells are not only located in the adult brain and retina, but also in the adult zebrafish spinal cord serving as a pool of stem cells. Some of these radial cells express commonly known glia-related proteins such as BLBP (brain lipid-binding protein), but not GFAP protein suggesting radial cells characterized by heterogeneity in the spinal cord (Kim et al., 2008).

RG cells change their morphology and transcriptome profiles depending on the situation (Lange et al., 2020) (Figure 4). These cells express glial markers such as GFAP (glial fibrillary acidic protein), Vimentin, S100β during homeostasis. On the other side, some radial glia undergoes inner kinetic nuclear migration (INM) by expressing GFAP, Vimentin, and s100b and BLBP proteins at very low levels in the subpallium of the telencephalon. Some of these

cells are also express olig2 distinguishedly, indicating the heterogeneity between radial glial cells in the same part of the brain. Radial glia cells are described as neural progenitors that are responsible for constitutive neurogenesis in the adult telencephalon (Chapouton et al., 2010). Some of the neurodegenerative diseases such as traumatic brain injury (TBI) and amyloid toxicity have a significant impact on radial cell transition (Y. Shimizu et al., 2018), (Cosacak et al., 2019). Activated radial cells differentiated into mature neurons to compensate for neuronal loss in the TBI model.

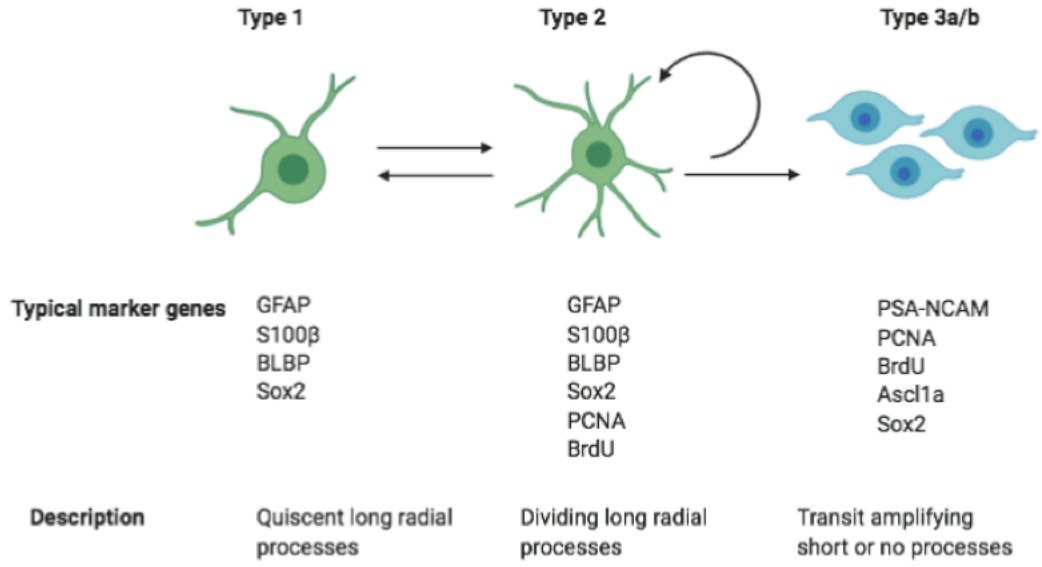


Figure 4: Types of radial glia cells and their markers (adapted from Schmidt et al., 2013)

2.3.1.2 Oligodendrocytes and Oligodendrocyte Progenitor Cells (OPC)

Oligodendrocytes and their progenitors are located predominantly in the parenchyma of the adult telencephalon in zebrafish (Schmidt, Beil, Strähle, & Rastegar, 2014). Olig2 is a transcription factor that is mostly expressed by both oligodendrocytes and their progenitors (Figure 5) (Ackerman & Monk, 2016). These two cell types can be distinguishable by their morphology and comparing the existence of additional myelin basic protein staining (MBP) (Ackerman & Monk, 2016). The functional roles of Oligodendrocytes and OPC are not well characterized in traumatic brain models in zebrafish, but both cell types mostly OPC are

abundant in the injured area transiently and even some of the OPC is proliferating in response to injury. This situation is similar to mammalian reactive gliosis.

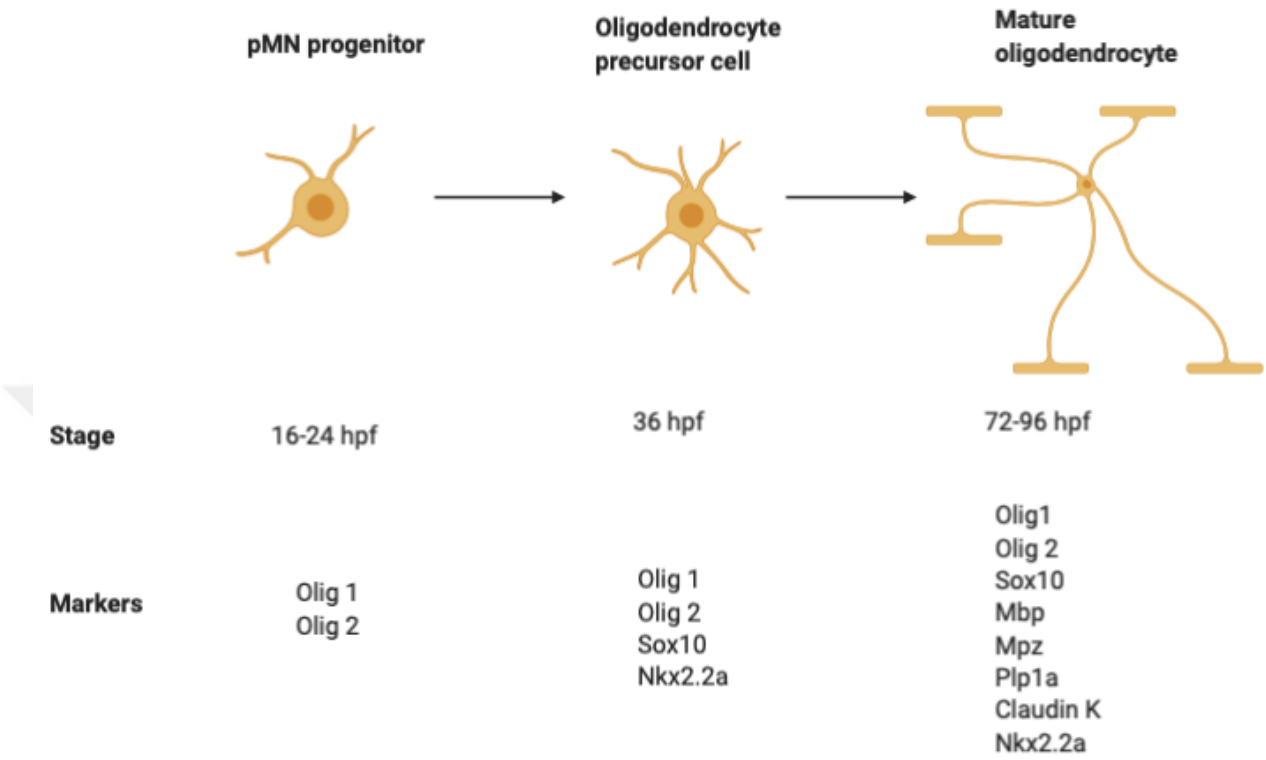


Figure 5: Oligodendrocyte markers (Adapted from Ackerman et al., 2017)

2.3.1.3 Microglia

Microglia are classified as tissue-resident macrophages that enter the brain before the blood-brain barrier established. Its roles include synaptic pruning, engulfment of apoptotic and necrotic cells, brain wiring, and maintaining blood-brain barrier integrity (Yenari, Xu, Tang, Qiao, & Giffard, 2006), (Herzog et al., 2019), (Lehrman et al., 2018). In zebrafish, not only a variety of transgenic lines have been developed but also antibodies such as L-plastin (lymphocyte cytosolic protein) and 4C4 are widely used for labeling microglia in embryonic and adult fish (Lehrman et al., 2018), (Var & Byrd-Jacobs, 2019). In addition to fluorescent labeling, neutral red staining is one of the most prevalent dye to detect microglia in developing zebrafish brain (Demy et al., 2017). Microglia activation is characterized by their morphology and production of proinflammatory cytokines such as il-1b, tnf-a in mouse and zebrafish (Tsarouchas et al., 2018), (Liu & Quan, 2018). Their shape is transformed from ramified to amoeboid morphology with the presence of enhanced proinflammatory cytokine activity in

neurodegenerative disease models in zebrafish (Kyritsis et al., 2012). The roles of microglia have been elucidated in central nervous system regeneration including olfactory bulb and telencephalon (Var & Byrd-Jacobs, 2019), (Kyritsis et al., 2012).

3. MATERIALS AND METHODS

3.1 Type of study

The type of research conducted in this study is experimental.

3.2 Time and Place of Study

All experiments were performed between December 2017- October 2019 at Izmir Biomedicine and Genome Center.

3.3 Materials of Study

3.3.1 Zebrafish

All experiments were done with transgenic 6xTCF/Lef-miniP:2dGFP Tg(6XTCF:dGFP) (N. Shimizu, Kawakami, & Ishitani, 2012) 6-9 months old adult male zebrafish. Zebrafish were provided by Izmir Biomedicine and Genome Center Zebrafish Core Facility.

3.3.2 Chemicals

Chemicals used in this study are listed in Table 1.

Chemical	Vendor	Cat. No.
50X TAE	Genemark	P00901
Acetic acid	Sigma-Aldrich	537020
Agorose	Sigma-Aldrich	A9539-500G
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	472301
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich	E6635
Gelatin	Merck	G1393
IWR-1	Sigma-Aldrich	I0161
Paraformaldehyde	Sigma-Aldrich	158127
Potassium Chloride	Sigma-Aldrich	P9541
RNAlater	Sigma-Aldrich	R0901
Sodium Chloride	Sigma-Aldrich	S9888
Sodium Citrate	Sigma-Aldrich	W302600
Sodium phosphate dibasic	Sigma-Aldrich	255793
Sodium phosphate monobasic	Sigma-Aldrich	S0751
Sucrose	Sigma-Aldrich	S0389
Tissue-Tek O.C.T	SAKURA	4583
Tricaine	Sigma-Aldrich	E10521
Tris	Sigma-Aldrich	252859
Triton X-100	Sigma-Aldrich	T8787
Chloroform	Merck	1.02431
Ethanol Absolute	Merck	1.00986
Methanol	Sigma-Aldrich	32213
Glycerol	Amresco	0854-1L

Table 1: List of chemicals.

3.3.3 Kits and Enzymes

Kits and enzymes used in this study are listed in Table 2.

Kit/Enzyme	Vendor	Cat. No.
100 bp DNA Ladder	New England Biolabs	N3231L
Agilent RNA 6000 Pico Kit	Agilent	5067-1512
DNA Ladder	Gene Ruler	SM1331
Deoxynucleotide (dNTP)	Genemark	GM007
Gel Loading Dye Blue (6X)	New England Biolabs	B7021S
GelRed	Biotium	41009
GoTaq qPCR Master Mix	Promega	A6001
miRNeasy Mini Kit	QIAGEN	217004
One Taq DNA Polymerase	New England Biolabs	M0480
ProtoScript® First Strand cDNA Synthesis Kit	New England Biolabs	E6300L
Safeview	ABM	G108

Table 2: List of kits and enzymes.

3.3.4 Instruments

Instruments used in this study are listed in Table 3.

Instrument	Vendor
2100 Bionalyzer Instrument	Agilent Technology
7500 Real-Time PCR- ABI 7500 FAST	Applied Biosystem
Basic power supply	Bio-rad
Cryostat	LEICA
Gel Doc™ XR+ System	Bio-rad
LSM 880 Laser Scanning Confocal Microscopy	ZEISS
Mini-Sub Cell GT system	Bio-rad
mySPIN 6 Mini Centrifuge	Thermo Fisher Scientific
Nanodrop 2000 Spectrophotometer	Thermo Fisher Scientific
pH meter	Hanna
Stemi 305 Compact Stereo Microscope	ZEISS
Thermal cycler	Thermo Fisher Scientific
Water bath and Lid	Nüve

Table 3: List of instruments.

3.3.5 Consumables

Consumables used in this study are listed in Table 4.

Consumables
1,5 ml eppendorf tubes
2 ml eppendorf tubes
50 mL centrifuge tubes
96 well plate
Glass bottle
Coplin Staining jar
Disposable microtome blades
Disposable scalpel
Disposable plastic pipette tips
Embedding Molds
Needle 30G 0,3x13 mm
Sterile vacuum filtration system

Table 4: List of consumables.

3.3.6 Primers

Primers used in qPCR were designed by using NCBI Primer Designing Tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers used in this study listed in Table 5. Primers were purchased from Oligomer Biotechnology, Ankara-Turkey. Primers were dissolved in NFW.

	Forward Primer (5'->3')	Reverse Primer (5'->3')
inhbab	ATCTTGGCAGAGGAGCAGAAC	CCACTATCAGATGGAGTAGGCG
fsta	TGGGAGGCTCGGTACATCTT	CAGACGCAGCGAGGTTTACT
prg4b	CGCTGCGGAGAGCCTTTTA	CTCAGAAGGTGTGGGCTCAG
klf11b	CACAGAAAGGACCACACCCC	GGTGGCACAAGCTAGGTGAT
ctgfa	CTACGGCTCCCCAAGTAACC	CAACCTACAGCACCGTCCAG
egfp	CCCGACAACCACTACCTGAG	AACTCCAGCAGGACCATGTG
rpl13a	CCCTCCACCTTATGACAAGAGA	CGTCCAAGCAGGGCAAATTT
actb	AGAGAGAAGATGACACAGATCA	TAGGCCATTTAAGGTGGCAA

Table 5: List of primers

3.3.7 Buffers

Buffers used in this study are listed in Table 6.

Buffer	Description
1X Phosphate buffer saline (PBS)	8 gr of NaCl, 0,2 gr of KCl, 1.44 gr of Na2HPO4, 0,24 gr of KH2PO4 added and dissolved in 800 mL of distilled water and pH is adjusted to 7.4 with HCl. Distilled water added to 1L.
1X Phosphate Buffered Saline Tween-20 (PBST)	8 gr of NaCl, 0,2 gr of KCl, 1.44 gr of Na2HPO4, 0,24 gr of KH2PO4, 2 ml of tween-20are added and dissolved in 800 ml of distilled water and pH is adjusted to 7.2 with HCl.
Sodium Citrate buffer	2,94 gr of Trisodium citrate (dehydrate) dissoved in 1L of distilled water and adjust pH 6.0 with HCl.

Table 6: List of buffers.

3.3.8 Antibodies

Antibodies used in this study are listed in Table 7.

Antibody	Host	Dilusion	Vendor	Code
GFAP	mouse	1/500	Abcam	ab154474
PCNA	mouse	1/500	Dako	Dako-M0879
S100β	rabbit	1/500	Dako	Dako-Z0311
Parvalbumin	mouse	1/500	Sigma-Aldrich	Sigma P3088
Acetylated tubulin	mouse	1/250	Sigma-Aldrich	T6793
Phospho β-catenin	rabbit	1/100	Cell signaling	D2F1
NeuroD1	mouse	1/300	Abcam	ab60704
Caspase-3	rabbit	1/500	Cell signaling	9664S

Table 7: List of antibody.

3.4 Variables of Study

In this research dependent variable is expression levels of reporter activity.

3.5 Tools for Data Collection

3.5.1 Stab Wound Assay

Stab wound assay is performed in 6-9 months old male transgenic Wnt reporter zebrafish. Before surgery, fish were anesthetized with Tricaine (MS-222, Sigma-Aldrich). A 30G needle was inserted through the left nostril into the end of the telencephalon. After that, fish were transferred into the tank with freshwater.

3.5.2 Drug treatment

After the application of the brain injury in transgenic 6XTCF:dGFP zebrafish reporter line, they immersed in 20 micromolar IWR-1 (Sigma-Aldrich) dissolved in DMSO (Sigma-Aldrich). IWR-1 solution was freshly prepared and changed daily.

3.5.3 RNA Isolation, cDNA synthesis and qPCR

3.5.3.1 RNA isolation

RNA isolation was performed with miRNeasy Micro Kit-QIAGEN according to manufacturer's instruction. RWT and RPE buffer supplied with kit. In detail, dissected telencephalon hemispheres were removed and transferred into 1,5 mL sterile eppendorf containing 700 µl QIAzol Lysis Reagent (QIAGEN). Brain tissues homogenized with RNase free pestles (sterilized with DEPC water). Then, 140 µl chloroform was added to the tube and shook vigorously for 15 seconds. After that, the homogenate was centrifuged for 15 minutes at 12,000 x g at 4°C. After centrifuge, the upper aqueous phase was transferred into new eppendorf tube. After adding 525 µl of 100% ethanol, solution mixed thoroughly by pipetting. In order to

bind DNA to the spin column, 700 µl of the sample was transferred into an spin column in a collection tube and centrifuged at 8,000 x g for 15 seconds at room temperature. After that, 350 µl of Buffer RWT was added to the spin column and centrifuged for 15 seconds at 8,000 x g to wash. Later, 80 µl of DNase I treatment was performed to eliminate DNA contamination and placed on the benchtop for 15 minutes. To remove DNase I mix, 500 µl of Buffer RPE was added to the column and centrifuged for 15 seconds at 8,000 x g. Then, 500 µl of 80% ethanol (prepared with absolute ethanol and diluted with DEPC) was added to the column and centrifuged for 2 minutes at 8,000 x g. Finally, RNA is eluted with RNase-free water.

3.5.3.2 cDNA Sythesis and qPCR

Isolated RNA samples were reverse transcribed by using ProtoScript II First Strand cDNA Synthesis Kit according to manufecturer's instruction in two steps (Table 8 and Table 9). 50 ng RNA is used for cDNA reaction. QPCR is performed by using GoTaq qPCR Master Mix (Promega) (Table 10). CDNA templates were diluted as 1:10 in NFW. Applied Biosystem 7500 Real-Time PCR thermal cycler was used for analysis.

Step 1

Component	Volume
Template RNA	variable
Random Primer Mix (60µM)	2 µl
Nuclease-free H_20	to a total volume of 8 µl

Table 8: Step 1 for cDNA synthesis reaction protocol

Step 2

Component	Volume
RNA-primer mix	8 µl
ProtoScript II Enzyme Mix (10X)	2 µl
ProtoScript II Reaction Mix (2X)	10 µl

Table 9: Step 2 for cDNA synthesis reaction protocol

Component	Volume per reaction (µl)
cDNA template	4
2X Master Mix	5
Forward Primer (10 µl)	0,5
Reverse Primer (10 µl)	0,5

Table 10: qPCR reaction protocol

3.6 Tissue preparation

For cryosection fixation protocol in Table 11. All procedures were performed at +4°C except embedding part. After fixation with 4% PFA, heads were transferred for decalcification and cryoprotection to sucrose/EDTA solution and brain within the skulls were embedded in 20% sucrose-7,5% gelatin. Then, immediately transferred the tissue embedding mold to dry ice. Brains sectioned at 14 µm by using cryostat (Leica) for further analyses including immunofluorescent staining.

Buffers	Time
4% PFA	48 hours
0,1M PB	3 x 30 minutes
20% Sucrose - 20%EDTA	48 hours
30% Sucrose - 20%EDTA	24 hours

Table 11: Tissue preparation protocol for cryosection

3.7 Immunohistochemistry and Imaging

Brain slices were dried for 20 minutes at room temperature. After that, all steps are performed at room temperature except primary antibody treatment (Table 12). Z-stack images are collected with LSM 880 Laser scanning confocal microscopy (ZEISS).

Solution	Time
PSTX	2 x 10 minutes at RT
1X PBS	2 X 5 minutes at RT
10 mM Sodium Citrate	15 minutes at 85°C
1X PBS	2 X 5 minutes at RT
PSTX	2 x 10 minutes at RT
Primary antibody diluted in PBSTX	Overnight at 4°C
PSTX	3 X 10 minutes at RT
Secondary antibody diluted in PBSTX	2 hours at RT
1X PBS	3 x 5 minutes at RT

Table 12: Immunofluorescence protocol

3.8 Evaluation of Isolated RNA Integrity

3.8.1 Agarose Gel Electrophoresis

Isolated RNA samples were reverse transcribed as described previously in cDNA sythesis part. To validate RNA integrity of isolated RNA, β-actin PCR was performed for each sample (Table 13). PCR condition is shown in Table 14.

Component	Volume per reaction (µl)
5X One Taq Buffer	5
dNTP (10 mM)	0,5
Forward Primer (10 mM)	0,5
Reverse Primer (10 mM)	0,5
One Taq Polymerase	0,125
Nuclease-free Water	13,375

Table 13: PCR reaction protocol for β-actin

Step	Temperature	Time	Cycles
Initial Denaturation	95°C	1 minute	1
Denaturation	95°C	5-10 seconds	
Annealing	60°C	30 seconds	30
Elongation	72°C	30 seconds	
Final Elongation	72°C	2 minutes	1
Cooling	4°C	1 minutes	1

Table 14: PCR conditions.

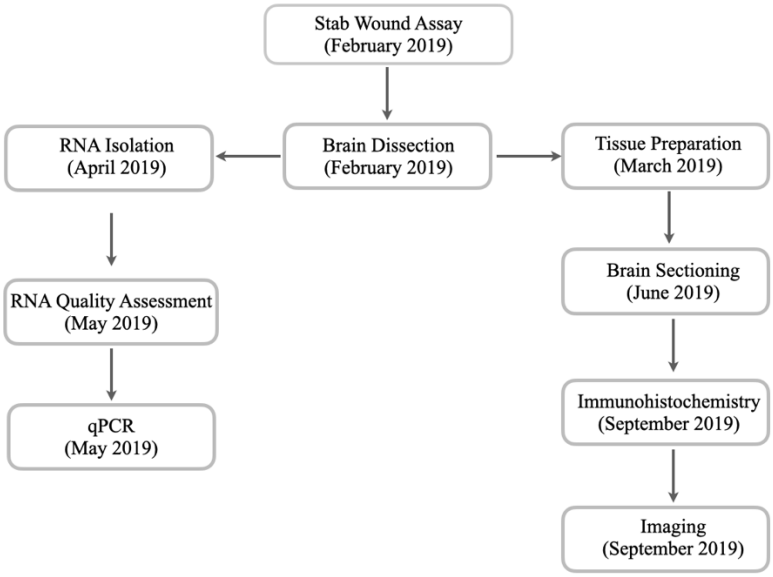
3.8.2 RNA Quality Assessment

Quality of RNA samples were determined by using Agilent 2100 Bioanalyzer (Agilent Technology). Only total RNA samples with RIN values ≥ 7.6 were used for further transcriptome analysis.

3.9 RNA Sequencing and Analysis

RNA samples were sent to EMBL-Genomics Core (Heidelberg) for sequencing. Differentially expressed gene analysis was performed by Ozhan Lab member Yeliz Demirci. Protein interaction network was generated using STRING database and visualized with Cytoscape 3.8.0 software (Shannon et al., 2003). Gene ontology analysis was performed with EnricheR web server (Kuleshov et al., 2016).

3.10 Study Plan and Calender



3.11 Data Evaluation

Quantitative-PCR results was visualized with Prism 8.0 (Graphpad) program. Man-Whitney U test is used for statistical analysis and this analysis was performed using paired or unpaired student's t test. Statistical significance is represented as *p<0.05; **p<0.01; ***p< 0.001.

3.12 Limitation of Study

The limitation of this study is the total amount of isolated RNA in telencephalon due to its size.

3.13 Ethics Committee Approval

Ethics protocol number is 2020-004.

4.RESULTS

4.1 Validation of Brain Injury Model in The Adult Brain

4.1.1 The Effects of Brain Injury on Neurogenesis and Mature Neurons

To validate the impact of injury on neuronal and glial cell types, immunohistochemistry staining was performed on adult zebrafish brain sections. For this purpose, 20 hours and 3 days after injury was chosen as a time points to reveal the altered localization and expressions of selected neuronal and glial markers including Acetylated tubulin, Neurogenic differentiation factor 1 (Neurod1), Parvalbumin (PV), Glial fibrillary acidic protein (GFAP), S100 calcium binding protein β (S100β), Proliferatincell nuclear antigen (PCNA).

Acetylated tubulin is one of the types of mature neurons in the adult telencephalon. Acetylated tubulin staining was performed to determine the effects of injury. It is observed that Acetylated tubulin positive neurons are localized homogeneously in the regions of telencephalon in the control brain while localization pattern of these neuronal subtype was significantly changed upon injury (Figure 6). These neurons were observed predominantly in the lateral nucleus of the ventral telencephalic area, central zone of the dorsal telencephalic area and the lateral zone of the dorsal telencephalic area where the number of neuronal stem cells called radial glia cells abundant. However, reduced Acetylated tubulin staining was detected in medial ventricular zone.

Neurod1 is a member of NeuroD gene family of basic helix-loop-helix transcription factor that involves in neuronal differentiation. Neurod1 staining was performed to demonstrate the impact of injury on neuronal differentiation in the traumatized brain. The expression pattern of Neurod1 is observed in the ventral nucleus of the ventral telencephalic area, medial and lateral olfactory tract. However, diminished expression of Neurod1 is detected after the application of injury (Figure 7).

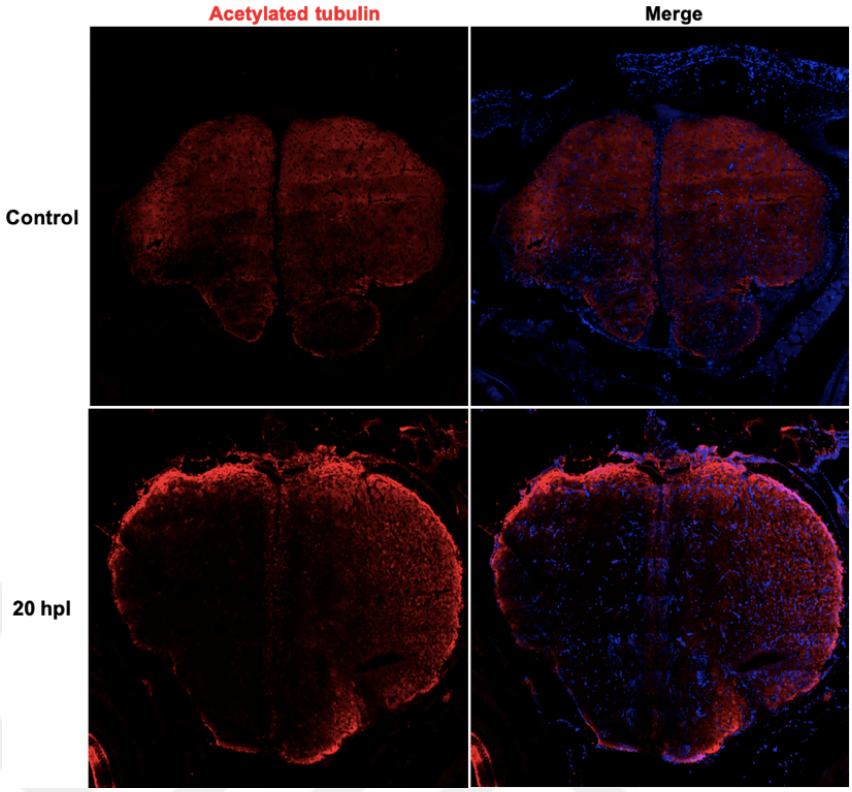


Figure 6: Altered localization of mature neurons in traumatized brain upon injury.

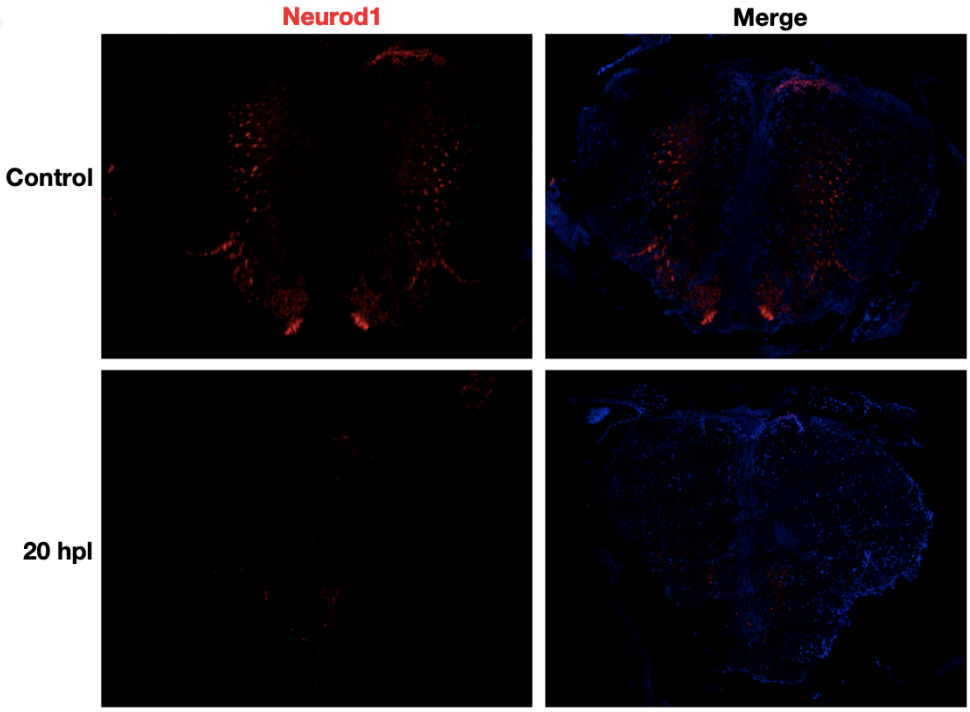


Figure 7: Neuronal differentiation is inhibited in the injured brain at the early stage.

One of the indication of injury-induced brain damage is neuronal cell death in the early stage. Parvalbumin (PV) is one of the Calcium-binding proteins that modulate intracellular calcium level in neurons. PV is widely used mature interneuron subtype marker in adult zebrafish brain. To investigate neuronal fate upon injury at early stage of brain injury, Parvalbumin and Cleaved-caspase 3 immunohistochemistry staining was performed in the lateral zone of the dorsal telencephalon. It is observed that Parvalbumin-positive neurons are also positive for cleaved-caspase 3 indicating that some of the mature neurons undergo apoptosis at the early stage of brain regeneration in the adult zebrafish brain (Figure 8).

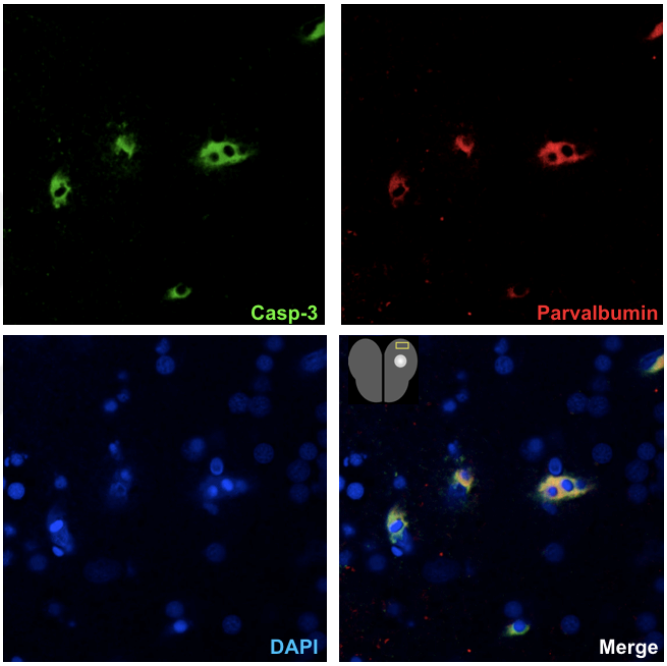


Figure 8: Parvalbumin and Cleaved-caspase 3 staining in the dorsal telencephalon. **(**Yellow box indicates region of interest).

4.1.2 The Effects of Brain Injury on Reactive Gliosis

PCNA staining was performed to specify the localization of injury-induced proliferating cells at 3 days post lesion in the injured telencephalon (Figure 9). It is observed that proliferating cells are located dominantly on the lateral region of ventricular zone and dorsal nucleus of the ventral telencephalic area of the injured telencephalic hemisphere. Conversely, proliferating cells are not detected in the central zone of the dorsal telencephalic area.

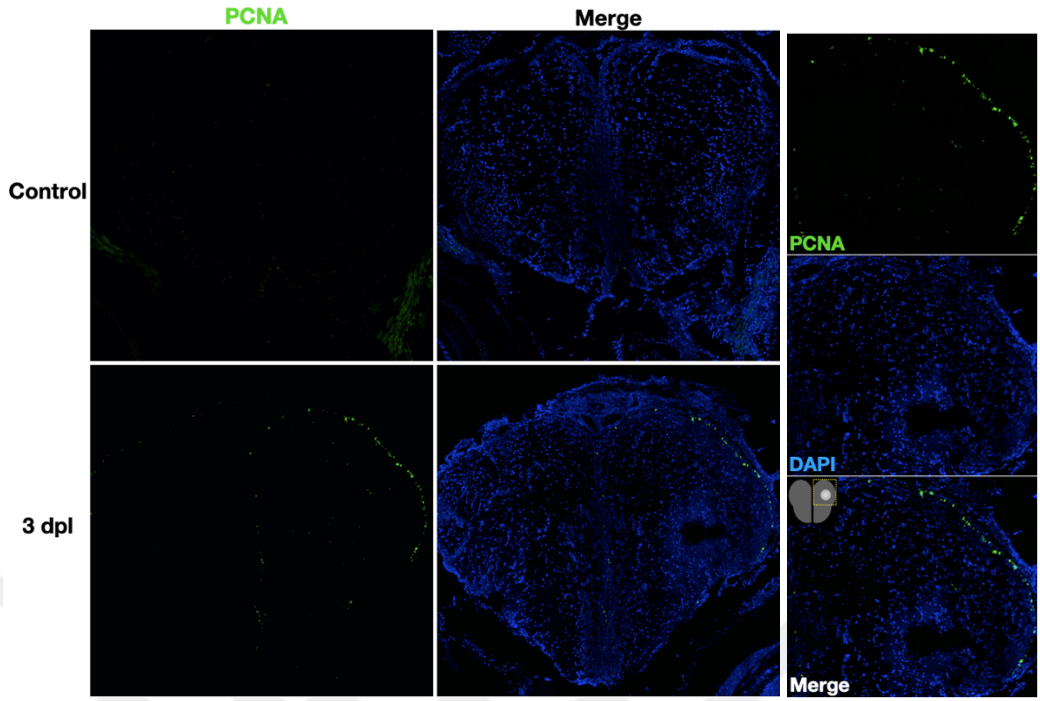


Figure 9: Proliferating cell nuclear antigen staining at 3 days post lesion. (Yellox box indicates magnified region of telencephalon)

It has been demonstrated that over expression of S100β exacerbates reactive gliosis in neurodegenerative diseases including traumatic brain injury and Alzheimer's disease. It is observed that S100β situated in dorsal ventricular zone in the intact brain (Figure 10). Also, injury-induced overexpression of S100β is detected in both dorsal ventricular zone, dorsal nucleus and ventral nucleus of the ventral telencephalic area at 3 days post lesion. Furthermore, slightly enhanced expression of S100β is noticed in entopedincular nucleus and posterior zone of the dorsal telencephalic area. On the contrary, S100β overexpression is not detected in the central zone.

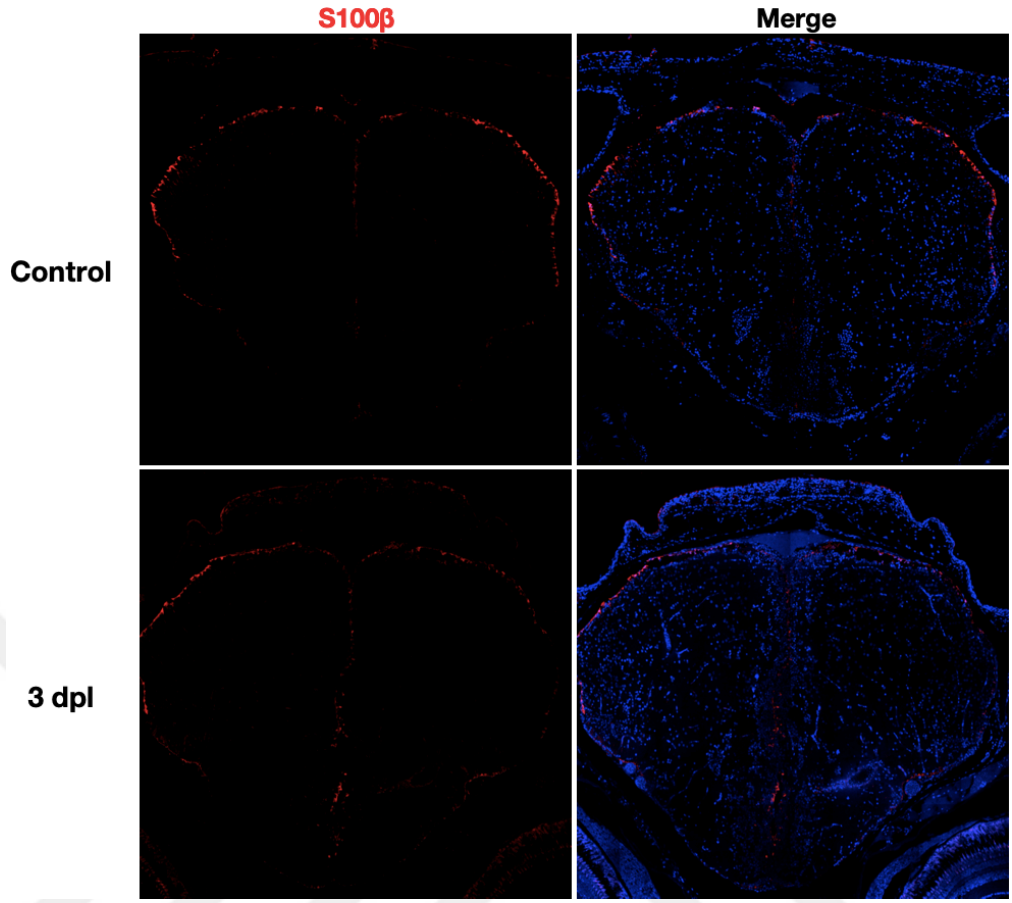


Figure 10: S100 calcium binding protein β staining at 3 days post lesion.

GFAP is a type III intermediate filament that is commonly used to indicative of reactive gliosis in the TBI model in model systems. Overexpression of GFAP was demonstrated in the early stage and proliferative stage of the brain regeneration in the adult zebrafish (Figure 11). It localized predominantly in the dorsal part of pallium in the intact brain while its expression was enhanced in the entopedincular nucleus instead of the dorsal part of telencephalon upon injury at 20 hpl. In addition to PCNA, proliferating neural stem cell express GFAP in reponse to injury at 3 days post lesion. GFAP overexpression is observed not only in the entopedincular nucleus, but also the posterior zone of the dorsal telencephalic area and the central zone of the dorsal telencephalic area where radial glia cell situated.

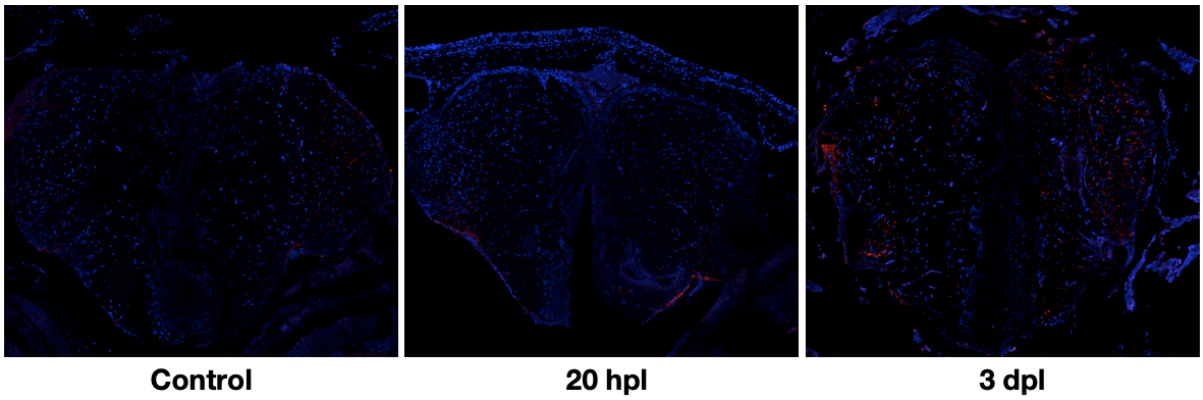


Figure 11: GFAP staining at 20 hours post lesion and 3 days post lesion.

4.2 Detection of Wnt/β-catenin signaling in the Brain Regeneration

After demonstrating the sufficiency of our injury model for regeneration, quantitative polymerase chain reaction (qPCR) is performed to detect the activation of Wnt/β-catenin signaling at 20 hours post lesion and 3 days post-lesion in injured and uninjurd hemispheres of the telencephalon in Wnt/β-catenin reporter Tg(6XTCF:dGFP) adult zebrafish (Figure 12). Based on qPCR results, Wnt/β-catenin signaling reporter activity is increased both in injured and uninjured hemispheres at 20 hpl. Additionally, increased reporter activity is observed only in the uninjured hemisphere at 3 days post lesion (dpl). Intriguingly, reporter activity is not altered in the injured hemisphere at 3 dpl.

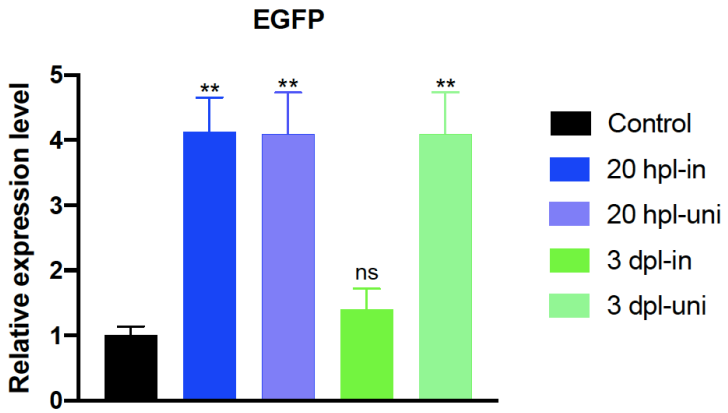


Figure 12: Wnt/β-catenin reporter activity at 20 hours post lesion and 3 days post lesion.

In order to determine the localization of Wnt/β-catenin signaling, phosphorylated β-catenin immunohistochemistry staining is performed at 20 hpl (Figure 13). Activation of Wnt/β-catenin signaling is observed predominantly in the injury area (Figure 14). In order to figure out whether activation of Wnt/β-catenin signaling is dependent on injury type-specific, telencephalon is injured through the skull (Figure 15). This injury model is a well-established method and known to be sufficient to induce brain regeneration. In this technique, the activation of Wnt/β-catenin signaling is observed near the injury as shown in the other injury model.

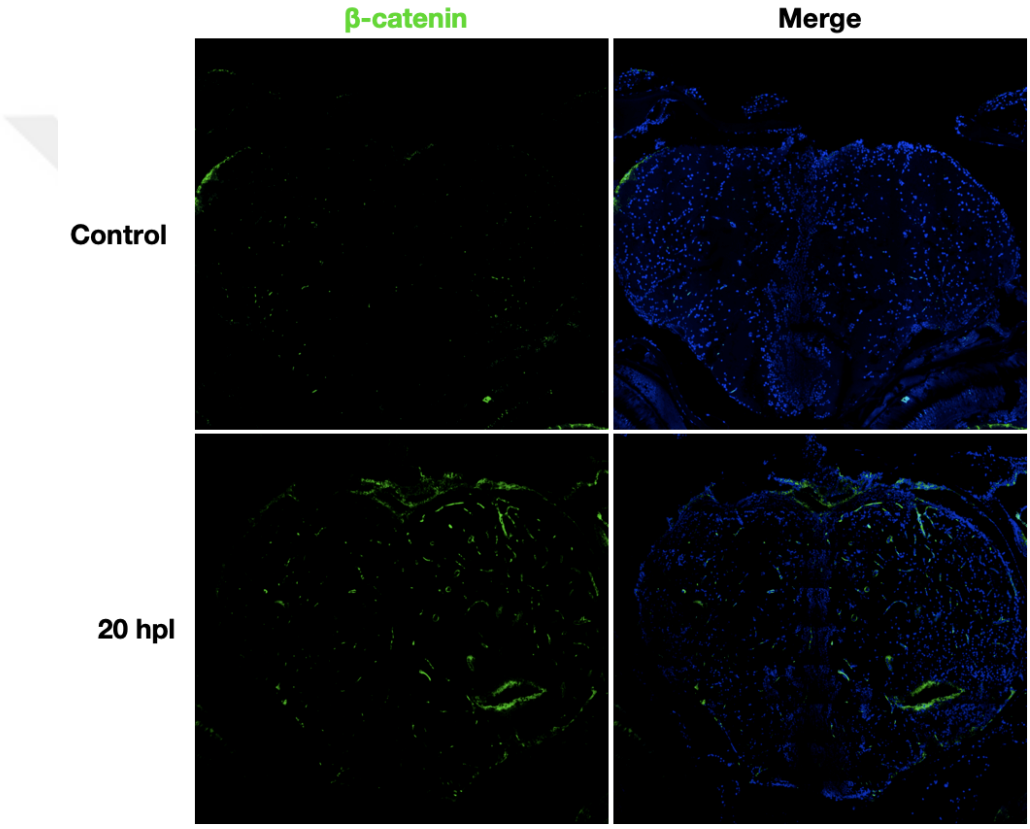


Figure 13: Localization of Wnt/β-catenin signaling at 20 hours post lesion.

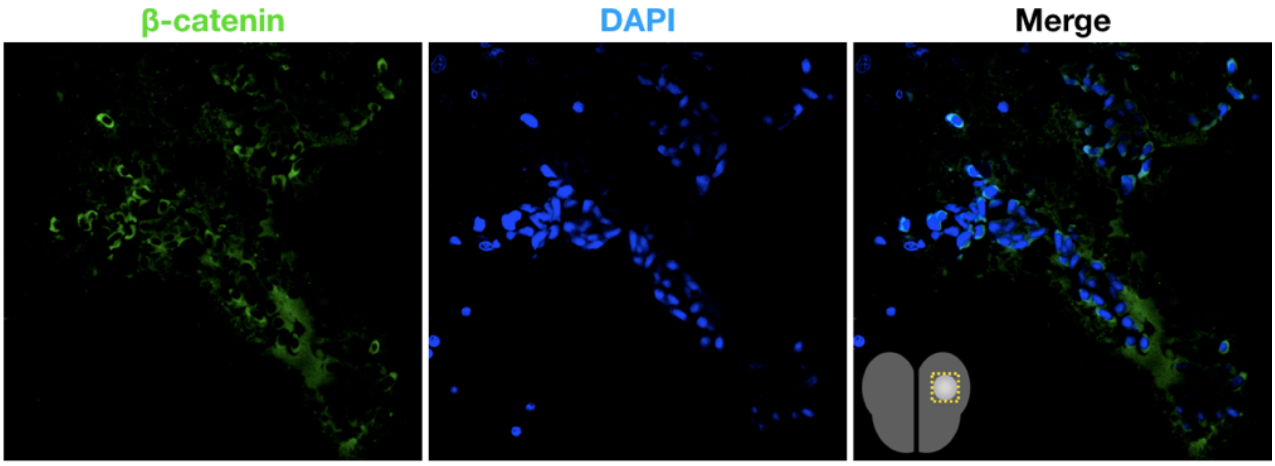


Figure 14: Wnt/β-catenin signaling activity in the injured area. (Yellox dashed box indicates magnified region)

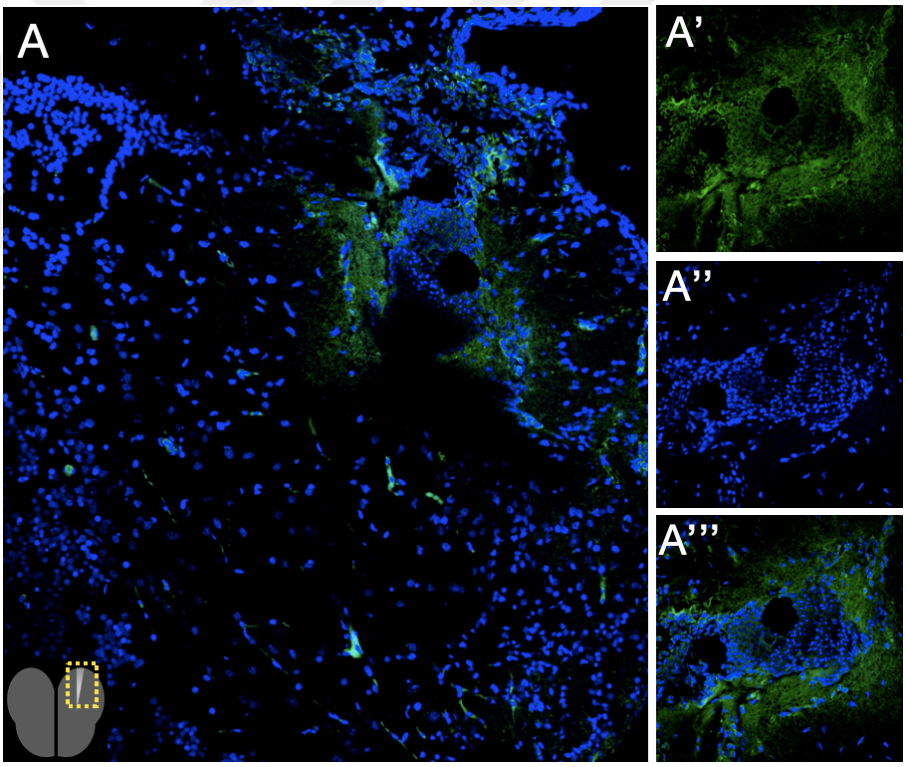


Figure 15: Localization of Wnt/β-catenin signaling activity in Schmidt model.

(A) Yellow dashed box indicates magnified region, (A') β-catenin staining, (A'') Counter staining with DAPI, (A''') Merge image.

4.3 Identification of Wnt/β-catenin signaling-related genes in brain regeneration

To identify Wnt/β-catenin signaling-associated genes in brain regeneration, transcriptome analysis was performed. For this aim, fish are injured and some of them are dissected after 20 hours and some of them are dissected after 3 days (Figure 16A). The expression level of LEF1, which is a downstream target of Canonical Wnt signaling was measured to determine the effective dose of IWR-1 (Figure 16-B). It is observed that 20 µM of IWR-1 efficaciously inhibits reporter activity in the intact brain. Reporter activity of injured fish and IWR-1 treated fish was measured by q-PCR (Figure 16C, Figure 16D).

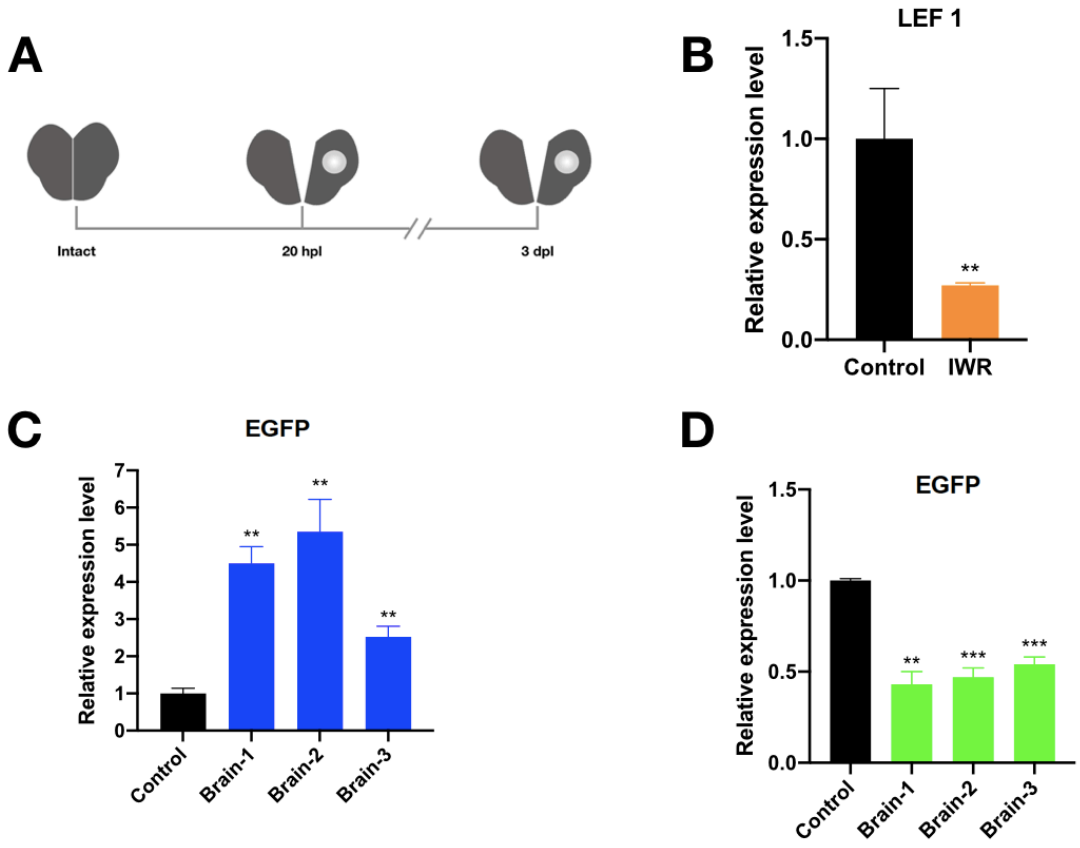


Figure 16: Sample preparation steps and validation for RNA-Seq samples.

(A) Scheme for experimental design. (B) Relative expression levels of LEF1 upon Wnt inhibition. (C) Relative reporter activity of injured hemispheres. (D) Relative reporter activity of injured hemispheres upon Wnt inhibition.

RNA integrity of transcriptome samples is validated by both Bionalyzer instrument and RT-PCR (Figure 17-33).

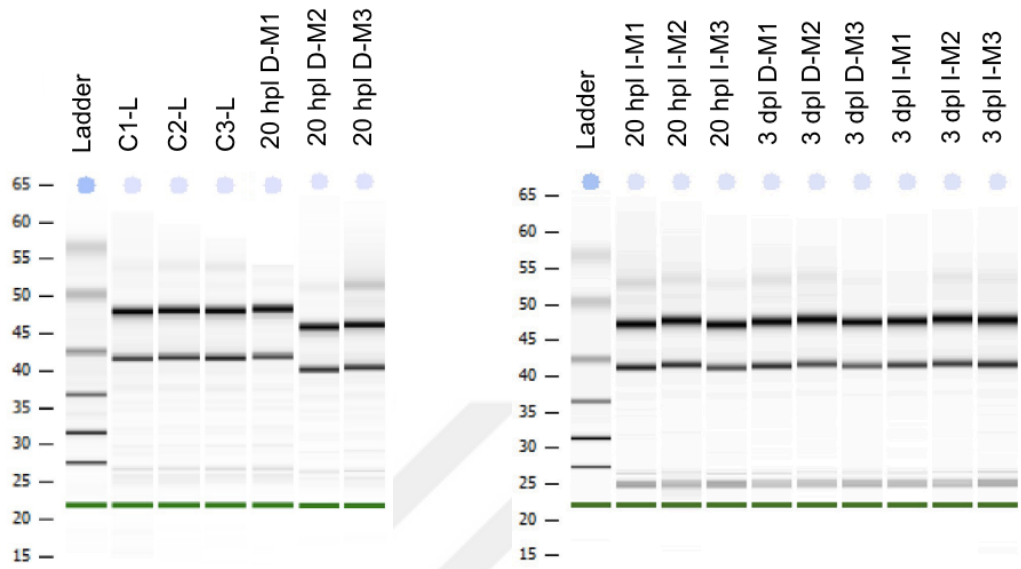


Figure 17: Electrophoresis summary of RNA samples.

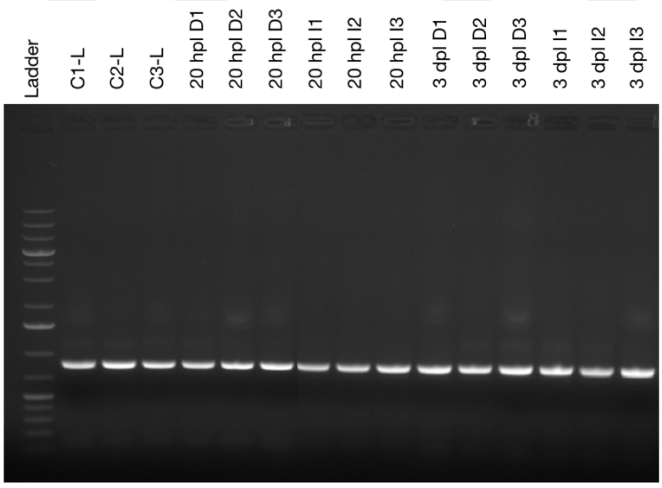


Figure 18: β-actin PCR products gel image for each samples.

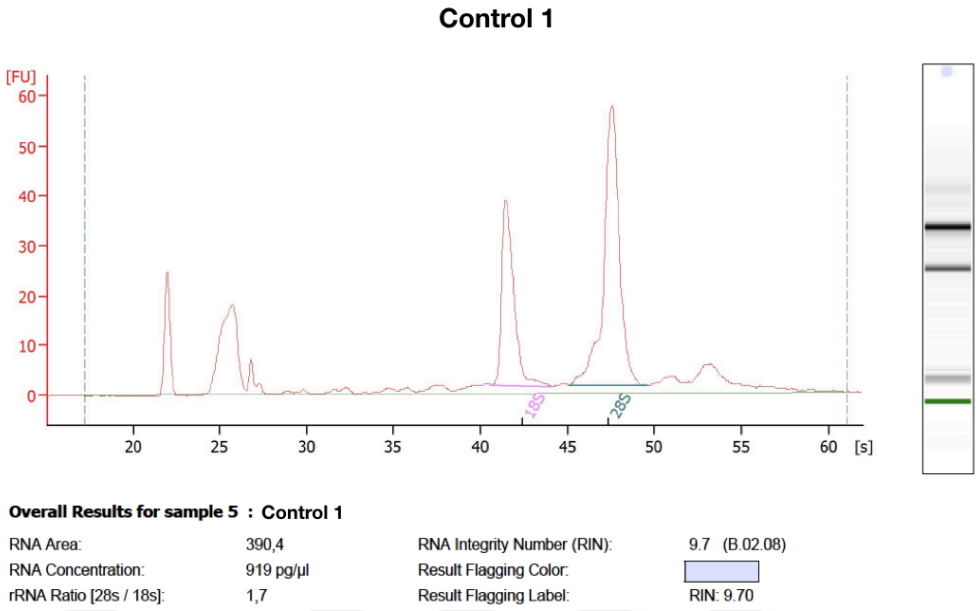


Figure 19: Electropherogram summary of Control 1 sample

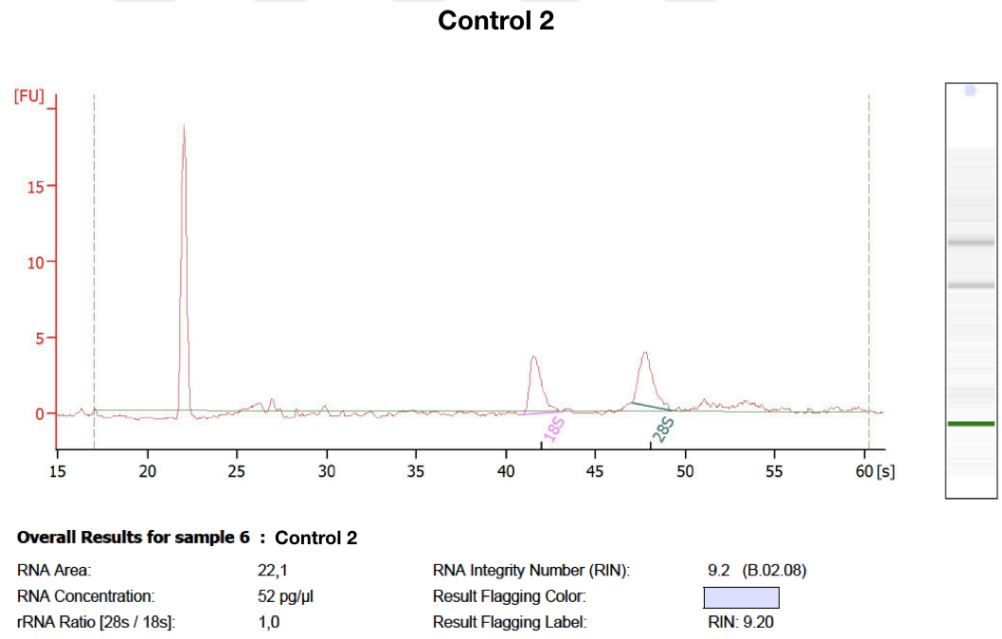
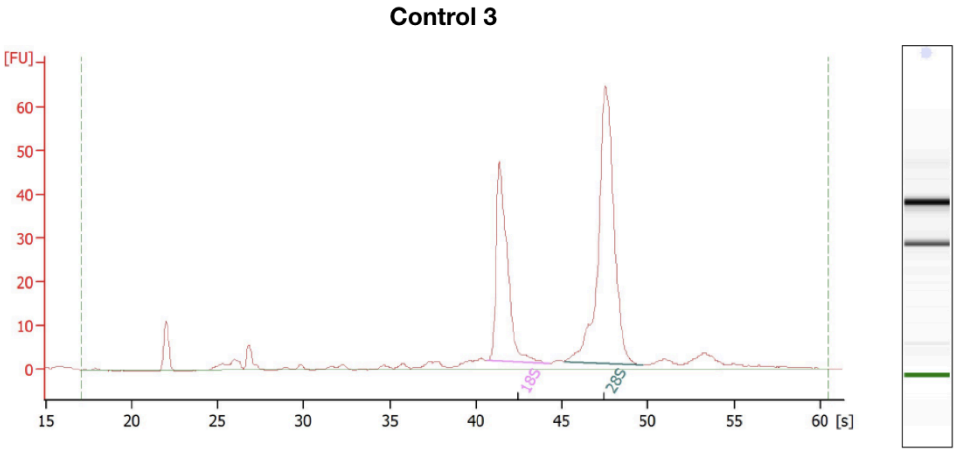


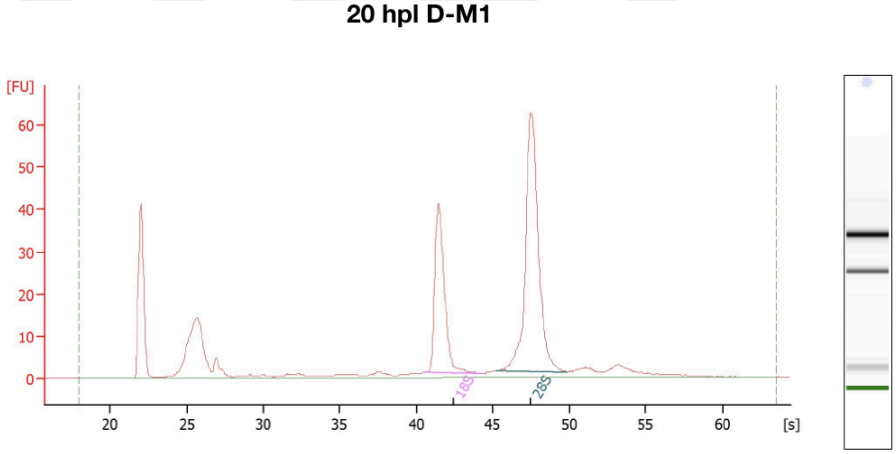
Figure 20: Electropherogram summary of Control 2 sample.



Overall Results for sample 7 : Control 3

RNA Area:	319,3	RNA Integrity Number (RIN):	10 (B.02.08)
RNA Concentration:	751 pg/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	1,7	Result Flagging Label:	RIN:10

Figure 21: Electropherogram summary of Control 3 sample.



Overall Results for sample 4 : 20 hpl D-M1

RNA Area:	343,7	RNA Integrity Number (RIN):	10 (B.02.08)
RNA Concentration:	645 pg/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	1,8	Result Flagging Label:	RIN:10

Figure 22: Electropherogram summary of 20 hpl D-M1 sample.

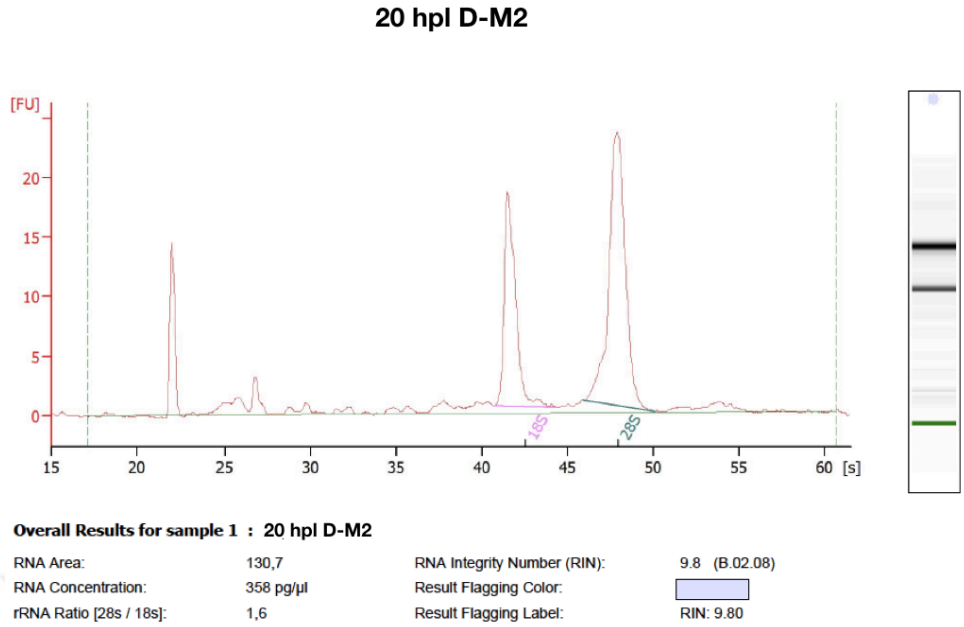


Figure 23: Electropherogram summary of 20 hpl D-M2 sample.

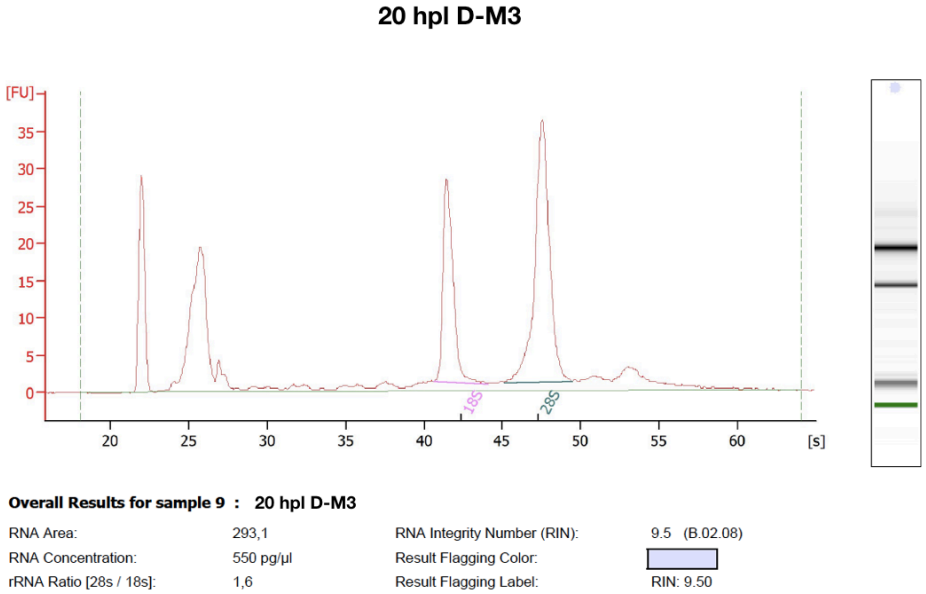
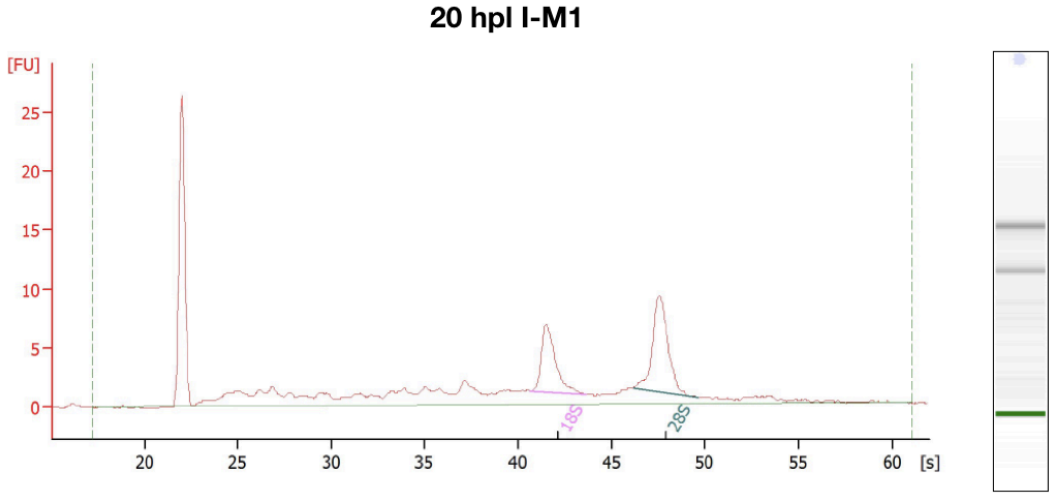


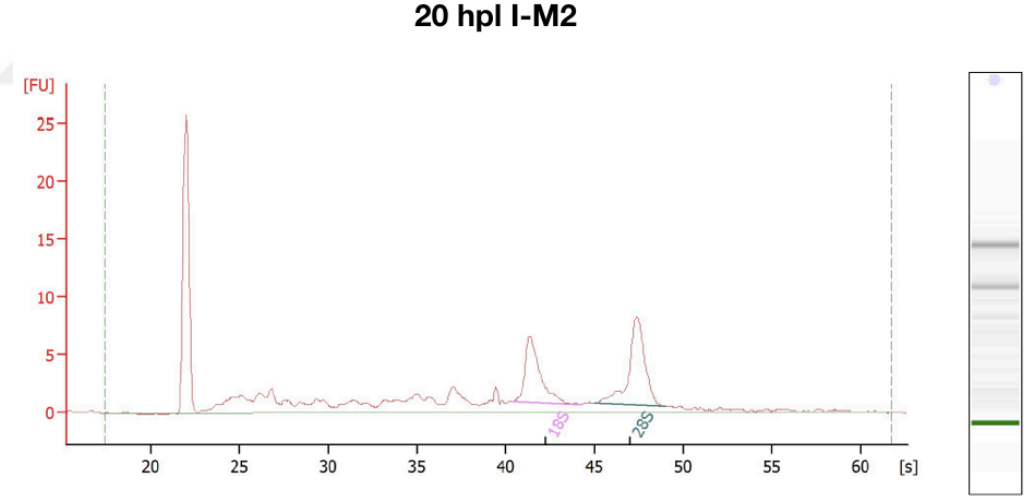
Figure 24: Electropherogram summary of 20 hpl D-M3 sample.



Overall Results for sample 8 : 20 hpl I-M1

RNA Area:	111,5	RNA Integrity Number (RIN):	7.6 (B.02.08)
RNA Concentration:	262 pg/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	1,3	Result Flagging Label:	RIN: 7.60

Figure 25: Electropherogram summary of 20 hpl I-M1 sample.



Overall Results for sample 9 : 20 hpl I-M2

RNA Area:	106,4	RNA Integrity Number (RIN):	7.8 (B.02.08)
RNA Concentration:	250 pg/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	1,3	Result Flagging Label:	RIN: 7.80

Figure 26: Electropherogram summary of 20 hpl I-M2 sample.

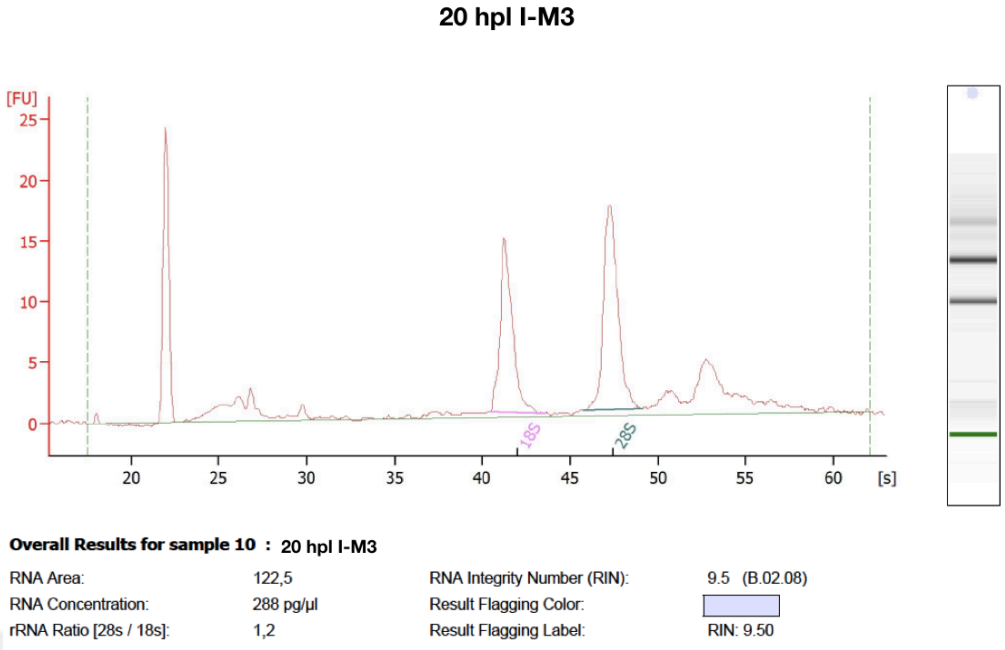


Figure 27: Electropherogram summary of 20 hpl I-M3 sample.

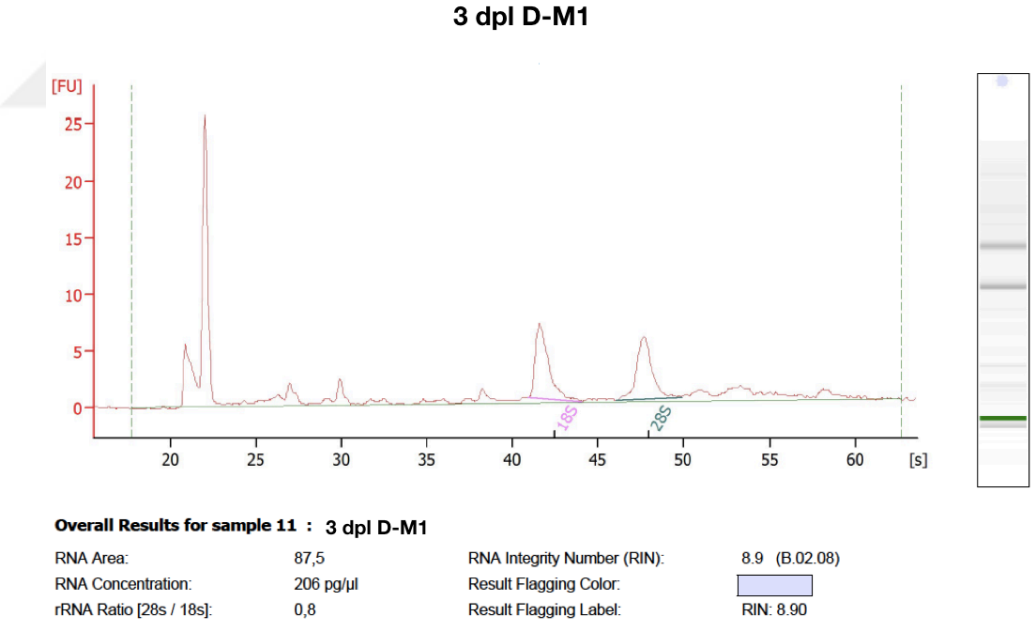
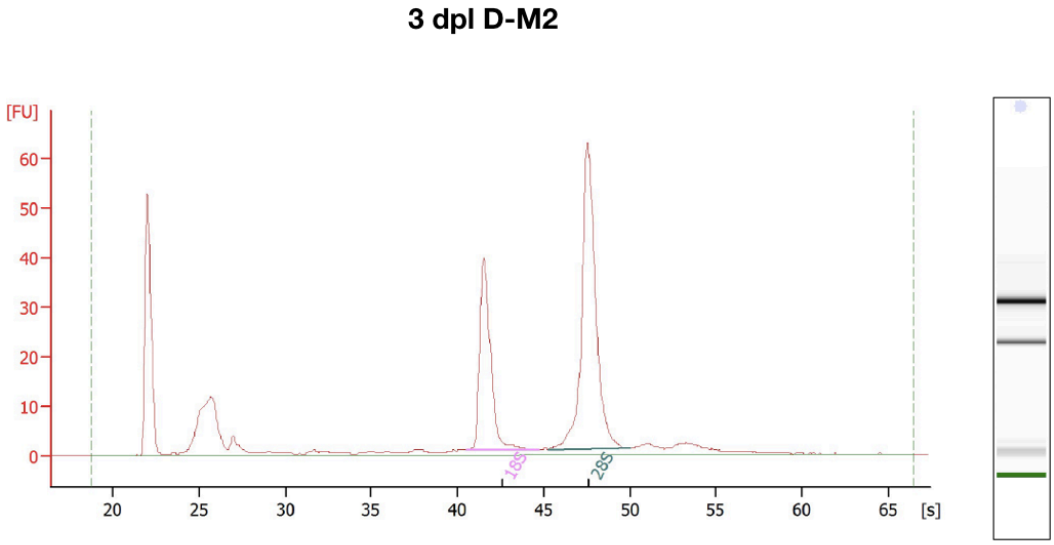


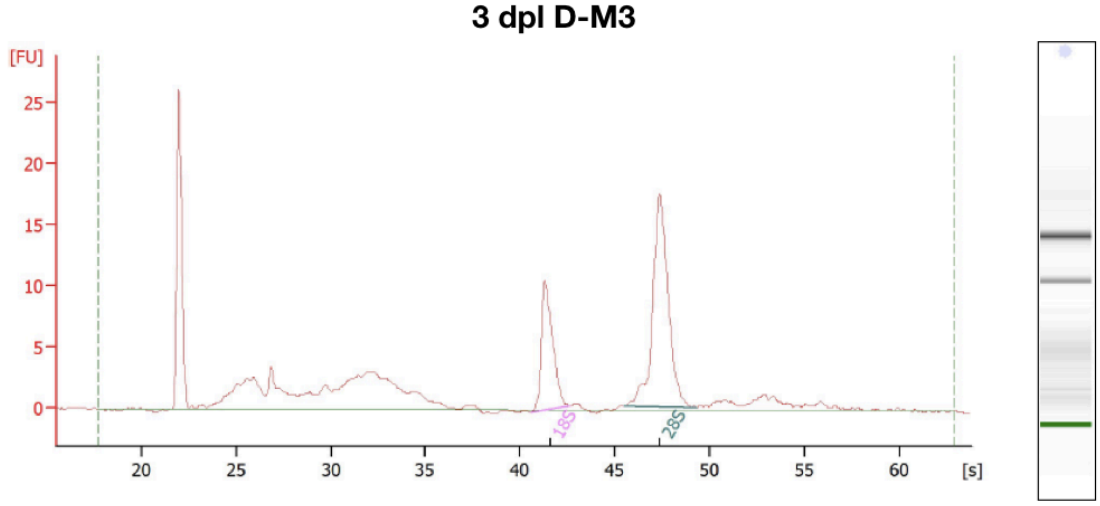
Figure 28: Electropherogram summary of 3 dpl D-M1 sample.



Overall Results for sample 1 : 3 dpl D-M2

RNA Area:	327,8	RNA Integrity Number (RIN):	10 (B.02.08)
RNA Concentration:	615 pg/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	1,8	Result Flagging Label:	RIN:10

Figure 29: Electropherogram summary of 3 dpl D-M2 sample.

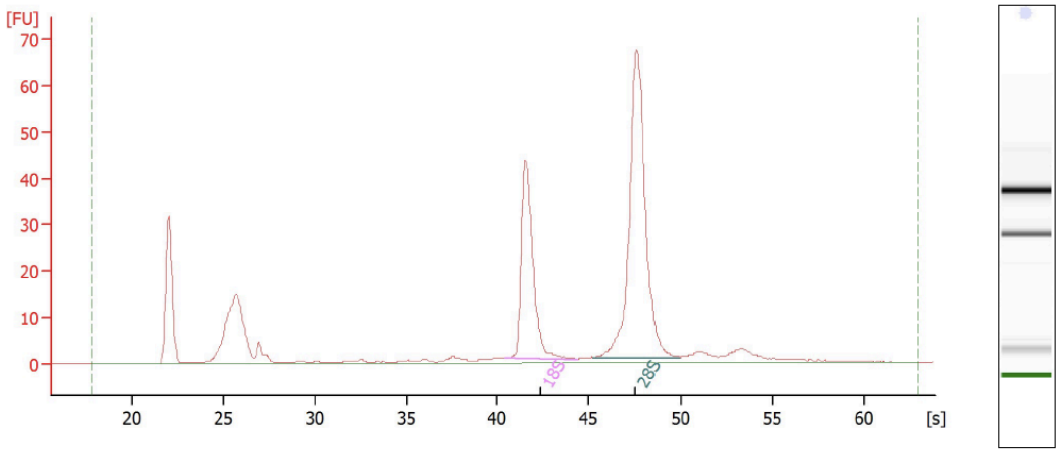


Overall Results for sample 3 : 3 dpl D-M3

RNA Area:	140,2	RNA Integrity Number (RIN):	8.1 (B.02.08)
RNA Concentration:	330 pg/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	1,9	Result Flagging Label:	RIN: 8.10

Figure 30: Electropherogram summary of 3 dpl D-M3 sample.

3 dpl I-M1

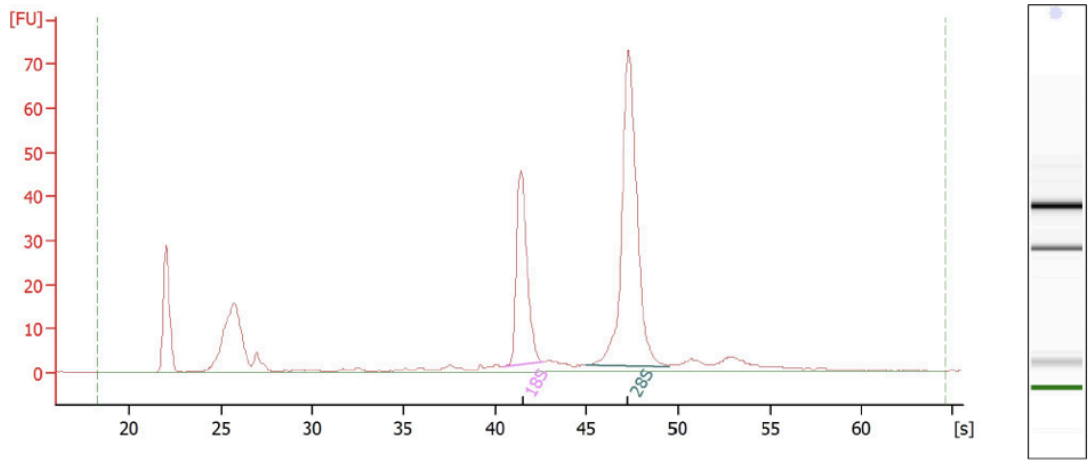


Overall Results for sample 7 : 3 dpl I-M1

RNA Area:	349,6	RNA Integrity Number (RIN):	10 (B.02.08)
RNA Concentration:	656 pg/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	1,8	Result Flagging Label:	RIN:10

Figure 31: Electropherogram summary of 3 dpl I-M1 sample.

3 dpl I-M2



Overall Results for sample 11 : 3 dpl I-M2

RNA Area:	373,9	RNA Integrity Number (RIN):	10 (B.02.08)
RNA Concentration:	702 pg/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	2,0	Result Flagging Label:	RIN:10

Figure 32: Electropherogram summary of 3 dpl I-M2 sample.

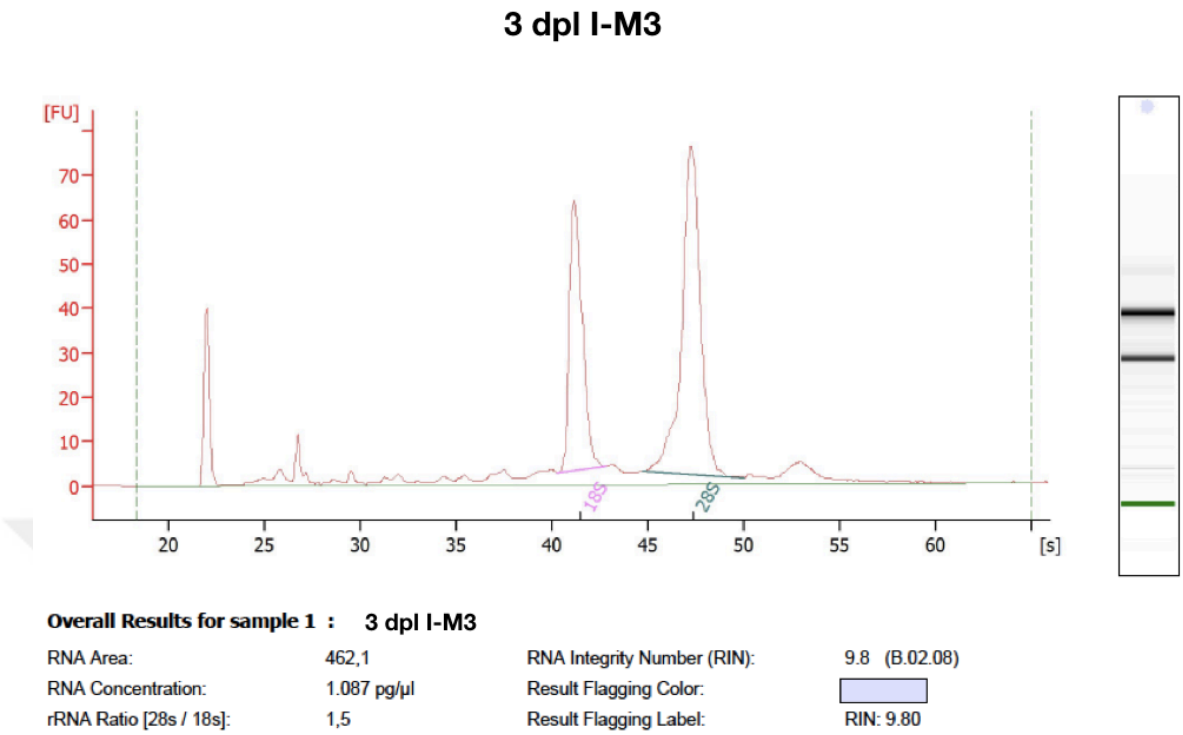


Figure 33: Electropherogram summary of 3 dpl I-M3 sample.

Transcriptome analysis showed that the numbers of differentially expressed genes (DEGs) are 829 and 1707 at 20 hpl and 3 dpl injured brain respectively. Their gene ontology terms are visualized according to their biological process (Figure 34). To identify Wnt/β-catenin-related genes at 20 hpl and 3 dpl, groups of the injured brain at 20 hpl and 3 dpl compared with their IWR treated counterparts. As a result, 117 genes have been identified at 20 hpl samples, while 8 genes have been identified at 3 dpl in injured hemisphere. Those genes are up-regulated upon injury and down-regulated with the application of IWR-1 in the injured brain (Figure 35). Some of the potential candidate genes (ctgfa, prg4b, fsta, inhbab, klf11b, pappaa) that involve in regeneration from DEGs list were validated by qPCR (Figure 36). In order to find out potential interacting proteins of candidate genes, co-expression analyses and co-expressed genes are visualized at 20 hpl (Figure 37). Grey nods indicate possible interacting protein with identified Wnt/β-catenin-related candidates in each time points. After the identification of candidate genes, their biological process and molecular functions are visualized (Figure 38). 8 genes are detected at 3 dpl as a Wnt-related genes (Figure 39). Co-expression analysis of these genes are visualized (Figure 40).

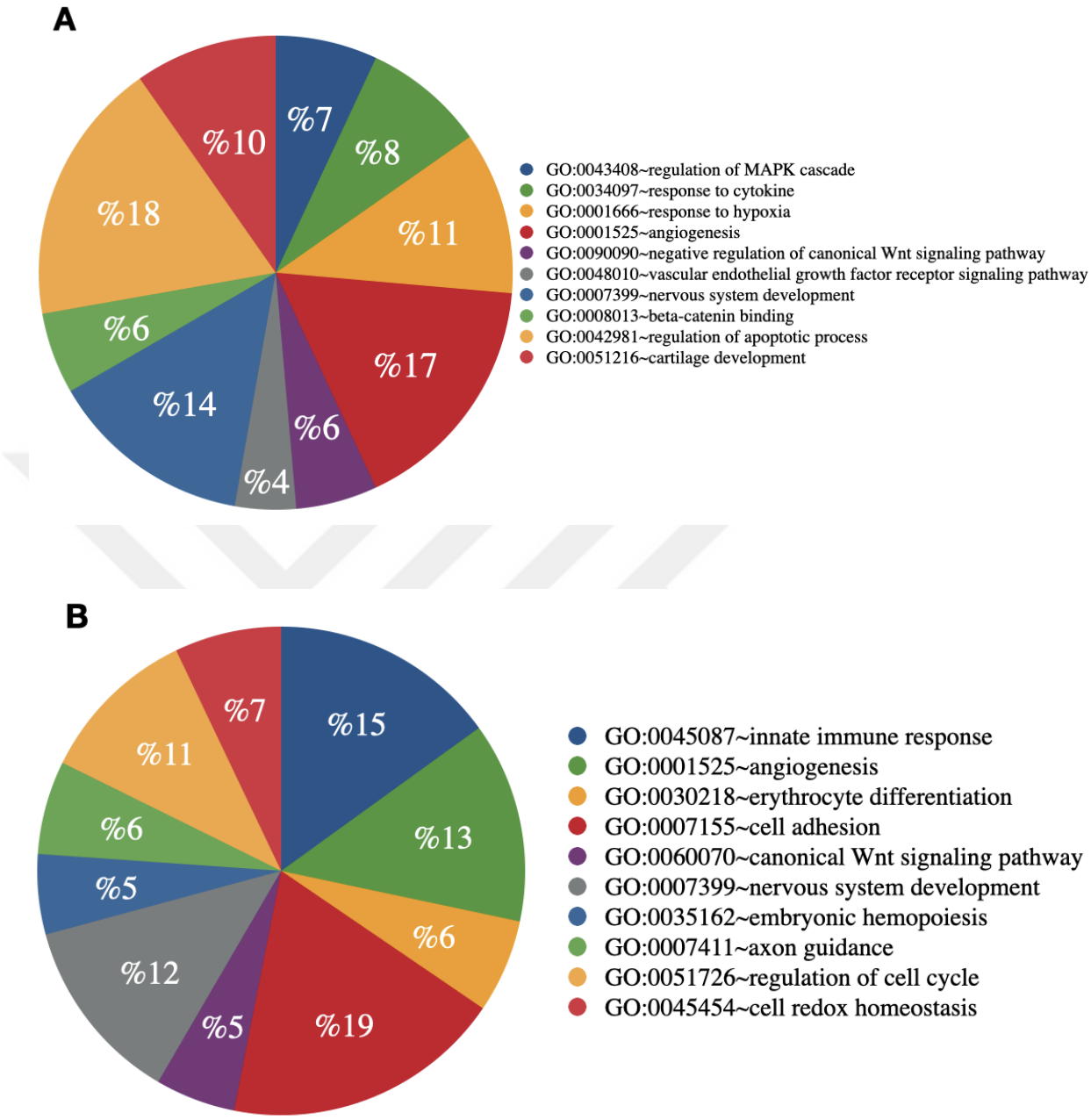


Figure 34: Biological terms of injured hemisphere at two stages. Biological terms at 20 hpl (A) and 3 dpl (B).

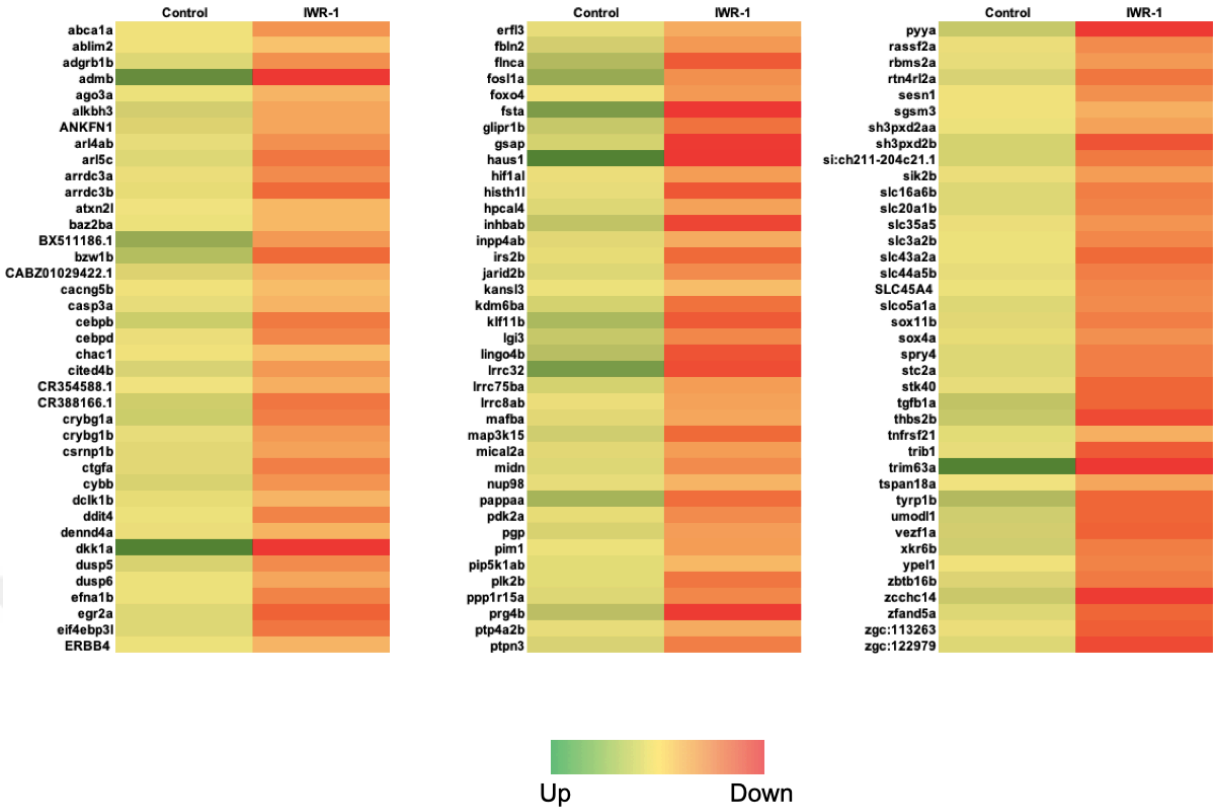


Figure 35: Heatmap of Wnt/β-catenin-related genes at 20 hpl.

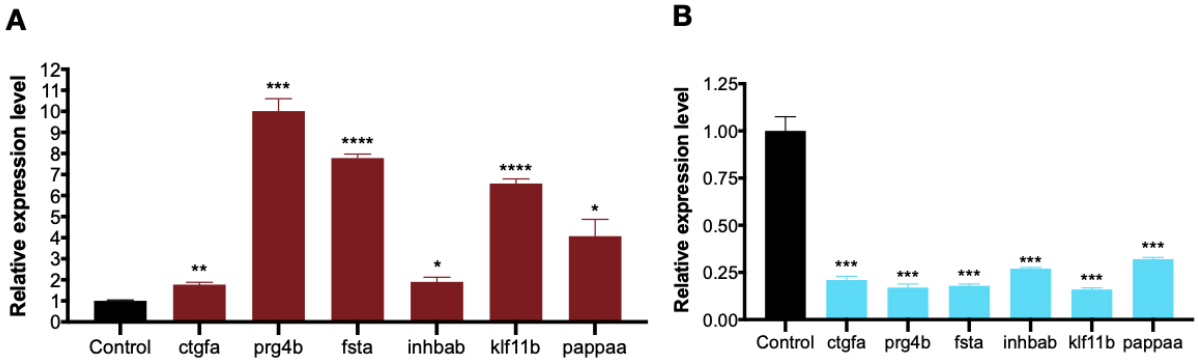


Figure 36: Validation of Wnt-related genes at 20 hpl. (A) Up-regulation of Wnt-related genes. (B) Down-regulation of Wnt-related genes upon Wnt inhibition.

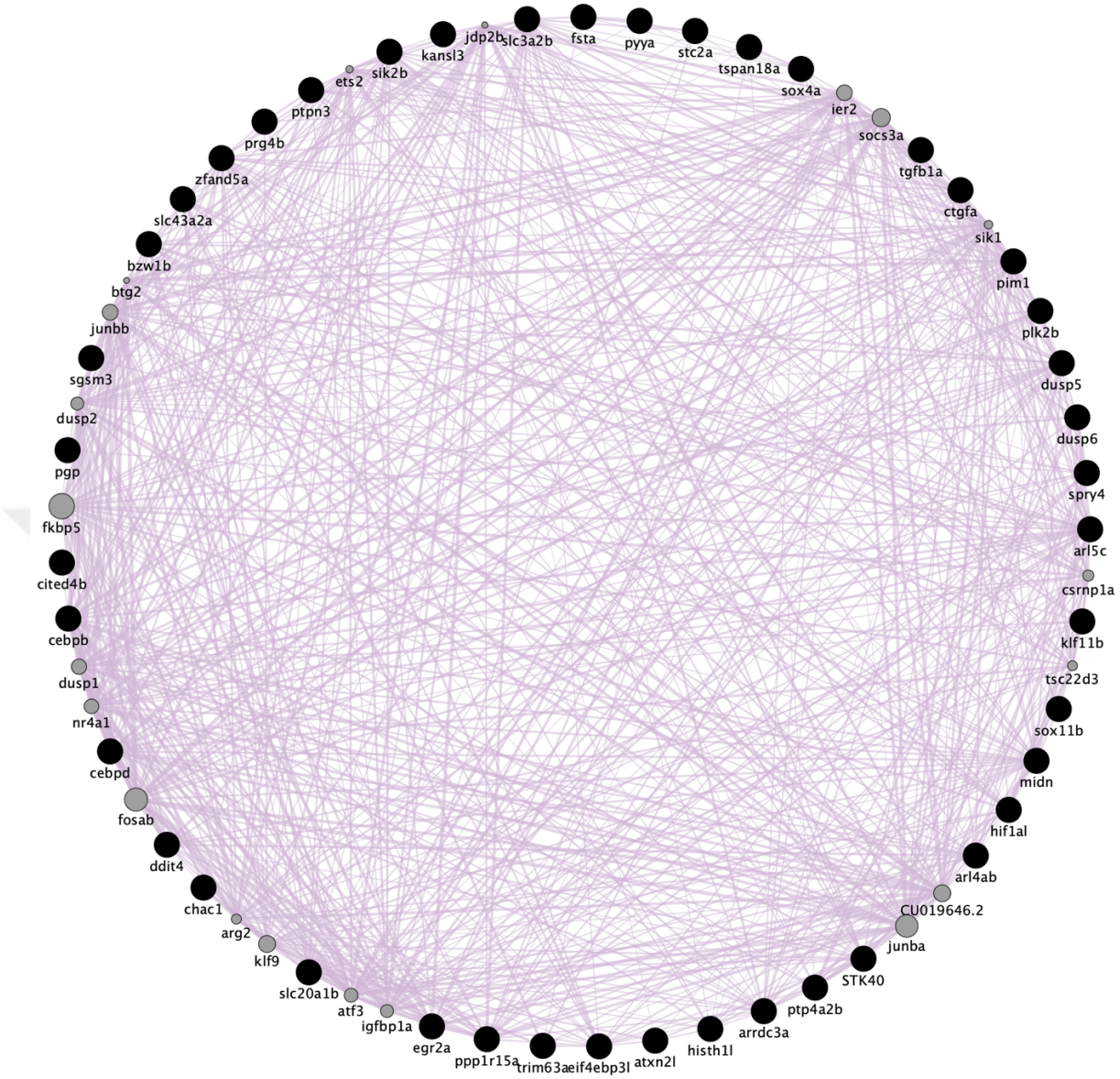


Figure 37: Co-expression analysis of some of Wnt/β-catenin-related genes.

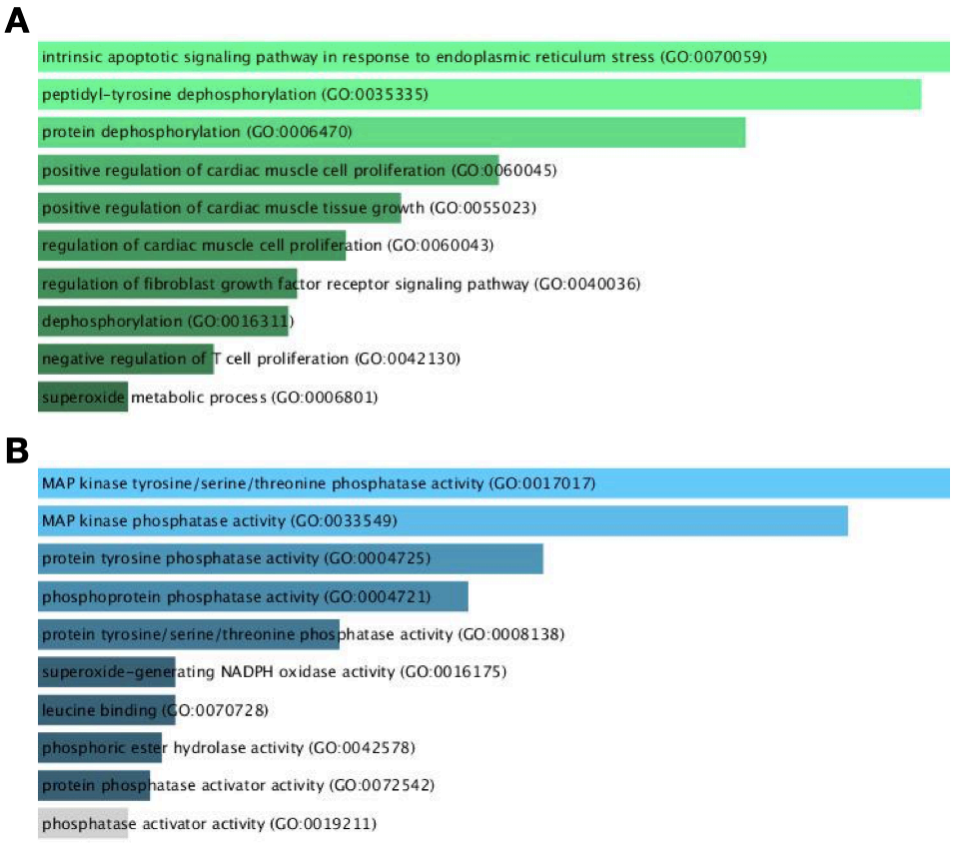


Figure 38: Biological process (A) and Molecular function (B) analysis.

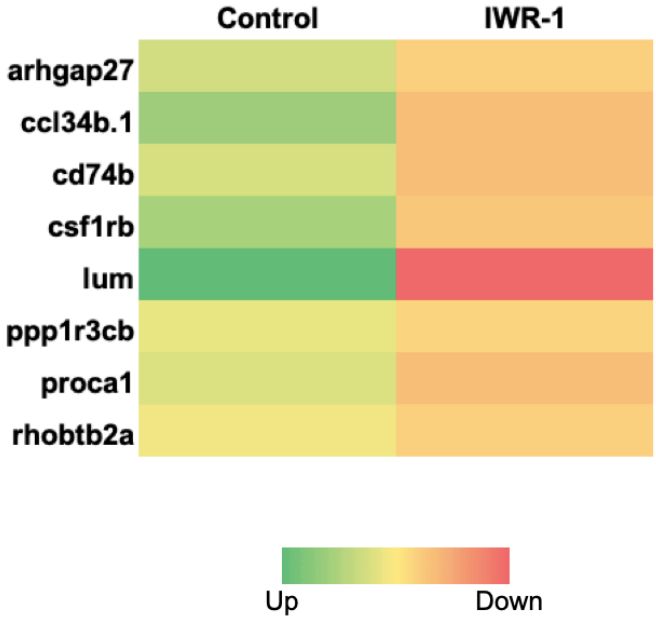


Figure 39: Heatmap of Wnt/β-catenin-related genes at 3 dpl.

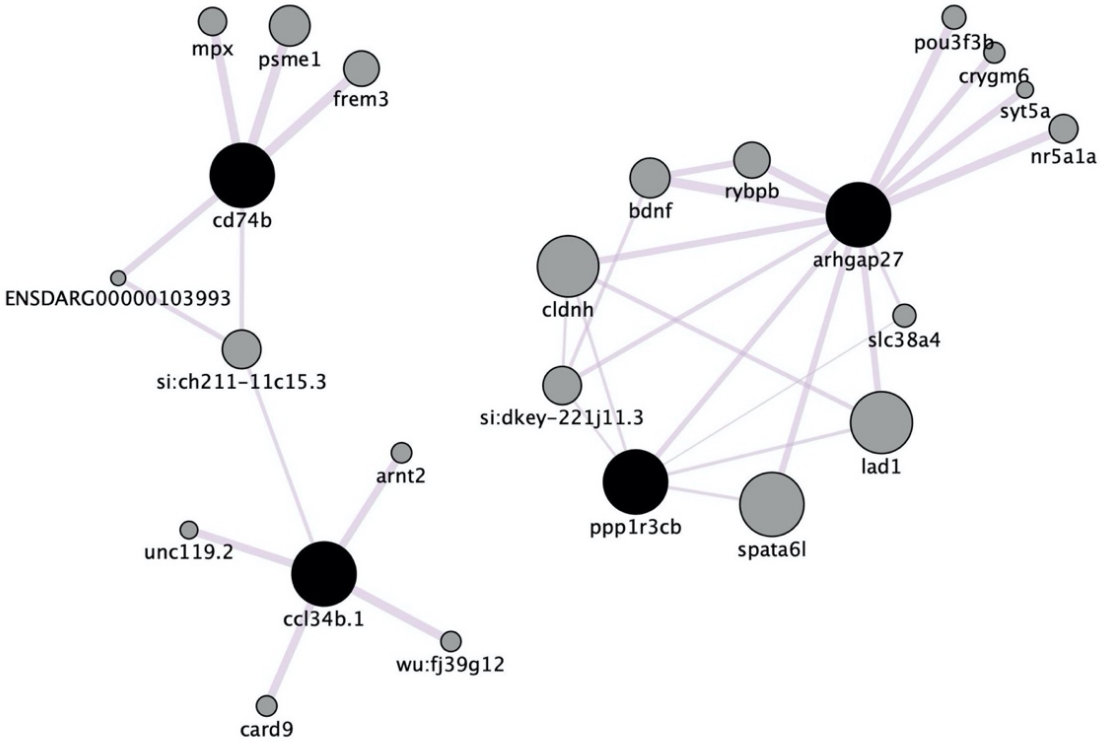


Figure 40: Co-expression analysis of Wnt/β-catenin-related genes at 3 dpl.

5. DISCUSSION

In this study, outcomes and underlying mechanisms of early and proliferative stages of brain regeneration are investigated and Wnt signaling target genes are identified in the adult zebrafish.

The early stage of brain regeneration is dominated by the activation of the inflammatory response and cell death. In our study, we observed that mature neurons undergo apoptosis indicating microenvironment does not support neuronal survival, also induces their apoptosis at the early stage of brain regeneration. Neuronal apoptosis has been never demonstrated in TBI model in zebrafish at early stage. Besides, the expression of neuronal differentiation factor neurod1 is reduced. These observations are consistent with previous research and support the idea that neuronal survival is inhibited caused by the excessive immune response which is required for proper tissue repair (Pavlov et al., 2015). Inhibition of neuronal survival and differentiation are not specific for zebrafish brain regeneration.

Glial cell activation is one of the hallmarks of various neurodegenerative diseases (Burda et al., 2014). Here in this study, widely known reactive gliosis markers, GFAP and S100β are observed in the situation of injury in adult zebrafish supporting the notion that activation of glial cell is part of neurodegeneration associated outcome in not only mammalian model systems, but also in the teleost model. In our study, reactive gliosis marker GFAP is detected in both early and proliferative stages of brain regeneration, so early detection of GFAP in the zebrafish TBI model was never mentioned. Interestingly, not only glial cells contain GFAP and S100β, in addition to glial cells, neural stem cells in adult zebrafish brain named as RG cells express both GFAP and S100β in response to damage too. Recent technologies such as Single-cell transcriptomics are valuable one for our deep understanding of RG progeny cells in the adult zebrafish brain. (Lange et al., 2020) showed that S100β is predominantly observed in quiescent RG cells. This shared feature of neurodegeneration in the different model systems could be a valuable target to induce brain regeneration with the help of zebrafish model.

Roles of Wnt signaling have been revealed in various studies, and its function is clarified in injury-induced brain regeneration in non-regenerating animal models. In our injury model, we detected the enhanced expression of Phospho-β-catenin (Ser675) at wound site which indicates the activation of Canonical Wnt signaling. In addition to immunohistochemistry staining, Wnt reporter activity is elevated in the injured reporter fish brain. According to (Zhao et al., 2019), Wnt signaling is also activated the wound site in the

damaged liver in mice model. This result is in line with our result and supports the idea that Wnt signaling is required for proper tissue regeneration and its activation observed in damaged area. Furthermore, Wnt signaling is also detected near the amputated organ regeneration. (Whner et al., 2014) showed that Wnt signaling is required for fin regeneration by modulating other signaling pathways including Fgf and Bmp near the wound side.

According to (Salehi et al., 2018), it is observed that Wnt signaling contributes vascular regeneration process and signaling activity is detected in perilesional vessels at early after injury. This result is partly consistent with our finding and supports the notion that the activation of Canonical Wnt signaling is a general concept at the early phase of damaged brain. Also, in this study, they showed that vascularization plays an essential role during brain regeneration in traumatized brain through Wnt signaling. Activation of Wnt signaling in endothelial cells, not only observed in brain regeneration, but also observed in other tissues to induce the healing process in damaged vessels. Enhanced Wnt signaling in endothelial cells induces the expression of certain tight junction genes such as claudin-1 and claudin-3 to maintain BBB integrity in the seizure model (Tran et al., 2016). In contrast, according to (Liebner et al., 2008), the expression of claudin-3 increased while claudin-1 expression is not affected by Wnt3a stimulation. In our study, vascular endothelial zinc finger 1a (vezf1a) is suggested to be a Wnt signaling related transcription factor that is expressed in traumatized brain. This transcription factor involves vascular development, angiogenesis and mainly expressed in endothelial cells (Miyashita et al., 2004). Until now, Wnt signaling dependent transcriptional regulation of vezf1a has never been mentioned. Manipulation of this gene may be a valuable target to treat for the vascular injury-dependent disease.

The interplay of the component of Wnt signaling and Hif-1α pathway is demonstrated in various conditions. For instance, neuronal maturation of glioblastoma stem cells is associated with the interaction of Hif-1α and downstream component of Canonical Wnt signaling TCF1 (Boso et al., 2019). Besides, Wnt signaling is linked to muscle formation by regulating Hif pathway in vivo research (Majmundar et al., 2015). Here, our transcriptome analysis revealed that the expression of one of the Hif-1 signaling related genes, hif1al (Hypoxia-inducible factor 1, alpha subunit, like) is regulated by Wnt signaling in brain regeneration.

According to our transcriptome analysis, the most up-regulated gene is trim63a (Tripartite motif-containing 63a) and increased with 8,86 (log2fold), upon Wnt inhibition the most downregulated gene is admb (Adrenomedullin b) and decreased with -4,26 (log2fold) at early

stage of brain injury. Positive regulatory role of trim63 in Wnt signaling has been elucidated by promoting phosphorylation of Gsk-3β in breast cancer, thus trim63 activates Wnt signaling and induces the development of breast cancer (Li et al., 2019). This result suggests that trim63 induces proliferation via Wnt signaling in different concepts including regeneration according to our result. Adrenomedullin has a role in the maintenance of vascularization and endothelial permeability. It is not surprising to observe a gene that involves vascular homeostasis and affected by the inhibition of Wnt signaling. Neuroprotective effects of admb are observed and strongly suggest that Wnt signaling is required for the maintenance and function of endothelial cell activity (Ochoa-Callejero, 2016).

Tissue remodeling is one of the main process that required for tissue regeneration. This process is mainly dominated by the dynamics of extracellular matrix (ECM). In the proliferative stage, the most up-regulated and down-regulated gene is lum (Lumican) and increased with 6,81 (log2fold), decreased with -4,82 (log2fold) upon Wnt inhibition. Lumican is a small leusine-rich proteoglycan which has a role in matrix remodeling. Our result indicates that Wnt signaling also has a regulatory role in ECM remodeling by regulating the components of ECM in brain regeneration. Functions of lum are demonstrated in tendon regeneration, but its direct regulation by Wnt signaling is not detected (Miyabara et al., 2014). In addition to lumican, our result includes another glycoprotein proteoglycan4b (prg4b) as a Wnt signaling dependent candidate genes that drive ECM remodeling.

6. CONCLUSION AND FUTURE PERSPECTIVE

As a conclusion, we have demonstrated that the early activation of Wnt/β-catenin signaling in brain regeneration and its regulatory roles in the regulation of regeneration-related genes in zebrafish model for the first time. For the future studies, these identified genes, which are involves in the regeneration of various organ pave the way for the treatment of numerous diseases.

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8. APPENDIX

8.1 Research Ethics Committee Approval

İZMİR BİYOTIP VE GENOM MERKEZİ
HAYVAN DENEYLERİ YEREL ETİK KURULU (İBG-HADYEK)
KARARI

Toplantı Tarihi	: 12/02/2020	**Toplantı Günü**	: Çarşamba
Toplantı Sayısı	: 2	**Toplantı Saati**	: 15:30

Sayın Doç. Dr. Güneş ÖZHAN BAYKAN,

2020-004 Protokol No'lu; yürütücüsü olduğunuz "Investigation of the role of Wnt/ß-Catenin signaling in brain regeneration using zebrafish as a model system" başlıklı projenin uygulanmasında etik açıdan sakınca olmadığına oy birliği ile karar verilmiştir.

Bilgilerinizi ve gereğini rica ederiz.

Doç. Dr. Güneş ÖZHAN BAYKAN **Başkan** (Katılmadı)	**Vet. Hek. Kerem ESMEN** **Başkan Yardımcısı**
Prof. Dr. H. Alper BAĞRIYANIK **Üye**	**Prof. Dr. Ensari GÜNELİ** **Üye** (Katılmadı)
Doç. Dr. Kasım DİRİL **Üye**	**Dr. Öğr. Üyesi Ayşe Banu DEMİR** **Üye**
Dr. Gülçin ÇAKAN AKDOĞAN **Üye**	**Vet. Hek. Umur KELEŞ** **Üye**
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Ülke	Üniversite	Fakülte/Enstitü	Öğrenim Alanı	Derece	Mezuniyet Yılı
TR	Ege University	Faculty of Science	Department of Biology	Bachelor of Science	2017
TR	DEU-IBG	Izmır International Bimedicine and Genome Institute	Molecular Biology and Genetics	Master	2020
TR	Anadolu University	Faculty of Economics	International Relations	Bachelor ’s degree	2020

AKADEMİK/MESLEKTE DENEYİM

Kurum/Kuruluş	Ülke	Şehir	Bölüm/Birim	Görev Türü	Görev Dönemi

UZMANLIK ALANLARI

Uzmanlık Alanları
Immunohistochemistry, Frozen sectioning, Confocal Imaging, qPCR, Microinjection

YAYINLARI

SCI, SSCI, AHCI indekslerine giren dergilerde yayınlanan makaleler

Demircan, N., Cucun, G., & Uzilday, B. (2020). Mitochondrial alternative oxidase (AOX1a) is required for the mitigation of arsenic-induced oxidative stress in Arabidopsis thaliana. *Plant Biotechnology Reports*, 1-11.

Diğer dergilerde yayınlanan makaleler

Hakemli konferans/sempozyumların bildiri kitaplarında yer alan yayınlar
