ANALYSIS OF MOLECULAR SIGNALING PATHWAYS REGULATING HB-EGF-INDUCED MITOTIC CELL DIVISION AND NEUROGENESIS IN THE ZEBRAFISH OLFACTORY EPITHELIUM

by

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B.S., Molecular Biology and Genetics, Boğaziçi University, 2020

Submitted to the Institute for Graduate Studies in
Science and Engineering in partial fulfillment of
the requirements for the degree of
Master of Science

Graduate Program in Molecular Biology and Genetics
Boğaziçi University
2023

ACKNOWLEDGEMENTS

As a person who is always skeptical and sees the glass as half empty, I am grateful to have had the people who could always show me the full part. Even though my desire was to work on a different topic, I have never regretted staying here for my Master's thanks to my supervisor, Stefan Fuss. He was always a human above all else. When I hated the world, he could direct me to a new hope, and he did not give up despite looking at a grumpy face with killer eyes. He taught me how to be a student, a scientist, and especially, a better person. The lab has always been a mess, but it would not have been possible to hang on there with all the failed experiments if it were not for the people who came to the rescue. Failing and cursing together with Zeynep has always been a treat. In the end, failure is the best way to learn, and we have always recovered together. Aysu was always there to help and cry together. Mehmet Can was the one to teach me almost all the techniques I used. My lovely intern, Su, was a greater help than I could have ever asked for. Also, I was especially lucky to have worked with an amazing group of undergrads; Sinem, Ecem, Ebru, and Hatice.

Last but not the least, I was so lucky to have my family, both small and big one. When I come back home after a horribly busy and usually disappointing day of the lab, my partner Baybars, who was always there to listen, and our crazy cats have always reminded me what life is actually about. Keeping my sanity would have been impossible without them. I would also like to thank my parents and my sister for always being there for support. Finally, I owe a lot to my hobbit support network, my people who were always there to help even in times that I could not ask for any help. Yağmur, Sinem, Çiğdem, Emren, Doğukan, Ulduz, Anna, Emre, Barış, Ece, Cem, and Dilşad always patiently listened my complaints.

The work presented in this thesis was supported by the Scientific and Technological Research Council of Turkey (TÜBİTAK) through research grant 119Z081 to SHF.

ABSTRACT

ANALYSIS OF MOLECULAR SIGNALING PATHWAYS REGULATING HB-EGF-INDUCED MITOTIC CELL DIVISION AND NEUROGENESIS IN THE ZEBRAFISH OLFACTORY EPITHELIUM

The zebrafish olfactory epithelium (OE) has an outstanding regenerative capacity among other nervous tissues. Two distinct modes of neurogenesis regulate the mechanism of olfactory sensory neuron (OSN) generation under physiological and injury conditions. Continuous division and differentiation of globose basal cells (GBCs) provide the lifelong turnover and maintenance of OSNs, whereas repair neurogenesis is achieved by the activation of horizontal basal cells (HBCs) to replenish the loss of OSNs following acute injury. Transcriptome analysis and *in situ*-hybridization of the injured OE showed a strong upregulation of the signaling factor heparin-binding epidermal growth factor-like growth factor (HB-EGF) shortly after damage. Previously, exogenous administration of recombinant HB-EGF to the OE was shown to increase proliferative activity in HBCs and the generation of OSNs. Inhibition of HB-EGF ectodomain shedding showed the opposite effect and reduced cell proliferation in response to injury. These findings suggested a critical role for HB-EGF during regeneration of the zebrafish OE. The work presented in this study further validates that HB-EGF is a key regulator of OSN neurogenesis in the zebrafish OE. Pharmacological inhibition of HB-EGF ectodomain shedding reduced OSN regeneration as well as cell proliferation. Similarly, direct inhibition of soluble HB-EGF suppressed HBC proliferation following injury and impaired OSN regeneration. The analysis of *hbegfa* and *ascl1a* expression by *in situ*-hybridization upon HB-EGF stimulation also showed direct activation of HBCs, which proliferate and give rise to neurons through the transient induction of intermediate GBC populations. In addition, the potential role of Leptin and IL-11 cytokines on cell proliferation and a possible synergism between HB-EGF and these cytokines were investigated. Exogenous stimulation with recombinant cytokines separately or in combination with HB-EGF did not point out an effect on cell proliferation.

ÖZET

ZEBRA BALIĞI OLFAKTÖR EPİTELİNDEKİ HB-EGF KAYNAKLI MİTOTİK HÜCRE BÖLÜNMESİ VE NÖROJENEZİNİ DÜZENLEYEN MOLEKÜLER SİNYAL YOLAKLARININ ANALİZİ

Zebra balığının olfaktör epiteli (OE) diğer sinir dokularına göre üstün rejenerasyon kapasitesi göstermektedir. Fizyolojik ve hasar şartlarında olfaktör duyu nöronlarının (OSN) üretim mekanizmasını iki ayrı nörojenez modu düzenler. Küresel bazal hücrelerin (GBC) sürekli bölünmesi ve farklılaşması OSN'lerin yaşam boyu dönüşümünü ve korunmasını sağlarken, akut hasardan sonra kaybedilen OSN'leri yenileyen onarım nörojenezine yatay bazal hücrelerin (HBC) aktivasyonu ile ulaşılır. Hasarlı OE'nin transkriptom analizi ve in situ hibridizasyonu, heparine bağlanan epidermal büyüme faktörü benzeri büyüme faktörünün (HB-EGF) ifadesinde hasardan hemen sonra ciddi bir artış gösterdi. Önceki çalışmalarda rekombinant HB-EGF'nin OE'ye dışarıdan uygulanmasının HBC'lerin bölünmesi ve OSN oluşumunu artırdığı gösterildi. HB-EGF'nin ectodomain kesilmesinin inhibisyonu ise hasardan sonra hücre bölünmesini azalttı. Bu bulgular, zebra balığının OE rejenerasyonunda HB-EGF için kritik bir rol öne sürmüştür. Bu çalışma, HB-EGF'nin zebra balığı OE'sindeki OSN nörojenezinin temel bir regülatörü olduğunu doğrulamaktadır. HB-EGF'nin ectodomain kesilmesinin farmakolojik inhibisyonu, hücre bölünmesini ve OSN rejenerasyonunu azaltmıştır. Çözünür HB-EGF'nin direkt inhibisyonu da hasarı takiben HBC bölünmesini ve OSN rejenerasyonunu düşürmüştür. HB-EGF stimülasyonunun ardından *hbegfa* ve *ascl1a* genlerinin in situ hibridizasyonu ile ifade analizi, ara GBC popülasyonlarının geçici indüksyonu üzerinden nöronları oluşturan HBC'lerin doğrudan aktivasyonunu göstermiştir. Ayrıca, Leptin ve IL-11 sitokinlerinlerinin hücre bölünmesi üzerindeki potansiyel rolleri ve HB-EGF ile olabilecek sinerjileri de araştırılmıştır. Rekombinant sitokinlerlerle ayrı ayrı veya HB-EGF ile birleştirilerek yapılan dışarıdan stimulasyonlar hücre bölünmesi üzerinde bir etki göstermemiştir.

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LIST OF SYMBOLS

G	Gauge
g	Gram
l	Liter
mg	Miligram
ml	Mililiter
mM	Milimolar
N	Normal
ng	Nanogram
µl	Microliter
µg	Microgram
µm	Micrometer
°C	Degree Celsius
±	Plus or minus

LIST OF ACRONYMS / ABBREVIATIONS

6-OHDA	6-hydroxydopamine
AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
aNSC	Adult neural stem cell
ATP	Adenosine triphosphate
bFGF	Basic fibroblast growth factor
BLBP	Brain-lipid binding protein
BMP 11	Bone Morphogenetic Protein 11
BrdU	5- Bromo-2'- Deoxyuridine
BUHADYEK	Institutional Ethics Board for Animal Experiments at Boğaziçi University
CNTF	Cilia neurotrophic factor
DAMP	Damage-associated molecular pattern
DCX	Doublecortin
ddH_2O	Double distilled sterile water
DG	Dentate gyrus
DT	Diphtheria toxin
EdU	5-Ethynyl-2'-Deoxyuridine
EGF	Epidermal growth factor
EGFR	EGF receptor
FGF	Fibroblast growth factor
GBC	Globose basal cell
GBC_{INP}	Immediate neuronal precursor
GBC_{MMP}	Multipotent GBC

GBC_{STEM}	GBC with stem cell properties
GBC_{TA}	Transit-amplifying GBC
GDF11	Growth and differentiation factor 11
GFAP	Glial fibrillary acid protein
HB-EGF	Heparin-binding epidermal growth factor-like growth factor
HBC	Horizontal basal cell
hpl	Hours post lesion
hpi	Hours post injection
hps	Hours post stimulation
HSD	Honest Significant Difference
HSP	Heat shock protein
IGF1	Insulin-like growth factor 1
ILC	Interlamellar curve
IP	Intraperitoneal
IPC	Intermediate progenitor cell
LIF	Leukemia inhibitory factor
MAPK	Mitogen-activated protein kinase
MeBr	Methyl bromide
MG	Müller Glia
MMP	Matrix metalloproteinase
MPP	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NE	Neuroepithelial
NFκB	Nuclear factor κB
NGF	Nerve growth factor
NO	Nitric oxide
NOS	Nitric oxide synthase

NP	Neuronal progenitor
NS	Non-sensory
NSC	Neural stem cell
OB	Olfactory bulb
OE	Olfactory epithelium
OMP	Olfactory marker protein
OR	Olfactory receptor
OSN	Olfactory sensory neuron
PKC	Protein kinase C
PSA-NCAM	Polysialylated neuronal cell adhesion molecule
RG	Radial glia
RGC	Radial glial cell
ROI	Regions of interest
SGZ	Subgranular zone
Shh	Sonic hedgehog
SNS	Sensory/non-sensory border
Sox2	Sex determining region Y-box 2
SSC	Saline Sodium Citrate Buffer
TAAR	Trace amine-associated receptor
TGFβ	Transforming Growth Factor-β
Trp63	Transcription factor p63
TrX	Triton X-100
v-SVZ	Ventricular-subventricular zone
VEGF	Vascular endothelial growth factor
VNO	Vomeronasal organ
VZ	Ventricular zone

1. INTRODUCTION

1.1. Adult Neurogenesis

Scientific understanding is molded by new insight that results in the formulation of dogmata and their subsequent destruction upon revealing additional evidence. This process depends on time, people, perspective, and experiments. The generation of new neurons during adulthood, also known as adult neurogenesis, was one of these concepts that changed over time and the long-accepted notion that neurons are immutable after birth is now considered to be a misconception in the history of science. Just like any other paradigm change, it took a long time for the scientific community to consider the possibility of adult neurogenesis.

The journey began when Santiago Ramon y Cajal collected his observations on neurons and suggested one of the strongest neuroscientific theories of his time in the early 1900s. While most of his observations were confirmed later by other scientists, an exception was his suggestion that the growth of neurons is completed at the end of embryogenesis and that nerve endings are fixed following the completion of post-natal development in mammals (Cajal, 1913). This notion that neurogenesis does not occur in adulthood was widely accepted for a long time. This view started to be challenged when Sidman developed a new method to observe dividing cells, which included the use of tritium-labeled thymidine (Thymidine-H^3) and detected dividing cells in the primitive ependymal zone of mouse embryos (Sidman *et al*, 1959). Joseph Altman was one of the first scientists to use this method to investigate cell proliferation in the brain of adult rats. Surprisingly, he observed dividing cells, possibly newly generated neurons, in the brains of 1-day to 2-month-old rats (Altman, 1962). He continued this research with a series of studies using Thymidine-H^3 in rats, cats, and guinea pigs. His results suggested the presence of newborn neurons specifically in the dentate gyrus (DG) of the hippocampus and the cortex (Altman, 1963; Altman and Das, 1965; Altman, 1967). However, the histological evidence at the time was not found to be convincing enough to change the rigid dogma that prevailed in the scientific community. Michael Kaplan was the next scientist who took up the challenge to chase the possibility of neurogenesis in adulthood. He also observed dividing cells in the DG, visual cortex, and olfactory bulb (OB) of 3-month-old rats and confirmed their neuronal identity

by electron microscopy (Kaplan and Hinds, 1977; Kaplan, 1981). He later showed thymidine-labelled cells to exist in the subependymal layers of the primate brain (Kaplan, 1983).

Meanwhile, Fernando Nottebohm was conducting studies on the vocal control nuclei of the canary brain, which revealed new neurons and glial cells after Thymidine-H^3 injection (Goldman and Nottebohm, 1983). A follow-up of this paper presented evidence for the seasonal integration of newborn neurons into functional brain circuits (Paton and Nottebohm, 1984). Another study on birds indicated postnatal neurogenesis in the ventricular zone (VZ) of canary brains, and identified a population of precursor cells in this proliferative zone (Alvarez-Buylla *et al*., 1988). Although groundbreaking, these studies still faced strong criticism because they involved a non-mammalian model. It was argued that mammals have more complex nervous structures and that the dogma still applies to higher species.

The pioneering studies mentioned above were criticized and rejected with a strong prejudice. Especially Pasko Rakic, a well-known neuroscientist of the time, was reluctant to accept the results of these experiments. In his studies, Rakic did not detect any newborn neurons in the brains of rats and rhesus monkeys (Rakic, 1985; Eckenhoff and Rakic, 1988). Even if adult neurogenesis would exist in mammals, the mechanistic aspect was hard to integrate into the nervous structure. It was argued that the addition of new cells to a fully developed brain would interrupt the pre-existing circuits. The basic connections in the brain were believed to be stable, in order to preserve the knowledge and abilities that were acquired over time to promote the survival of the animal. While the debate continued, a study of neural grafts in intact and damaged rat brains showed integration and differentiation of grafted cells, which indicated that also the process of generating new neurons could continue with their functional integration into the existing circuits (Bjorklund and Gage, 1985). In another study, Nestin-expressing cells were isolated in culture from the striatum of adult mouse brains in the presence of epidermal growth factor (EGF). These cells were able to divide to form ball-shaped structures called neurospheres and to differentiate into astrocytes and neurons *in vitro* (Reynolds and Weiss. 1992). A similar paper of the same year showed that precursor cells isolated from adult mice were able to generate neurons in the presence of basic fibroblast growth factor (bFGF) and astrocyte glia population (Richards *et al*, 1992).

At the other end of the discussion, there were arguments against the use of the Thymidine-H^3 label, since it was a radioactive reagent with low visibility. Delivery methods were criticized due to the potential risk of damage and limits of the blood-brain barrier. The absence of established neuronal markers was another limitation for confirming the identity of proliferating cells as neurons. When a new thymidine analog, Bromodeoxyuridine (BrdU), was developed, it offered lower toxicity, higher penetration, and less interference with differentiation and migration of dividing cells, since it was a non-radioactive molecule that can be easily detected by antibodies (Miller and Nowakowski, 1988). In 1990s, a series of studies further confirmed the presence of adult neurogenesis with this method. Isolated BrdU-labelled cells from rat hippocampus showed a proliferation response to fibroblast growth factor (FGF) *in vitro* and the fate of these cells could be traced upon grafting into the brain. Lineage tracing showed the potential of progenitor cells in the hippocampus to generate new neurons (Gage *et al*., 1995).

Elizabeth Gould opened another path forward in adult neurogenesis research with her seminal work on adult-generated neurons in DG of the rat brain. The investigation of adult neurogenesis with BrdU-labelling showed an inverse relationship between adrenal steroid hormone levels and adult neurogenesis in the hippocampus (Gould *et al*., 1992, 1994; Cameron *et al*., 1993). During the same period, supporting studies came from rats, replicating the data on adult neurogenesis in rat brains and suggesting a decrease in the proliferating cells in the brain with aging (Kuhn *et al*, 1996). Research in birds continued to support the concept of adult neurogenesis. A possible role of adult neurogenesis was suggested to support learning, the modification of existing memories, and thus, the ability to produce new or improved mating songs in birds (Alvarez-Buylla and Kirn, 1997). Gould continued her research by showing the presence of newly generated neurons in marmoset monkeys, as well as an inverse relationship between the number of dividing cells in the brain and stress (Gould *et al*. 1998). Later, she added a fresh perspective on the relationship between hippocampus-dependent learning and the number of newborn neurons in the brain, by showing a direct correlation with BrdU-labeling. (Gould *et al*. 1999). Around the same time, Eriksson was the first one to provide evidence for the presence of adult neurogenesis in human brains, also using BrdU-labeling technique. In his study, he administered BrdU to cancer patients to analyze the division patterns of tumor cells. When the hippocampus and SVZ of postmortem tissues were examined, he clearly observed and presented progenitor

cells and newborn neurons in the DG and SVZ of human brains (Eriksson *et al*. 1998). These studies finally provided a strong counter argument to the central dogma of neuroscience, which rejected neurogenesis in adult mammals for nearly 100 years. All these evidence from various animal models led to a shift in the view on adult neurogenesis and proved that neurons are not fixed after birth and new neurons continue to be produced in adulthood.

Even though the debate continued on the function, mechanism, and effects of adult neurogenesis, the notion of adult-generated neurons was now widely accepted. Even the strong critics of adult neurogenesis concept, such as Pasko Rakic, accepted the presence of precursor cells in DG and SVZ of the adult primate brains (Kornack and Rakic, 1999; Rakic, 2002). With the widespread recognition of the concept, addition of new information on the molecular and cellular aspects of the process accelerated quickly. Especially with the development of new techniques and the availability of immunohistochemical agents to identify cell types and major signaling factors, we came to a detailed understanding of how new neurons are generated in adult brains of both invertebrates and vertebrates, to which regions they contribute, and how they functionally integrate into the existing circuits.

Following the initial observation, several attempts were made to verify the presence of adult generated neurons in the human brain, and to understand the mechanism. First, a study confirmed the expression of a rodent neurogenesis marker doublecortin (DCX) in post-mortem human brains in the age spectrum of 0 to 100 (Knoth *et al*., 2010). Another study caught evidence of newborn neurons in SGZ and their migration to the OB, in early childhood. However, the group was not able to detect adult generated neurons (Sanai *et al*., 2011). A milestone in the research of adult neurogenesis in human brains was the study examining the brains of a population exposed to different levels of the radioactive C^{14} isotope that emerged from nuclear bomb tests. They detected considerable amount of hippocampal neurogenesis by C^{14} dating (Spalding *et al*., 2013). Another group of studies, however, could not detect newborn neurons in aged human brains, while confirming neurogenesis in younger brains (Dennis *et al*., 2016; Sorrells *et al*., 2018). As these various papers indicate, the debate if and for how long over the life span adult neurogenesis exists in human brains still continues.

It is now widely accepted that continuous generation of new neurons, also called constitutive neurogenesis, takes place in two specific regions of the mammalian brain,

mainly ventricular-subventricular zone (v-SVZ), which resides on the lateral ventricles and the subgranular zone (SGZ) of the hippocampal DG (Kempermann *et al.,* 2015). In addition, there is evidence for adult generated neurons in the striatum, amygdala, hypothalamus, and substantia nigra (Parent *et al*., 1995; Betarbet *et al*., 1997; Evans *et al*., 2002; Shapiro *et al*., 2009; Bernier *et al*., 2002; Zhao *et al*., 2003). According to the current model in mammals, there are various morphologically and molecularly distinct types of neural stem cells (NSCs). The biggest NSC population are the B1 cells, an astrocyte-like cell population, found in v-SVZ niche. B1 cells originate from radial glia (RG) cells and they retain some of radial glia properties, such as contact with apical surface at the ventricle, the presence of basal processes that contact blood vessels, and expression of glia factors like glial fibrillary acid protein (GFAP) and brain-lipid binding protein (BLBP). When activated, B1 cells divide asymmetrically for the self-renewal and generation of transient-amplifying C cells, also called intermediate progenitor cells (IPCs). After a series of divisions, type C cells generate A cells, which are known as neuroblasts and express the DCX marker. Type A cells then migrate to the OB to become inhibitory interneurons (Lois *et al*., 1996; Doetsch *et al*., 1997; Anthony *et al*., 2004; Mirzadeh *et al*., 2008). The activation of B1 cells tightly depends on intercellular and niche-derived signals. For instance, GABA release from A cells can inhibit the activation of B1 cells and might function as a feedback signal that signifies that a sufficient number of cells have been generated (Liu *et al*., 2005; Alfonso *et al*., 2012). Growth factors such as mitogens fibroblast growth factor 2 (FGF-2), epidermal growth factor 2 (EGF2), and vascular endothelial growth factor (VEGF), which may be supplied from the blood circulation or the ventricular cerebrospinal fluid, positively regulate NSC activity (Morita *et al*., 2005; Jin *et al*., 2002).

The mechanism of adult hippocampal neurogenesis follows a route similar to v-SVZ. Radial glia-like cells (also called as type 1 cells) of the DG give rise to IPCs upon activation. Type 1 cells express the glial marker GFAP and neuronal progenitor marker Nestin (Seri *et al*., 2001). A subset of type 2 cells differentiates and becomes type 3 cells, which are neuroblasts expressing polysialylated neuronal cell adhesion molecule (PSA-NCAM; Seki *et al*., 2002). With the contribution of GABA-ergic signaling, neuroblasts complete neuronal maturation and integrate into the circuitry in the hippocampus (Ge *et al*., 2006). The second pool of NSCs in the hippocampus is composed of non-radial precursors. These cells do not express GFAP; however, they are known to show sex determining region Y box 2 (sox 2)

expression (Lugert *et al*., 2010). They can divide either to self-renew and to expand the pool of NSCs or to generate neurons or glia. The lineage relationship of non-radial precursors to RG-like cells is still not fully understood (Kronenberg *et al*., 2003; Steiner *et al*., 2006). A crucial point, however, is the role of Sox2 in the regulation of adult hippocampal neurogenesis. NSCs in the hippocampus are now known to be activated by promotion of sonic hedgehog (Shh) and inhibition of Wnt-signaling through Sox2 expression (Favaro *et al*., 2009; Kuwabara *et al*., 2009).

The functional ramifications of constitutive adult neurogenesis in v-SVZ and SGZ are still under investigation. v-SVZ born neurons that migrate to the OB are supposed to have a function in the olfactory response and plasticity of odor discrimination (Magavi, 2005; Gheusi *et al*., 2000). Adult neurogenesis in SGZ is known to increase synaptic plasticity, which in turn reflects onto several functions of the hippocampus (Snyder *et al*., 2001). Behavioral analyses strongly suggest that there is a direct correlation between the number of adult generated neurons in the hippocampus and its function, such as spatial learning, memory organization and consolidation (Kee *et al*., 2007; Jessberger *et al*., 2009; Kitamura *et al*., 2009;); as well as cognitive behaviors, stress response and depression (Lagace *et al*., 2010; Anacker *et al*., 2018; Ngwenya *et al*., 2015; Snyder *et al*., 2011).

Regenerative neurogenesis is the second mode of adult neurogenesis, and it is usually activated by injuries or trauma. It appears to follow a different route from the process of constitutive adult neurogenesis described above. Traumatic brain injury is shown to enhance endogenous cell proliferation in DG of rodents (Sun *et al*., 2005; Gao *et al*., 2013). An increase in the cell proliferation can be observed for up to a year (Chen *et al*., 2003). However, whether these proliferated cells have a neuronal fate is not clear. There have been several studies with contradicting results (Gao *et al*., 2013; Sun *et al*., 2007; Villasana *et al*., 2014). While the use of different injury models in these studies could explain the diversity of newly generated cells, the bases of regenerative neurogenesis in the brain is still largely elusive.

1.2. Adult Neurogenesis in Zebrafish

The capacity for adult neurogenesis displays a gradual decline when we move from lower vertebrates to mammals, as more primitive vertebrates show stronger regenerative

capacities in general. Birds and teleost fish species have been extensively researched to unravel the molecular mechanisms of adult neurogenesis and develop models to better understand human adult neurogenesis system. Tracing experiments showed that approximately 0.003% of brain cells are renewed in adult rodents, while this rate is increased to 0.06% in zebrafish (Hinsch and Zupanc, 2007; Cameron and Mckay, 2001; Herculano-Houzel and Lent, 2005). Thus, the zebrafish is a prominent candidate to study adult neurogenesis due to its high regeneration capacity and the resemblance of certain brain regions to the brain of mammals (Anand and Mondal, 2017).

Zebrafish can regenerate almost any tissue from head to tail. In addition to high regenerative capacity of the heart, fin, and retina; it retains the potential to regenerate neurons of both the brain and spinal cord (Gemberling *et al*., 2013). To date, 16 regions of constitutive neurogenesis have been discovered in the zebrafish brain (Grandel *et al*., 2006). Among those, the dorsal telencephalon (pallium) is considered to be a homologous region of the mammalian v-SVZ. A significant difference of the zebrafish telencephalon adult neurogenesis from mammals is that NSCs of the zebrafish proliferate not only to compensate for the loss of neurons but also to expand the pool of progenitors during adulthood (Than-Trong *et al*., 2020). Radial glial cells (RGCs), non-radial neuronal progenitors, and neuroepithelial progenitor cells are the predominant cell types within the telencephalon. RGCs constitute the main progenitor niche, and they differ from mammalian RGs by the preservation of proliferative capacity into adulthood. RGCs are the major stem cell population during development of mammals but they cease to exist after completion of embryonic development, by transforming into B1 and radial glia-like cells in the adult v-SVZ and DG (Doetsch, 2003). Therefore, the zebrafish RG population is both morphologically more similar to the mammalian hippocampal NSCs, but functionally resembles v-SVZ cells. RGCs express glial factors GFAP and BLBP, as well as progenitor markers *her-4* and Sox2 (März *et al*., 2010). The progeny of RGCs can be traced to the OB, with their migration through the rostral migratory stream. Most of the RGC population is quiescent, while around 5% is active under physiological conditions. Active RGCs can divide symmetrically to self-renew and to enlarge the pool of progenitors. Under injury conditions, however, their mode of action changes towards asymmetric division that generate neurons, as proven by neuronal marker expression in their progeny (Barbosa *et al*., 2015; Rothenaigner *et al*., 2011). Non-radial neuronal progenitors of the telencephalon lack

the expression of glial markers; however, they are positive for Sox2. Non-radial neuronal progenitors show the characteristics of transient-amplifying cells of the mammals, such as their origin of RG, localization among RGs, and neuronal differentiation (März *et al*., 2010; Than-Trong *et al*., 2020). Neuroepithelial progenitor cells, on the other hand, do not display any glial properties or proliferation behavior, and their morphology is distinct from other progenitor niches. Neuroepithelial progenitors are hypothesized to be the embryonic roots of RGs in the telencephalon, and a function in the regeneration of optic tectum was suggested (Dirian *et al*., 2014; Galant *et al*., 2016).

In addition to the telencephalon, the olfactory bulb and the optic tectum are among the most studied neurogenic zones of the zebrafish brain. Similar to the mammalian system, migration of neuroblasts from the telencephalon to the olfactory bulb is observed. PSA-NCAM expressing cells, which are the functional orthologs of mammalian type A cells, can be traced to the olfactory bulb. Origin of these cells are RGCs in the ventral part of the telencephalon, which is a more active region of proliferation. After division and commitment to the neuronal fate, PSA-NCAM^{+} cells migrate to the olfactory bulb through the rostral migratory system, as seen in mammals (Grandel *et al*., 2006; Byrd and Brunjes, 2001). The optic tectum is populated mainly with NE progenitor cells, which are considered to be non-canonical progenitors that divide in a fast-cycling manner. In addition, there is a small population of RGCs in the optic tectum, which is thought to function in the replenishment and maintenance of the stem cell pool (Ito *et al*., 2010).

As in the mammalian brain, there are two distinct modes of neurogenesis in zebrafish: constitutive and repair. Pulse-chase studies using BrdU labeling showed that there is a change in the division patterns and stem cell behavior in the intact and damaged brain (Grandel *et al*., 2006). In the intact brain, adult neural stem cells (aNSCs) of the telencephalon regularly divide with a low rate. aNSCs are usually in a quiescent state. When a small portion of aNSCs is activated to maintain the constitutive neurogenesis rate, they can divide either symmetrically or asymmetrically. If they divide symmetrically, they expand the pool of progenitors. In the case of asymmetric division, on the other hand, they give rise to new neurons, either by direct conversion or via intermediate progenitors, while keeping the number of progenitor cells constant. Under injury conditions, the mode of action changes. A subpopulation of RGCs is activated to divide in a faster rate and produces new neurons. After amplification of IPCs, they differentiate into neurons and the newborn

neurons migrate to the injured area of the brain. There is a significant increase in the proliferation of aNSCs after damage. However, it does not seem to affect the constitutive neurogenesis (Grandel *et al*., 2006; Barbosa *et al*., 2015; Kroehne *et al*., 2011). In other regions of the CNS, such as retina, dedifferentiation of glial cells to serve as progenitors has been observed (Bernardos *et al*., 2007; Thummel *et al*., 2008).

Several methods have been developed to study regeneration of the nervous system in zebrafish. The most commonly used methods are based on injury induction. Creating physical lesions by stab-wound injuries can be easily made in the brain with a needle or surgical blade, and immunohistology methods can be used to investigate the regeneration capacity. (Zupanc, 2008; Schmidt *et al*., 2014). Chemically induced injuries via neurotoxins can also be employed to selectively damage a brain region. Agents such as 1-methyl-4-phenylpyridinium (MPP+), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) are known neurotoxins that can inhibit specific neuron populations in the zebrafish brain (Anichtchik *et al*., 2003; Sallinen *et al*., 2009). As a relatively new approach, genetic manipulations started to be employed to analyze specific neuron types and niches. One of these was the “Brainbow” method, which allows the imaging of individual circuitries via Cre-LoxP recombination strategy (Weissman *et al*., 2011; Pan *et al*., 2011). With a broad spectrum of methods, zebrafish offers a valuable advantage in the research of AN.

Neurogenesis is regulated by a number of factors. As mentioned previously, diverse signaling pathways and messengers are involved, such as Notch and Shh signaling, growth factors, and several transcription factors (refer to 1.1 Adult Neurogenesis). Notch signaling is known to regulate the activity rate and fate of progenitor cells. High Notch activity also drives RG cells into a quiescent state (Park and Appel. 2003; Chapouton *et al*., 2010). On the other hand, canonical Wnt signaling components induce neurogenesis in adult brains (Lie *et al*., 2005). Fibroblast growth factors have also been shown to be critical in the activation of proliferation for the progenitor cells of the zebrafish brain (Kaslin *et al*., 2009; Ganz *et al*., 2010). Corticosterone negatively modulates neurogenesis by inhibiting the cell proliferation and differentiation of progenitors in the hippocampus (Wong and Herbert. 2006; Murray *et al*., 2008). In addition, a link between inflammation upon damage and the regenerative neurogenesis activity was found. In this context, IL4/STAT6 pathway was shown to activate proliferative activity (Bhattarai *et al*., 2016). Loss of Cxcr5, the receptor

of the chemokine Cxcl13, promotes neurogenesis in the brains of aged mice (Fritze *et al*., 2020). Cxcr5 is also expressed in the radial glia cells of zebrafish brain and it was found to modulate the proliferative activity and neuronal fate of the generated cells (Kizil *et al*., 2012). Upregulation of cxcr5 activates neuroinflammation and suppresses neurogenesis in the adult hippocampus of mouse (Shen *et al*., 2020).

1.3. The Olfactory System

Olfactory system, which underlies smell sensation, is the one of the most primitive sensory systems and, yet, has made one of the biggest contributions to the evolution of life. Although vision has largely overruled chemosensation in higher vertebrates, it remains to be an important sensory modality that warns the animals against danger, signals the presence of toxic chemicals, and indicates the presence of predators and mating partners. Smell sensation not only protects the animal, but also takes part in learning, memory formation, and navigation (Wiedenmayer *et al*., 2000; Olofsson *et al*. 2020). Odors determine the choices, reactions, and even the mood of an organism, from basic preferences of food to the more important survival and reproduction principles, like mate choice and progeny protection behaviors (Daghfous *et al*., 2018; Kontaris *et al*., 2020). The olfactory system was one of the first parts of the nervous system that was studied in detail (Cajal, 1892). It has always been considered as a bridge between the peripheral and central nervous systems, since the olfactory epithelium (OE) is located in the periphery, and the synaptic targets, olfactory bulb (OB) and olfactory cortex, are found in the brain. Additional peripheral chemosensory structure complement the main olfactory system of many vertebrates, such as the vomeronasal organ (VNO) or the Grüneberg ganglion (D'Aniello *et al*., 2017; Fleischer and Breer, 2010). However, some vertebrates do not have these structures, instead, the function of the VNO is carried out by the OE (Jones and Rog, 1998).

OE is the most peripheral unit of the olfactory processing that samples the environment for chemical cues. In mammals, it is found in the nasal cleft, which is localized in the superior medial area of the nasal region. Mammalian OE is composed of olfactory sensory neurons (OSNs), basal cells, and supporting cells (Kimmelman, 1993; Graziadei, 1973). OSNs are bipolar neurons which extend dendrites to the apical surface where they contact the air and sense chemicals. Mature OSNs are localized more apical to the immature OSNs in the OE. They send axons through the basal layer to carry information to the OB (Mombaert, 2001).

Basal cells are comprised of two separate stem/progenitor cell populations, horizontal basal cells (HBCs) and globose basal cells (GBCs). They localize to the most basal strata and take part in the renewal of OSNs and the continuous regeneration of the OE (Graziadei, 1973; Suzuki *et al*., 2013). Supporting cell populations of the OE are the sustentacular (Sus) cells and microvillar cells. Sus cells have microvilli at the apical surface and their cellular structure extend to the basal OE. They have glial properties and support tissue integrity with possible roles in detoxification, phagocytosis, and tissue maintenance (Chen *et al*., 1992; Suzuki *et al*., 1995; Graziadei and Graziadei, 1979). Microvillar cells, on the other hand, are shown to be necessary for the tissue integrity and maintenance of OSN function, along with their role in stimulating Sus cells (Miller *et al*., 1995; Ogura *et al*., 2011).

OSNs are ciliated bipolar neurons that express olfactory receptor (OR) genes that enable response to a diverse spectrum of odors. OR proteins are G-protein coupled receptors found in the outer ciliary membrane of OSNs, and the total number of unique ORs varies largely across species (Buck and Axel, 1991). Including pseudogenes, the mouse genome contains over 1400 OR genes, whereas around 800 genes, 50% of which are pseudogenes can be found in humans. Thus, anywhere between 1000 and 350 genes are able to be expressed and to generate functional ORs (Niimura *et al*., 2005). For such a high number of genes, the receptor distribution in the OE and recognition of different odors by specific OSNs were initially puzzling concepts. The one neuron-one receptor rule is now an established concept. Each OSN is restricted to express one specific OR gene (Chess *et al*., 1994; Serizawa *et al*., 2004). The axons of OSNs that express the same OR expression converge together and target the same glomerulus in the OB, where they form synapses with mitral/tufted cells. Thus, each glomerulus represents a specific type of OR (Mombaerts *et al*., 1996). Mitral and tufted cells then carry the information to the olfactory cortex. The coding of the odor information was shown to depend on different combinations of activated ORs. Multiple different chemicals are detected by multiple ORs in a combinatorial fashion to encode certain odors, while any single OR respond to a number of different chemicals. With the high number of OR genes, the number of possible combinations is near limitless (Malnic *et al*., 1999).

The second peripheral organ of the olfactory system is VNO in some species. It is considered as an accessory chemosensory structure that is found at the base of the nasal cavity. The cellular structure of VNO shows great similarities with the OE and consists of

receptor neurons, supporting cells and basal stem cells. However, VNO receptor neurons differ from OSN receptors and react to different chemicals, mainly pheromones (Keverne, 1999; Dulac, 1997). Another critical difference is the processing region of VNO information in the brain. VNO receptor neurons relay information to the accessory olfactory bulb (AOB), which is found in the dorsal part of the OB (Halpern, 2007). Although the presence of VNO and AOB structures in humans is an ongoing debate (Meisami *et al*., 1998; D'Aniello *et al*., 2017), it has been clearly observed in many species such as amphibians, reptiles, and non-primate mammals (Døving and Trotier, 1998).

1.4. Neurogenesis in the Vertebrate OE

The high regeneration capacity of both OE and OB makes olfactory system an exceptional model among sensory systems and for the nervous system in general. The central unit of this system, the OB, is one of the main regions displaying constitutive adult neurogenesis via SVZ-derived new neurons as explained above. Constitutive adult neurogenesis of the OB contributes to the plasticity of local circuits, which is necessary for the adaptation to various environments and sensory stimuli (Lepousez *et al*., 2013). The peripheral units of the olfactory system, however, particularly face the danger of toxic and structural damage since they are directly exposed to the outer environment. While the VNO is protected within a cartilaginous capsule (Keverne, 1999), the OE is always at the risk of damage due to the direct contact to potentially harmful environmental substances. The life-long functionality of the OE is preserved because of the high capacity for regeneration. Different stem cell populations in the basal epithelium enable the continuous maintenance of the structure, as well as its recovery after acute injury (Schwob, 2002). Surprisingly, despite the remarkable regeneration capacity, the OE does not show high rates of tumor formation (Bailey and Barton, 1975). The complex regulation of stem cell behavior, injury response and neuronal turnover ensures the balance of this system.

Maintenance of the OE under intact or injured conditions is achieved by several mechanisms governed by the basal stem/progenitor cells. Similar to the AN neurogenesis of the brain, two distinct modes of OE neurogenesis can be observed. Our current understanding suggests that repair neurogenesis differs from maintenance neurogenesis with the mode of action. Accordingly, behavior of the basal cells with different potency levels changes in the two types of neurogenesis (Schwob *et al*., 2017). Analyses in both intact and

damaged OEs were carried out to determine the molecular regulation of adult neurogenesis in the OE. The most commonly used methods of adult neurogenesis research in the intact OE have been lineage tracing with retroviral vectors, selection of cell subtypes by FACS followed with transplantation, and also olfactory bulb ablation (Goldstein *et al*., 1998; Chen *et al*., 2004; Carr and Farbman, 1992). In order to examine the behavior of different cell types of the mammalian OE in response to injury, bulbectomy and chemical lesion are often utilized. Exposure to methyl bromide (MeBr), methimazole, dichlobenil, zinc sulfate, or Triton X-100 (TrX) can be employed to generate lesions in the OE (Schwob *et al*., 1995; Bergman *et al*., 2002; Genter *et al*., 1995; Cancalon, 1982, 1983).

HBCs comprise the reserve stem cell pool of the OE (Graziadei and Graziadei, 1979). They are located at the most basal region of the OE, forming the first layer above basal lamina. HBCs have a characteristic elongated morphology and can be identified by the expression of keratins 5 and 14, as well as the stem cell marker Sox2 (Holbrook *et al*., 1995; Guo *et al*., 2010). This cell population is quiescent in the intact OE, which is under the control of the transcription factor p63 (Trp63) that belongs to the p53 gene family. p63 is known to be a critical factor for the maintenance of stem cell pool in epithelial tissues (Yang *et al*., 1999). HBCs of the OE are capable of producing both glial and neuronal cell types in vitro (Carter *et al*., 2004).

HBC formation in the OE is dependent on p63 expression during late embryonic development. The absence of p63, however, does not disrupt the structural integrity of the OE, even though it results in the lack of HBCs (Packard *et al*., 2011). p63 expression not only promotes the formation of HBCs, but also maintains their mitotically quiescent state. Upon injury, p63 expression is downregulated and is followed by the activation of HBCs. It functions as a molecular switch that regulates the balance of dormancy, self-renewal, and differentiation (Fletcher *et al*., 2011).

Lineage tracing experiments indicated that HBCs do not directly give rise to neurons. Instead, GBCs were found to be the neuronal progenitor of the OE (Caggiano *et al*., 1994). However, these studies did not rule out the possible lineage relationships between HBCs and GBCs. Lesions created with the toxic gas Methyl bromide (MeBr) leading to the near-complete loss of both neurons and neuronal precursors, initiates the activation of an HBC population, which in turn gives rise to progenitor GBCs (Leung *et al*., 2007). In other injury

models such as bulbectomy or partial neural depletion, however, HBCs keep the dormant state while GBCs are activated to replenish the neuronal loss (Schwob *et al*., 2017). Lineage analysis of HBCs at different developmental stages and under various injury conditions led to a hypothesis which states that HBCs are capable of both self-renewal and production of non-neuronal cells. According to this model, when activated, HBCs mainly divide to create progenitor GBCs, and, thereby indirectly replenish OSNs. In addition, they divide in a lower frequency to differentiate into Sus cells or Bowman's duct/gland cells (Iwai *et al*., 2008).

In the search of an explanation for different modes of action following various injury conditions, the effect of specific Sus cell depletion was examined. Targeted elimination of Sus cells resulted in the activation of HBCs, possibly through a change in Notch1 signaling in HBCs. Sus cells are known to express the Notch ligand Jagged 1, which positively modulates p63 in HBCs. With the loss of Notch1 signaling, HBCs are activated to repair the damage in the tissue (Herrick *et al*., 2017). Taken together, these results suggest that HBCs comprise the reservoir stem cell pool of the OE and are not activated until an extensive injury, which destroys glial and progenitor populations, occurs.

The second basal cell population of the OE is the GBCs. They have a spherical morphology and are located apically to HBCs. GBCs display a heterogeneity in molecular signatures and behavior. The GBC population can be dissected into cells that show stem cell properties, but also multiple progenitor capacity, transit-amplification, and immediate neuronal precursor characteristics. The GBC population showing stem cell (GBC_{STEM}) characteristics is distinguished by its capacity for self-renewal. They retain the thymidine analog label, which is an indication of mitotic quiescence and considered as a stem cell property (Jang *et al*., 2014). GBC_{STEM} population is distinguished by the expression of the transcription factors Sox 2 and Pax 6 (Guo *et al*., 2010). Multipotent GBCs (GBC_{MMP}) are progenitor cells that can give rise to neurons, Sus and Bowman's duct/gland cells. GBC_{MMP} also expresses Sox2 and Pax6 like GBC_{STEM}, as well as the stem cell marker Lgr5 (Chen *et al*., 2014). Lgr5 marks active stem cells in other tissues such as intestine, cochlea, and tongue (Barker *et al*., 2007; Shi *et al*., 2012; Yee *et al*., 2013). The $Lgr5^+$ GBC population is multipotent and activated by Wnt signaling, contributing to epithelial regeneration after injury (Chen *et al*., 2014). Upon activation, GBC_{MMP} can give rise to glial progenitors via upregulation of Hes1 or a neuronal progenitor by the upregulation of the proneuronal gene Ascl1. GBC_{MMP} differentiates into transit-amplifying GBC (GBC_{TA}), which are committed

progenitors. GBC_{TA} that gives rise to Sus cells maintain the expression of Sox2, Pax6, and Hes1, and continue their maturation (Manglapus *et al*., 2004). GBC_{TA} that produces neurons, however, lose the expression of stem cell markers Sox 2 and Pax6, along with the neuronal progenitor marker Ascl1 (Manglapus *et al*., 2004; Krolewski *et al*., 2013). Loss of *ascl1* is shown to inhibit OSN generation from GBC_{TA}, while not affecting the production of Sus cells (Krolewski *et al*., 2012). With the upregulation of early neuronal markers NeuroD1 and Neurog1, this cell type becomes an immediate neuronal precursor (GBC_{INP}) (Cau *et al*., 2002; Packard *et al*., 2011). After GBC_{INP} gains a neuronal fate, generation of mature neurons is characterized by the OMP expression.

It is well established that GBCs are the constitutively active progenitor cells of the OE, contributing to maintenance neurogenesis. HBCs, on the other hand, are dormant under physiological conditions and activated only upon extensive injury. The dual model of OE neurogenesis displays complex properties, most of which are yet to be investigated. However, as our current knowledge suggests, tight regulation of signaling plays a crucial role in the behavior of progenitors and the program of events.

1.5. Zebrafish OE and OSN Neurogenesis

Because of its lifelong perseverance, the vertebrate OE presents an exceptional model to study adult neurogenesis. Conservation of the OE structure, cell types and most molecular interactions also allow the use of less complex animals as models. The zebrafish is a prominent AN model among vertebrates, due to the high regeneration capacity, short life cycle, easy maintenance, and considerable homology to humans (Sakai *et al*., 2018; Howe *et al*., 2013).

The peripheral olfactory system of the zebrafish is composed of symmetrically arranged two OEs, which are embedded in the nasal cavities that are located anterodorsally to the eyes. Similar to the mammalian counterpart, the zebrafish OE is highly folded and consists of stacked epithelial tissue that provides a larger surface area (Calvo-Ochoa and Byrd-Jacobs, 2019). The OE is composed of stacks that are called lamellae, which emerge from the median raphe to form an oval structure that resembles a flower. Each lamella is further organized in a particular pattern where different cell types occupy specific regions. A horizontal cross-section of the OE provides a pair of flattened oval sheets, which is called

an epithelial fold. A common pattern of cell type-specific regions can be observed in every epithelial fold. These regions include a U-shaped curve in the medial part, a long flat area in the center of the fold, and another, wider U-shaped closure at the lateral part (Byrd and Brunjes, 1995; Hansen and Zeiske, 1998). The medial curve where two lamellae merge were termed the interlamellar curve (ILC). The central, flat epithelial tissue comprises the core sensory region that is mainly populated by OSNs. The wider lateral curve is composed of non-sensory cells, hence named as the non-sensory region (NS). In addition, there is an imaginary border where OSNs cease to exist and non-sensory cell population occupy the rest of the epithelium, which is called the sensory-non-sensory border (SNS) (Figure 1.1; Bayramli *et al*., 2017).

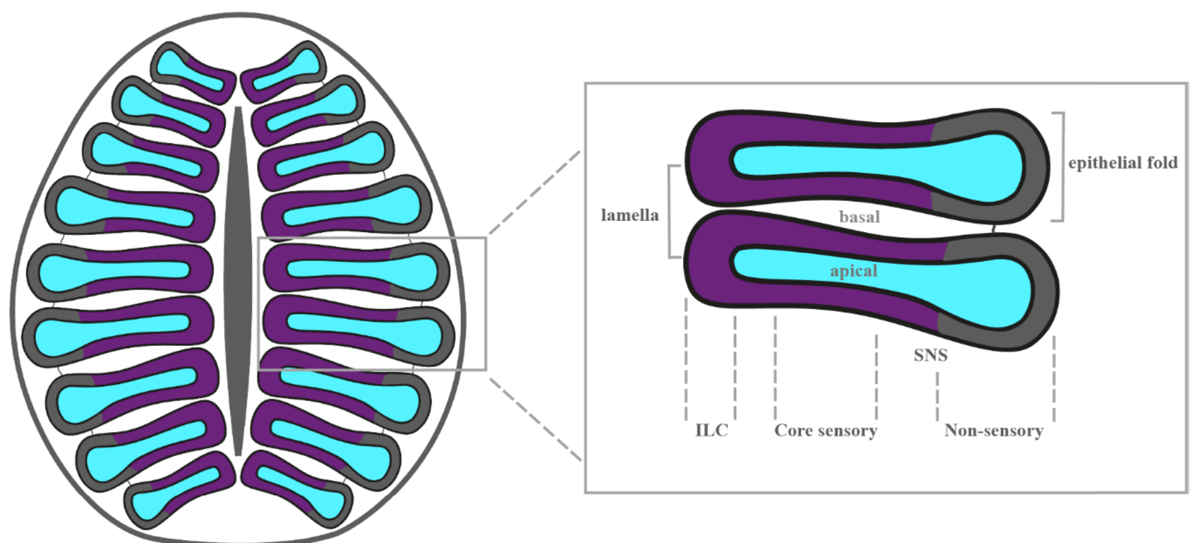

Figure 1.1. Structure of the zebrafish OE.

Each epithelial fold has a layered structure, but the organization of layers is different from the mammalian OE. The gap between epithelial sheets in the middle of folds is open for the flow of water, and corresponds to the apical side. The other end of the sheet is connected to the basal lamina in the center of the lamella (Hansen and Zeiske, 1998). Basal layers are occupied by HBCs, while GBCs are only present in the ILC and SNS. OSNs populate most of the tissue up to the apical layers. From medial to the lateral side, a lamella is populated with different cell populations. Whereas OSNs cover almost 2/3 of the radial axis starting from ILC, supporting populations like goblet cells occupy the rest of the lateral part (Figure 1.2; Byrd and Brunjes. 1995; Iqbal and Byrd-Jacobs, 2010). Zebrafish OE has five types of OSNs, that can be classified by their morphology and receptor types.

Microvillous and Ciliated OSNs are the dominant OSN types in the zebrafish OE. Both types of OSNs send dendrites from basally located somata to the apical surface. They differ in morphology and expression profiles. As the names indicate, the former has microvilli, and the latter has cilia structures at the apical surface. While microvillous neurons are identified by expression of TRPC2 and V2R-like receptors, ciliated OSNs express ORs, trace amine associated receptors (TAARs), and olfactory marker protein (OMP) (Sato *et al*., 2005; Hussain *et al*., 2009; Niimura and Nei, 2005). The remaining groups of OSNs, crypt, kappe, and pear neurons exclusively occupy apical locations. Crypt neurons have sunken cilia and are located apically with their globular soma (Hansen and Zeiske, 1998; Biechl *et al*., 2016). They express *ora4* from the family of V1R genes and have been implicated in kin recognition (Oka *et al*., 2012.). Kappe neurons are also located apically and have microvillar extensions. They differ from crypt neurons with their projection to a distinct glomerulus in the OB and lack of tubulin expression, which is necessary to form cilia (Ahuja *et al*., 2014). Pear neurons are named due to their peculiar pear shape. They were recently categorized with their A2c receptors which responds to adenosine (Wakisaka *et al*., 2017).

Basal cells of the zebrafish OE resemble their counterparts in rodents. HBCs are flat cells located superior to the basal lamina and span the most basal layer of each lamella. They can be identified by expression of Sox2, keratin 5, and p63. GBCs, on the other hand, are recognized by the same globular morphology as seen in mammals, and distinguished by the expression of the markers Sox2, Ascl1, and NeuroD1 at different stage of their lineage. While their location along the apical-basal axis is the same with rodent OE, their specific pattern at the mediolateral axis draws a major difference. GBC_{TA} reside specifically in the ILC and SNS, as the *ascl1* expression indicates (Bayramli *et al*., 2017).

Sustentacular cells of the zebrafish OE have not been investigated in detail. However, they are known to play the supporting role in tissue maintenance and neurogenesis. They can be identified with a columnar morphology, basal soma, and expression of markers Sox2 and cytokeratin 2 (Demirler *et al*., 2020).

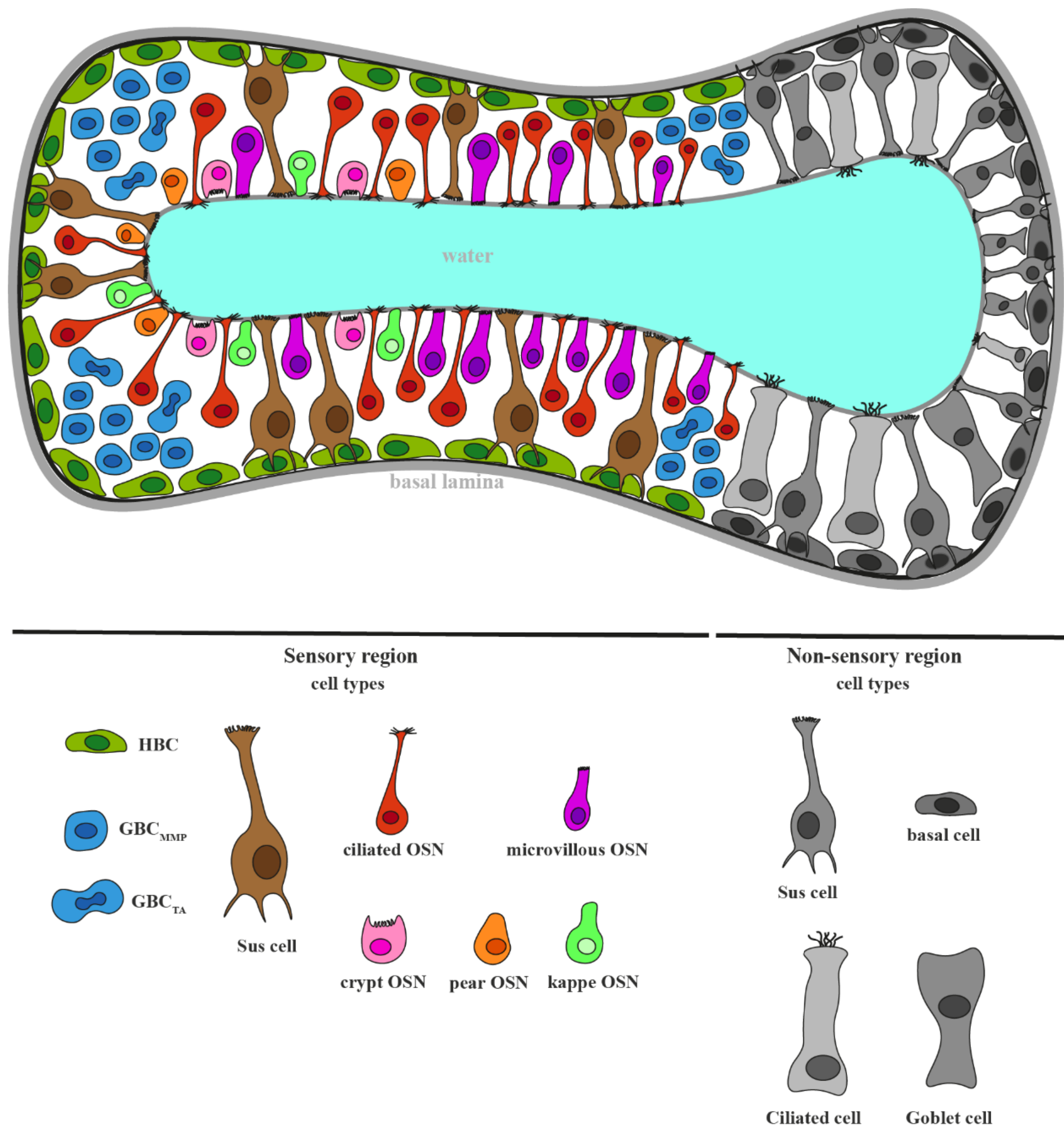

Figure 1.2. Cell types of the zebrafish OE.

Adult neurogenesis of the zebrafish OE can follow one of the two routes, quite similar to the mechanism in rodent OE. Constitutive turnover of neurons is achieved by the activity of GBCs. BrdU pulse-chase experiments showed that average lifespan of adult zebrafish OSNs is only 28.5 days, creating a need for constitutive neurogenesis to maintain the OSN population. GBCs are the main progenitor population responsible for maintenance neurogenesis. At any given moment, the division pattern in the intact OE shows a bimodal distribution, where dividing GBCs are localized at the ILC and SNS. Continuous division of

GBC_{MMP} at a rate that supplies new OSNs for the rather fast loss of mature neurons, is followed by migration of newborn OSNs radially along the lamella (Bayramli *et al*., 2017).

Apart from constant and fast neuronal turnover due to maintenance neurogenesis, zebrafish OE also displays a remarkable capacity for the repair in response to acute injury conditions. After severe injury with TrX, almost the entire OSN population of the OE is ablated. Surprisingly, the OE can be repopulated almost completely with newborn OSNs in 5-7 days (Iqbal and Byrd-Jacobs, 2010; Kocagöz *et al*., 2022). Repeated pulses of the detergent disrupt the circuitry of olfactory system with extended effects observed in glomeruli of the OB (White *et al*., 2015). Yet, axonal connections from new OSNs are form within approximately 10 days (Paskin and Byrd-Jacobs, 2012). Eventually, the whole structure and olfactory function is regained in a very short time, compared to the approximately 2 months regeneration period in rodents (Matulionis, 1975; Schwob *et al*., 1995). In contrast to maintenance neurogenesis, HBCs are mainly responsible for the tissue repair. While the rodent counterpart comprises a quiescent stem cell population, HBCs of the zebrafish OE also proliferate under steady-state conditions. However, the rate of mitotic division is low, and regionally distinct to the ILC- and SNS-specific HBC populations (Kocagöz *et al*., 2022). Upon injury, on the other hand, the main mitotically active population is the HBCs. A uniform pattern of $BrdU^{+}$ cells at the most basal part can be observed as soon as 24 hours after the chemical lesion with TrX. This cell population is identified with the marker keratin 5, which is an indicative of HBCs (Demirler *et al*., 2020). At 48 hours post lesion (hpl), higher numbers of ascl1a expressing GBC_{TA} are observed mainly in the ILC and SNS, along with the small number of cells in the core sensory region. Combined with the observation of newly generated neurons five days after chemical lesion, this mode of action suggests a neurogenic route (Kocagöz *et al*., 2022). Together, these data suggest that HBCs are the predominant cell population regulating repair neurogenesis through GBCs, resulting in the generation of new OSNs.

1.6. Molecular Signals of the OSN Neurogenesis

OSN neurogenesis is a conserved phenomenon that contributes to the lifelong capacity of olfaction. Since it is also conserved among vertebrates, unraveling the mechanisms governing this exceptional capacity could pave the way for the adult neurogenesis research and the study of neurodegenerative disease mechanisms. OSN neurogenesis is a highly

regulated process. Critical signaling pathways also known for developmental functions such as Wnt (Fletcher *et al*., 2017) and TGFβ signaling (Wu *et al*., 2003), growth factors and various molecules such as nitric oxide (Sülz *et al*., 2009), retinoic acid (Rawson and LaMantia, 2006), and dopamine (Féron *et al*., 1999) were suggested to play roles in the regulation of neurogenic activity in the OE.

Notch signaling was shown to contribute to OSN neurogenesis regulation via control over p63 expression in the rodent OE. Both the dormancy of HBCs and the route of neuronal differentiation are regulated by Notch signaling (Herrick *et al*., 2017). Downregulation of Notch signaling pathway was observed to result in the downregulation of p63, which breaks dormancy in HBCs of the OE in contrast to the p63-inhibitory role of Notch signaling in other tissues (Herrick *et al*., 2017; Laurikkala *et al*., 2006; Nguyen *et al*., 2006; Saravanamuthu *et al*., 2012). Expression of the Notch ligand Jagged1 in Sus cells, and activation of HBCs upon selective Sus cell damage together support the hypothesis that Notch signaling is involved in the regulation of progenitor cell behavior in the injured OE (Herrick *et al*., 2017).

Wnt/β-catenin signaling was shown to increase progenitor proliferation and neuronal differentiation in the SVZ of adult mice, which results in the increase of newborn neurons in the OB (Adachi *et al*., 2007). Wnt signaling is also involved in HBC differentiation and neuronal fate promotion in the mouse OE (Fletcher *et al*., 2017). Similarly, activation of Wnt/β-catenin signaling is necessary for HBC induction in the lesioned zebrafish OE. Inhibition of the pathway reduces the number of proliferating cells significantly in both intact and lesioned OE. Yet, it does not completely abolish the restoration upon injury, which indicates the presence of other components in the regulation of repair neurogenesis (Clevers *et al*., 2014; Kocagöz *et al*., 2022).

Damage-associated molecular patterns (DAMPs) are molecules that are known with major roles in the cell metabolism under physiological conditions and acquire additional roles in response to damage. They can act as signaling molecules for the initiation of tissue repair (Vénéreau *et al*., 2015). Heat shock proteins (HSPs), calreticulin, and adenosine triphosphate (ATP) are among DAMPs that were shown to have effects on cell proliferation and migration (Krysko *et al*., 2012; Bianchi, 2007; Idzko *et al*., 2014). In addition, ATP was shown to promote neurogenic proliferation in the v-SVZ and hippocampus in adult brains

(Suyama *et al.*, 2012; Cao *et al.*, 2013). Recently, it was also shown to regulate GBC behavior in the zebrafish OE. Exogeneous ATP stimulation activates GBC_{TA} population while exhibiting no noticeable effect on HBCs. Therefore, ATP is hypothesized to be released from dying OSNs under physiological conditions and direct maintenance neurogenesis in the zebrafish OE (Demirler *et al.*, 2020).

Various other molecules, growth and neurotrophic factors have been shown to play roles in the regulation of AN in the OE. Nitric oxide (NO), a product of nitric oxide synthase (NOS), was suggested to have a role in AN. Normally, NO acts as a diffusible transmitter in the nervous system (Garthwaite, 1991). High expression of NOS in developing OE and induction of NOS after bulbectomy suggests a considerable role for NO in the regulation of OSN generation (Jane *et al.*, 1994). Additionally, inhibition of NOS decreases proliferative activity of progenitors while promoting neuronal differentiation in the OE (Sülz *et al.*, 2009). Cilia neurotrophic factor (CNTF) is hypothesized to be involved in basal cell behavior and neuronal fate regulation (Buckland and Cunningham, 1998). Neurotrophin-3 signaling was shown to suppress progenitor cell proliferation and neuronal differentiation in the mouse OE (Simpson *et al.*, 2003).

Transforming Growth Factor-β (TGFβ) superfamily proteins are involved in the embryonic neuronal differentiation and also known to play an inhibitory role in the AN (Wang *et al.*, 2014). Growth and differentiation factor 11 (GDF11/BMP11) from TGFβ signaling was shown to suppress the progenitor activity and negatively regulate OSN neurogenesis in the OE (Wu *et al.*, 2003; Gokoffski *et al.*, 2011). Another group of growth factors, on the other hand, have been shown to promote OSN neurogenesis. Epidermal growth factor (EGF) activates division of basal cells by acting as a mitogen (Mahanthappa and Schwarting, 1993). Fibroblast growth factor 2 (FGF2) and insulin-like growth factor 1 (IGF1) have been shown to activate progenitor cells and increase the total number of OSNs in the mouse OE (Fukuda *et al.*, 2018). Additionally, nerve growth factor (NGF) is among OSN neurogenesis regulator candidates, due to its receptor expression by the basal cells of the OE and upregulation of this expression upon OE lesion (Trojanowski *et al.*, 1991; Gong *et al.*, 1994).

1.7. HB-EGF / EGFR Signaling

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is a transmembrane protein that belongs to the EGF family of proteins, initially identified in a macrophage-like human culture cell line (Higashiyama *et al*. 1991). It consists of several domains including a conserved EGF-like domain, a cytoplasmic part, and a transmembrane domain. HB-EGF is synthesized as a transmembrane protein, also called as proHB-EGF, which can function in juxtacrine signaling (Higashiyama *et al*., 1992). It also acts as a receptor for diphtheria toxin (DT) (Iwamoto *et al*., 1994). proHB-EGF is cleaved upon stimulation, by a process called "ectodomain shedding", which is facilitated by matrix metalloproteinases (MMPs), predominantly of the ADAM (a disintegrin metalloprotease) family metalloproteases. MMPs 3 and 7, as well as ADAMs 9, 10 and 12 were shown to cleave proHB-EGF (Suzuki *et al*., 1997; Izumi, Y. 1998; Asakura *et al*., 2002; Yan *et al*., 2002; Yu *et al*., 2002). Mitogen activated protein kinase (MAPK) and protein kinase C (PKC) are known to contribute the activation of pro HB-EGF through regulation of metalloproteases (Izumi, 1998; Gechtman *et al*., 1999). The soluble ectodomain part of the proHB-EGF, which is cleaved, then functions in autocrine or paracrine signaling by binding to the EGF receptor (EGFR, also known as ErbB1), and ErbB4, which belong to the family of receptor tyrosine kinase (Elenius, 1997; Prenzel *et al*., 1999; Paria *et al*., 1999). Upon binding of HB-EGF, tyrosine phosphorylation of EGFR results in the homodimerization of subunits. Following EGFR activation, critical downstream signaling pathways including MAPK, PKC, JAK/STAT and PI3K/AKT are triggered (Figure 1.3; Yarden and Sliwkowski, 2001; Wee and Wang, 2017). In addition to HB-EGF, EGFR responds to a variety of ligands, including EGF, TGFα, amphiregulin, betacellulin, epiregulin, and epigen (Harris *et al*., 2003). EGFR family and its ligands are integral to the embryonic development and also known to promote cell proliferation, growth, and motility while inhibiting apoptosis (Wee and Wang, 2017).

HB-EGF is a critical component of development, suggested by the embryonic lethality of HB-EGF null mice. Partial loss of HB-EGF function results in the enlargement of the cardiac ventricles and disruption of heart function (Iwamoto *et al*., 2003). A significant contribution of HB-EGF is reported in wound healing. Expression of *hbegf* is upregulated in keratinocytes of the wounded epidermis and activation of HB-EGF stimulate migration (Mathay *et al*., 2008; Stoll *et al*., 2012). Inhibition of HB-EGF, on the other hand, suppresses

keratinocyte migration, without altering the proliferation response (Stoll *et al.*, 2012; Shirakata *et al.*, 2005). Additionally, wound closure in corneal epithelium was shown to be significantly reduced in keratinocyte specific HB-EGF null mutants, whereas the effect was rescued by administration of HB-EGF (Yoshioka *et al.*, 2010).

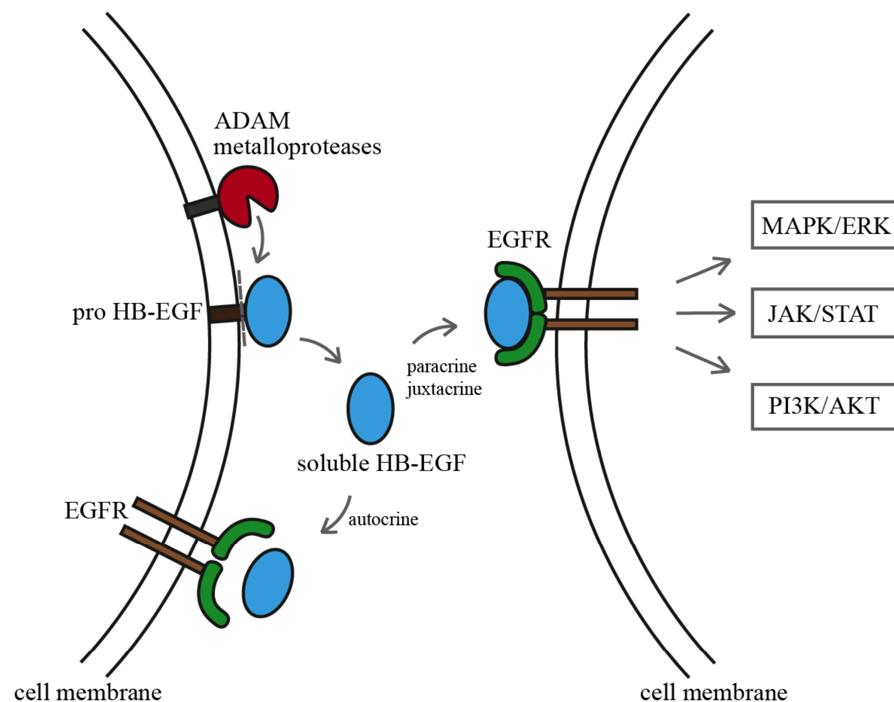

Figure 1.3. HB-EGF / EGFR signaling.

HB-EGF mRNA expression was shown in both glial and neuronal populations of the rat brain, located in various regions including the amygdala, hippocampus, and cortex (Mishima *et al.*, 1996). Expression of HB-EGF is upregulated in response to ischemic brain injury, suggesting a positive regulatory role in repair. BrdU^{+} cell number decreases in the SVZ of HB-EGF-knockout mice after ischemic injury (Oyagi *et al.*, 2011). In another study, exogeneous administration of HB-EGF increased the activity of neuronal progenitors and reduced the infarcts following ischemic injury in rats (Jin *et al.*, 2004.) Intranasal HB-EGF administration was shown to promote neurogenesis, indicated by an increase of BrdU^{+}/DCX^{+} cells in the SVZ of the mouse brain (Jin *et al.*, 2003). Taken together, these data suggest a neurotrophic and neurogenesis-promoting role for HB-EGF in the brain.

EGFR and ErbB4, the receptors of HB-EGF, are expressed in p63^{+} basal cells of the OE, suggesting a role in HBC behavior (Holbrook *et al.*, 1995; Krishna *et al.*, 1996; Duan

et al., 2016). Inhibitors of MMPs and EGRF have been shown to reduce HBC proliferation in olfactory organoids as well as the lesioned mouse OE (Chen *et al*., 2020). Additionally, HB-EGF was shown to activate Müller Glia (MG) dedifferentiation through Wnt/β-catenin signaling in the intact zebrafish retina, which eventually increases number of newborn neurons. Inhibition of HB-EGF ectodomain shedding or EGFR activation diminishes the induction of repair response and proliferation of precursors (Wan *et al*., 2012).

Previous studies from our lab showed that exogeneous stimulation of the OE with HB-EGF significantly increases $BrdU^+$ cell number in the intact OE. Conversely, inhibition of MMPs results in a reduction of proliferation in the lesioned OE (Kocagöz, 2021). Moreover, inhibition of EGFR decreases the number of newly generated neurons after OE injury, without showing a considerable effect on the proliferation or maintenance neurogenesis (Alkiraz, 2019).

1.7. The Role of Cytokines in Tissue Regeneration and Neurogenesis

Tissue repair after traumatic injuries may also involve the contribution of immune system, especially by means of inflammation, release of cytokines and chemokines, and recruitment of immune cells. Macrophage and neutrophil activity, as well as cytokine release are required for the clearance of cellular debris, host defense, wound healing, and repair. Precise regulation of immune activity was shown to be critical for the balance between pro-inflammatory responses and the course of regeneration in various tissues and systems such as heart, skeletal muscle, skin, and CNS (Aurora and Olson, 2014). In addition to repair, immune activation is also involved in neurogenesis in the brain. With their contribution to cytokine signaling, microglia and astrocytes have emerging roles in the regulation of stem cell activity and determination of the neuronal fate (Carpentier and Palmer, 2009).

Several studies suggested profound roles for the immune system in both maintenance and repair neurogenesis. Selective depletion of macrophages in the mouse OE causes reduction in the number of proliferating basal cells and mature OSNs in the uninjured or bulbectomized OE (Borders *et al*., 2007). The acute injury response of the OE is accompanied with inflammation through recruited immune cells. While the short-term inflammation stimulates HBCs for repair, long-term exposure to cytokines and chemokines upregulates stemness and dormancy genes in HBCs. Specifically, the upregulation of nuclear

factor kB (NFkB) creates a molecular switch to immune defense in the basal cell population. Induction of inflammation also causes an upregulation in the expression of cytokines and chemokines from HBCs, which suggests a position for HBCs at the crossroad of inflammation and regeneration (Chen *et al*., 2019).

Cytokines and chemokines and their signaling components have been shown to regulate progenitor cell activity in the OE. Leukemia inhibitory factor (LIF) expression is upregulated in the lesioned OE, especially localizing in the recruited macrophages. Released LIF subsequently activates GBCs for the repair process (Nan *et al*., 2001; Getchell *et al*., 2002). While LIF knockout mice displays deficiency in cell proliferation, exogeneous administration of LIF increases newborn cells in the OE (Bauer *et al*., 2003). Injury-derived inflammation and pro-inflammatory cytokines can also suppress progenitor cell differentiation to neurons and mobility of adult generated neurons, directing the precursors for glial differentiation (Picard-Riera *et al*., 2002; Taga and Fukuda, 2005). The pro-inflammatory cytokine IL1β was found to decrease hippocampal proliferation, whereas inhibition of IL1β eliminated the stress-dependent blockage of neurogenesis (Koo and Duman, 2008). Stimulation with another pro-inflammatory cytokine, TNFα, increases the proliferation and neuronal differentiation of mouse SVZ progenitor cells *in vitro* (Bernardino *et al*., 2008). Together with IL-1β, TNFα stimulates the upregulation of *hbegf* in vascular endothelial cells (Yoshizumi et al., 1992).

Expression of IL-6 and its receptor was shown to be upregulated in the mouse OE after olfactory bulbectomy (Nan *et al*., 2001). Over production of IL-6 by astrocytes was shown to reduce hippocampal neurogenesis in the adult mice (Vallières *et al*., 2002). Chronic inflammation, microglia activation, and high IL-6 levels are associated with reduced hippocampal neurogenesis. The blockage of inflammation and microglia activity, on the other hand, restores the decrease in neurogenesis in adult rats (Monje *et al*., 2003). Surprisingly, knock-out of IL-6 also diminishes progenitor survival and proliferation in the hippocampus and SVZ of the mice brain (Bowen *et al*., 2011). Moreover, IL6 family cytokines, which all target the common gp130-coupled receptor system, and Leptin were found to act in MG dedifferentiation in zebrafish retina upon injury. Exogeneous stimulation with IL11, CNTF, and Leptin were shown to activate proliferation and MG dedifferentiation in the uninjured retina. Neurogenesis-promoting effect of these cytokines were further increased when combined, suggesting a synergistic mechanism (Zhao *et al*., 2014). Another

study from the same group showed that among these cytokines, IL-11 presents a crosstalk with HB-EGF, which results in an enhanced rate of MG proliferation in the uninjured retina (Wan *et al*., 2014). Previous studies from our lab indicated a cytokine storm following chemical lesion to the OE, indicated by the transcriptome data. Immunohistochemical analysis of exogeneous IL-6 stimulation showed an increase in the proliferating cell number in the uninjured OE. injury. Furthermore, the number of activated and dividing HBCs were shown to increase in the sensory region with IL-6 administration (Demirler, 2021).

2. PURPOSE

The zebrafish OE maintains a lifelong capacity for OSN turnover and to respond to tissue injury with high activity of resident neuronal progenitor cells. This capacity is driven by a dual system that resides in the basal OE. Maintenance of sporadically dying OSNs is regulated mainly by GBC activity, while regenerative responses to extensive injuries are directed by HBCs.

Previous work by our research group has shown that components of HB-EGF/EGFR signaling are upregulated shortly after OE lesion. Inhibition of this pathway using the pharmacological MMP inhibitor Marimastat resulted in the reduction of cell proliferation at 24 hpl. Exogeneous HB-EGF administration, on the other hand, caused an increase in OSN generation. These findings suggest a pivotal role for HB-EGF in the regulation of injury-induced neurogenesis in the zebrafish OE. In addition, transcriptome analyses of the lesioned OE also showed a transient upregulation of the cytokines IL-6, IL-11, and Leptin. Among these, IL-6 was recently shown to induce HBC proliferation upon external stimulation with human recombinant protein.

The main objective of this study was to further investigate the role of HB-EGF and the potential interaction between HB-EGF and cytokines in the mitotic neurogenic activity of the OE after injury. The first aim was to establish whether HB-EGF is necessary for repair neurogenesis. Loss-of-function analysis of HB-EGF was performed by using pharmacological inhibitors of the matrix metalloproteases Marimastat and GM6001. In addition, the HB-EGF sequestering agent CRM197 was used to directly block HB-EGF activity. The effects of these inhibitors were analyzed by immunostainings against the neuronal marker HuC/D and the proliferation marker BrdU. To evaluate the effects on cell proliferation, a quantitative analysis of BrdU^{+} cells was performed on intact, injured, and inhibitor-treated OEs. In order to understand the effects on OE regeneration, the number of HuC/D^{+} cells was analyzed.

The second objective of this project was to further determine the route of HB-EGF action on the mitotic activity of the OE. For this purpose, changes in the expression of *hbegfa* and the GBC marker *ascl1a* were analyzed upon external stimulation of the OE with

recombinant HB-EGF by nasal irrigation. For the cellular characterization of *hbegfa* expression upon HB-EGF stimulation, staining against the dormancy marker tp63, which selectively labels HBCs, was performed in addition to *in situ*-hybridization against *hbegfa* and *ascl1a*.

Lastly, the potential role of IL-11 and Leptin in cell proliferation and their synergistic effects with HB-EGF were investigated by the administration exogenous human recombinant proteins. In order to determine the effects of the gain-of-function of these cytokines, five sets of OE stimulation experiments were carried out. Individual effects of these cytokines, as well as their combinations with HB-EGF were investigated by immunostaining against HuC/D, tp63, and BrdU/EdU at 24 hps.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Animals

Adult zebrafish (6 month or older *Danio rerio*) of AB/AB or wild type (derived from a local pet shop and bred in the facility) genetic background were used throughout this study. All fish were maintained in the Animal Facility of Bogazici University Center for Life Sciences and Technology.

3.1.2. Equipment and Supplies

A complete list of all equipment and disposable/non-disposable supplies with their respective brand and manufacturer names used during this study is attached in Appendix A.

3.1.3. Buffers and Solutions

A list of buffers, solutions, antibodies, and reagents used in the experiments of this study is attached in Appendix B. All standard zebrafish maintenance buffers used during this study were prepared according to the recipes found in “The Zebrafish Book” (Westerfield, 2007). The solutions and buffers used in molecular laboratory techniques were prepared according to the recipes described in their respective methods section or in Table 1.3.

3.2. Methods

3.2.1. Zebrafish Maintenance and Husbandry

Zebrafish were kept in an artificial aquatic habitat system built in a special room that has a 27 °C constant temperature, 37% humidity, and a 14/10 light/dark cycle. Adult zebrafish were maintained in 1L, 3L, or 10L tanks that are connected to the continuous flow of the artificial freshwater (regularly prepared with 2.0 g sea salt, 7.5 g Sodium Bicarbonate, and 0.84 g Calcium Sulfate in 100 L reverse osmosis water). Aquatic systems of these tanks are equipped with heater, water and air pumps, UV filters ensuring the quality of the circulating artificial fresh water. Maintenance and cleaning of the aquatic systems were

carried out regularly. Fish were fed twice a day. Flakes and dried egg yolk were provided in the morning and evening feedings, and frozen artemia brine shrimp cubes were added to this mix in the afternoon.

Approval for the use of zebrafish in experiments was taken from the Institutional Ethics Board for Animal Experiments at Boğaziçi University (BUHADYEK) under the project title 2020/17 ("The role of heparin-binding epidermal growth factor (HB-EGF) signaling during regenerative neurogenesis in the zebrafish olfactory epithelium"). All international, national, and institutional regulations regarding the care and use of animals were obeyed, including the National Animal Protection Act (Turkish national law number 5199, "Hayvanları Koruma Kanunu", published 24.06.2004), the directive 2010/63/EU of the "European Parliament and the Council of 22. September 2010 on the Protection of Animals Used for Scientific Purposes" and the "Guide for the Care and Use of Laboratory Animals" (NRC2011) of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

3.2.2. Chemical Lesion to the OE

Fish was anesthetized with 160 mg/ml MS222 dissolved in artificial fish water until opercular movement slowed down and tail reflexes disappeared. The animal was placed into a wet sponge to stabilize the head and positioned for the dorsal part to be visible under the stereo microscope. 1% Triton X-100 solution was prepared in 1X PBS, and 0.1% Phenol red was used as an indicator of application. Approximately 1 µl of this solution was placed into a fine glass capillary tube and applied to one OE for 90 seconds by using a microinjector. The contralateral OE did not get any treatment to serve as an internal control. After TrX treatment, the lesioned OE was flushed with fresh tank water and the fish was placed back in the tank.

3.2.3. Intraperitoneal Injection of Inhibitors and Reagents

160 mg/ml MS222 prepared with tank water was used to anesthetize the fish. After the temporary loss of tail reflexes, the fish was taken into a sponge which holds the animal stable in a position so that the ventral part of the body is open for application. Respective inhibitor solutions were prepared and injected into the periton by an insulin syringe at a position proximally to the anal fins. Leakage of the solution was prevented by holding the needle

stable for approximately 30 seconds following the intraperitoneal (IP) injection. The fish was taken back to the tank for recovery after the application.

For the effects of inhibition with Marimastat on 5d regeneration period, 80 µg/g Marimastat dissolved in DMSO/1X PBS (50 µg Marimastat, 2.5 µl DMSO, 27.5 µl PBS) was used for each dose. Control animals were injected with a solution containing 2.5 µl DMSO vehicle and 27.5 µl 1X PBS. Fish received a total of four doses of the inhibitor: a priming injection 4 h before, at the time of TrX lesion, at 12 hpl and 36 hpl. For the effect of GM6001 on regeneration at 5dpl, 50 µg/µl GM6001 (50 µg GM6001, 12.5 µl DMSO, 37.5 µl PBS) dissolved in DMSO/1X PBS was used for each dose. The injection routine was the same as Marimastat experiment. The control group animals received a vehicle solution composed of 12.5 µl DMSO and 37.5 µl PBS. For the analysis of CRM197 inhibition at 24hpl and 5dpl, 1 µg/µl CRM197 was used for each injection. The injection routine of 24 hpl analysis included the administration of two CRM197 doses, the first one 6h before the lesion and the second one at the time of the TrX lesion. For the analysis of CRM197 inhibition at 5dpl, four doses of the inhibitor were injected. Priming injection was applied 4h before the lesion. Fish received the following injections together with the lesion, 24 hpl, and 48 hpl.

In addition to the inhibitors, Leptin was also administered to the fish by IP injections. Fish received one dose of 2 µg/µl Leptin (2 µg Leptin, 2 µl of 0.1% BSA, 28 µl of 1X PBS) for indirect stimulation of the OE. Control fish received an injection of the solution containing 2 µl of 0.1% BSA and 28 µl of 1X PBS. For this experiment, no lesion was induced in the OE.

3.2.4. BrdU and EdU Incorporation Assays

In order to label proliferative activity, thymidine analogs 5- Bromo-2’- Deoxyuridine (BrdU) and 5-Ethynyl-2’-Deoxyuridine (EdU) were used. The day of labeling and the choice of the thymidine analog depended on the analysis. For BrdU incorporation, the fish was placed in a tank filled with 30 mg/l BrdU dissolved in fresh artificial fish water for 24 hours. Exposure to the light was avoided. At the end of the BrdU incubation, the fish was transferred to the fresh tank water. For the detection of proliferation signal, anti-rat BrdU primary

antibody was used in combination with anti-rat cy2 secondary antibody during immunohistochemistry.

EdU, on the other hand, was injected intraperitoneally. The fish was anesthetized with the regular dose of MS222 and placed into a sponge for the IP injection. 25 µl of 10 mM EdU was injected into the periton with an insulin syringe. The fish was transferred back to the tank after the injection. EdU signal was detected by utilization of the Click-iT EdU Cell Proliferation Kit. Following antibody staining, a cocktail composed of 212 µl sterile water, 25 µl of 10X Click-iT EdU reaction buffer, 10 µl CuSO4, 0.6 µl Alexa-Fluor 488-azide, and 2.5 µl of 10X Click-iT EdU buffer additive were prepared. This detection mix was applied to the slide and incubated for 30 minutes in a dark chamber at room temperature. The slide was washed with PBST for 10 minutes to stop the reaction of detection and tissues were analyzed.

3.2.5. Stimulation of the OE with HB-EGF or Cytokines

Fish were anesthetized with 160 mg/ml MS222 solution until tail reflexes disappeared and placed into a sponge in the same position used for the chemical lesion method. A lower dose of the anesthetic was prepared with 80 mg/ml MS222 in fresh tank water. This solution was continuously perfused through the mouth of the fish by using a micropipette tip, in order to keep the animal under anesthesia throughout the stimulation period of 30 minutes. The body of the fish was covered with a wet towel to prevent drying and preserve temperature. Nasal cavities were drained and depending on the experiment, approximately 1 µl of HB-EGF (200 ng/ µl) and/or cytokines (100 ng/ µl IL-11 or 1 µlg/µl Leptin) were injected into one nasal cavity with the microinjector by using a fine capillary glass. The contralateral nasal cavity was filled with 1 µl of the vehicle solution of 0.1% BSA. The tip of the glass capillary was never in close proximity to the OE to ensure that no lesion was induced. Reagents were injected into the nasal cavities for 3 sets, 10 minutes apart to prevent drying out of the OEs. At the end of the nasal irrigation, fish were transferred to fresh tank water for recovery.

3.2.6. Dissection of the OE

Fish were euthanized by transferring into cold MS222 solution and keeping on ice for approximately 15 minutes. The observation of the loss of opercular movements was followed by decapitating fish with a surgical blade. The head was transferred into ice-cold 1X PBS in

a dissecting pad placed under the stereo microscope. By using dissection forceps with fine tips, first the epithelia covering the nasal cavities were lifted. Then, the head was dissected from the midline in a manner that exposes cranial bones. OEs were scraped from the bones and transferred into clean cold PBS. After ensuring that there are no bones attached to OEs, tissues were taken into a mold containing OCT medium. Molds were frozen at -20 ℃ for about 30 minutes and covered with aluminum foil to prevent the shrinkage of the OCT.

3.2.7. Cryosectioning of the OE

OEs embedded in the frozen OCT were taken to the cryostat, for which the chamber temperature is -20 ℃. The frozen OCT brick was transferred into a stage covered with OCT medium, ensuring the brick did not melt at any point. After the brick stuck onto the stage and was stabilized, the stage was placed into the object groove of the cryostat. Object temperature was kept stable at -18 ℃. The OCT brick was trimmed to make a smaller rectangular region covering both OEs and 12µm sections were taken onto a Superfrost® Plus Slide. Tissues were oven-dried at 65 ℃ for 2 hours for heat fixation. Depending on the circumstances, slides were either directly used in immunohistochemistry/*in situ* hybridization protocols or stored in airtight boxes at -80 ℃ up to 3 months.

3.2.8. Immunohistochemistry

OEs were stained with neuronal, cellular, and proliferation markers for further analysis by using immunohistochemistry. Heat-fixed slides were directly used in the protocol, while an extra 15 minutes of incubation at 65℃ were used for the slides stored at -80 ℃. Before starting the procedure, a frame that covers the edges of the slide was drawn with the hydrophobic blocker PAP pen, in order to prevent the leakage of solutions in later steps. OEs were first rehydrated by immersing slides into a Coplin jar filled with 1X PBS (pH: 7.4) for 5 minutes. Then, 1 ml of 4% paraformaldehyde (PFA; in 1X PBS, pH:7.4) was poured onto the slide for fixation at room temperature for 15 minutes. Tissues were washed with 1X PBST (0.1% Tween-20 in 1X PBS) for 10 minutes at room temperature and the wash step was repeated with fresh PBST for three times. For the permeabilization of nuclei, OEs were treated with 4N HCl solution at room temperature for 15 minutes. Slides were washed 3 times with fresh PBST, for 10 minutes each. For the blocking of antibody binding to unspecific proteins, 1 ml of 3% BSA (bovine serum albumin) was poured onto the slide and

incubated at room temperature for 1 hour. Following the blocking step, a total of 250 µl primary antibody mix was prepared in 3% BSA according to their respective dilution ratios (1:500 for mouse anti-HuC/D, rat anti-BrdU, or rabbit anti-tp63). Slides were placed in a humidity chamber to prevent evaporation during incubation. Primary antibody mix was poured onto the slide and a coverslip was used to homogeneously distribute the solution. Slides were incubated in the 4°C room overnight (16-18 hours). On the next day, primary antibodies were collected from slides and stored at -20°C for reuse in further experiments. Slides were washed in Coplin jars filled with PBST for 15 minutes. The wash step was repeated three times with fresh PBST. A total volume of 250 µl secondary antibody solution was prepared in 3% BSA with respective dilutions (1:800 for anti-mouse Alexa Fluor 555 and 1:200 for anti-mouse cy 5 and anti-rat cy2). Following the washes, the slides were transferred to the humidity chamber again. The secondary antibody solution was poured onto the slide, and a coverslip was used to distribute the solution. OEs were incubated with secondary antibodies in the dark chamber for 2 hours at room temperature. Following the incubation, slides were washed with 1X PBS for 10 minutes and the wash step was repeated for 3 times. Then, OEs were analyzed by confocal microscopy. After imaging, slides were stored in jars containing 1X PBS at 4 °C.

3.2.9. *In situ*-hybridization

In order to detect *hbegfa* or *ascl1a* mRNA, in situ hybridization was performed. All equipment used for *in situ* hybridization was cleaned and sterilized beforehand. The room was cleaned with 70% ethanol and the procedure was carried out cautiously by wearing masks, in order to prevent RNA degradation due to RNAses. Also, all solutions used throughout the procedure were freshly prepared with double distilled sterile water (ddH$_2$O) treated with the RNAse inhibitor DEPC (1:1000). After sectioning the OEs, if the heat-fixed slides were stored at -80°C used, a hair dryer was used to quickly evaporate water droplets emerging due to the sudden contact with room temperature. Then the slides were oven-dried for another 15 minutes before starting the procedure. If the slides were directly used after heat-fixation, the procedure was started at the end of the 2 hour-long oven-dry. OEs were rehydrated with 1X PBS$_{DT}$ (DT for DEPC-treated) for 2 minutes. Fixation was performed by immersing the slides into a jar filled with freshly prepared 4% PFA for 10 minutes at room temperature (pH:7.4; dissolved in 1X PBS$_{DT}$). Two consecutive washes with 1X PBS$_{DT}$, the first one for 1.5 minutes and the second wash for 5 minutes followed the fixation.

Slides were transferred to a jar filled with preheated 0.05% Proteinase K solution (2 µl proteinase K in 40 ml 0.1 M Tris-Cl_{DT}, pH: 8.0), which is placed in the water bath for 7.5 minutes at 37℃. In order to stop Proteinase K activity, slides were taken back to the 4% PFA at room temperature for 5 minutes. OEs were washed again with 1X PBS_{DT} consecutively for 1.5 minutes and 5 minutes at room temperature. Slides were incubated in 0.2M HCl (diluted in ddH_2O_{DT}) for 10 minutes for nuclear permeabilization. Standard 1X PBS_{DT} washes for 1.5 and 5 minutes were carried out afterward. Tri-ethanolamine (TEA) buffer was prepared freshly by mixing 49.1 µl ddH_2O_{DT}, 662.5 µl TEA, 112.5 µl 1M HCl, and 125 µl acetic anhydride in the given order. Slides were incubated in TEA buffer at room temperature for 10 minutes. Then, slides were washed with 1X PBS_{DT} for 1.5 and 5 minutes consecutively. Meanwhile, the hybridization mixture was prepared and heated in an 85 ℃ thermomixer for 20 minutes before the addition of the probes. At this step, the hybridization mixture (total volume 250 µl/slide) contained 50% formamide (v/v), 5X saline sodium citrate buffer (SSC), 50 µg/ml heparin, 500 µg/ml total yeast tRNA, 9.2 mM citric acid, 0.05% Tween-20, up to 250 µl ddH_2O_{DT}. After the mix is heated up, a final concentration of 3 ng/µl digoxigenin-labeled riboprobes to detect *hbegfa* (488 ng/µl; 821 nt fragment of ENSDART00000109138.4; positions 347 – 1.168) or *ascl1a* (517.9 ng/µl; 422 nt fragment of ENSDART00000056005.5; positions 638 – 1060) were added and riboprobes were denatured at 85℃ for 5 minutes. During this preparation, slides were kept in fresh 1X PBS_{DT}. The hybridization chamber was covered with a towel immersed in a 50% formamide/50% ddH_2O_{DT} solution. Slides were taken out of PBS and the edges of the slides were drawn with PAP pen to create a hydrophobic barrier. After the slides were placed in the hybridization chamber, hybridization mixture with denatured probes was poured onto the slides. Plastic coverslips were used to distribute the solutions homogeneously on the slides. The hybridization chamber was placed into the oven set to 65 ℃ and incubated overnight.

On the second day of *in situ* hybridization, slides were washed with gradually decreasing concentrations of SSC buffer at 65 ℃. All SSC buffers were prepared beforehand and preheated in the jars placed in the water bath. Slides were taken out of the 65 ℃ oven and transferred to 5X SSC for 2-3 minutes and coverslips were removed in this solution. Then, they were washed in a mixture of 2X SSC/50% formamide for 30 minutes. Consecutive two washes with 2X SSC for 20 and 10 minutes followed this. Lastly, slides were washed with 0.2X SSC for 10 minutes. Following the last wash, slides were directly

transferred to DIG buffer (150 mM NaCl, 100 mM Tris-Cl_{DT}, pH:7.5) and incubated for 5 minutes at room temperature. Meanwhile, 1X blocking solution was prepared by dilution from 10X stock in maleic acid buffer. Slides were transferred to a humidity chamber and 1 ml of 1 X blocking solution was poured onto each slide. After 2 hours of blocking at room temperature, primary antibody mixture was prepared by using anti-digoxigenin-alkaline phosphatase fab fragments (1:750) and rabbit anti-tp63 (1:500; if combined with IHC) dissolved in 1X blocking solution. Slides were covered with a total of 250 µl primary antibody solution and plastic coverslips were placed. Primary antibodies were incubated in the 4 °C room overnight.

On the third day of the protocol, primary antibodies were collected from the slides and stored at -20 °C for reuse. Slides were washed 3 times with DIG Buffer containing 0.05% Tween-20 for 5, 15, and 15 minutes. Subsequently, slides were transferred to the detection buffer (100 mM NaCl, 100 mM Tris-Cl, pH: 8.0, 100 mM $MgCl_2$) and incubated for 10 minutes. Meanwhile, a fresh detection mixture of HNPP/Fast Red (3 µl of freshly prepared and filtered 25 µg/µl Fast Red, 3 µl HNPP dissolved in 300 µl detection buffer per slide) was prepared. Slides were taken back to the humidity chamber and the detection mixture was poured onto each slide. Light exposure was avoided during the detection step. Development of the enzymatic reaction was checked after approximately 90 minutes. The detection mixture was collected into a tube. If the signal is detected, the reaction was stopped by washing slides in ddH_2O_{DT}. If not, the collected detection mixture was placed on the slides again and another 90 minutes were given. Following the detection of the *in situ* signal, slides were washed three times with ddH_2O_{DT} for 10 minutes each. If anti-tp63 were used in the primary antibody incubation, the procedure was continued with the secondary antibody incubation. Anti-rabbit Alexa Fluor 647 (1:800) was diluted in 3% BSA (total volume 250 µl) and incubated for 2 hours in the dark at room temperature. Slides were washed 3 times with 1X PBS_{DT} for 10 minutes. OEs were analyzed under the confocal microscope. After the analysis, slides were stored in jars filled with ddH_2O_{DT} at 4 °C.

3.2.10. Confocal Microscopy Imaging

For the OEs stained against BrdU, HuC/D and tp63 by immunohistochemistry, 1024x1024 digital images were taken with 20X water immersion lens of the Leica SP5-AOBS system. For the OEs treated with *in situ* hybridization, 2048x2048 digital images

were taken with the 40X water immersion lens of the Leica TCS SP8 system. All images were taken with 400 Hz frequency and smart offset up to 3%. For the inhibitor experiments and analyses of cytokine stimulations, the images of at least 5 OE sections from each group were taken for the statistical analysis.

3.2.11. The Processing and the Analysis of Images

Images taken with the confocal microscope were transferred to FIJI (Image J) Software in lif file format. Stacks of the images were projected onto a single image by maximum projection. Brightness and contrast of the maximum projections were adjusted, and these images were saved in Tiff file format. For the analysis of BrdU/EdU positive cells, a rectangular region with 2/3 width/length ratio was cropped from the central part of each hemi-OE, in order to avoid the complications arising from the oval shape of the tissue. Left hemi-OEs were flipped horizontally to keep the direction correct, from ILC towards the peripheral edge. BrdU/EdU channel of the images were separated and saved. BrdU/EdU positive signals were counted by a custom macro on FIJI, which divides the entire region to 10 compartments and counts the number of cells (based on the pixel size of signals) in each compartment. These counts were then corrected for the cells falling into the borders of the compartments by using an R script. The compartments corresponded to specific regions of the OE as follows: the 1st compartment is the ILC, 3rd, 4th, and 5th compartments labeled the core sensory, 6th-8th compartments were counted as the SNS, and 8th-10th compartments comprised the non-sensory region. The final numbers for the cell count were used for further statistical analyses.

For the analyses of HuC/D area, whole OE images were used. HuC/D channel was split and saved in Tiff file format. HuC/D^{+} area was selected by using the threshold tool in FIJI software. This area was measured in the unit of pixels. Then, the borders of the OE were selected with the polygon drawing tool, and the area within these borders was measured in pixels. The area of HuC/D^{+} cells was divided by the measured total area. After this procedure was carried out for all images in the experiment, results of each group were normalized to the average of the control group.

All statistical analyses were carried out by using GraphPad Prism Software (GraphPad v7, USA). One-way ANOVA and following post hoc Tukey's Honest Significant Difference

(HSD) test were used for comparisons between experimental and control groups in the experiments described in sections 4.1.1, 4.1.2, 4.1.3, and 4.1.4. Unpaired, two-tailed t-test was used for the analyses that were mentioned in sections 4.2.1, 4.3.1, 4.3.2, 4.3.3, 4.3.4, and 4.3.5. All graphs were created in the GraphPad Prism Software and all figures were prepared using Adobe Illustrator (CS6, Adobe, USA).

4. RESULTS

The zebrafish OE possesses a high capacity of regeneration with continuous turnover of neurons during steady state conditions, as well as rapid generation of neurons after injury. The OE is capable of recovering from the loss of a large number of neurons within a very short time. Following an injury and near complete loss of OSNs, 80% of the diminished neuronal population can reform within only five days. Degeneration of neurons in injured OEs activates HBCs, which proliferate to repopulate the tissue (Kocagöz *et al*, 2022). Whereas cellular dynamics of repair neurogenesis are understood to a certain extent, the inter-, and intracellular signals that drive this process remain largely elusive.

HB-EGF is a membrane bound signaling molecule, which is known to play roles in proliferation, growth, and regeneration. Studies in the zebrafish retina showed that HB-EGF triggers Müller Glia dedifferentiation upon damage and thus, takes part in initiating neurogenesis (Wan *et al*, 2012). Transcriptome studies from our lab showed that *hbegfa* is transiently upregulated after damage (Demirler, 2020) and a sharp peak of expression is observed within the tissue as early as 4 hpl (Güler, 2021). These findings suggested that HB-EGF may be a key regulator of the OE regeneration. The studies conducted in the framework of this thesis aimed to understand the role, the intercellular signaling mechanism, and potential interaction partners of HB-EGF during zebrafish OE neurogenesis.

The studies conducted in this thesis are organized into three main parts: an analysis of HB-EGF loss-of-function after injury, the examination of the OE response to external stimulation with human recombinant HB-EGF, and the investigation of a possible synergism between HB-EGF and cytokines during OE regeneration. The first section reports the effects of the inhibition of HB-EGF activation by Marimastat, GM6001, and CRM197. The second section presents the changes in *hbegfa* and *ascl1a* expression after stimulation of the OE with human recombinant HB-EGF. Lastly, the third section offers some insight into the individual effects of cytokines IL11 and Leptin, as well as the combined effects of the cytokines with HB-EGF on cell proliferation in the OE.

4.1. Effects of Inhibition of HB-EGF Activation on Zebrafish OE Regeneration and Repair Neurogenesis

HB-EGF is a transmembrane protein which is activated by ectodomain shedding and soluble form of the protein is known to trigger proliferation (Nishi and Klagsburn, 2004; Ito *et al*, 1994). Recent studies from our lab have found that inhibition of HB-EGF shedding by the metalloprotease inhibitors Marimastat and GM6001 leads to a significant decrease in cell proliferation at 1 dpl (days post lesion; Kocagöz, 2021). In this section, the results of pharmacological inhibition of HB-EGF activation with different agents will be reported. The examination focuses on the effects of inhibition by metalloprotease inhibitors Marimastat and GM6001 at 5 dpl, and the effects of the HB-EGF-masking agent CRM197 at 1 dpl and 5 dpl.

4.1.1. Effects of the Metalloprotease Inhibitor Marimastat on Proliferative Activity and OSN Neurogenesis of the Injured OE

HB-EGF is cleaved by matrix metalloproteases and in particular by ADAM proteases following activation. The soluble part of the protein works both as an autocrine and paracrine signal to stimulate cells expressing the cognate EGF receptor EGFR (Prenzel *et al*., 1999). In order to understand whether HB-EGF ectodomain shedding functions as a key step in repair neurogenesis, the broad-spectrum metalloprotease inhibitor Marimastat was used. Marimastat was administered to the fish by intraperitoneal injections (80 µg/g for each dose, dissolved in DMSO). Fish (n=2) were injected with Marimastat 4 h before 1% Triton-X-100 (TrX) treatment for priming. A second dose of inhibitor was injected at the time of the lesion and additional injections were carried out at 12 hpl (hours post lesion) and 36 hpl, respectively. Fish were incubated in 30 mg/l BrdU between 48 – 72 hpl to label dividing cells and OEs were analyzed at 120 hpl (5dpl). The same experimental procedure was employed for a control group (n=2), which received injections of the vehicle DMSO.

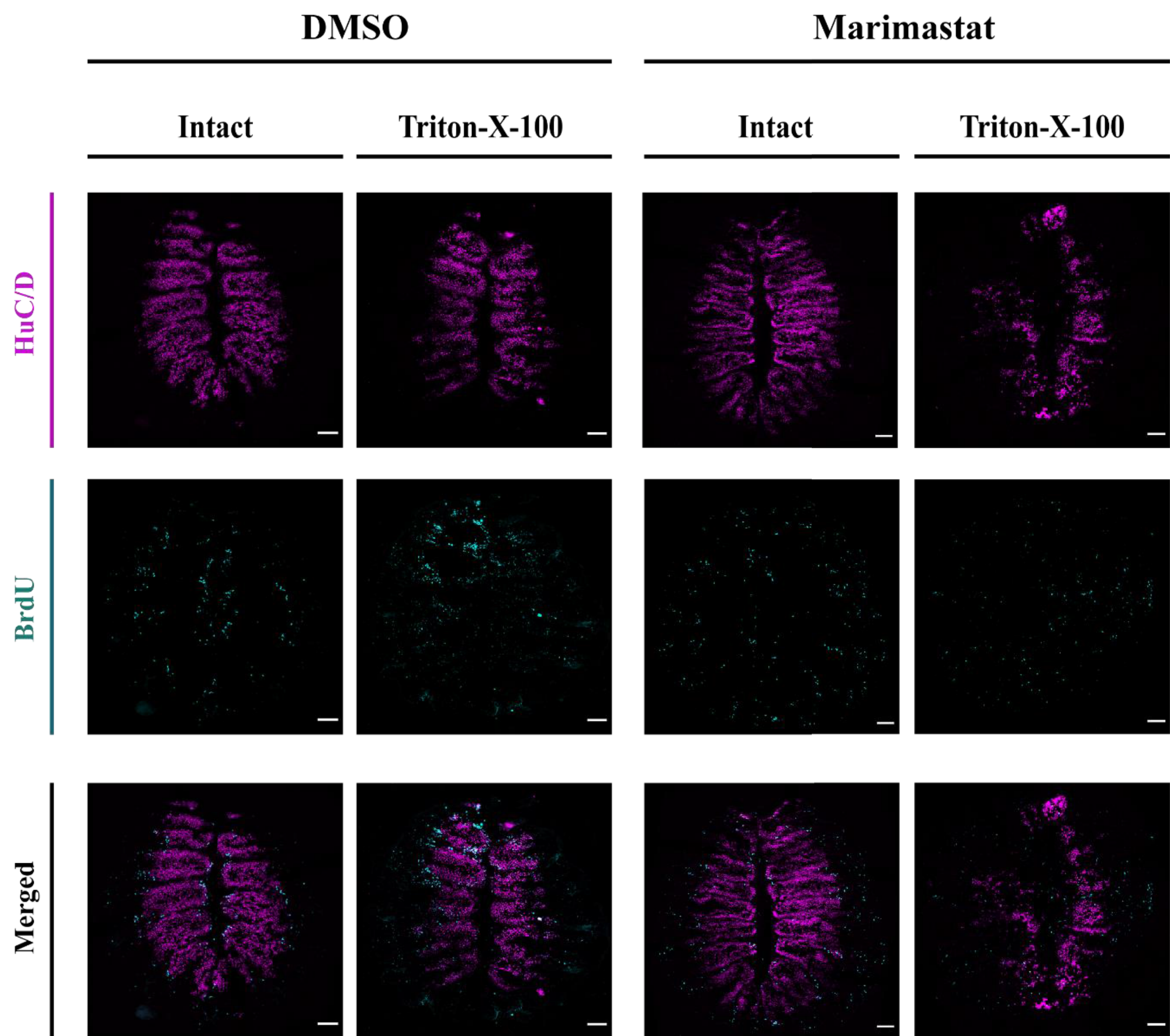

Figure 4.1. Effects of metalloprotease inhibition by Marimastat on OE regeneration at 5dpl. Immunohistochemistry against HuC/D (magenta) and BrdU (cyan) in intact and 1% TrX-treated OE of DMSO- and Marimastat-injected fish. Scale bars are 50 µm.

Figure 4.1 displays 12 µm sections of intact and TrX-treated OE sections of DMSO- (left) and Marimastat-injected fish (right), stained against the neuronal marker HuC/D (magenta) and BrdU (cyan) for the analysis of cell proliferation at 5 dpl. Intact OEs of both DMSO- and Marimastat-treated fish show the typical intact distribution of neurons that starts from the ILC and covers approximately 2/3 of the tissue along the radial dimension. Proliferative activity in the intact OEs of both control and inhibitor-treated groups is limited to the ILC and SNS, consistent with the bimodal distribution of cell proliferation that has been described previously (Bayramli *et al.*, 2017; Kocagöz *et al.*, 2022).

Analysis at 5 dpl showed that the TrX-lesioned OE of the DMSO-injected control group regained most of its neuronal population as confirmed by analysis of the HuC/D signal. The injured OE of Marimastat-treated fish, on the other hand, is characterized by a significantly smaller area covered by neurons, indicating reduced recovery of OSNs following injury.

For a quantitative analysis of proliferation, the central region of each hemi-OE was cropped from images of whole OE sections. Due to the elliptic shape of the OE, regions of interest (ROIs) were chosen that cover the tissue between ILC and the periphery of each hemi-OE, and in which sensory lamellae are oriented largely perpendicular to the midline axis of the OE. ROIs were standardized to a 2 by 3 to the length ratio. $BrdU^+$ cells were counted for 20 hemi-OE from controls and 20 hemi-OE from the Marimastat group with a custom macro in Fiji software. The macro divides the image into 10 equally sized bins along the x-axis and automatically counts cells for each region. These counts are then corrected for cells that fall onto the borders of regions using R scripts. The statistical analysis of cell counts was performed using GraphPad Prism software. The same analysis method was employed for all the $BrdU^+$ or EdU^+ cell counts in the experiments that are described in this study.

As seen in Figure 4.2a, the intact OE of Marimastat-treated fish (orange) shows a uniform decrease in the number of $BrdU^+$ cells along all positions of the radial index. In Figure 4.2c, a significant decrease of $BrdU^+$ cells can be seen at the ILC from 27.9 ± 2.9 (SEM) in intact OE of the DMSO controls to 9.6 ±1.3 in Marimastat-injected group (ANOVA: $F_{(3, 76)} = 10.8$, $p < 0.0001$, Tukey HSD $p = 0.0025$). A similar change can be observed in the SNS of intact OEs, from 45.2 ± 8.3 in controls to 19.9 ± 3.6 in the inhibitor group (ANOVA: $F_{(3, 76)} = 17.99$, $p < 0.0001$, Tukey HSD $p < 0.0001$). The slight reduction of $BrdU^+$ cells in the core sensory regions from 18.6 ± 2.9 to 8.6 ± 1.4, did not reach significance (ANOVA: $F_{(3, 76)} = 17.99$, Tukey's adjusted p-value =0.7463). Similarly, the number of $BrdU^+$ cells changed from 20.2 ± 4.3 to 11.4 ± 1.9 in the NS of intact OEs, which was also non-significant (ANOVA: $F_{(3, 76)} = 3.941$, Tukey HSD p-value = 0.1296).

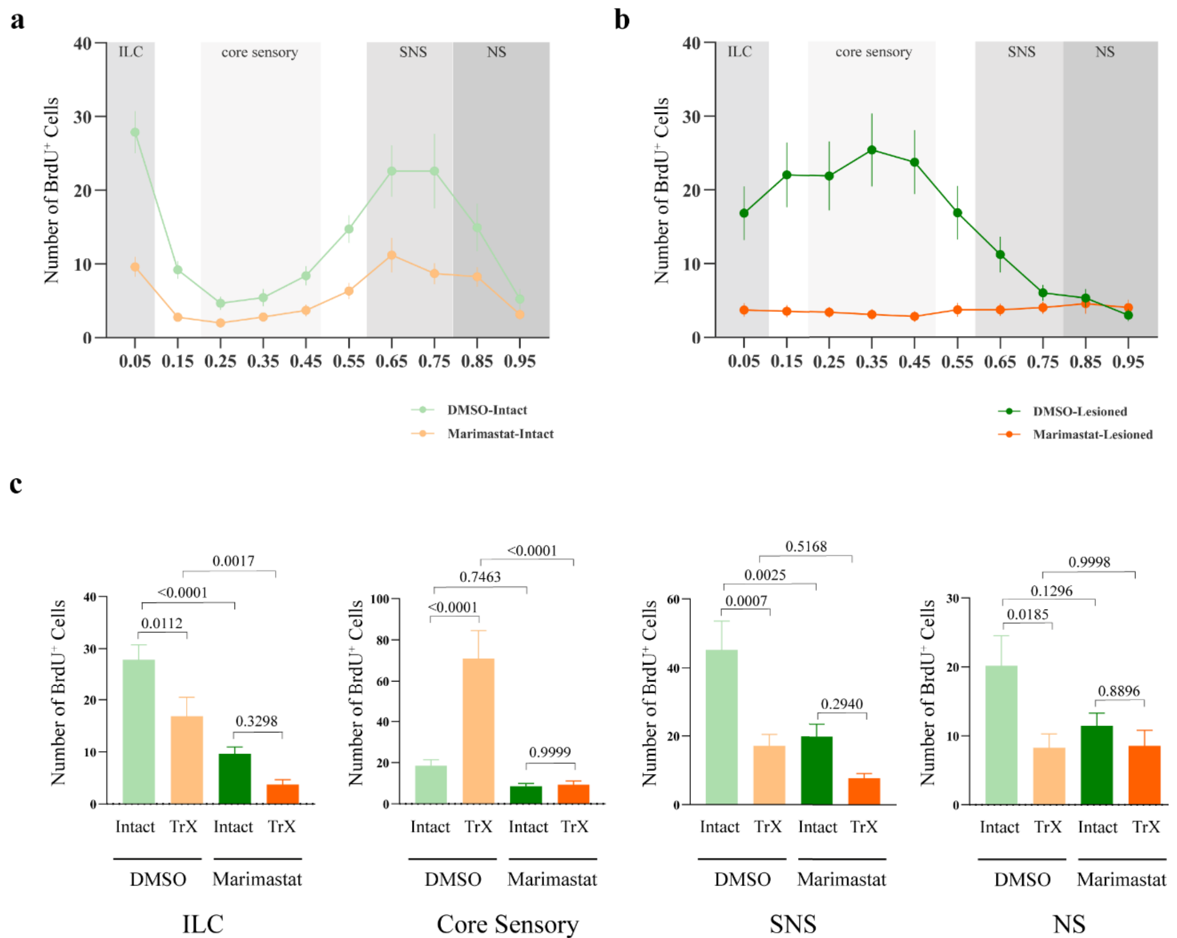

Figure 4.2. Positional profiling of BrdU$^+$ cells in intact (a) and 1% TrX-treated (b) OEs of DMSO and Marimastat injected fish at 5dpl. The graphs at the bottom detail the analysis of dividing cells separately for relevant OE regions (c).

Figure 4.2b shows the change in the number of BrdU$^+$ cells between TrX-lesioned OEs of DMSO- (dark green) and Marimastat-injected fish (dark orange). The injury induces high proliferative activity in the DMSO control group, which is uniformly distributed across the tissue but includes a high number of BrdU$^+$ cells in the core sensory region, that is largely devoid of dividing cells in the intact OE. In contrast, the Marimastat-injected group displays an extremely low number of BrdU$^+$ cells across all regions of the OE. The reduction in the number of proliferating cells was confirmed to be highly significant by statistical analysis (ANOVA: $F_{(3, 36)} = 8.559$, p=0.0002 Tukey HSD p-value=0.0009; ILC: from 16.9 ± 3.6 in DMSO-control to 3.7 ± 0.9 in Marimastat group, Core sensory: from 71.0 ± 13.6 to 9.3 ± 1.9, SNS: from 17.2 ± 3.3 to 7.7 ± 1.4, NS: from 8.3 ± 2.0 to 8.6 ± 2.2). The notable reduction

in proliferative activity in the presence of Marimastat suggests that inhibition of HB-EGF ectodomain shedding impairs the efficiency of the OE regeneration.

The recovery of neurons at 5 dpl was analyzed as the area covered by HuC/D^{+} cells relative to the overall dimension of the OE. The HuC/D signal was thresholded in FIJI, the area measured and divided by the total area of the OE. This ratio was calculated for each section (10 sections from DMSO-intact, DMSO-TrX, Marimastat-intact and Marimastat TrX groups) and results of all groups were normalized to the average HuC/D ratio of the DMSO-intact group. The same analysis method was also employed for all other inhibitor experiments that are described in the sections below.

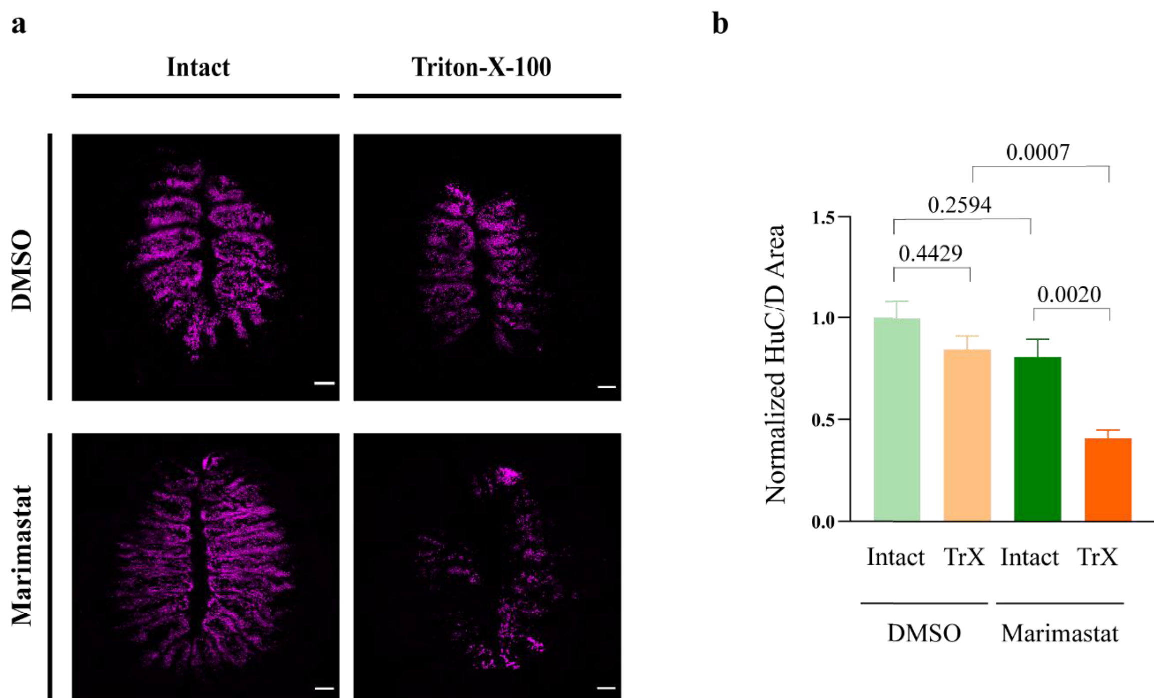

Figure 4.3. Comparison of the area covered by HuC/D^{+} cells in intact and 1% TrX-treated OE of DMSO and Marimastat injected fish at 5dpl (a). Analysis of normalized HuC/D area in each group (b).

The analysis of OSN recovery is presented in Figure 4.3. As seen in Figure 4.3a, TrX-treated OEs of Marimastat-injected fish (bottom right) shows a clearly irregular pattern of neurons that are distributed in isolated patches, and the HuC/D^{+} area is significantly reduced compared to the TrX-treated OE of the DMSO-group. In Figure 4.3b, the recovery rate of the DMSO group amounts to 85% (0.85 ± 0.06) at 5 dpl, while this number is reduced to

41% in the Marimastat-treated group (0.41 ± 0.04). A substantially smaller coverage of neurons is observed in the presence of the metalloprotease inhibitor Marimastat (ANOVA: $F_{(3, 36)} = 12.22$, $p < 0.0001$, Tukey's adjusted p-value = 0.0007). Even though OSN recovery is significantly impaired, it is not completely abolished. Therefore, the analysis indicates that the inhibition of HB-EGF shedding reduces the efficiency of repair neurogenesis after chemical injury, either by a delay or arrest in the repair mechanism.

4.1.2. Effects of GM6001 on Proliferative Activity and Recovery of the Injured OE

In order to confirm the effects of metalloprotease inhibition on regeneration and recovery of neurons, an additional metalloprotease inhibitor, GM6001 (Ilomastat) was used. Even though both compounds are classified as broad-spectrum MMP inhibitors, they exhibit different target spectra. Marimastat has been shown to have an inhibitory effect on MMPs 1, 2, 3, 7, and 9, whereas GM6001 inhibits a broader spectra of MMPs (Rasmussen and McCann, 1997; Schultz *et al*, 1992). It is known that GM6001 suppress wound healing and epithelial regeneration (Ågren *et al.*, 2001). The same experimental procedure that was used for Marimastat was employed for GM6001. Fish were injected intraperitoneally with GM6001 (50 µg/µl for each dose, dissolved in DMSO) 4 h before, at the time, 12h after and 36 h after the lesion. Fish were incubated in BrdU-containing water between 48-72 hpl and OEs were analyzed for HuC/D and BrdU at 120 hpl. The same protocol was used for control animals, which were injected with the DMSO vehicle.

OE sections were stained against HuC/D and BrdU, to check for proliferation at 3 dpl and neurogenesis at 5 dpl. Representative images are shown in Figure 4.4. Intact OEs of both conditions show the regular borders of neurons and bimodal pattern of proliferation. There is no visible difference in the sense of cellular distribution between intact OEs of DMSO control and GM6001-injected fish. On the other hand, TrX treated OEs of the GM6001 group exhibits clearly a reduced coverage of neurons. In addition, HuC/D$^+$ cells show a disrupted patchy distribution pattern similar to the results of Marimastat treatment. Proliferative activity also showed a notable decrease in the TrX-treated OE of the GM6001-injected fish relative to the control animals. While TrX-treated OE of the DMSO group presents a high number of BrdU$^+$ cells across all regions of the tissue, only a small number of BrdU$^+$ cells was visible in the TrX-treated OE of the GM6001 group.

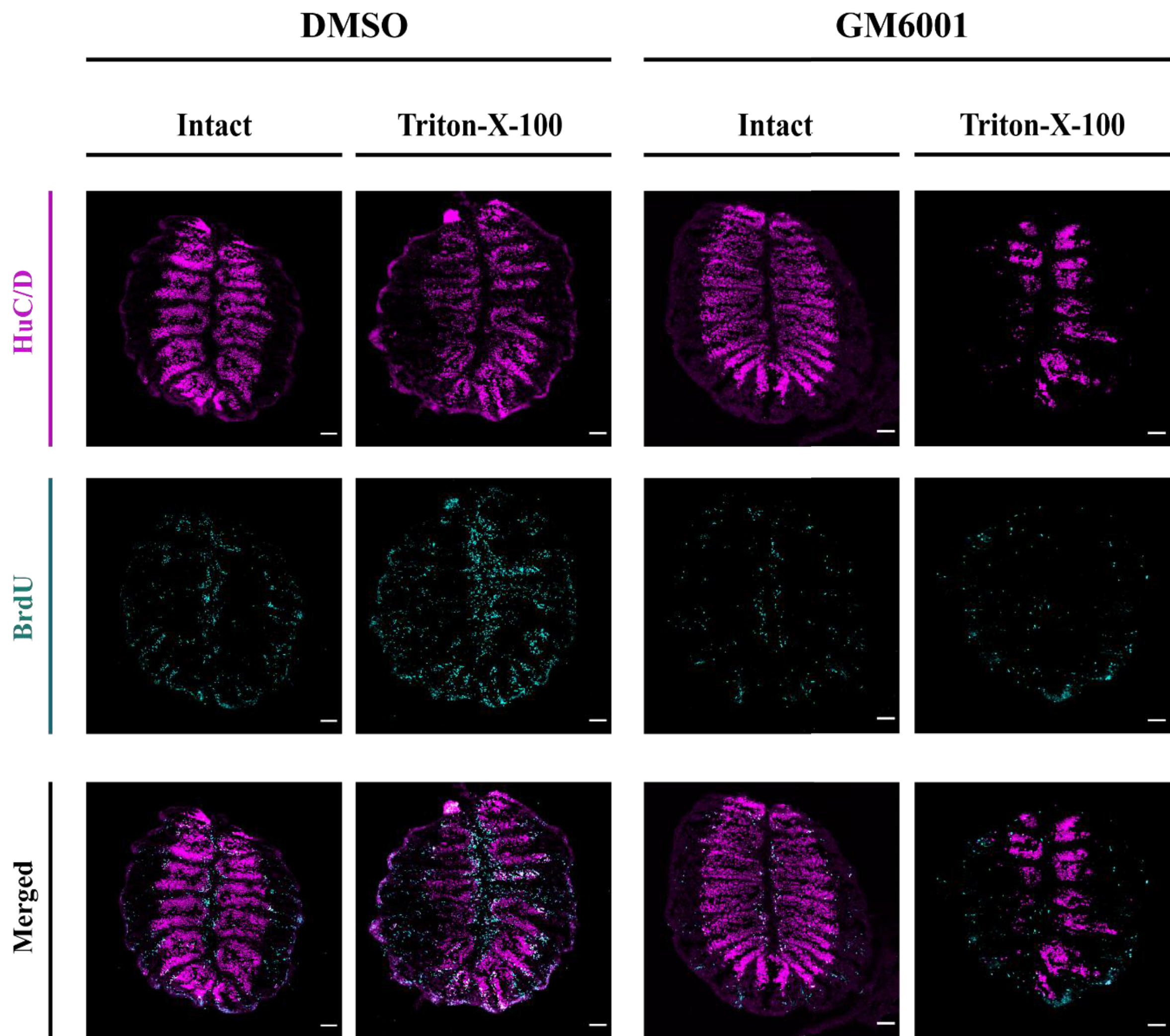

Figure 4.4. Effects of metalloprotease inhibition by GM6001 (Ilomastat) on OE regeneration at 5dpl. Immunohistochemistry against HuC/D (magenta) and BrdU (cyan) in Intact and 1% Triton-X-100 treated OE of DMSO and GM6001 injected fish. Scale bars: 50 µm.

In order to understand whether GM6001 impairs proliferative activity in response to injury, $BrdU^+$ cells were counted from ROIs, with the same method as explained above for Marimastat procedure. Comparisons of $BrdU^+$ cell counts are shown in Figure 4.5a, b. In Figure 4.5a, intact OEs of the DMSO- (green) and GM6001-injected (orange) fish are shown. DMSO-injected fish displays the regular bimodal pattern of $BrdU^+$ cells that two peaks at the ILC and the SNS. The intact OE of GM6001-treated fish shows a similar distribution of cells, however, the number of dividing cells was significantly reduced at the ILC and in the non-sensory region. As Figure 4.5c shows, $BrdU^+$ cells in the ILC declined from 33.2 ± 2.9 in the control to 11.3 ± 1.4 in GM6001-injected fish (ANOVA: $F_{(3, 116)}$ =

19.85, Tukey HSD p-value <0.0001). In the non-sensory region, a significant change from 18.4 ± 2.6 to 7.0 ± 1.1 (ANOVA: $F_{(3, 116)} = 9.845$, Tukey HSD p-value = 0.0010) can also be detected. The core sensory and SNS, on the other hand, do not display any change in the number of proliferating cells (Core sensory: from 9.7 ± 1.2 to 16.3 ± 4.3, ANOVA: $F_{(3, 116)} = 34.43$, Tukey HSD p-value = 0.6507, SNS: from 17.9 ± 2.4 to 13.0 ± 1.9, ANOVA: $F_{(3, 116)} = 7.366$ Tukey HSD p-value = 0.557). Since increased proliferation in the core sensory region is a characteristic of repair neurogenesis, the difference between DMSO- and GM6001-injected groups does not seem to affect maintenance proliferation pattern.

Figure 4.5b shows the $BrdU^+$ cell comparison along the radial index between injured OEs of DMSO- (dark green) and GM6001-injected (dark orange) groups. The injured OE of the control group displays a high rate of proliferation across the entire tissue, while the injured OE of the GM6001-injected group only has a low number of $BrdU^+$ cells. The comparison of individual regions in Figure 4.5c indicates that there is a uniform and substantial reduction in the proliferation rate in the GM6001-treated group. Analysis of $BrdU^+$ cells in all regions were found to be statistically significant (ANOVA: $F_{(3, 36)} = 9.845$, p <0.0001, ILC: from 28.8 ± 5.4 in control to 3.4 ± 0.6 in GM6001-treated group, Tukey HSD p-value <0.0001, Core sensory: from 16.3 ± 4.3 to 4.8 ± 0.8, Tukey HSD p-value <0.000, SNS: from 21.3 ± 4.1 to 4.9 ± 1.1, Tukey HSD p-value = 0.0001, NS: from 13.0 ± 3.0 to 3.8 ± 0.6, Tukey HSD p-value = 0.0105). The noteworthy decline in the number of $BrdU^+$ cells of GM6001 treated OEs after lesion suggests that the inhibition of metalloproteases by GM5001 reduces injury-induced cell proliferation, which is consistent with the results of Marimastat treatment.

Effects of GM6001 on the recovery of neurons at 5 dpl were analyzed by HuC/D staining and thresholding of the signal as detailed above. Representative images of HuC/D^+ cells in the intact (left) and injured (right) OEs of the control (top) and GM6001-treated (bottom) fish are shown in Figure 4.6a. The difference of the HuC/D area between intact OEs of DMSO- and GM6001-treated fish was not significant (Figure 4.6b; 0.9 ± 0.07 in the intact OE of GM6001 group, normalized to the intact OE of the control, p = 0.5014). Threshold analysis of the HuC/D signal determined that the recovery rate in the DMSO-injected group was 68% (0.68 ± 0.05), while TrX-treated OE of the GM6001 fish showed 47% (0.47 ± 0.07) neuronal coverage. Even though the difference was not as high as for Marimastat treatment, inhibition with GM6001 also resulted in a significant decline

(ANOVA: $F_{(3, 56)} = 17.65$ $p < 0.0001$, Tukey HSD p-value = 0.0381) in OSN neurogenesis at 5 dpl.

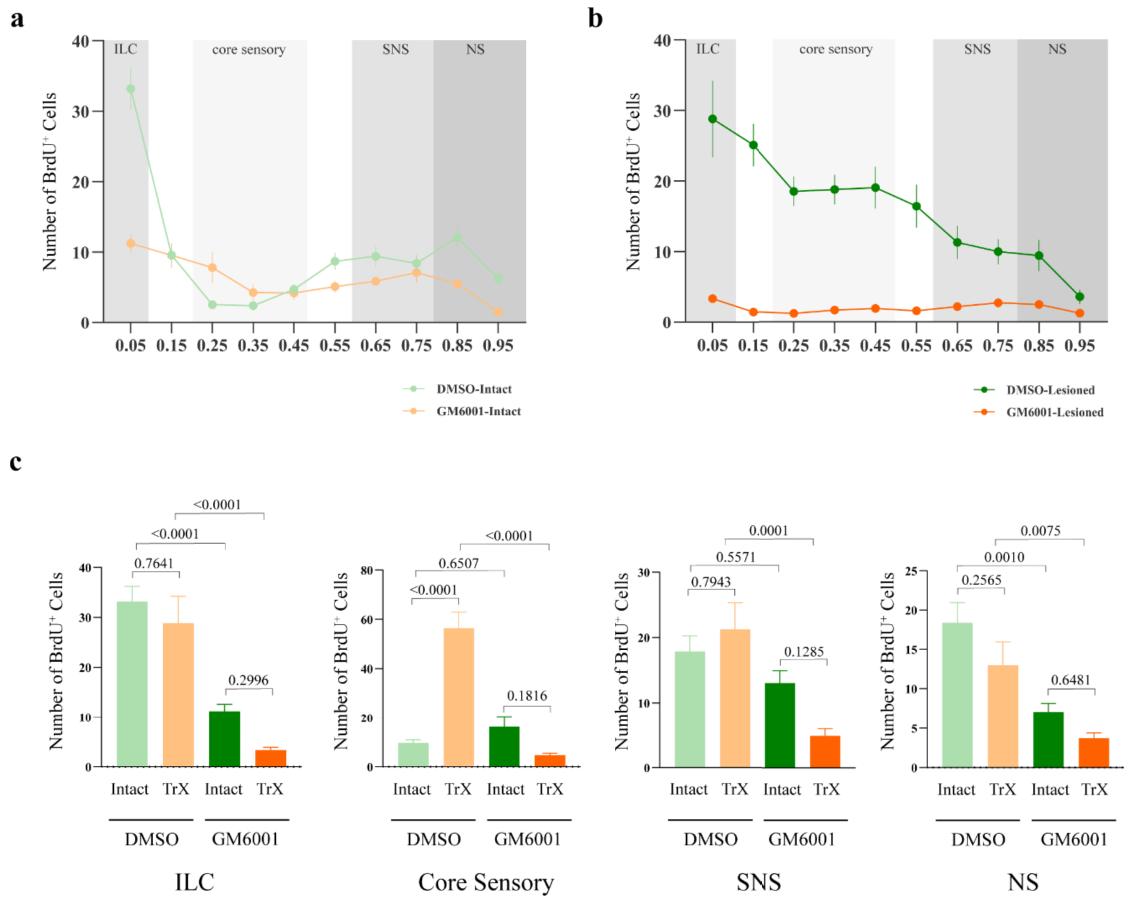

Figure 4.5. Positional profiling of BrdU^{+} cells in Intact (a) and 1% Triton-X-100 treated (b) OE of DMSO and GM6001 injected fish at 5dpl. The graphs at the bottom detail the analysis of dividing cells separately for relevant OE regions (c).

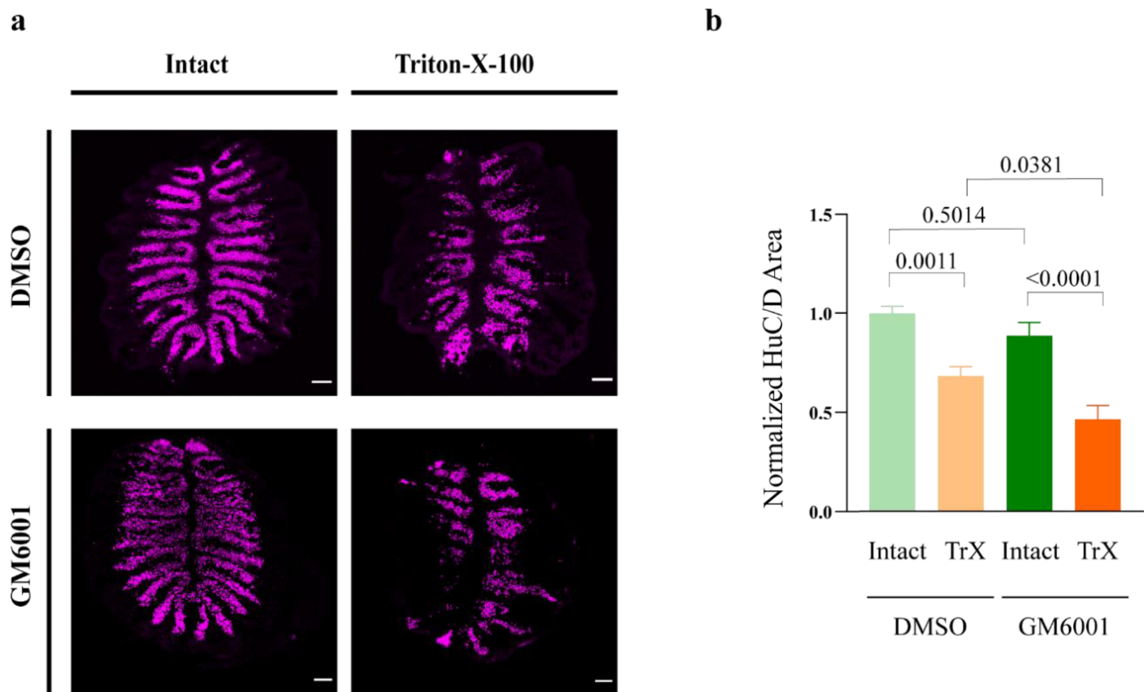

Figure 4.6. Comparison of the area covered by HuC/D$^+$ cells in the intact and 1% Triton-X-100 treated OE of DMSO and GM6001 injected fish at 5dpl (a). Analysis of normalized HuC/D area in each group (b).

4.1.3. Effects of CRM197 on Proliferative Activity of the Injured OE

Results of inhibition of ectodomain shedding showed that the cleavage of HB-EGF might be a critical step during OE regeneration. In order to more directly address the role of HB-EGF in regeneration and to rule out that other signaling molecules regulated by metalloproteases are responsible for the effect, the soluble form of HB-EGF was sequestered. CRM197 (Cross-reactive material 197) is a diphtheria toxin mutant that has a single amino acid change from glycine to glutamic acid in position 52. CRM197 binds to the soluble form of HB-EGF and masks the activity without creating toxicity for the animal (Mitamura *et al*, 1994). In this section, the effect of CRM197 during an early time point following OSN degeneration were assessed. CRM197 was injected into the fish intraperitoneally (1 µg/µl dissolved in HEPES Buffer, 29 µl 1X PBS was added for total volume of 30 µl for each dose) 6 h before the lesion for priming and another dose of the inhibitor was given at the time of 1% TrX-treatment. Fish (n=3) were incubated in BrdU-containing water after the lesion and kept until analysis at 24 h. The same injection protocol

was used for the control group, which was injected with the vehicle HEPES Buffer (1 µl in 29 µl of 1X PBS for each dose).

Figure 4.7 shows OEs of HEPES buffer- (left) and CRM197-injected (right) fish at 23 hpl. Immunostaining against HuC/D for neurons and BrdU for proliferation are presented. Intact OEs of both groups display the characteristics patterns of the maintenance neurogenesis with high proliferative activity at the ILC and SNS, as expected. Similar to results shown above, HuC/D signal is severely reduced in both groups following injury. TrX-treated OEs of the control fish show a higher number and a uniform distribution of proliferating cells. TrX-treated OE of the CRM197-injected fish, however, show a reduced number of dividing cells with a higher density around ILC.

For the analysis of cell proliferation, images of hemi-OEs were cropped as before, $BrdU^+$ cells were counted with a custom macro in FIJI and results were analyzed for statistical significance. $BrdU^+$ cell profiles can be observed in Figure 4.8a for intact OEs, and in Figure 4.8b for TrX-treated OEs of HEPES Buffer- and CRM197-injected fish.

The proliferation curves of intact OEs of the control (green) and CRM197-injected (orange) fish look very similar as shown in Figure 4.8a. Figure 4.8c displays the statistical analysis of each experimental group for separate regions of the OE. The comparison of $BrdU^+$ cells between intact OEs of the control and CRM197-injected fish did not indicate a significant change in the ILC (from 18.2 ± 1.8 to 17.9 ± 1.2, Tukey HSD p-value = 0.9997), core sensory (from 13.2 ± 2.0 to 9.8 ± 1.5, Tukey HSD p-value = 0.9657) and SNS (from 34.0 ± 3.9 to 20.0 ± 2.0, Tukey HSD p-value = 0.1454). The only difference was found in the NS (from 29.0 ± 4.5 to 14.8 ± 0.8, ANOVA: $F_{(3, 116)} = 6.308$, $p = 0.0005$, Tukey HSD p-value = 0.0222), which, however, does not contribute to neurogenesis.

The comparison between injured OEs of the control and CRM197-treated fish, however, showed a clear difference in the number of dividing cells (ANOVA: $F_{(3, 36)} = 10.28$, $p < 0.0001$, Tukey HSD, p-value = 0.0001). The change in the ILC (from 16.3 ± 2.8 control value to 11.6 ± 1.8 CRM197-treated, Tukey HSD adjusted p-value = 0.3344) and NS (from 20.8 ± 5.1 to 8.7 ± 1.0, Tukey HSD adjusted p-value = 0.0687) regions were not statistically significant. On the other hand, the core sensory (from 60.7 ± 9.6 to 22.1 ± 2.1, Tukey HSD adjusted p-value <0.0001) and SNS (from 41.4 ± 8.0 to 11.5 ± 1.2, Tukey HSD

adjusted p-value <0.0001) presents a notable decline in proliferation. The reduced number of BrdU$^+$ cells in especially the core sensory and SNS indicates that inhibition of HB-EGF with CRM197 negatively affects the proliferation during the early phase of repair.

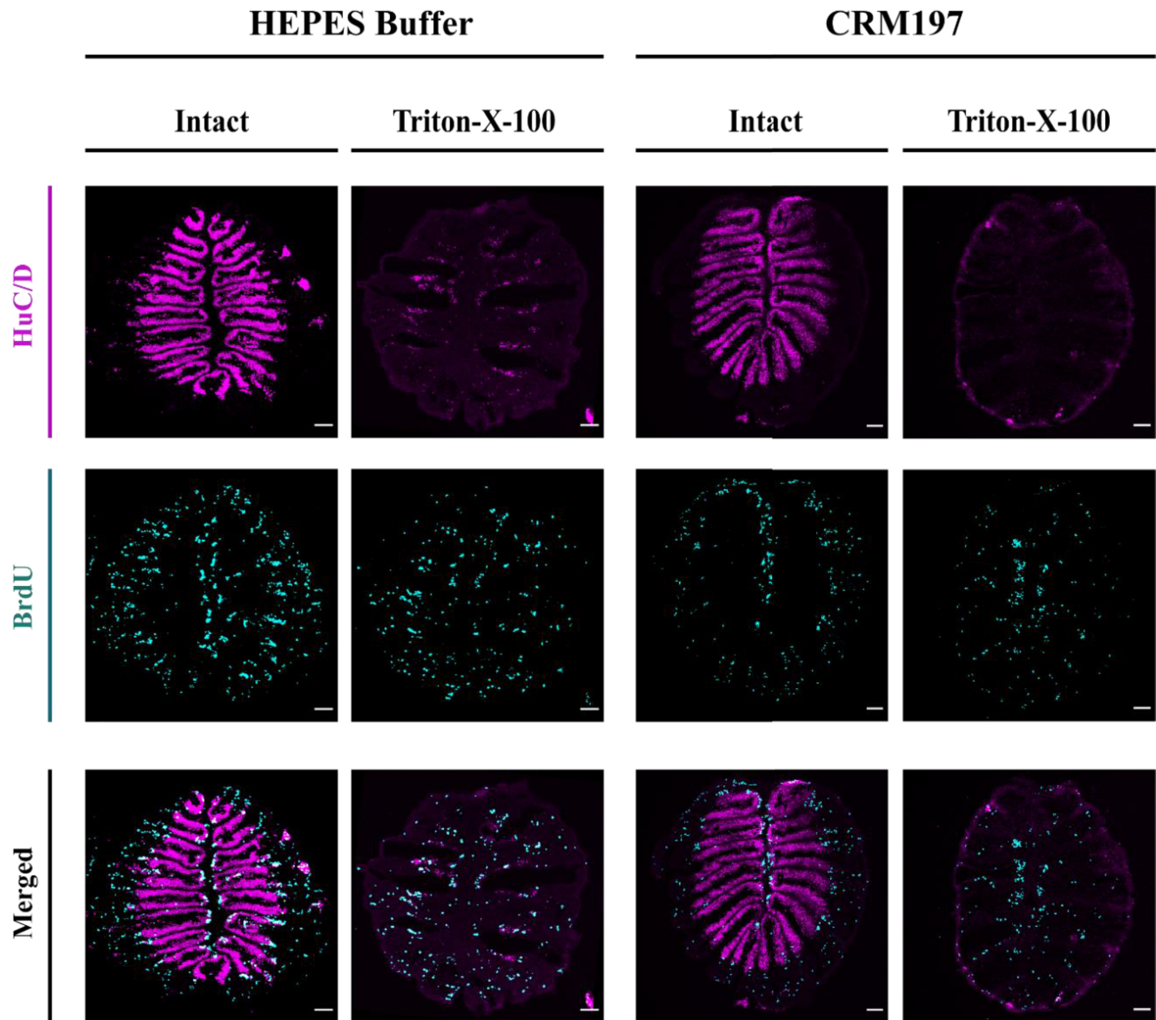

Figure 4.7. Effects of HB-EGF inhibition with CRM197 on OE at 1dpl. Immunohistochemistry against HuC/D (magenta) and BrdU (cyan) in Intact and 1% Triton-X-100 treated OE of HEPES Buffer and CRM197 injected fish. Scale bars: 50 µm.

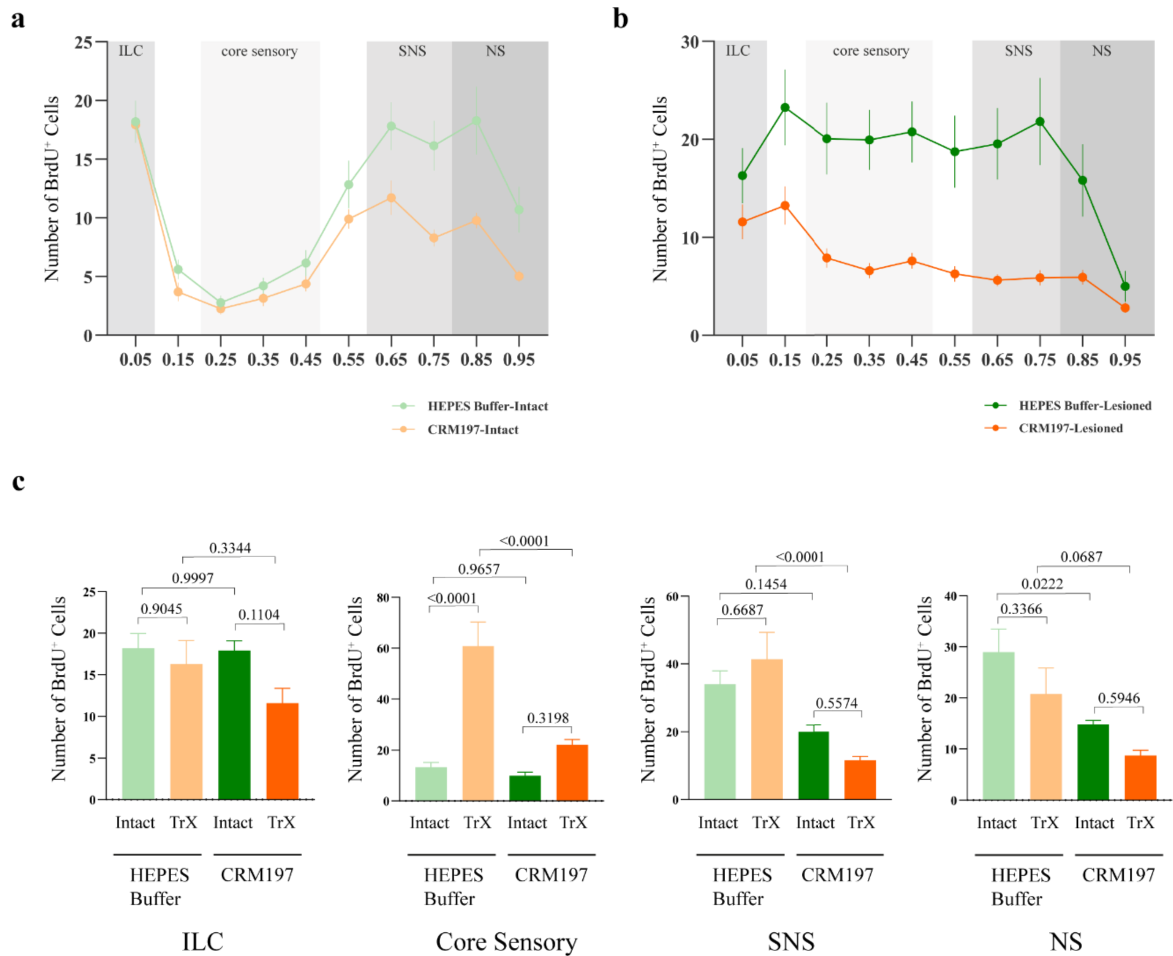

Figure 4.8. Positional profiling of BrdU$^+$ cells in Intact (a) and 1% Triton-X-100 treated (b) OE of HEPES Buffer and CRM197 injected fish at 1dpl. The graphs at the bottom detail the analysis of dividing cells separately for relevant OE regions (c).

4.1.4. Effects of CRM197 on Repair Neurogenesis in the Injured OE

Results of the CRM197 inhibition at 1 dpl support the hypothesis that HB-EGF is involved in regeneration of the OE. To see the effects of sequestering HB-EGF at a later time point, fish were subjected to a different routine of CRM197 injections and given more time to recover similar to the metalloprotease inhibition experiments described above. CRM197 (1 µg/µl dissolved in HEPES Buffer, 29 µl 1X PBS was added for total volume of 30 µl for each dose) was injected to the fish intraperitoneally 4 h before the lesion for priming, at the time of the lesion, at 24 hpl, and at 48 hpl. Fish were injected with EdU (10 mM in PBS) intraperitoneally at 48 hpl to label proliferative activity between 48 – 72 hpl. OEs were dissected at 120 hpl and analyzed for OE regeneration and mitotic activity. The

same experimental procedure was used for control animals, which were injected with HEPES buffer (1 µl in 29 µl of 1X PBS for each dose).

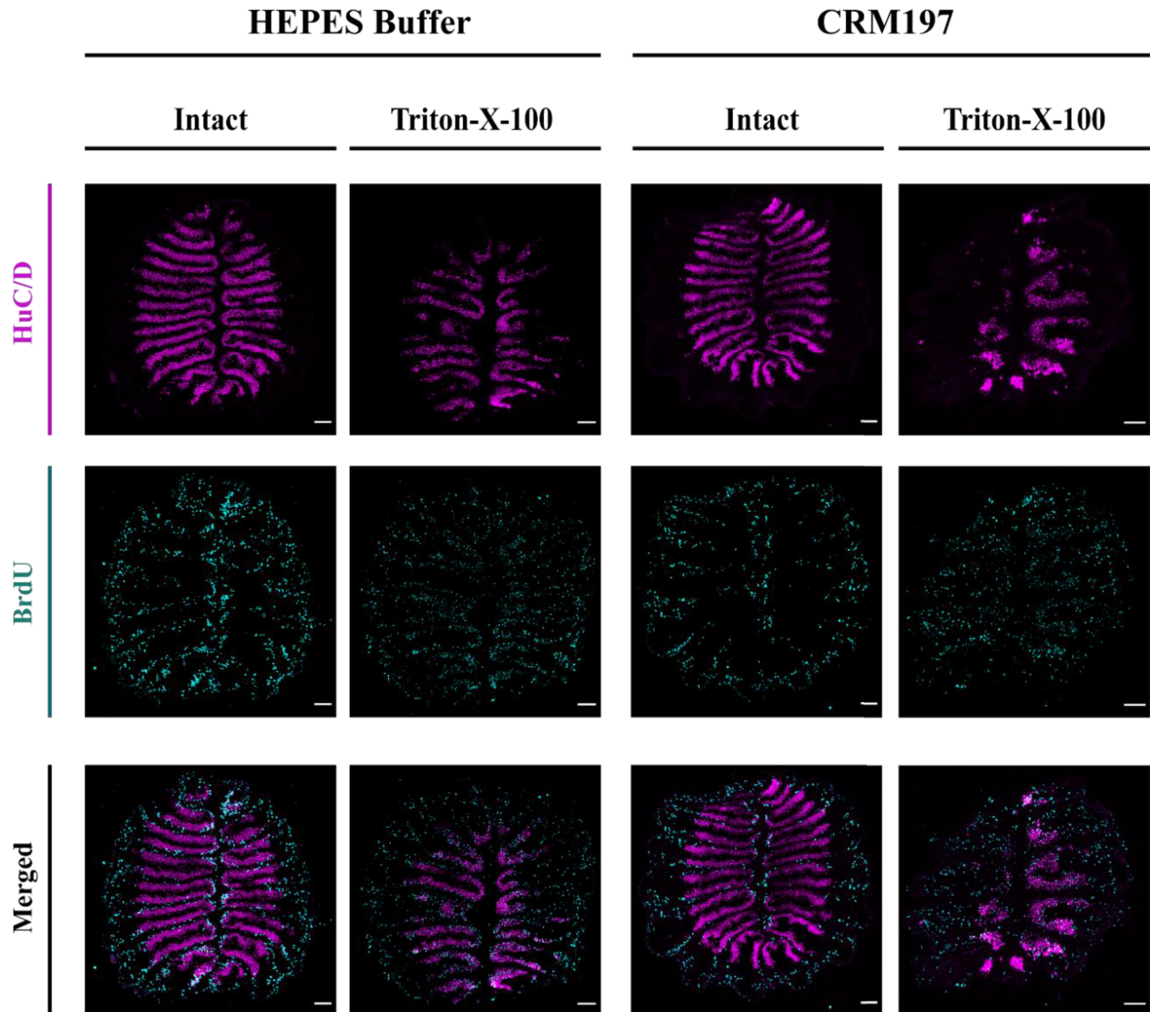

Figure 4.9. Effects of HB-EGF inhibition with CRM197 on OE regeneration at 5 dpl. Immunohistochemistry against HuC/D (magenta) and BrdU (cyan) in Intact and 1% Triton-X-100 treated OEs of HEPES Buffer and CRM197 injected fish. Scale bars: 50 µm.

Representative images of the intact and TrX-treated OEs of HEPES buffer- (left) and CRM197- injected (right) fish at 5 dpl are shown in Figure 4.9. OEs were stained with HuC/D for the detection of neurons and BrdU for proliferation analysis. Intact OEs of both groups display the expected distribution of proliferating cells at the ILC and SNS. There is no disruption in the neuronal profile. The injured OE of the control group shows an incomplete but substantial recovery, and most of the neurons are regained. In addition, a

high number of cells are labelled with BrdU, indicating a strong proliferation response. TrX-treated OEs of the CRM197 group, on the other hand, show a dispersed pattern of neurons and incomplete regeneration. The density of the HuC/D signal is low, suggesting a less efficient recovery compared to control OEs. Proliferating cells, however, appear as dense as in the control group.

The changes in proliferative activity were assessed by counting $BrdU^+$ cells with the method mentioned above. Figure 4.10a, b shows the distribution of proliferating cells in the intact and injured OEs of HEPES Buffer- (green and dark green) and CRM197-treated (orange and dark orange) fish, respectively. Both the intact and the injured OEs of CRM197-treated fish present similar, almost identical curves to the control group animals. No significant difference between the proliferation rates of control and CRM197-treated animals were found (For intact OEs; ILC: from 33.0 ± 2.4 to 22.7 ± 2.1, Tukey HSD adjusted p-value = 0.3264, Core sensory: from 21.2 ± 2.0 to 15.0 ± 1.2, Tukey HSD adjusted p-value = 0.9707, SNS: from 44.4 ± 3.3 to 39.2 ± 3.6, Tukey HSD adjusted p-value = 0.8278, NS: from 36.8 ± 3.2 to 42.5 ± 2.7, Tukey HSD adjusted p-value = 0.6578) (For injured OEs; ILC: from 66.9 ± 4.8 to 59.4 ± 6.4, Tukey HSD adjusted p-value = 0.6054, Core sensory: from 186.0 ± 15.1 to 157.9 ± 12.7, Tukey HSD adjusted p-value = 0.1935, SNS: from 61.0 ± 5.5 to 54.4 ± 4.5, Tukey HSD adjusted p-value = 0.6958, NS: from 46.2 ± 4.6 to 36.1 ± 3.1, Tukey HSD adjusted p-value = 0.1775). CRM197 treatment did not affect the proliferation in intact or injured OEs.

In order to see if there is a change in the number of newborn neurons, $HuC/D^+/BrdU^+$ cells were counted and the distribution of double positive cells are displayed in Figure 4.10 c, d. While the distribution of double-positive cells for intact OEs of control (green) and CRM197-injected (orange) groups are nearly the same, the injured OE of the CRM197-injected (dark orange) fish shows a notable decline in the number of $HuC/D^+/BrdU^+$ cells. Among all regions, only the SNS showed a significant difference between intact OEs of the two animal groups (ILC: from 22.1 ± 2.4 to 15.6 ± 1.2, Tukey HSD adjusted p-value = 0.5218, Core sensory: from 14.2 ± 1.9 to 10.0 ± 1.0, Tukey HSD adjusted p-value = 0.9858, SNS: from 14.2 ± 3,1 to 3,7 ± 0.9, Tukey HSD adjusted p-value = 0.0032, NS: from 5.9 ± 2.2 to 1.1 ± 0.7, Tukey HSD adjusted p-value = 0,1439).

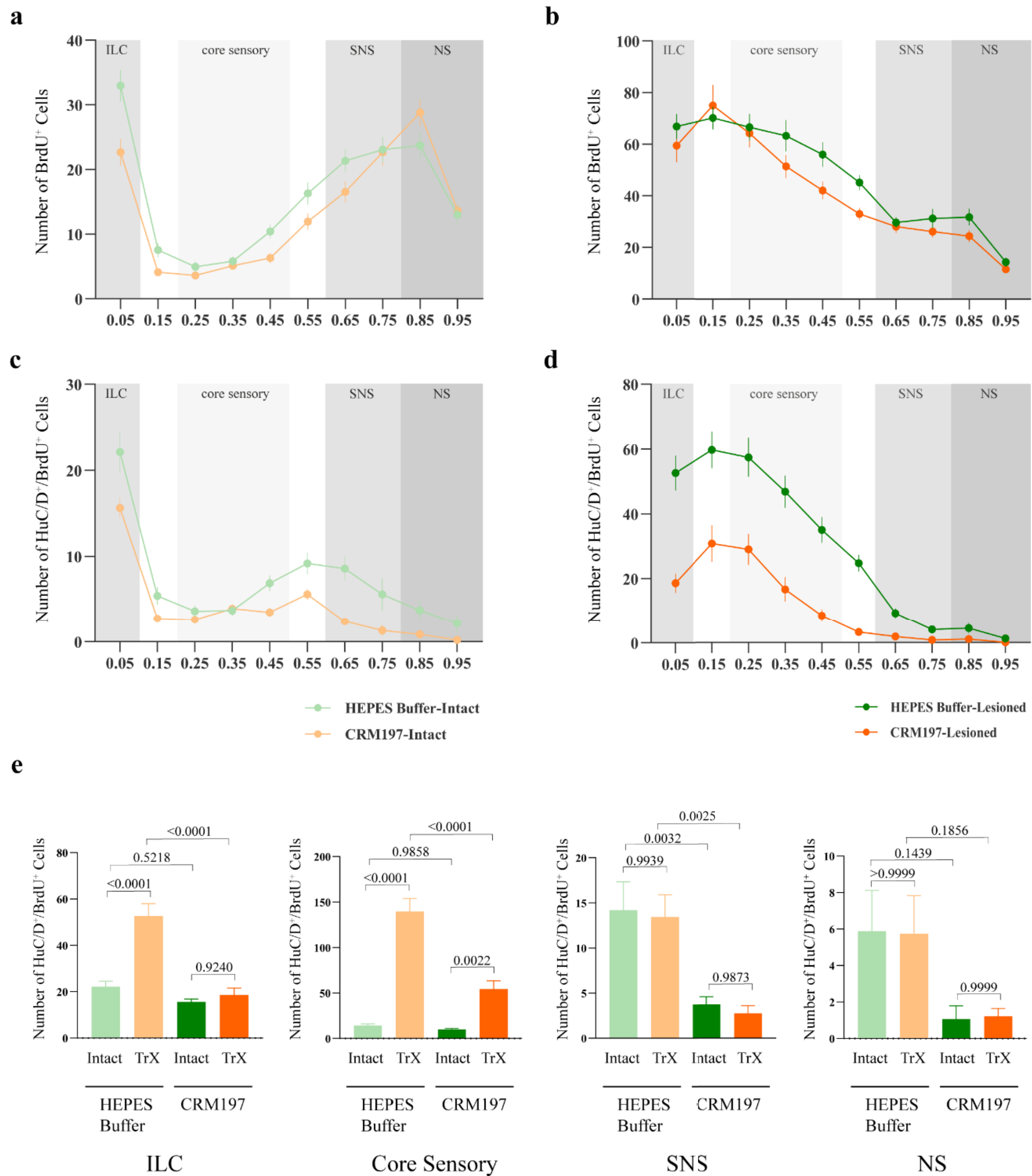

Figure 4.10. Positional profiling of BrdU$^+$ cells in Intact (a) and 1% Triton-X-100 treated (b) OE of HEPES Buffer and CRM197 injected fish at 5 dpl. Positional profiling of BrdU$^+$/HuC/D$^+$ cells in Intact (c) and 1% Triton-X-100 treated (d) OE of HEPES Buffer and CRM197 injected fish at 5 dpl. The graphs at the bottom detail the analysis of BrdU$^+$/HuC/D$^+$ cells separately for relevant OE regions (e).

On the other hand, a uniform and notable decline in the number of HuC/D$^+$/BrdU$^+$ cells were observed in the ILC (from 52.6 ± 5.4 to 18.6 ± 3.0, Tukey HSD adjusted p-value

<0.0001), core sensory (from 139.6 ± 14.3 to 54.4 ± 9.2, Tukey HSD adjusted p-value <0.0001), and SNS (from 13.4 ± 2.5 to 2.7 ± 0.9, Tukey HSD adjusted p-value = 0.0025) of the injured OE of CRM197-treated fish. Only the NS did not show a significant change between injured OEs (from 5.8 ± 2.1 to 1.2 ± 0.4, Tukey HSD adjusted p-value = 0,1439). The reduced number of newborn neurons in CRM197-treated fish at 5 dpl suggested that HB-EGF signaling is necessary for the differentiation of proliferating cells to neurons after injury.

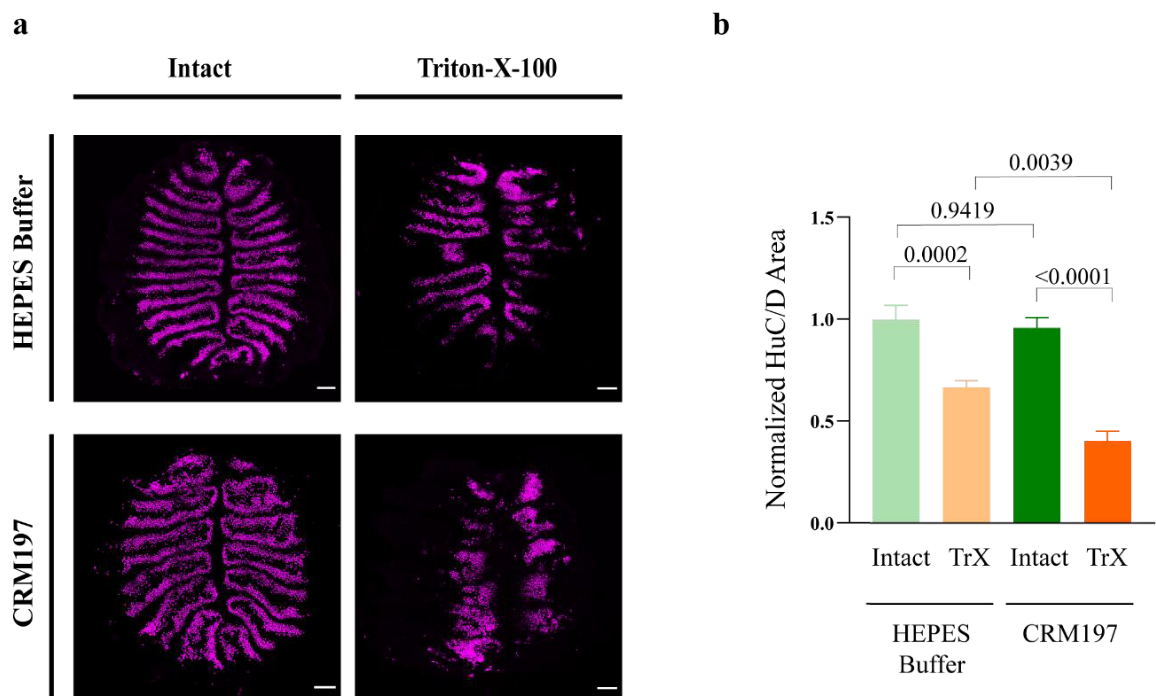

Figure 4.11. Comparison of the area covered by HuC/D^{+} cells in Intact and 1% Triton-X-100 treated OE of HEPES Buffer- and CRM197-injected fish at 5dpl (a). Analysis of normalized HuC/D area in each group (b).

For the analysis on the effects of CRM197 on OSN recovery, HuC/D^{+} signal was measured and compared to the controls. In Figure 4.11a, neurons present a more organized pattern at 5 dpl in the control group. However, CRM197 treated group shows an evidently smaller number of neurons at the same time point after injury. Values of normalized HuC/D area for different groups are shown in Figure 4.11b. While the injured OE of the control group shows 67% (0.67 ± 0.03 normalized to the intact OE of the control) recovery of neurons, the number is reduced to 40% (0.40 ± 0.05) in the CRM197-treated group. These results suggests that masking of HB-EGF activity results in a significantly reduced rate of

neuronal recovery at 5 dpl (ANOVA: $F_{(3, 56)} = 28.58$, $p<0.0001$, Tukey HSD adjusted p-value = 0.0039).

4.2. Effects of External HB-EGF Stimulation on *hbegfa* and *ascl1a* Expression

In the first part of this study, an analysis of HB-EGF loss-of-function at two different steps of HB-EGF signaling was presented. Both inhibition of HB-EGF shedding and intercellular HB-EGF signaling resulted in the reduction of repair neurogenesis in the OE, suggesting that HB-EGF signaling is necessary for the induction of the regenerative response. To understand whether HB-EGF is also sufficient to induce OSN neurogenesis, gain-of-function experiments were carried out to analyze if increased HB-EGF activity positively affects regeneration. Previously, the effects of intranasal stimulation with human recombinant HB-EGF on proliferation and the rate of newborn neurons were investigated in our lab. The results indicated that external HB-EGF administration to the OE was sufficient to trigger an increase in proliferation and number of newborn neurons (Kocagöz, 2022).

It is already known and shown in our lab that *hbegfa* expression is upregulated shortly after damage (Guler, 2022). Studies in the zebrafish retina also showed that HB-EGF signaling is involved in the repair of the retina via a positive feedback mechanism (Wan *et al*, 2012.). In addition, external stimulation with HB-EGF resulted in enhanced proliferation (Kocagöz, 2022). In the light of this knowledge, we aimed to find out more about the mechanism regulating increase of HB-EGF levels and to test if there is a similar feedback system of HB-EGF signaling in the OE.

4.2.1. Effects of External HB-EGF Stimulation on *hbegfa* Expression

To test whether a positive feedback mechanism of HB-EGF expression exists in the zebrafish OE, OEs were stimulated with human recombinant HB-EGF and the expression of *hbegfa* was analyzed by *in situ* hybridization. One OE of the fish was irrigated with 200 ng/µl human recombinant HB-EGF (dissolved in 0.1% BSA) for 30 min, and the contralateral OE was irrigated with 0.1% BSA to serve as the internal control. Fish were kept alive by constant perfusion with a reduced concentration of the anesthetic MS222 over the course of the experiment for 30 min. Nasal cavities were filled with a total of approximately 3 µl HB-EGF or BSA solution for 30 min of irrigation, to prevent OEs from drying out over

the duration of the experiment. OEs were dissected 4 hours after nasal irrigation and analyzed by *in situ* hybridization against *hbegfa* combined with tp63 immunostaining.

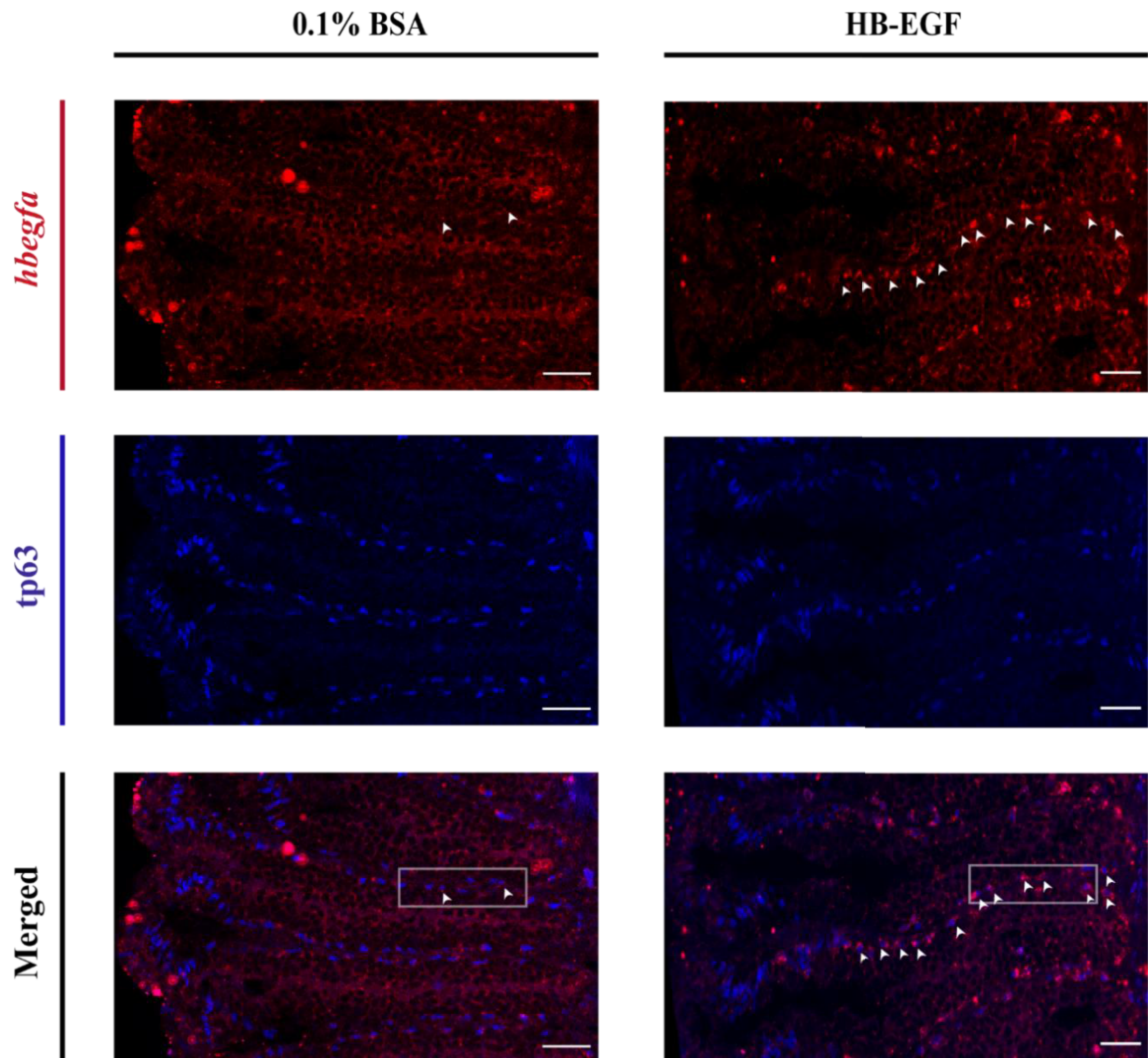

Figure 4.12. *In situ* hybridization against *hbegfa* (red) 4 h after stimulation with human recombinant HB-EGF (right) and 0.1% BSA as control (left). Immunostaining against tp63 (blue) for detection of HBCs. Scale bars are 30 µm.

Figure 4.12 shows the changes in *hbegfa* expression (red) after stimulation with human recombinant HB-EGF protein. BSA control presents an image consistent with the steady-state expression of *hbegfa*, with a low number of isolated cells that can be observed in the most basal layers of the epithelial folds (shown with arrowheads). Tp63 staining (blue) selectively labels HBCs, which are colocalized with the small number of *hbegfa*$^{+}$ cells in the BSA control group. HB-EGF stimulated OEs, on the other hand, show a substantial increase

in the number of *hbegfa* expressing cells, especially in HBCs, as indicated by increased colocalization with tp63$^+$ structure. These results indicated that HB-EGF stimulation of the OE causes an upregulation of *hbegfa* expression in HBCs.

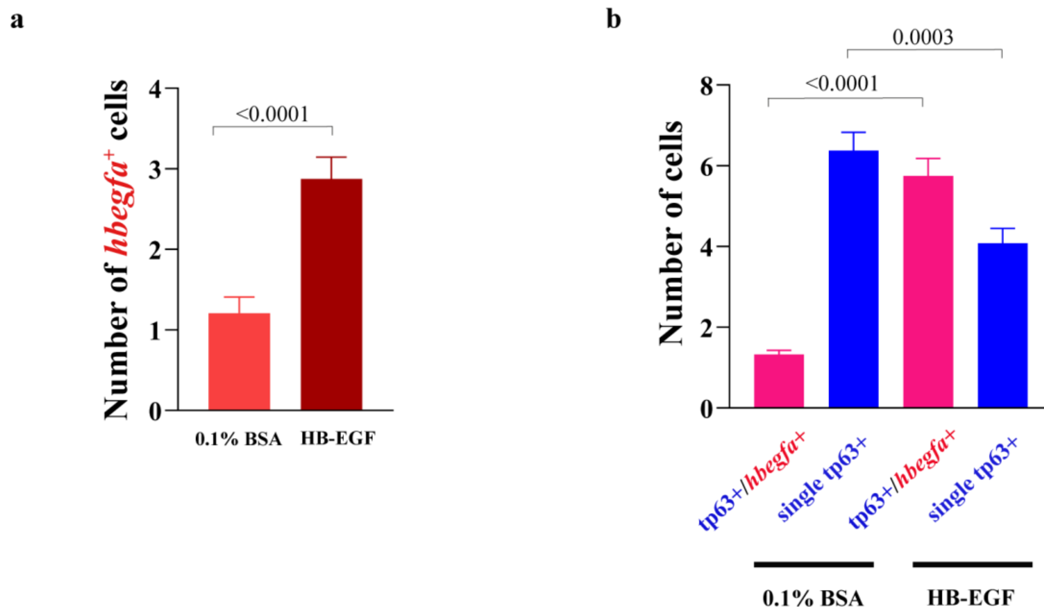

Figure 4.13. Counts of *hbegfa*$^+$ (a), tp63$^+$, and *hbegfa*$^+$/tp63$^+$ cells (b) in BSA control and HB-EGF stimulated OEs after at 4 hps (hours post stimulation).

To determine the statistical significance of *hbegfa* upregulation after HB-EGF stimulation, a region of 80 µm between ILC and SNS was cropped from each epithelial fold. 24 folds were counted for 2 fish (12 folds per fish). Comparison of the counts of *hbegfa*-expressing cells in BSA- and HB-EGF- irrigated OEs are shown in Figure 4.13a. The number of *hbegfa*$^+$ cells increased 2.3-fold from 1.2 ± 0.2 to 2.9 ± 0.3, which was found to be significant by unpaired, two-tailed t-test (t_{46}=4.950, $p < 0.0001$).

The analysis of tp63$^+$/*hbegfa*$^+$ double positive cells are shown in Figure 4.13b. The number of tp63$^+$/*hbegfa*$^+$ double positive cells increased from 1.3 ± 0.1 in the BSA control to 5.8 ± 0.4 in HB-EGF stimulated OEs, which was a notable increase (unpaired, two-tailed t-test, t_{46}=9.993, $p < 0.0001$). The comparison between single tp63$^+$ cells was also complementary with these results. The number of single tp63$^+$ cells decreased from 6.4 ± 0.5 in the control to 4.1 ± 0.4 in HB-EGF stimulated OEs as the number of double-positive cells increased by the same rate (unpaired, two-tailed t-test, t_{46}=3.912, $p = 0.0003$). These

results supported the hypothesis that HB-EGF stimulation induces *hbegfa* upregulation in HBCs by a positive feedback mechanism.

4.2.2. Effects of External HB-EGF Stimulation on *ascl1a* Expression

HBCs respond to the injury with cell proliferation to generate a transient population of ascl1-positive cells of the early OSN lineage (Kocagöz et al., 2022). In order to examine whether *hbegfa* upregulation upon HB-EGF stimulation also results in an increase of proliferating and differentiating GBCs, in situ hybridization against *ascl1a* was performed at 48 hps. The stimulation procedure was the same as previous section. One OE was irrigated with 200 ng/µl HB-EGF, while the other OE was irrigated with 0.1% BSA as internal control. Irrigation continued for 30 min, with the continuous administration of solutions to prevent drying of the OEs. This procedure was repeated once after 24 h and 48 hours after the second stimulation, OEs were dissected, and analyzed by *in situ* hybridization against *ascl1a*.

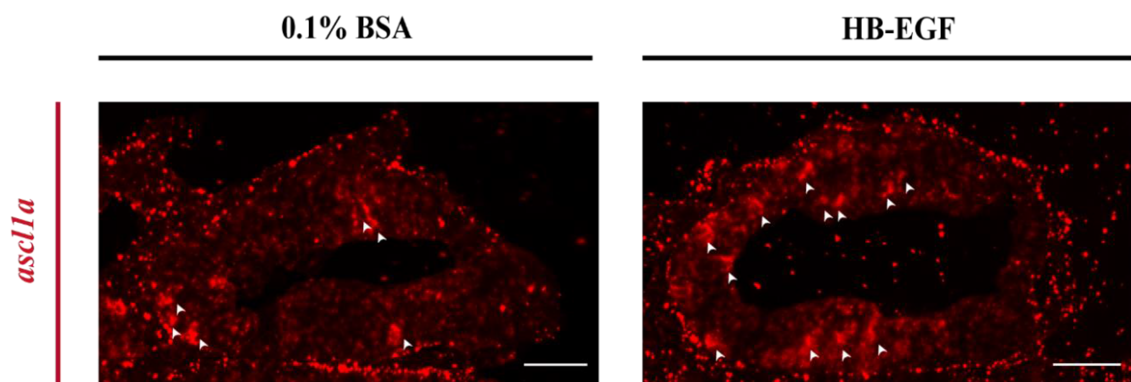

Figure 4.14. *In situ*-hybridization against *ascl1a* (red) 48h after stimulation with human recombinant HB-EGF (right) and 0.1% BSA as control (left). Scale bars are 30 µm.

The change in *ascl1a* expression at 48 hours after HB-EGF stimulation is presented in Figure 4.14. As shown in the figure, the control OE that was irrigated with BSA shows the regular pattern of *ascl1a* expression with positive cells that can be found at the ILC and SNS (Bayramli et al., 2017; arrowheads). For the BSA control OE, arrowheads show the *ascl1a*$^+$ cells that are located in the ILC and SNS, where transit-amplifying GBCs are found under steady-state conditions. In the HB-EGF stimulated OE, more cells expressing *ascl1a* could be observed. Besides the increase in number, *ascl1a*$^+$ cells were found to be localized in the sensory region in addition to the ILC and SNS regions. Since there was no staining against

an HBC or GBC marker, the identity of these cells could not be determined further. However, taken together with the previous section, it could be speculated that HB-EGF stimulation increases the number of transit-amplifying GBCs by inducing HBC proliferation.

4.3. Effects of External Stimulation with Cytokines on OE Proliferation

The experiments described above suggest that HB-EGF is critically involved in the tissue response to injury by inducing repair neurogenesis. However, the mechanism of HB-EGF induction and the potential synergistic effects with other signaling molecules is unknown. Transcriptome data of the regenerating OE revealed that specific cytokines are also strongly and transiently upregulated shortly after OE damage. Although the role of the immune system in repair neurogenesis in the OE has not been studied, it was interesting to understand whether cytokines function only as messenger molecules or they have a crucial regulatory role in the repair of the injured OE. The strongest and most robust upregulated cytokines after damage to the OE are IL-6, Leptin, and IL-11. Effects of IL-6 on proliferation were investigated recently in our lab and IL-6 was found to cause an induction in the total proliferation rate and most likely by inducing HBC activity (Demirler, 2021). There is also supporting evidence from other studies that Leptin and IL-11 work synergistically with HB-EGF in the regulation of cell proliferation and adult neurogenesis in the zebrafish retina (Zhao *et al*, 2014). Considering these findings, the main aim of this part was to examine whether Leptin and IL-11 have individual and/or synergistic effects with HB-EGF on cell proliferation in the OE.

4.3.1. Effects of Intranasal Leptin Stimulation on Proliferation in the OE

For the examination of the potential effect of Leptin on OE proliferation, an intranasal irrigation method was used. One OE was stimulated with approximately 3 µl of 1 µg/µl Leptin (dissolved in 0.1% BSA) while the other OE served as an internal control and irrigated with about 3 µl of 0.1% BSA. Nasal cavities were filled with these solutions and OEs were irrigated in regular intervals for 30 minutes, to prevent drying out of the tissue. To label proliferating cells, fish were kept in BrdU-containing water for the first 24 hours after the stimulation. OEs were dissected at 24 hps and analysis was carried out (n=3).

OE sections were stained against the pan-neuronal marker HuC/D, the proliferation marker BrdU, and the HBC marker tp63. Confocal images of the sections are shown in

Figure 4.15a. The comparison of control (left) and Leptin-treated (right) OEs does not show any obvious difference for the visual analysis. As seen from the figure, the area covered by HuC/D^+ is representative of the intact OE and shows the regular pattern of neurons from the ILC until the SNS, which means nasal irrigation did not result in damage to the neurons. The $BrdU^+$ cells also show the typical maintenance pattern of cell proliferation with activity at the ILC and SNS. The signals of tp63 are localized in the most basal parts of the folds, which is observed in steady state, for both the control and Leptin-treated groups.

In order to check if there is any significant change in the proliferation with Leptin treatment, the number of $BrdU^+$ cells were positionally counted from cropped ROIs of hemi-OEs. Results of the analysis are presented in Figure 4.15b, c. The distribution of $BrdU^+$ cells along the radial axis of the OE can be seen in Figure 4.15b. While BSA control group (green) displays the standard bimodal distribution with peaks in the ILC and SNS, Leptin-treated group (orange) shows a very similar image.

The number of $BrdU^+$ cells in each region of the hemi-OEs were compared individually, to determine the changes in proliferative activity. As Figure 4.15c clearly demonstrates, no significant change in the number of $BrdU^+$ cells were found in any defined regions (unpaired, two-tailed t-test; ILC: from 17.2 ± 1.1 in the control to 17.4 ± 1.1 in the Leptin-treated OEs, t_{58}=0.1162, p = 0.9079. Core sensory: from 9.7 ± 1.0 to 11.1 ± 1.5, t_{58}=0.7759, p = 0.4409. SNS: from 24.0 ± 1.3 to 27.5 ± 1.9, t_{58}=1.471, p = 0. 1468. NS: from 13.7 ± 1.8 to 16.0 ± 1.3, t_{58}=1.090, p = 0.2803.).

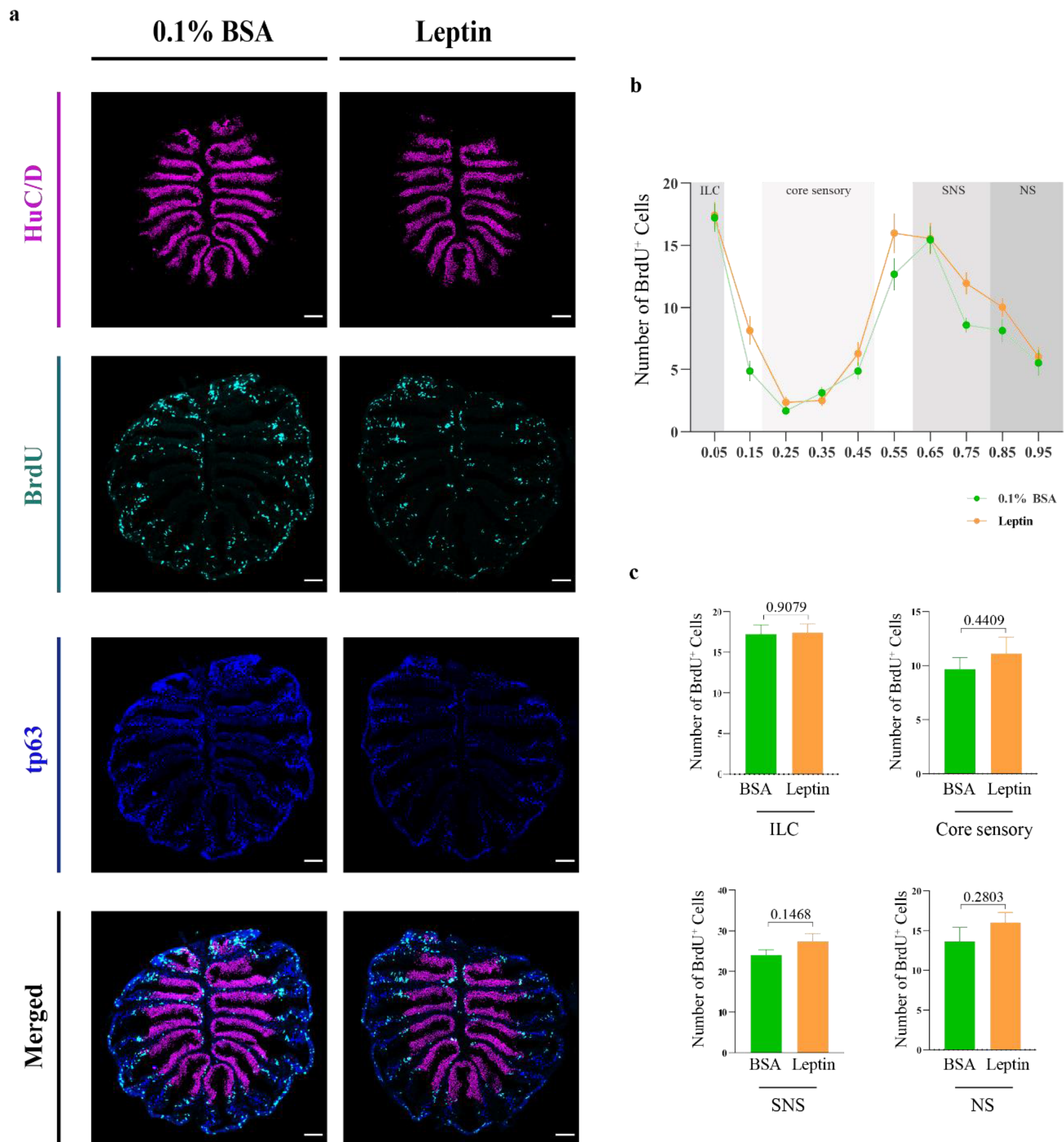

Figure 4.15. Effects of intranasal Leptin stimulation on OE proliferation at 24 hps (a). Immunohistochemistry against HuC/D (magenta), BrdU (cyan), and tp63 (blue) in BSA and Leptin treated OEs. Scale bars: 50 µm. Positional profiling of BrdU$^+$ cells in BSA and Leptin treated OEs at 24 hps (b) with the analysis of BrdU$^+$ cells separately for relevant OE regions (c).

4.3.2. Effects of Intraperitoneal Leptin Administration on Cell Proliferation in the OE

Nasal irrigation with Leptin did not show any significant effects on cell proliferation. However, this observation does not generally rule out any potential role of the Leptin in

regeneration, and the failure to detect effects may be due to limitations of the stimulation procedure. One disadvantage is that the intranasal administration of any substance to the OE is leaky because the fluid in the nasal cavity has a natural flow (Reiten *et al*., 2017), and the administered solution gets diluted once entered to the cavity. In addition, the cytokine may not be able to penetrate the tissue to reach relevant HBCs in the basal OE. Yet, it was still a preferrable method since it enables the direct stimulation of the OE, bypassing the limitations of the bloodstream. Considering the factors affecting the efficiency of intranasal administration of Leptin, another delivery method was tested. For this part, 2 µg of Leptin combined with 10 mM EdU was intraperitoneally injected to the fish (n=2). Animals were incubated in fresh fish water for 24 hours and the analysis was carried out at 24 hpi. The same procedure was repeated with 0.1% BSA injection for the control group.

Results of the IP injections of Leptin can be found in Figure 4.16. OE sections stained against HuC/D (magenta) and EdU (cyan) are shown in Figure 4.16a. The HuC/D staining pattern displays an intact pattern and indicates that there was no damage in the OE. Similarly, EdU^+ cells show the maintenance proliferation pattern for the BSA group. The OE of the Leptin-injected group has slightly more EdU^+ cells in the ILC, with the regular pattern of proliferation in the rest of the tissue. EdU^+ cells were counted from hemi-OE crops for the statistical analysis. EdU^+ cell profiles of BSA- and Leptin-injected OEs are shown in Figure 4.16b. Graphs of each treatment group appeared to be very similar to each other, even though the cell counts at the ILC for Leptin-injected animals is shifted slightly towards the margins of the tissue. However, no significant change between two groups were found in the statistical analysis (unpaired, two-tailed t-test, $p = 0.6866$, $t_{18}=0.4100$.).

The number of EdU^+ cells for each determined region of the OE is shown in Figure 4.16c. Leptin-injected OEs showed a minor decrease in all regions except SNS. However, analysis indicated that the decrease was not significant for regions other than the core sensory (unpaired, two-tailed t-test, ILC: from 15.0 ± 1.2 to 12.3 ± 1.1, $t_{38}=1.656$, $p = 0.1059$. Core sensory: from 7.3 ± 0.8 to 4.4 ± 0.8, $t_{38}=2.590$, $p = 0.0135$. SNS: from 23.9 ± 1.9 to 26.2 ± 3.1, $t_{38}=0.6462$, $p = 0.5220$. NS: from 13.7 ± 2.4 to 11.3 ± 1.7, $t_{38}=0.8235$, $p = 0.4153$.). Together these results indicated that Leptin has no detectable individual effect on the proliferation rate in the OE.

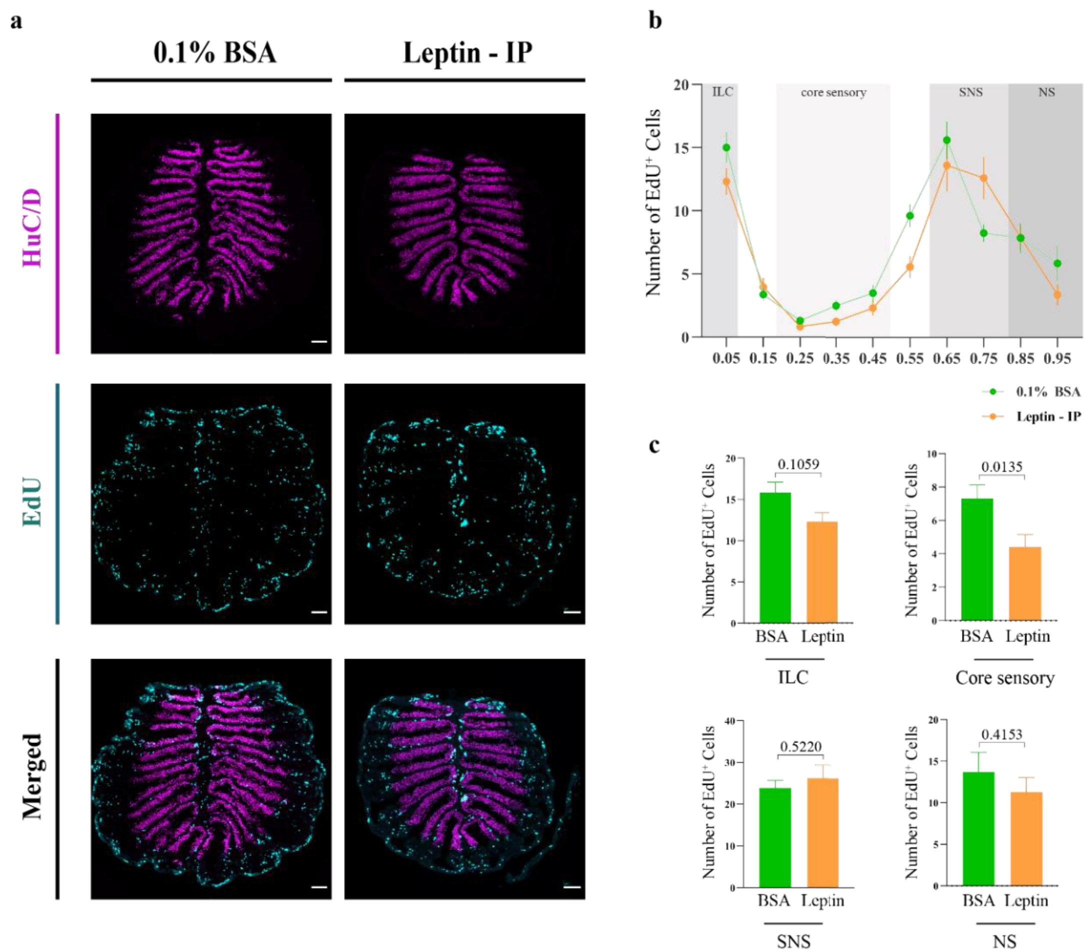

Figure 4.16. Effects of intraperitoneal Leptin injection on OE proliferation at 24 hpi (a). Immunohistochemistry against HuC/D (magenta) and EdU (cyan) in OEs of BSA- and Leptin- injected fish. Scale bars: 50 µm. Positional profiling of EdU$^+$ cells in OEs of BSA- and Leptin- injected fish at 24 hpi (b), with the analysis of EdU$^+$ cells separately for relevant OE regions (c).

4.3.3. Effects of IL-11 Stimulation on Proliferation in the OE

IL-11 is a cytokine in the family of IL-6 proteins. In addition to its role in inflammatory responses and development of hematopoietic cells, IL-11 is also known to have pro-regenerative effects (Tsujioka et al, 2017). Previously, it was reported to work synergistically with other IL-6 family cytokines and HB-EGF in a model zebrafish retina regeneration (Zhao *et al*, 2014). It is also one of the cytokines that is transiently and strongly upregulated in the OE after damage (Kocagöz et al., 2022). These findings have led to the hypothesis that Il-11 could have a regulatory role in the regeneration of zebrafish OE upon damage.

To understand whether IL-11 has a role in the proliferation in the OE, a nasal irrigation method was employed. One of the OEs was stimulated with approximately 3 µl of 250 ng/µl IL-11 (dissolved in 0.1% BSA) and the other OE was irrigated with 0.1% BSA as the control (n=3). Both OEs were continuously irrigated with the respective solutions for 30 minutes. Animals were kept in BrdU containing fish water for 24 h following stimulation. OEs were dissected at 24 hps and the analysis was carried out.

Figure 4.17 presents results of the proliferation analysis on the effects of IL-11. BSA- and IL-11-treated OEs stained for HuC/D (magenta), BrdU (cyan), and tp63 (blue) can be observed in Figure 4.17a. Both the control and IL-11-treated groups show the expected phenotype of neurons and proliferating cells for the intact condition. There is no visible difference between $BrdU^+$ cells of the two conditions. HuC/D signal is not interrupted, which rules out the possibility of mechanical damage to the OE. $tp63^+$ cells are observed at the most basal parts of the folds, as expected.

$BrdU^+$ cells were for further analysis. Distribution of proliferating cells along the radial index can be seen in Figure 4.17b. Similar to the Leptin stimulation, graphs of the BSA- (green) and IL-11- treated (orange) OEs resemble each other. There is no noticeable difference from the control. The statistical analysis also did not indicate any significant change in IL-11-treated OEs (unpaired, two-tailed t-test, $p = 0.8547$, $t_{18}=0.1858$.).

The distribution of $BrdU^+$ cells in designated regions of the OE is shown in Figure 4.17c. A distinct change in the core sensory region can be observed, the number of proliferating cells go down from 14.7 ± 2.2 to 8.9 ± 1.0 in the IL-11-treated OE (unpaired, two-tailed t-test, $p = 0.0193$, $t_{58}= 2.407$). The other significant change is detected in the NS, with 16.6 ± 1.4 in the control, increasing to 24.8 ± 2.9 in the IL-11-treated OE ($p = 0.0141$, $t_{58}= 2.530$). The other regions did not show any noteworthy changes (ILC: from 18.2 ± 1.4 to 16.3 ± 1.1, $t_{58}=1.058$, $p = 0.2945$. SNS: from 24.2 ± 1.6 to 28.2 ± 2.2, $t_{58}=1.483$, $p = 0.1436$.). While the change in the core sensory region could be meaningful for the change of proliferation mode, the significance is not high enough to make a strong claim. These results were not convincing enough to suggest that IL-11 have a role in proliferation in the OE.

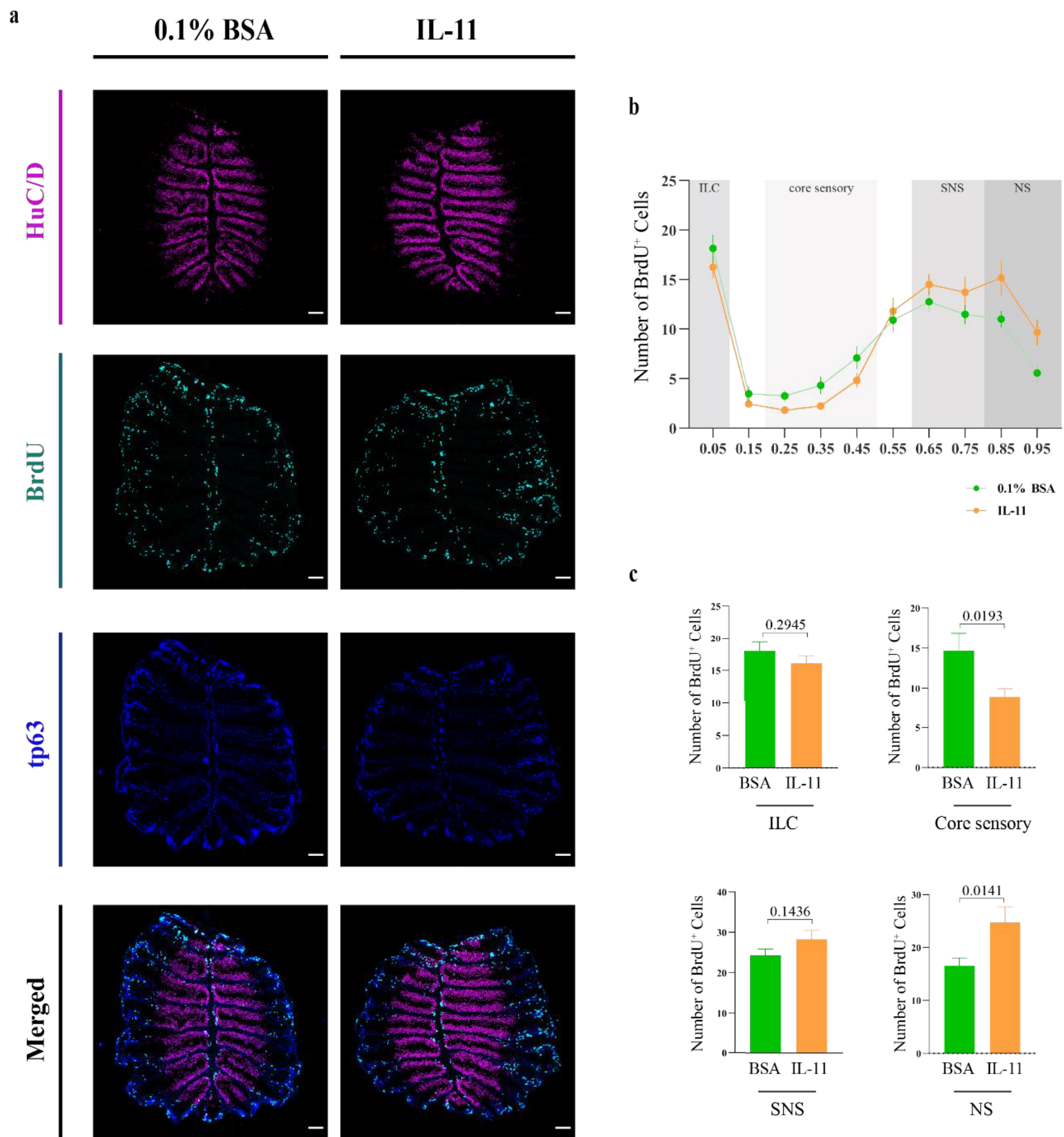

Figure 4.17. Effects of intranasal IL-11 stimulation on OE proliferation at 24 hps (a). Immunohistochemistry against HuC/D (magenta), BrdU (cyan), and tp63 (blue) in BSA and IL-11 treated OEs. Scale bars: 50 µm. Positional profiling of BrdU$^+$ cells in BSA and IL-11 treated OEs at 24 hps (b), with the analysis of BrdU$^+$ cells separately for relevant OE regions (c).

4.3.4. Effects of Leptin and HB-EGF Combination on Proliferation in the OE

In order to investigate the possible interactions and synergism between cytokines and HB-EGF, a combined stimulation approach was followed. Animals were injected

intraperitoneally with 2 μg of Leptin, and one OE was simultaneously stimulated with approximately 3 μl of 200 ng/μl human recombinant HB-EGF. The other OE was stimulated with the same amount of 0.1% BSA for the internal control. The nasal irrigation procedure was the same as single HB-EGF stimulation protocol. Right after the HB-EGF stimulation and Leptin injection, animals were injected intraperitoneally with EdU, in order to label proliferating cells. Animals (n=2) were sacrificed at 24 hps and OEs were dissected.

OEs were stained against HuC/D (magenta) and EdU (cyan) as shown in Figure 4.18a. HuC/D signal confirmed that no damage was given to OEs in either condition. HuC/D$^+$ cells show an uninterrupted pattern, indicating that OEs were not damaged. EdU$^+$ cells displayed comparably similar patterns and amount in BSA control and Leptin+HB-EGF stimulated OEs. In order to check if there was a quantifiable difference, EdU$^+$ cells were counted. The graph in Figure 4.18b shows the radial distribution of EdU$^+$ cells in BSA (green) and Leptin+HB-EGF stimulated (orange) OEs. A minor reduction can be seen in the stimulated OEs, especially visible in the core sensory and SNS. However, no statistically significant difference could be found when the total numbers of control and stimulated OEs were compared (unpaired, two-tailed t-test, t_{18}=0.1858, p = 0.1501.).

Number of EdU$^+$ cells in separate regions are presented in Figure 4.18c. A noteworthy reduction in the EdU$^+$ cells was found in all regions except NS. The number of EdU$^+$ cells decreased from 17.2 ± 1.3 to 11.8 ± 1.0 in the ILC (t_{38}=3.302, p = 0.0021). A similar reduction was observed in the core sensory region, from 12.4 ± 1.3 in the control to 7.0 ± 0.9 in the stimulated group (t_{38}=3.387, p = 0.0017). The most drastic decrease was found in the SNS, from 22.9 ± 1. 7 to 13.6 ± 1.6 (t_{38}=4.056, p = 0.0002). NS did not show any critical changes (from 24.7 ± 2.0 to 19.7 ± 2.0, t_{38}=1.781, p = 0.0829). Since we previously showed that HB-EGF stimulation increases the proliferation, these results might indicate that Leptin does not work in parallel to HB-EGF, if they interact at all.

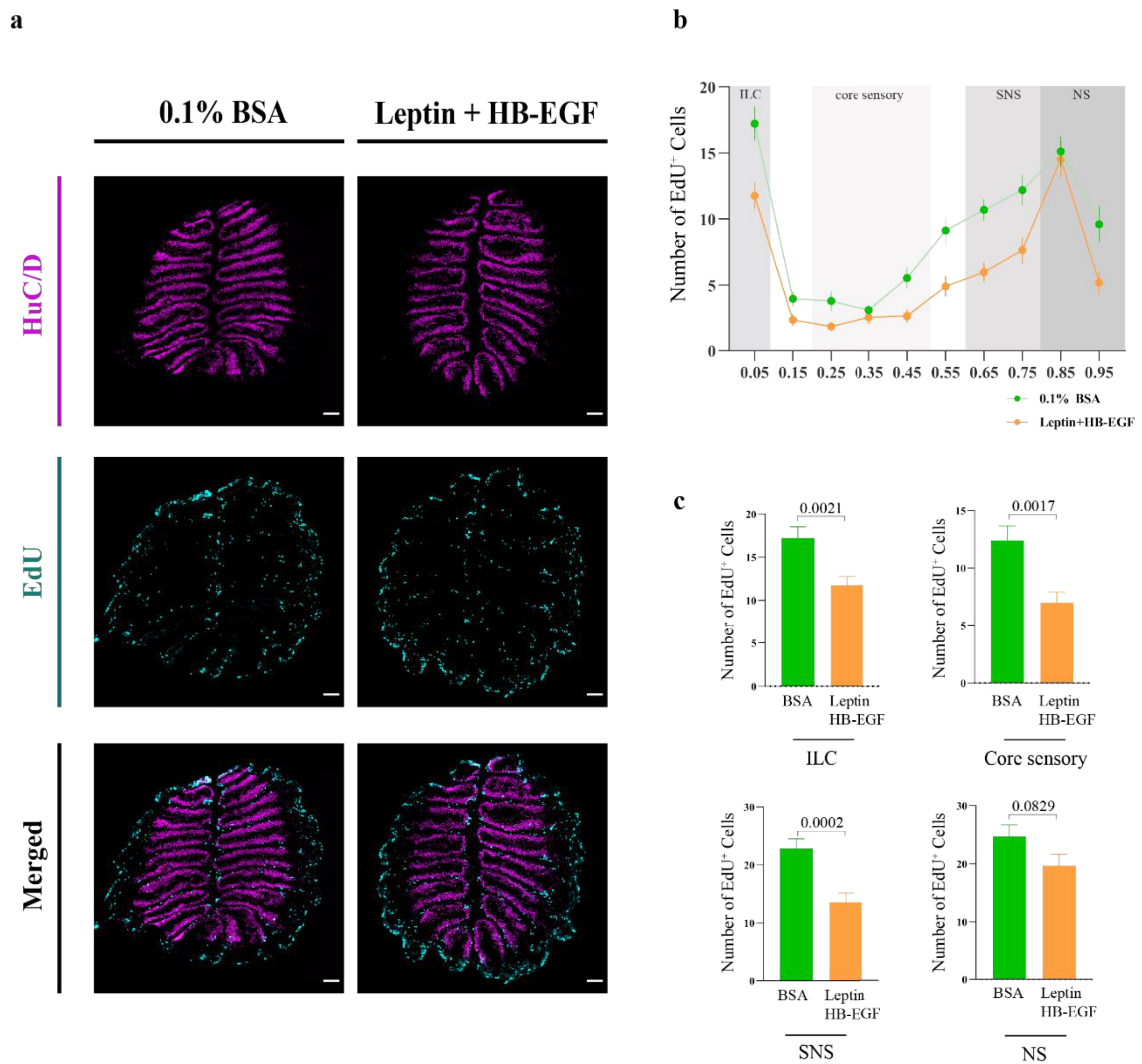

Figure 4.18. Effects of stimulation with the combination of Leptin and HB-EGF on OE proliferation at 24 hps (a). Immunohistochemistry against HuC/D (magenta) and EdU (cyan) in OEs of BSA and Leptin+HB-EGF stimulated fish. Scale bars: 50 µm. Positional profiling of EdU^+ cells in OEs of BSA and Leptin+HB-EGF stimulated fish at 24 hps (b), with the analysis of EdU^+ cells separately for relevant OE regions (c).

4.3.5. Effects of Cytokines in Combination with HB-EGF on OE Proliferation

In order to see whether HB-EGF and cytokines have synergistic effects, a stimulation was carried out by using a mixture of IL-11, Leptin, and HB-EGF. 1 µl from each stock of 1 µg/µl Leptin, 250 ng/µl HB-EGF, and 250 ng/µl IL-11 were combined and administered to one OE by nasal irrigation for 30 minutes. The procedure was the same that was applied in previous experiments of this section. The control OE was irrigated with 0.1% BSA as it

was the vehicle for all proteins. At the end of the 30 minutes-long stimulation, fish (n=3) were injected intraperitoneally with 25 µl of 10 mM EdU. OEs were dissected at 24 hps and analysis was carried out via immunostainings.

Figure 4.19a shows the staining against HuC/D and EdU in the control OE (left) and treated OE (right). The pattern of HuC/D^+ cells appears typical for uninjured OE in both groups. EdU^+ cells are mostly localized in the ILC and SNS in the control and treated OEs. There is no considerable change in the number of EdU^+ cells when the control and treated OEs are compared. In order to understand there is indeed no quantitative difference, EdU^+ cells were counted by the standard method. Figure 4.19b displays the comparison of the EdU^+ cells along the radial index for the control (green) and treated OEs (orange). As seen in the figure, the number of EdU^+ cells are almost the same for the whole curve. The two curves are almost aligned. There is no statistically significant difference between the control OE and treated OE for the Leptin+IL-11+HB-EGF stimulation (unpaired, two-tailed t-test, t_{18}=1.503, p = 0.9797).

The number of EdU^+ cells in separate regions was analyzed to see the changes in the mode of proliferation. However, no region showed any notable change (ILC: from 17.3 ± 2.5 to 16.0 ± 2.2, t_{58}=0.4, p = 0.6979. Core sensory: from 10.3 ± 2.4 to 10.2 ± 3.2, t_{58}=0.02907, p = 0.9769. SNS: from 18.5 ± 3.2 to 20.4 ± 3.0, t_{58}=0.4335, p = 0.6662. NS: from 13.4 ± 2.6 to 9.7 ± 1.7, t_{58}=1.182, p = 0.2420.). These results indicated that stimulation of the OE with the combination of IL-11, Leptin, and HB-EGF did not affect the proliferation at 24 hps.

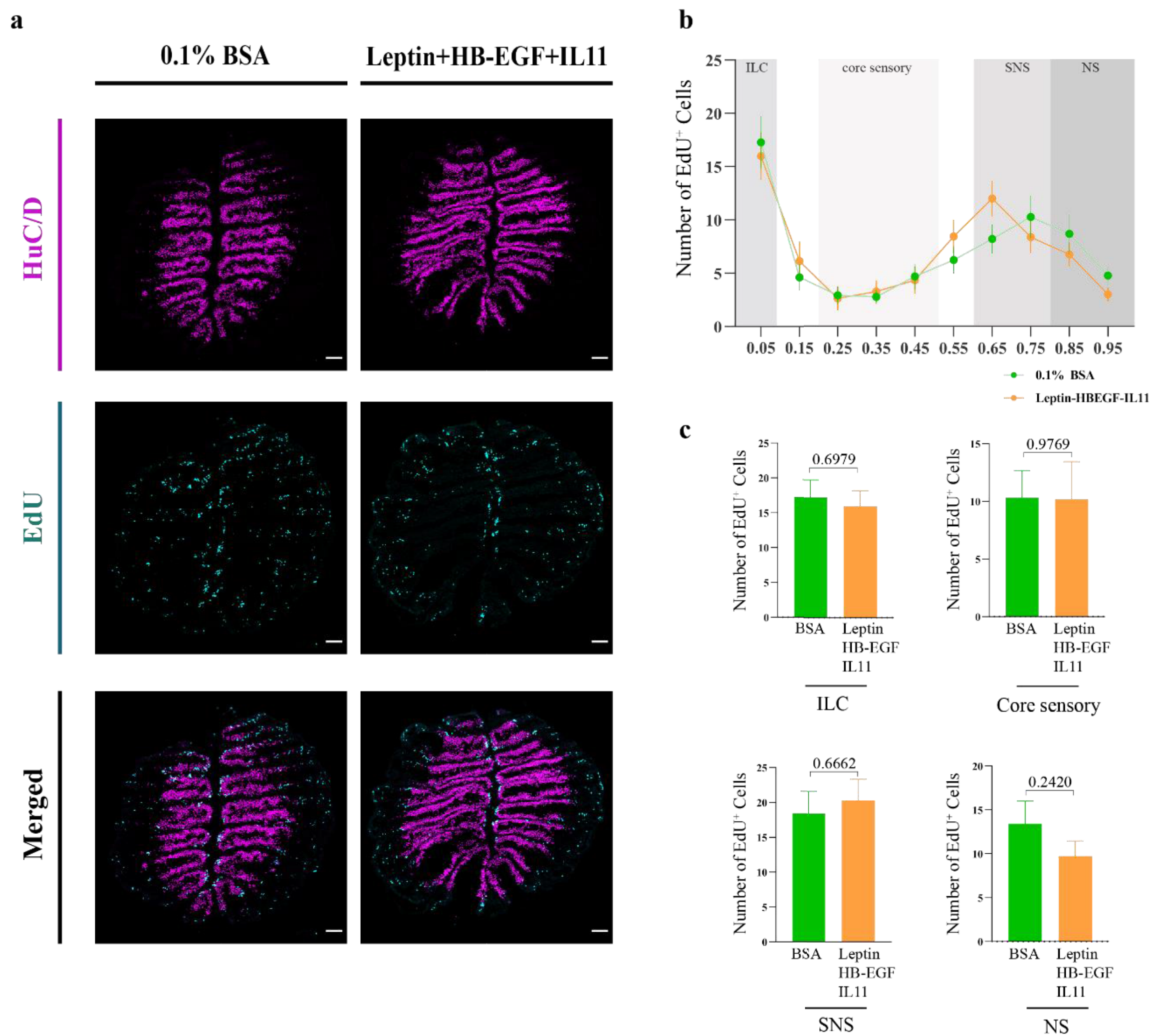

Figure 4.19. Effects of stimulation with the combination of Leptin, IL-11, and HB-EGF on OE proliferation at 24 hps (a). Immunohistochemistry against HuC/D (magenta) and EdU (cyan) in OEs of BSA and Leptin+IL11+HB-EGF treated fish. Scale bars: 50 µm. Positional profiling of EdU$^+$ cells in OEs of BSA and Leptin+IL-11+HB-EGF stimulated fish at 24 hps (b), with the analysis of EdU$^+$ cells separately for relevant OE regions (c).

5. DISCUSSION

The preservation of an intact OSN population by the lifelong turnover of neurons, the ability to prompt efficient injury response, and the tight regulation of these two modes of neurogenesis in the OE are curios phenomena that may help to better understand the mechanisms and cellular dynamics during adult neurogenesis. This study was conducted to further contribute to this understanding by gaining insight into the molecular regulation of adult neurogenesis in the zebrafish OE.

In the first part of this thesis, the role of HB-EGF signaling during OE regeneration was analyzed by loss-of-function studies. Pharmacological inhibition of HB-EGF ectodomain shedding by the MMP inhibitors Marimastat and GM6001 resulted in a significant impairment of OSN generation and OE regeneration at 5 dpl. Similarly, sequestration of available soluble HB-EGF by CRM197 also showed a reduction in cell proliferation at 24 hpl and severely affected OSN formation after injury at 5 dpl. These results suggest that HB-EGF signaling is necessary for the development of a full injury response in the OE.

Gain-of-function analyses with exogeneous HB-EGF stimulation, on the other hand, resulted in an upregulation of *hbegfa* expression specifically in HBCs at 4 hps, which suggests the existence of a positive feedback mechanism. In order to determine whether the upregulation of *hbegfa* expression is followed by the generation of neurons, expression of *ascl1a*, a marker for neuronally committed intermediate progenitor cells, was analyzed at 48 hps upon HB-EGF administration. *In situ* hybridization showed an induction of *ascl1a*$^+$ cells across the sensory region of the OE, suggesting that HB-EGF triggers a neurogenic response through the activation of GBC_{TA} population, which is initiated by the HBC activity.

The second objective of this study was to identify potential interaction partners of HB-EGF signaling during repair neurogenesis of the zebrafish OE. Due to their strong upregulation in the transcriptome data after injury and their involvement in other neuronal injury models, Leptin and IL-11 were investigated. Human recombinant Leptin, IL11, and combinations of the cytokines with HB-EGF were administered to the OE to determine whether they are sufficient to create a response in stem cell proliferation. However,

exogenous stimulation with cytokines, separately or in combinations, did not show any significant change in the mitotic activity of the intact OE.

5.1. Inhibition of HB-EGF Ectodomain Shedding Disrupts the Repair Response

The zebrafish OE is a constitutively active zone of adult neurogenesis, and comprises a dual progenitor system constituted by HBCs and GBCs that are active under different tissue conditions. Under physiological conditions, OSNs of the OE have a lifespan of around 29 days, which creates a need for the continuous renewal of OSNs (Bayramli *et al*., 2017). Maintenance neurogenesis is driven by GBC activity, which is localized to the ILC and SNS (Demirler *et al*., 2020). Repair neurogenesis, on the other hand, is a distinct mode of action required for a robust injury response that mainly depends on HBC activity. HBCs can be considered to be the reserve stem cells of the OE, which are located in the most basal tissue and remain mitotically quiescent under physiological conditions but become activated in response to global injury (Kocagöz *et al*., 2022). The OE can recover from injuries that destroy nearly the entire OSN population and can replenish approximately 80% of these neurons within only five days (Kocagöz *et al*., 2022). This high capacity for OSN regeneration over such a short time requires to be tightly and timely regulated by defined molecular signals. In the rodent OE, the regulation of p63 expression, Notch and Wnt signaling pathways were shown to contribute to the regulation of HBC activity, which is critical for the injury response (Fletcher *et al*., 2011; Herrick *et al*., 2017; Fletcher *et al*., 2017). In addition, several growth factors including FGF2, IGF1, and EGF were found to act as mitogens to promote OSN neurogenesis in the rodent OE (Mahanthappa and Schwarting, 1993; Fukuda *et al*., 2018).

In the search for molecular regulators of zebrafish OE repair neurogenesis, a transcriptome analysis at 4 hpl showed the transient upregulation in the expression of *hbegfa*, *egfra*, *mmp9*, and *adam15*, all of which have critical functions during HB-EGF/EGFR signaling (Demirler, 2021; Dao *et al*., 2018). The HB-EGF precursor is a membrane-anchored protein belonging to the EGF family of proteins, which commonly have an EGF-like extracellular domain. The membrane-bound form of the protein is also known as pro-HB-EGF, which has roles in the suppression of apoptosis, promotion of cell survival, and complex formation with integrins for epithelial cells (Kinugasa *et al*., 2007; Miyoshi *et al*., 1997; Nakamura *et al*., 1995). The transmembrane domain of pro HB-EGF can be cleaved

by MMPs upon activation by the process of ectodomain shedding (Nishi and Klagsbrun, 2004). Soluble HB-EGF has been shown to signal in various autocrine or paracrine ways in a wide range of events such as wound healing in keratinocytes, cell growth in myeloma cells, the proliferation of hepatocytes (Tokumaru *et al*., 2000; Wang et al., 2002; Ito et al., 1994). It was also found that HB-EGF functions in the induction of cerebral neurogenesis in mouse brains, the repair of ischemic injuries in the rat brain, and the regulation of progenitor cell activity and regeneration in the zebrafish retina (Jin *et al*., 2003; Oyagi *et al*., 2011, Wan *et al*., 2012).

Previous work from our lab has shown that MMP inhibition via the pharmacological agent Marimastat reduced the number of proliferating cells significantly at 24 hpl (Kocagöz, 2021). The first part of this study aimed at the further investigation of the effects of MMP inhibition on regeneration and neurogenic mitotic activity of the OE and the ability to restore the OSN population at a later time point after the lesion. For this purpose, two different MMP inhibitors were used. Marimastat has been shown to inhibit MMPs 1, 2, 3, 7, and 9, whereas GM6001 (Ilomastat) is used as a more competent broad-spectrum MMP inhibitor (Rasmussen and McCann, 1997; Schultz et al., 1992). Disruption of HB-EGF signaling by Marimastat or GM6001 led to a severe impairment of OSN regeneration in the lesioned OEs at 5 dpl (Figure 4.1). The analysis of mitotically active BrdU$^+$ cells labeled at 3 dpl also showed a significant reduction upon MMP inhibitor treatment, which is consistent with the low levels of regeneration (Figure 4.2). The neuronal area measured by the HuC/D signal at 5 dpl was reduced substantially compared to the lesioned OEs of vehicle-injected fish (Figure 4.3) suggesting that MMP activity is required for OE regeneration.

Inhibition by Marimastat also showed a decrease in cell proliferation at the ILC and SNS of the intact OE, as well as a uniform reduction of mitotic activity in the entire OE after injury (Figure 4.2). The typical maintenance neurogenesis pattern of the zebrafish OE, which is observed with a bimodal distribution of proliferating cells at the ILC and SNS (Kocagöz *et al*., 2022), was changed following Marimastat treatment. The reduction of BrdU$^+$ cells at the ILC and SNS might indicate a disruption of the maintenance neurogenesis process, in addition to its effects on repair neurogenesis. MMPs have been shown to play roles in the regulation of neuronal precursors in the developing CNS, in addition to functions in the neurogenic repair of ischemic injuries in the brain (Frölichsthal-Schoeller *et al*., 1999; Wójcik-Stanaszek *et al*., 2011; Lu *et al*., 2008). From these findings, a possible role for

MMPs in the regulation of maintenance neurogenesis can be suggested, although future investigation is needed to shed more light onto which signaling pathways are affected.

Inhibition of ectodomain shedding by the alternative MMP inhibitor GM6001 further corroborated the results of Marimastat inhibition. Similar to Marimastat, the area covered by neurons at 5 dpl was significantly smaller compared to control animals (Figure 4.6) and the rate of cell proliferation at 3 dpl displayed a uniform decrease in the lesioned OEs in GM6001-treated fish (Figure 4.5). The intact OEs, on the other hand, showed a similar cell proliferation to the controls, except for a slight decrease at the ILC. Thus, inhibition of MMPs by GM6001 is clearly detrimental to the repair neurogenesis, whereas there is not enough data to further speculate on its effects on the maintenance neurogenesis.

Even though inhibition of MMP activity by either inhibitor significantly reduced the rate of cell proliferation and the OSN generation at 5 dpl, it did not completely abolish the repair response (Figure 4.1; 4.4). The use of pharmacological agents raises the question of whether the inhibition was in fact continuous throughout the entire observation period. In addition, the dosage of the inhibitor injections might not have been sufficient to suppress MMP activity completely, especially at later time points. Thus, the possible partial ineffectiveness of the inhibitor treatment protocol may explain the residual repair activity. Alternatively, other molecular regulators that are independent of MMP activity could be involved in the process and function in a redundant manner to activate HBC proliferation.

5.2. HB-EGF Signaling is Necessary for Tissue Repair

The MMP inhibitors Marimastat and GM6001 have broad target spectra and may inhibit proteases that are involved in signaling pathways different from HB-EGF. In order to affect HB-EGF activity directly, a pharmacological approach was employed by using the inhibitor CRM197. CRM97 is a non-toxic diphtheria toxin mutant, which has a single-point mutation of glycine to glutamic acid on the 52nd position (Giannini *et al.*, 1984). Diptheria toxin is a molecule produced by Corynebacterium diphtheriae, which binds to HB-EGF and thereby can enter HB-EGF-expressing cells by receptor-mediated endocytosis (Naglich *et al.*, 1992). Due to the interaction of diphtheria toxin with HB-EGF and its known masking effect, CRM197 is widely employed in loss-of-function experiments on HB-EGF in human cell lines. In rodents, however, it was argued that CRM197 does not bind to HB-EGF due to

differences in the protein sequence (Mitamura *et al*., 1995). However, there is also recent evidence of receptor-mediated internalization of CRM197 into murine cells (Fellermann *et al*., 2020). Although CRM197 has never been reported in zebrafish to inhibit HB-EGF, diphtheria toxin is known to induce heart defects in zebrafish (Wang, et al. 2016), which indicates a possible conservation of its binding to HB-EGF. The EGF-like domain of HB-EGF that binds to EGFR shows approximately 63% similarity between human and zebrafish, with the conservation of critical residues (Schneider and Wolf, 2009). Molecular docking studies and dynamic modelling of the HB-EGF from human, mouse, and zebrafish interaction with diphtheria toxin and CRM197 showed that most of the tight binding interactions between receptor and the ligand are conserved in zebrafish but not in mouse (Sögünmez, personal communication). Moreover, inhibition of HB-EGF activity with morpholinos is known to create heart defects such as pericardial edema and ventricle enlargement in embryos, which results in lethality (Friedrichs *et al*., 2009). We showed in our lab that continuous CRM197 exposure for the first 4 days after fertilization results in the enlargement of ventricular chambers of the heart, consistent with the shown effect of HB-EGF morpholinos (unpublished data, Şireci). Together, these data support the idea that CRM197 efficiently inhibits HB-EGF activity by masking the transmembrane domain binding in zebrafish, similar to its effect in human cell lines.

In this study, inhibition of HB-EGF activity by CRM197 showed a reduction in the number of proliferating cells, especially in the core sensory region at 24 hpl. While CRM197 did not affect the proliferation pattern in uninjured OEs, it decreased the number of $BrdU^+$ cells in TrX-treated OEs (Figure 4.8). In order to investigate the effects of HB-EGF inhibition on repair and generation of OSNs, two additional doses of CRM197 were injected at 24h intervals and OEs were analyzed at 5 hpl to allow more time for regeneration. The analysis of $BrdU^+$ cells labeled at 3 dpl with CRM197 treatment did not show any significant change compared to control animals (Figure 4.10). This finding was surprising, given the reduction of proliferating cells with CRM197 treatment at 24 hpl. When $BrdU^+$/HuC/D^+ cells were counted to answer the question of whether these proliferating cells differentiated into neurons, the results indicated a reduction in the number of newborn neurons (Figure 4.10). Taken together, the low number of $BrdU^+$/HuC/D^+ cells suggests that the mitotic activity upon injury was not neurogenic at the time of the analysis. Inhibition of HB-EGF might have delayed or decelerated the repair response, which resulted in a lower number of newborn

neurons at 5 dpl, despite the normal proliferation rate at 3 dpl. The other explanation could be that there is a specific time window for the neurogenic response to be activated after injury. If the activity of HB-EGF is inhibited at that time window, the proliferating cells might not be able to differentiate into neurons in the following period. Given that neurogenesis is tightly regulated in a timely manner, lesioned OEs should be investigated at a later time point to understand which explanation is more viable.

Previously, the effects of the EGFR inhibitor PD153035 were investigated under injury conditions (Alkiraz, 2019). Similar to the effects of CRM197, PD153035 treatment caused a decrease in the neurogenic proliferation at 5 dpl, while the total number of BrdU$^+$ cells was comparable to controls (Alkiraz, 2019). The common lack of change in the cell proliferation after injury in both CRM197 and PD153035-treated OEs indicates that HB-EGF might play a role in the neurogenic fate, rather than the cell proliferation. HB-EGF is known to direct migration via an autocrine loop in keratinocytes during wound healing, without causing proliferation (Shirakata *et al.*, 2005). It was also found that during enteric neural stem cell transplantation, HB-EGF protects against necrotizing enterocolitis by promoting migration in addition to positively modulating cell proliferation (Wei *et al.*, 2015). Together with these data, the findings of this study suggest that HB-EGF might direct the neurogenic fate of the progenitors during the repair of the OE. It might also suggest a separate route of direct differentiation for HBCs without induction of proliferation after damage, which could explain the unchanged total number of proliferating cells in the less-regenerated OEs of CRM197 treatment.

Even though CRM197 treatment created a decrease in the rate and efficiency of repair, it did not affect the maintenance neurogenesis in the short or long term (Figures 4.7; 4.10). The numbers of BrdU$^+$ cells at the ILC and SNS were comparable to those of control OEs for both 2-dose and 4-dose injection schedules of the inhibitor. The number of proliferating cells in the core sensory region, on the other hand, was significantly lower than the control at 24 hpl. Together with the knowledge that GBCs do not exist in the core sensory region unless there is an injury and HBC response (Kocagöz *et al.*, 2022), this is an indication that HB-EGF plays a role mainly in the behavior of HBCs, not GBCs. Hence, HB-EGF seems to regulate the repair neurogenesis mechanism, while not showing an effect on the maintenance neurogenesis.

5.3. Positive Feedback of HB-EGF on HBC Induction

HBCs have been classified as the reserve stem cell population of the OE (Graziadei and Graziadei, 1979), which can be distinguished by the expression of Sox2 and Keratin 5/14 (Carter *et al*., 2004; Guo *et al*., 2010). HBCs in the rodent OE are mitotically quiescent under physiological conditions. They also express *tp63*, which acts as a molecular switch of mitotic activity (Packard *et al*., 2011). The expression of *tp63* in HBCs has been correlated with their state of dormancy. Upon injury, *tp63* expression is downregulated, resulting in a proliferative state. The loss of *tp63* expression was also shown to suppress self-renewal and promote differentiation of HBCs (Fletcher *et al*., 2011). Under injury conditions, HBCs are activated to replenish the OSN population that has been destroyed. After the repair is completed, the expression of *tp63* goes back to basal levels and HBCs reenter the quiescent state (Schwob *et al*., 1995; Packard *et al*., 2011).

In the zebrafish OE, the HBC profile is similar to that of their rodent counterparts. They occupy the most basal layer and present a flat morphology that is in direct contact with the basal lamina. Most of the HBC population is dormant under physiological conditions, with the exception of a small number of actively dividing cells towards the ILC and SNS, which are hypothesized to contribute to maintenance neurogenesis. Upon injury with TrX treatment, HBCs exit the state of dormancy, however, downregulation of *tp63* could not be established in the zebrafish OE. The activation of HBCs is followed by the proliferation and generation of an *ascl1a*-expressing GBC_{TA} population, which are committed to a neurogenic fate (Kocagöz *et al*., 2022).

In the mouse OE, expression of *hbegf* is upregulated specifically in HBCs after injury (Gadye et al., 2017). Under physiological conditions, however, *hbegfa* expression is very low in the rodent and zebrafish OE (Demirler, 2021), and *in situ*-hybridization showed only basal expression in sporadic cells, mostly GBCs (Güler, 2021). Upon injury, however, *hbegfa* expression is highly upregulated, which is observed in all major cell types of the OE (Güler, 2021). However, only HBCs and some OSNs are known to express *egfra* (Güler, 2020), which suggests that the increased proliferation observed after HB-EGF stimulation could be a result of a cascade through HBCs or OSNs. To further investigate the relationship between HB-EGF exposure and *hbegfa* expression, the effects of HB-EGF stimulation on the expression levels of *hbegfa* were investigated.

Nasal irrigation with human recombinant HB-EGF resulted in the upregulation of *hbegfa* at 4 hps, as observed by *in situ*-hybridization analysis. Expression of *hbegfa* showed a 3-fold increase specifically in HBCs (Figure 4.13). The apical regions, which are composed of OSNs, did not show noticeable *hbegfa* expression or changes after stimulation (Figure 4.12). This finding suggests autocrine and paracrine signaling of HB-EGF between , which results in the amplification of the signal via upregulation of its own expression in HBCs. That mechanism might underlie the global activation of HBCs. Upon synthesis of more HB-EGF from HBCs, it can also signal in a paracrine way to stimulate more apical cells, such as Sus cells. Analysis of *egfra* expression showed that Sus cells might not express the EGFR receptor, at least not the ErbB1 subunit (Güler, 2021).

5.4. The Role of HB-EGF in OSN Generation

HB-EGF has been shown to promote neurogenesis in the SVZ of the mouse brain (Jin *et al*., 2003). It was also shown that HB-EGF induces proliferation and contributes to repair neurogenesis in the SVZ and SGZ after ischemic injury (Jin et al., 2002). In another study, HB-EGF was found to function as a key regulator in the regeneration of zebrafish retina by activating the dedifferentiation of Müller glia and promoting the proliferation of the Müller glia-derived progenitor cells through Notch signaling (Wan *et al*., 2012). Studies from our lab showed that stimulation with human recombinant HB-EGF is sufficient to increase neurogenic mitotic activity in the zebrafish OE without any injury (Kocagöz, 2021). Moreover, an increase in the number of $Krt5^+/EdU^+$ and $Sox2^+/EdU^+$ double-positive cells was observed 48h after exogenous HB-EGF stimulation (Dokuzluoğlu and Balcıoğlu, unpublished data). Together, these data suggest that the route of HBC proliferation to GBC expansion is followed upon HB-EGF stimulation.

In order to understand whether the increase in $BrdU^+/HuC/D^+$ cells that is observed in response to HB-EGF stimulation (Kocagöz, 2021) is due to the increased activity of GBC_{TA} population, *in situ*-hybridization against *ascl1a* was performed 48h after HB-EGF stimulation. Observation of *ascl1a* upregulation along the core sensory region indicated repair-like neurogenic activity in response to HB-EGF stimulation and confirmed the increase of GBC_{TA} activity upon HB-EGF (Figure 4.14). Taking into consideration that *hbegfa* expression is upregulated in HBCs upon HB-EGF stimulation, these findings suggest that *hbegfa* upregulation after damage initiates a cascade that is sufficient to create a

proliferation response in HBCs, which results in an increase of GBC_{TA} population and contributes to the generation of new OSNs.

The reduction in the number of HuC/D^+ cells after MMP inhibition by Marimastat or GM6001 supports the critical role of HB-EGF in the generation of new neurons upon injury. In addition, the decrease in the number of $BrdU^+/HuC/D^+$ cells at 5 dpl of CRM197 treatment indicate that loss of HB-EGF signaling results in the impairment of OSN generation. Taken together, these findings suggest that HB-EGF is necessary and sufficient for the generation of OSNs upon injury.

5.5. The Potential Role of Other EGFR Ligands and Signaling Molecules in OE Repair

Even though a significant impairment in the repair neurogenesis was observed after CRM197 treatment, the injury-response was not completely abolished. The leakiness of IP injection system or the short half-lives of the pharmacological reagent may have influenced the efficiency of the manipulation. However, the failure to completely block the injury response could also suggest the presence of other molecular signals that are involved in the process. As the lower numbers of $BrdU^+$ cells observed in MMP inhibitor treatments suggest, those other signals could be other signaling molecules that are activated by MMPs. For instance, MMP17 and ADAM17 can activate the pro-inflammatory cytokine TNF-α, which has important roles in synaptic activity (English *et al*., 2000; Sternlicht *et al*., 2001). TNF-α was also recently shown to regulate HBC proliferation upon injury to the mouse OE (Chen *et al*., 2017). Nerve growth factor and brain-derived neurotrophic factor are cleaved by MMPs 3 and 7 for activation (Lee *et al*., 2001). MMP9 was shown to promote embryonic neural stem cell proliferation and migration via canonical Wnt/β catenin signaling (Ingraham *et al*., 2011). In addition, Notch receptors are also cleaved by ADAM10, which increases the effectiveness of signaling (Yang *et al*., 2006).

Epiregulin is another member of the family of EGF-like proteins and has proliferation-stimulatory effects through binding to EGFR (Sasaki *et al*., 1997). It can induce proliferation through MEK/Erk and JNK signaling in mesenchymal stem cells (Cao *et al*. 2013). In the liver, Epiregulin is upregulated upon injury and promotes the proliferation of liver progenitor cells (Tomita *et al*., 2014). It also acts as a mitogen by activating EGFR during epithelial

regeneration after renal injuries (Zhuang *et al*., 2007). An autocrine role of Epiregulin in the induction of other EGF-family proteins, including HB-EGF, was detected to play roles in the proliferation of human corneal epithelial cells (Morita *et al*., 2007). In the transcriptome data of the regenerating zebrafish OE, an upregulation in the expression of epiregulin was observed at 4 hpl (Demirler, unpublished data). With the roles of Epiregulin in the activation of proliferation in several other systems mentioned above, a potential interaction of Epiregulin and HB-EGF can be suggested. However, future investigation is needed. This notwithstanding, CRM197 does not bind to Epiregulin and largely blocks at least the initial response of the tissue to injury, suggesting that Epiregulin does not play a major role.

Neural-glial-related cell adhesion molecule (NrCAM) has also been described to be a non-canonical ligand of EGFR and to target of Wnt/β catenin signaling (Sakurai, 2012). It requires the activity of MMPs for the known effects on cell proliferation, motility, and transformation in tumor formation (Conacci-Sorrell *et al*., 2005). NrCAM was recently shown to be specifically expressed in HBCs and stimulate the proliferation of HBCs in the mouse OE upon injury. Inhibition of MMP or EGFR activity was found to suppress HBC activity in olfactory organoids, and delay the repair (Chen *et al*., 2020). While this data supports the role of EGFR signaling in OE regeneration, it does not explain the results of MMP inhibition and the proliferative activity observed at 3 dpl of CRM197 inhibition, because *nrcama* expression is downregulated in the lesioned zebrafish OE and not upregulated as in the mouse (Demirler, unpublished data).

Wnt/β-catenin signaling is a known regulator of adult neurogenesis, in fact of all major mitotic events. In the adult hippocampus, Wnt/β-catenin signaling regulates neural stem cell behavior. Activation of Wnt3 was shown to induce hippocampal progenitor cells, whereas inhibition of Wnt/β-catenin signaling has detrimental effects on neurogenesis in the brain (Lie *et al*., 2005). In another study, Wnt7A was found to stimulate neural stem cell expansion and self-renewal in the adult olfactory bulb (Moreno-Estellés *et al*., 2012). Wnt signaling has also been shown to contribute to repair neurogenesis of the zebrafish OE by regulating the proliferative activity of HBCs. Activation of Wnt/β-catenin signaling with LiCl was found to increase the number of proliferating HBCs upon injury, while inhibition of Wnt signaling reduced the number of proliferating cells, even though not completely abolishing the repair response (Kocagöz *et al*., 2022). In another study, HB-EGF was shown to preserve Wnt/β-catenin signaling in intestinal stem cells and contribute to the repair after ischemic

injury (Chen *et al*., 2014). In the zebrafish retina, synergistic effects of HB-EGF, IGF, and Insulin stimulate β-catenin expression, whereas inhibition of EGFR reduces the expression of β-catenin (Wan *et al*., 2014). Taken together, these data could suggest a role for Wnt/β-catenin signaling in the repair neurogenesis of the zebrafish OE, which could be linked to the effects of HB-EGF.

Taking into consideration that Notch1 maintains the expression of tp63 and suppresses HBC activity in the rodent OE (Herrick *et al*., 2017), Notch signaling could be another candidate for regulating the repair neurogenesis. The interaction of Notch and EGFR signaling was previously shown to modulate the balance between neural stem cell self-renewal and proliferation (Aguirre *et al*., 2010). HB-EGF activates Notch signaling in addition to the Wnt/β-catenin pathway in Müller glia cells of human, as in the case of zebrafish retina (Angbohang *et al*., 2016). In the injured zebrafish retina, Notch1 inhibits *hbegfa* and *ascl1a* expression in a feedback system that is hypothesized to limit the proliferative response upon injury. Also, inhibition of Notch signaling induces HB-EGF activation in this system (Wan *et al*., 2012). Another study shows that HB-EGF administration to astrocyte cell culture shows upregulation in the expression of Notch ligand Dll1 and downregulation of Hes1, which is a main effector of Notch signaling (Puschmann *et al*., 2014). In turn, Notch signaling was also found to stimulate the activity of ADAM12, which is a sheddase for HB-EGF, and as a result, increase the soluble HB-EGF levels in cancer cells (Díaz *et al*., 2013). In summary, crosstalk between Notch signaling and HB-EGF can be suggested to have roles in the regulation of neurogenic activity in the OE.

5.6. A Proposed Model for the Role of HB-EGF Signaling in OE Repair

Stimulation of the OE with HB-EGF stimulates HBCs, which is followed by the neurogenic fate of GBC_{TA} population. Inhibition of HB-EGF signaling results in a severe reduction in the generation of new OSNs upon lesion. The findings of this study suggest that HB-EGF is a critical regulator of repair neurogenesis in the zebrafish OE. With the previous findings indicating that gain-of-function of HB-EGF results in the increase of HBC proliferation (Dokuzluoğlu, unpublished data) and eventually significantly higher numbers of newborn neurons in unlesioned OEs (Kocagöz, 2021), support the notion that HB-EGF is necessary and sufficient for the OSN neurogenesis in zebrafish OE.

The globally high levels of *hbegfa* expression at 4 hpl (Güler, 2020) can be explained with a layered stimulation system. As the findings of this study on the upregulation of *hbegfa* upon HB-EGF administration suggests, HBCs are probably the first responders to soluble HB-EGF. The binding of the exogenous HB-EGF to EGFR in HBCs creates a positive feedback mechanism through the upregulation of *hbegfa*. As more HB-EGF is synthesized and released, the neighboring cells are activated, which creates a spreading wave of *hbegfa* expression globally in the tissue. Expression of *egfra* in some OSN types (Güler, 2020) can explain the robust *hbegfa* upregulation upon damage.

The question of how HBCs are stimulated in the first place still remains. The source of HB-EGF in the OE might be dying OSNs and Sus cells, which reside apical to HBCs. Findings in rodents suggest that Sus cell depletion is a strong signal for breaking dormancy in HBCs (Herrick *et al*., 2017). The loss of interaction between Jagged1 on Sus cells and Notch1 on HBCs is hypothesized to suppress tp63 expression, directing HBCs to exit dormancy. In addition, the inverse relationship between Notch1 and HB-EGF levels in several other systems supports the hypothesis that the downregulation of Notch due to damage in Sus cells could activate HB-EGF signaling (Aguirre *et al*., 2010; Puschmann *et al*., 2014; Wan *et al*., 2012).

Since HBCs are in direct contact with the basal lamina, which is the only layer standing between HBCs and the bloodstream, there is also a possibility that HBCs are stimulated by resident or recruited immune cells (Mollamustafoğlu, 2023). Regulatory macrophages with immunosuppressive functions were previously identified with *hbegf* expression (Edwards *et al*., 2009). A paracrine loop of the cytokine GM-CSF and HB-EGF was also found to regulate HB-EGF release from macrophages during cancer cell growth (Rigo *et al*., 2010). In the rheumatoid arthritis model, *hbegf*-expressing macrophages were shown to induce fibroblast motility and neutrophil recruitment to the inflammation area through EGFR activation (Kuo *et al*., 2019). Moreover, in a study of head and neck cancer, M2 macrophages were shown to activate EGFR by secreting HB-EGF (Fu *et al*., 2020). Considering the involvement of macrophages in the inflammatory response after injury and the previous findings suggesting *hbegf*-expressing macrophages, the source of HB-EGF that activates HBCs could underlie the macrophage activity.

Here, I propose a model for the role of HB-EGF signaling in the repair neurogenesis of zebrafish OE (Figure 5.1). Soluble HB-EGF possibly released from Sus cells, dying OSNs or macrophages feeds back to HBCs to upregulate *hbegfa* expression. The resulting increase in the HB-EGF levels activates neighboring cells via paracrine or juxtacrine signaling and creates global upregulation of *hbegfa*. Increased HB-EGF activity results in the activation of EGFR and promotes the repair response through the induction of HBCs to proliferate and differentiate into GBCs. The increase in the GBC_{TA} population results in the generation of new OSNs. Other EGFR ligands, specifically Epiregulin, and HB-EGF-independent signaling pathways, such as Notch signaling, might work synergistically to further increase the effects of HB-EGF on the neurogenic mitotic activity.

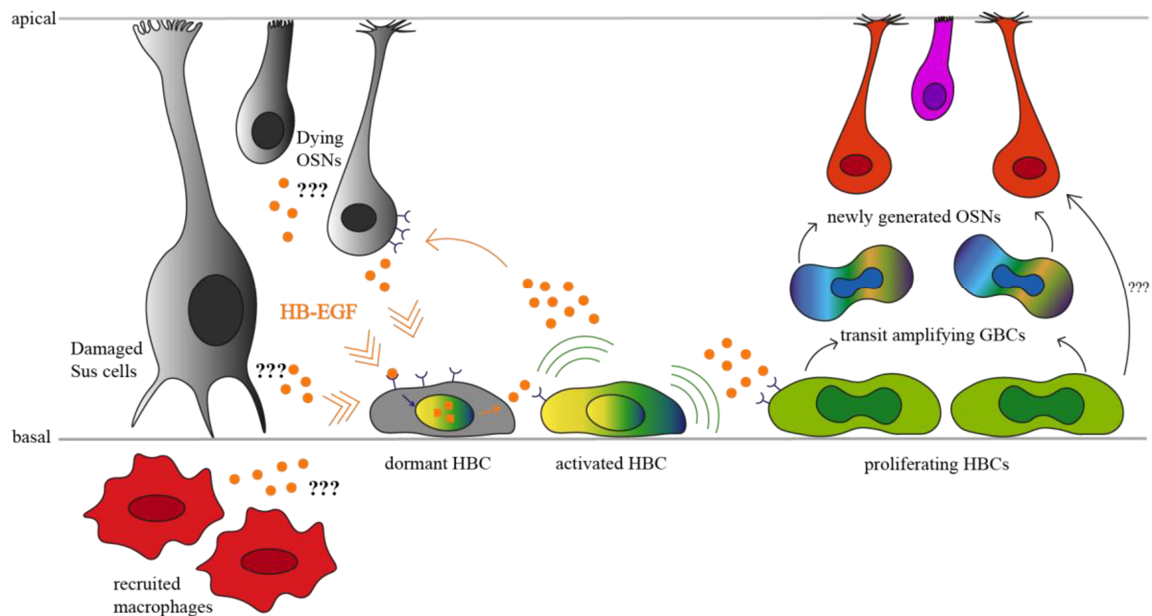

Figure 5.1. A proposed model for the role of HB-EGF in OE regeneration.

5.7. The Possible Role of Leptin and IL-11 in OE Regeneration

Cytokines and chemokines are the messengers of inflammatory processes. They are also known regulators of progenitor cell activity and neurogenesis (Borsini *et al*., 2015). For instance, IL-6 stimulates the neuronal differentiation of progenitors in the adult hippocampus (Oh *et al*., 2010). IL-11 was shown to be required for the induction and maintenance of progenitor cells in the tail regeneration of Xenopus tadpoles (Tsujioka *et al*., 2017). It also participates in dopaminergic neuron generation by promoting the differentiation of

mesencephalic stem cells in vitro (Ling *et al*., 1998). TGFβ and IL-1 were shown to induce MMP-9 expression in the wound healing of corneal epithelial cells (Gordon *et al*., 2009). Leptin promotes neurogenesis in the adult hippocampus through the activation of Akt and STAT3 (Garza *et al*., 2008). It also reduces neurodegeneration due to Alzheimer's disease and contributes to the induction of neurogenesis in the mouse model (Calió *et al*., 2021). IL-6 and HB-EGF work synergistically to promote the growth and proliferation of human myeloma cells (Wang *et al*., 2002). In another study, IL-11 and Leptin were shown to stimulate cell proliferation in the zebrafish retina. In addition to their separate stimulatory effects, they were found to work synergistically with HB-EGF in Müller Glia dedifferentiation and neurogenesis in the zebrafish retina (Wan *et al*., 2014).

RNA-Seq analysis of lesioned OEs showed an upregulation in the expression of *lepa, lepb*, *il6*, *il11b* their receptors *gp130* and *lifrb* at 4 hpl, which is very transient and expression levels drop down quickly back to the basal levels as soon as 12 hpl (Demirler, 2021). Upregulation of these cytokines concomitantly with HB-EGF led to the hypothesis that cytokines might be working redundantly or synergistically with HB-EGF in the regulation of repair response. These observations are interesting, especially in the light of the potential involvement of innate immune cells in OSN repair neurogenesis outlined above. Previously, stimulation of the OE with intranasal administration of IL-6 showed an increase in the number of BrdU$^+$ cells in the intact OE. In addition, these proliferating cells stained positive for Krt5, which is an HBC marker (Demirler, 2021).

In this study, the effects of exogenous human recombinant IL-11 and Leptin administration were analyzed to investigate their role in the regulation of repair neurogenesis. Stimulation with either Leptin or IL-11 by nasal irrigation method did not result in a change in the cell proliferation at 24 hps (Figures 4.15; 4.17). Due to the limitations of the nasal irrigation method, IP injection of Leptin was tested as an additional administration method. However, a single dose of Leptin injection did not result in a notable change in the number of BrdU$^+$ cells at 24 hps (Figure 4.16).

To test for potential synergisms between cytokines and HB-EGF, two sets of experiments were conducted. First, the combination of Leptin and HB-EGF was administered to fish by IP injection and nasal irrigation, respectively. The analysis of unlesioned OEs at 24 hps showed a slight decrease in the number of proliferating cells across

the sensory region (Figure 4.18). This finding was unexpected, since HB-EGF alone was shown to increase cell proliferation in the unlesioned OEs (Kocagöz, 2021). This finding could argue against the hypothesis of synergism. The upregulation of both molecules around the same time does not necessarily imply synergistic effects in the tissue. On the contrary, their activity may regulate the balance between inflammatory and proliferative responses. However, the difference in the number of BrdU$^+$ cells is not very high to make a strong argument. Additionally, only 2 fish were analyzed for this experiment. Further investigation is needed to determine the role of Leptin in the regeneration of the zebrafish OE, if the increase in the transcriptome data indeed has an additional effect in the neurogenic activity.

Lastly, the combination of IL-11, Leptin, and HB-EGF was administered to OEs to further investigate a possible synergism. All of the proteins were mixed, and this solution was applied directly to the nasal cavities by the standard nasal stimulation method. The analysis of OEs at 24 hps did not show any change in the number of BrdU$^+$ cells compared to the control OEs, contrary to the reduction of proliferating cells in the combination (Figure 4.19). The expectation was to observe at least some degree of increase in the proliferation since the mixture included HB-EGF, which is known to activate proliferation in the OE. Two possible explanations can be made for the lack of any increase in cell proliferation. Effects of Leptin and IL-11 might be suppressing the activity of HB-EGF, resulting in a balance so that no proliferation response is triggered. However, this experiment by itself is not sufficient to make that argument, due to the limitations of the intranasal delivery method and possible leaks of the procedure. The second explanation depends on the time of analysis. The proliferative effects of HB-EGF were observed at the 3rd day of a two-stimulation schedule. 24 hps might be too early to detect the change in the number of BrdU$^+$ cells, if the response is time-dependent. In addition, a second stimulation might be needed to boost the effect. However, this second explanation can be argued with the results of the previous experiment, which caused the reduction of cell proliferation by using the same method.

In summary, external administration of IL-11 or Leptin did not show any change in the cell proliferation, maintenance neurogenesis, or the typical OE structure. Despite the minor reduction of cell proliferation observed after the administration of Leptin and HB-EGF mixture to fish, the combination of all three molecules did not result in a considerable difference in the proliferating cells. However, intrinsic flaws with the nasal irrigation method and the lack of information on the loss-of-function assays make it difficult to eliminate the

possible role of these cytokines in neurogenesis. Having no change after the gain-of-function of cytokines does not necessarily rule out the possible role they might be playing. The lower proliferation rate observed with MMP inhibitors compared to direct inhibitors of HB-EGF/EGFR signaling with CRM197 and PD153035 suggest that other molecules activated by MMPs might play a role in the repair mechanism. It has been shown that MMPs function in the activation of cytokines and chemokines (Van Lint and Libert, 2007). Based on the upregulation of cytokines and their receptors after damage, the question of whether Leptin and IL-6 family cytokines are involved in the regulation of neurogenic repair response still remains elusive.

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APPENDIX A: EQUIPMENT AND SUPPLIES

Table A.1. List of disposable and non-disposable equipment and supplies.

Equipment	Manufacturer
4ºC Room	Birikim Elektrik, Turkey
-20ºC Freezer	Uğur, Turkey
-80ºC Freezer	Uğur, Turkey
Aquatic Habitats	Pentair Aquatic Eco-systems Inc. USA
Capillary glasses	Warner Instruments, USA
Confocal Microscope, SP5-AOBS	Leica Microsystems, USA
Confocal Microscope, TCS SP8	Leica Microsystems, USA
Coplin jars	VWR, USA
Cryostat CM3050S	Leica Biosystems, Germany
Drying oven	Nüve, Turkey
Forceps	Dumont, Switzerland
Glassware (Bottles, cylinders, beakers)	Isolab, Germany
Glass Slides – Superfrost® Plus	Thermo Scientific, USA
Incubator	Nüve, Turkey
Laboratory hybridization oven	Agilent Technologies, USA

Table A.1. List of disposable and non-disposable equipment and supplies (cont.).

Liquid Blocker PAP Pen	Hurst Scientific, Australia
Magnetic stirrer and heater	Nüve, Turkey
Microinjector, FemtoJet	Eppendorf, Germany
Micropipettes (2-1000 µl)	Eppendorf, Germany
Micropipette tips (2-1000 µl)	CAPP, Germany
Oil Microinjector	Narishige, Japan
Orbital shaker, Rotamax 120	Heidolph, Germany
P-97 Micropipette Puller	Sutter Instrument, Co., USA
Petri dishes	Fıratpen, Turkey
pH-meter	Mettler Toledo, Switzerland
Plastic coverslips	Electron Microscopy Sciences, USA
Refrigerator	Arçelik, Turkey
Stereo microscope	Zeiss, Germany
Surgical blade	Swann-Morton, UK
Syringe filter, 0.22 µm	TPP, Switzerland
Syringe filter, 0.45 µm	TPP, Switzerland
Sponges	Parex, Turkey
Thermomixer	Eppendorf, Germany
Vortex – Genie 2	Scientific Industries Inc., USA

Table A.1. List of disposable and non-disposable equipment and supplies (cont.).

Waterbath, WNB 7	Memmert, Germany
U-100 insulin needle (30G)	Beckon Dickinson, USA

APPENDIX B: BUFFERS AND SOLUTIONS

Table B.1. List of chemicals and reagents.

Chemical/Reagent	Manufacturer
4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI)	Thermo Scientific, USA
5- Bromo-2'- Deoxyuridine (BrdU) BioChemica A2139, 0005	AppliChem, Germany
5-Ethynyl-2'-Deoxyuridine (EdU)	Thermo Scientific, USA
α-DIG-Alkaline Phosphatase Fab Fragments	Roche, Germany
Acetic Anhydride	Merck, Germany
Anti-mouse Alexa Fluor® 647	Life Technologies, USA
Anti-mouse cy5 antibody	Jackson Immuno, UK
Anti-rabbit Alexa Fluor® 555	Life Technologies, USA
Anti-rabbit Alexa Fluor® 647	Life Technologies, USA
Anti-rat cy2 antibody	Jackson Immuno, UK
Mouse anti-HuC/D antibody	Life Technologies, USA
Rabbit anti-tp63 antibody	Genetex, USA
Rat anti-BrdU antibody	Abcam, UK
Blocking reagent	Roche, USA

Table B.1. List of chemicals and reagents (cont.).

Bovine Serume Albumin (BSA)	New England Biolabs, USA
Citric acid	Sigma-Aldrich, USA
Calcium Sulfate	Alfa Caeser, Germany
CRM197	Santa Cruz Biotechnology, USA
Diethylpyrocarbonate (DEPC)	Sigma-Aldrich, USA
Dimethyl Sulphoxide (DMSO)	Sigma-Aldrich, USA
EdU Click-IT™ Detection Kit	Thermo Scientific, USA
Fast HNPP Fluorescent Detection Kit, 11 758 888 001	Sigma-Aldrich, USA
Formamide	Roche, Germany
GM6001	Tocris, UK
HEPES	AppliChem, USA
Human recombinant HB-EGF	R&D Systems, USA
Human recombinant IL-11	R&D Systems, USA
Human recombinant Leptin	Sigma-Aldrich, USA
Hydrochloric acid	Merck, Germany
Magnesium chloride, A3511	Promega, USA
Maleic acid, 141882	Pancreac Quimica SA, Spain
Marimastat	Sigma-Aldrich, USA

Table B.1. List of chemicals and reagents (cont.).

Optimum Cutting Temperature Compound (OCT), 4583	Sakura Finetek, Holland
Parafilm, PM-996	Sigma-Aldrich, USA
Paraformaldehyde, P6148	Sigma-Aldrich, USA
Phenol Red, A761501001	AppliChem, Germany
Potassium Chloride, P9541	Sigma-Aldrich, USA
Potassium Phosphate Monobasic	Thermo Scientific, USA
Phosphate Buffer Saline Tablet, P4417	Sigma-Aldrich, USA
Proteinase K	Roche, USA
Sodium Bicarbonate	Aquatic Habitats, USA
Sodium Chloride, S7653	Sigma-Aldrich, USA
Sodium Phosphate Dibasic	Sigma-Aldrich, USA
Tricaine (MS222)	Sigma-Aldrich, USA
Triethylamine (TEA)	Merck Millipore, USA
Triton X-100, A4975	AppliChem, Germany
Trizma Base, T6066	Sigma-Aldrich, USA
Tween®20, 11332465001	Roche, USA

Table B.2. List of solutions and buffers.

Buffer/Solution	**Recipe**
3% BSA	- Measure 3 g BSA and dissolve it in 80 ml of 1X PBST. - Stir until all the crystals are dissolved. - Complete the volume up to 100 ml by adding 1X PBST. - Filter with 0.22 syringe filters. - Make aliquots and store at -20℃.
4% PFA Solution	- Measure 4 g PFA powder. - Add around 70 ml ddH_2O. - Add 200 µl 10N NaOH and place it in the oven set to 65℃. - Wait around 15 minutes until the solution gets clear. If not, add more NaOH gradually. - After all the powder is dissolved, take the beaker to room temperature. - Add 10 ml of 10X PBS. - Arrange pH to 7.4. - Complete the volume to 100 ml by adding water. - Check the pH again and set it to the final of 7.4. - Filter with 0.45 syringe filter. - Store at 4℃.

Table B.2. List of solutions and buffers (cont.).

1 M Maleic Acid Buffer$_{DT}$	- Measure 0.876 g NaCl and 1.161 g maleic acid. - Dissolve in 80 ml ddH_2O. - Set the pH to 7.5 by adding NaOH tablets directly. - Complete the volume up to 100 ml by adding ddH_2O. - Add 100 µl DEPC and stir overnight. - Autoclave, and check the pH afterwards.
10X PBS	- Measure 80 g NaCl, 2 g KCl, 14.4 g Sodium phosphate dibasic, and 2.4 g Potassium phosphate monobasic. - Dissolve in 800 ml ddH_2O. - Arrange pH to 7.4. - Complete the volume up to 1 L. - Filter and autoclave.
1 M Tris-Cl$_{DT}$	- Measure 30.27 g Trizma base. - Dissolve it in 200 ml ddH_2O. - Set the pH to either 7.5 or 8.0 by only adding HCl, depending on the need. - Complete the volume up to 250 ml with ddH_2O. - Add 250 µl DEPC and stir overnight.

	- Autoclave and arrange the pH again.
20X SSC_{DT}	- Measure 43.82 g NaCl and 22.05 g sodium citrate. - Dissolve in 200 ml ddH_2O. - Set the pH to 7.0. - Complete the volume to 250 ml. - Add 250 µl DEPC and stir overnight. - Autoclave and use after checking pH again. - Store at room temperature.