

Büşra ACAR

Ph.D. Thesis

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DEVELOPMENT OF BRAIN ORGANOID
MODEL TO TEST MULTI-TARGETED
PHARMACEUTICAL AGENTS FOR
MULTIPLE SCLEROSIS AND
COGNITIVE IMPAIRMENT AND STUDY
ALTERNATIVE EXTRACELLULAR
MATRIX FOR BRAIN ORGANOID
GENERATION

Ph.D. THESIS

SUBMITTED TO THE DEPARTMENT OF BIOENGINEERING
AND THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE
OF ABDULLAH GUL UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

By

Büşra ACAR

October 2025

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Büşra ACAR

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Ph.D. thesis titled Development of Brain Organoid Model to Test Multi-Targeted Pharmaceutical Agents for Multiple Sclerosis and Cognitive Impairment and Study Alternative Extracellular Matrix for Brain Organoid Generation has been prepared in accordance with the Thesis Writing Guidelines of the Abdullah Gül University, Graduate School of Engineering & Science.



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ABSTRACT

DEVELOPMENT OF BRAIN ORGANOID MODEL TO TEST MULTI-TARGETED PHARMACEUTICAL AGENTS FOR MULTIPLE SCLEROSIS AND COGNITIVE IMPAIRMENT AND STUDY ALTERNATIVE EXTRACELLULAR MATRIX FOR BRAIN ORGANOID GENERATION

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Brain organoids offer a valuable platform for modeling immune-mediated neurodegenerative disorders. Multiple sclerosis (MS), characterized by CNS-targeted autoimmunity, neuroinflammation, and demyelination, lacks robust in vitro models and effective therapies. To address this, MS-modelled brain organoids were developed using commercial extracellular matrix (Matrigel) and GelMA; LPS treatment induced MS pathology, and fingolimod derivatives were evaluated for therapeutic potential on the organoids. The results were analyzed by light microscopy, immunofluorescent microscopy, Raman spectroscopy, qRT-PCR, and ELISA. It was found that GelMA is effective as Matrigel to generate brain organoids with diverse neuron types (astrocytes, neural progenitor cells, mature neurons) but different spatially organized cell population, MS can be modelled with LPS induction, causing decreased myelination (2.4-fold in CNPase expression) and increased inflammation (1.7-fold in IBA1, 1.6-fold for GFAP, 4.4-fold NF- κ B and 6.7-fold for IL-6 expression) on brain organoids, and at least one of the compounds, ST-1505 would be a promising drug candidate because it increases myelination (2.9-fold MBP expression), decreases the inflammation (2.6-fold GFAP, 4-fold TNF- α , 2.3-fold FOXP3, 2.5-fold CSF-1 expression) and decreases the expression of cognitive impairment related markers (1.9-fold TAU expression).

Keywords: Brain organoid, Multiple sclerosis, Multi-targeted drug, Methacrylated gelatin, Extracellular matrix

ÖZET

MULTİPL SKLEROZ VE BİLİŞSEL BOZUKLUKTA ÇOK HEDEFLİ FARMASÖTİK İLAÇLARI TEST ETMEK İÇİN BEYİN ORGANOİD MODELİNİN GELİŞTİRİLMESİ VE BEYİN ORGANOİDİ OLUŞTURULMASI İÇİN ALTERNATİF EKSTRASELÜLER MATRİSİN İNCELENMESİ

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Beyin organoidleri, immün aracılı nörodejeneratif hastalıkların modellenmesi için değerli bir platform sunar. Merkezi sinir sistemini hedef alan otoimmünite, nöroenflamasyon ve demiyelinizasyon ile karakterize edilen multipl skleroz (MS), güçlü in vitro modellerden ve etkili tedavi seçeneklerinden yoksundur. Bu eksiklikleri gidermek amacıyla, ticari ekstraselüler matriks (Matrigel) ve metakrilatlı jelatin (JelMA) kullanılarak MS modellenmiş beyin organoidleri oluşturulmuş; LPS uygulaması ile MS patolojisi indüklenmiş ve fingolimod türevleri terapötik potansiyelleri açısından incelenmiştir. Bulgular ışık ve immüno Floresan mikroskobu, Raman spektroskopisi, qRT-PCR ve ELISA ile analiz edilmiştir. Sonuçlara göre, JelMA'nın, farklı hücre organizasyonları sergilemesine rağmen, astrositler, nöral progenitör hücreler ve olgun nöronlar gibi çeşitli nöron tiplerine sahip beyin organoidleri oluşturmakta Matrigel kadar etkili olduğu belirlenmiştir. LPS ile MS modeli başarıyla indüklenmiş; bu da miyelinleşmede azalma (CNPaz'da 2,4 kat) ve inflamasyonda artış (IBA1'de 1,7 kat, GFAP'ta 1,6 kat, NF- κ B'de 4,4 kat ve IL-6'da 6,7 kat) ile sonuçlanmıştır. Test edilen bileşiklerden en az biri, ST-1505, miyelinleşmeyi artırdığı (MBP'de 2,9 kat), inflamasyonu azalttığı (GFAP'ta 2,6 kat, TNF- α 'da 4 kat, FOXP3'te 2,3 kat, CSF-1'de 2,5 kat) ve bilişsel bozulma ile ilişkili belirteçlerini düşürdüğü (TAU, 1,9 kat) için umut verici bir ilaç adayı olarak öne çıkmıştır.

Anahtar kelimeler: Beyin organoid, Multipl skleroz, Çok hedefli ilaç, Metakrilatlanmış jelatin, Ekstraselüler matris

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LIST OF ABBREVIATIONS

3D	3-Dimensional
ANOVA	Analysis of Variance
BDNF	Brain-Derived Neurotrophic Factor
bFGF	Basic Fibroblast Growth Factor
BME	β -Mercaptoethanol
BMP4	Bone Morphogenetic Protein 4
cAMP	Cyclic Adenosine Monophosphate
cDNA	Complementary DNA
CHIR99021	6-[[2-[[4-(2,4-Dichlorophenyl)-5-(5-Methyl-1H-Imidazol-2-Yl)-2-Pyrimidinyl]Amino]Ethyl]Amino]-3-Pyridinecarbonitrile
CNPase	2',3'-Cyclic-nucleotide 3'-phosphodiesterase
DAPI	4',6-Diamidino-2-Phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
D-PBS	Dulbecco's Phosphate-Buffered Saline
EB	Embryoid Body
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
GelMA	Gelatin Methacrylate
GFAP	Glial Fibrillary Acidic Protein
GPCR	G-Protein-Coupled-Receptor
HEK	Human Embryonic Kidney
hESC	Human Embryonic Stem Cell
HOG	Human Oligodendroglioma Cells
IBA1	Ionized Calcium-Binding Adaptor Molecule 1

IF	Immunofluorescent
IFN	Interferon
IGF-1	Insulin-Like Growth Factor
IL	Interleukin
iMEF	Inactivated (Mitotically) Mouse Embryonic Fibroblast
iPSC	Induced Pluripotent Stem Cells
KOSR	Knock-Out Serum
LAP	Lithium Phenyl-2,4,6-Trimethylbenzoylphosphinate
MBP	Myelin Basic Protein
MEF	Mouse Embryonic Fibroblast
min	Minute
MAPK/ERK	Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase
MS	Multiple Sclerosis
NBT/BCIP	Nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate, toluidine-salt
NEAA	Non-Essential Amino Acids
NIM	Neural Induction Medium
OCT	Optimum Compound Temperature
OPC	Oligodendrocyte Progenitor Cells
PBS	Phosphate Buffered Saline
PDGF-AA	Platelet-Derived Growth Factor-AA
PI3K	Phosphatidylinositol 3-kinase
PFA	Paraformaldehyde
PLP1	Proteolipid Protein 1
RNA	Ribonucleic Acid
ROCK	Rho-Associated Protein Kinase
rpm	Revolutions Per Minute
RRMS	Relapsing-Remitting Multiple Sclerosis
RT-PCR	Real-Time Polymerase Chain Reaction
S1PR	Sphingosine-1-Phosphate Receptor
SATB2	Special AT-Rich Sequence-Binding Protein 2

SB-431542	4-[4-(3,4-Methylenedioxyphenyl)-5-(2-Pyridyl)-1H-Imidazol-2-Yl]Benzamide, Dihydrate, 4-[4-(1,3 Benzodioxol-5-Yl)-5-(2-Pyridyl)-1H-Imidazol-2-Yl]Benzamide
sec	Second
SEM	Scanning Electron Microscopy
SH-SY5Y	Human-Derived Neuroblastoma
T3	3,3',5-Triiodo-L-Thyronine
TAU	Tubulin-Associated Unit
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor





*To the 16-year-old me who dreams about
today and the future*

CHAPTER 1

Development of brain organoid model to test multi-targeted pharmaceutical agents for multiple sclerosis and cognitive impairment

1.1 Introduction

This chapter evaluates brain organoids for disease modelling and multiple sclerosis (MS). Brain organoid application compared to the traditional two-dimensional (2D) culture systems and animal models will be introduced, and then MS will be summarized in the context of disease etiology, its clinical features, immunopathology, and current therapies.

1.1.1 Disease modeling

Disease modeling is essential to understand the underlying mechanisms of various diseases and to develop effective therapeutic approaches. Replicating the specific pathology of a disease under a controlled environment through in vitro systems (two-dimensional (2D) culture and three-dimensional (3D) culture), animal models, or computational simulations helps researchers to investigate how the disease is initiated, progresses, and responds to the treatments. Improvements of the models provide a critical insight into molecular and cellular processes, support investigations of potential drug targets and biomarkers, and allow for the development of new therapies [1], [2]. Although animal models are one of the primary tools used in health research, including the development of new drugs and therapies, and they provide a complex human-like physiology under controlled conditions, they do not accurately reflect human physiology and raise ethical considerations [3], [4]. The ethical issues led Russell and Burch to present a universal principle known as the 3Rs, “Reduction, Refinement, and Replacement,” in 1959. Today, the 4th R, as a responsibility, is also considered by animal ethics committees [5], [6]. Ongoing research focuses on three-dimensional (3D) cell cultures within microenvironments that mimic the extracellular matrix (ECM) to enhance the physiological relevance of in vitro models for evaluating therapeutic strategies. These advanced culture systems are expected to improve in vitro–in vivo correlations while also contributing to the reduction of animal usage in research, supporting the 3Rs [7], [8].

1.1.1.1 2D disease modeling

Two-dimensional cell culture systems are used to model diseases at the preliminary stage of human-targeted applications. The models include primary cells or cell lines grown as a monolayer on a flat surface (culture dish or cell culture plate) or in a suspension to be exposed to various agents, such as small molecules, drugs, siRNAs, etc. This approach allows for the measurement of cellular responses to various physiological changes using biological assays that specifically assess changes in the expression levels of cellular components. Primary cell lines are preferable in research because of their physiological relevance compared to the immortalized cell lines. However, their limited proliferation capacity and availability restrict their usability. Multipotent adult stem cells and induced pluripotent stem cells (iPSCs) overcome these limitations by ensuring an

indefinite propagation capacity and transforming into somatic cells. Also, reporter cell lines, as engineered cells, are widely utilized for their suitability in high-throughput applications, offering advantages in scalability, reproducibility, and cost-efficiency over stem cell-based approaches [2], [9], [10].

Despite the advantages of the 2D culture systems, they have considerable limitations. They are unable to mimic the natural microenvironment around cells in tissue because the niche, one of the main determinants of the cell fate, is not present in monolayer culture (Figure 1.1.1). The niche also maintains the cell-cell and cell-extracellular environment interaction, which 2D culture lacks. Furthermore, cells lose their polarity and their diverse phenotypic characteristics because they are propagated from one cell. [2], [10], [11]. Nevertheless, they allow for the control of gene and protein expression upon treatment with a relatively low cost and shorter experiment duration, and it makes the 2D culture system preferable for the initial phase of the studies [12]. For example, retinoic acid addition to neuronal cell lines such as neuroblastoma (SH-SY5Y) or brain-derived growth factor addition to immature neuronal cell lines such as NT2 make them suitable models for studying the mechanism of axonal damage in MS. Similarly, OLN-93, which originated from rats, and HOG, which originated from humans, are oligodendrocyte cell lines, and they are able to differentiate under low serum conditions. When the maturation markers are presented on the cell surface, the cell lines are used as a 2D model for mechanistic studies about myelination [13], [14].

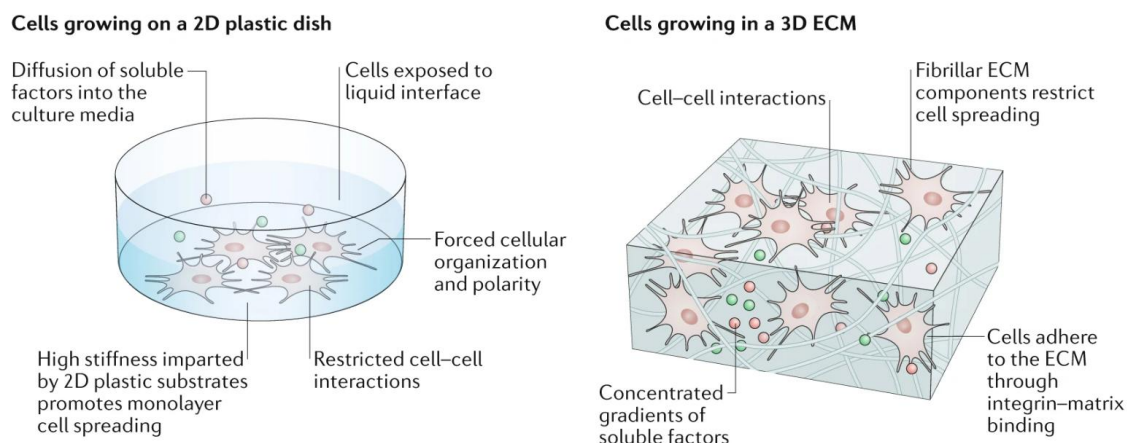


Figure 1.1.1 Comparison of cells growing in 2D and 3D culture [15]

1.1.1.2 Animal models

Animals provide a complex, controllable experimental environment with organ systems similar to human physiology. Therefore, bioavailability, toxicity, and therapeutic efficiency of the molecules are primarily tested on animals before clinical trials [7]. Animal models could be invertebrates such as *Caenorhabditis elegans* (worm) and *Drosophila melanogaster* (fruit fly) [16] or vertebrates such as rats, mice, rabbits, chimpanzees, fish, and frogs [17]. Modelling a disease in animals may involve the complete animal or particular organs/tissues/cells that mimic the pathology of the disease. Animal models provide a representation of whole-body reactions, allow for the identification of behavioral changes, and enable the modeling of various diseases, including autoimmune and neurodegenerative diseases. However, all human biological responses cannot be translated to animal models, as animal models are not time-efficient and are costly. Also, the genetic models' variability is limited [16], [17]. Nevertheless, they are commonly used in research studies. For example, there are MS-modelled animals that are generated by virus induction (Semliki Forest virus and Theiler's murine encephalomyelitis virus), toxin induction (cuprizone, lysophosphatidylcholine, lipopolysaccharide, and ethidium bromide), and transgenic mouse models [13]. The scientific basis of the models is similar, as they aim to either induce demyelination or induce inflammation directly or indirectly [3], [18], [19], [20].

1.1.1.3 Organoids

Most of the data for the drug candidates for clinical trials is obtained from animal-derived data; however, most of the drugs fail, although their efficiency and safety are proven [2]. The translational gap between preclinical studies and clinical trials underscores the necessity for more representative disease models. To enhance the physiological relevance of conventional systems and better mimic human tissue architecture and pathology, 3D culture models that replicate the native microenvironment are increasingly being developed. Organoids, one of the 3D culture models, are self-organizing, embryonic stem cell-derived, organ-like structures recapitulating human tissue and organ cytoarchitecture in an extracellular matrix. Organoid formation is guided by a growth factor cocktail whose composition depends on the targeted organ [2], [7]. The "organoid" term was first used in 1946 to define cystic teratoma; however, the

consensus about the nomenclature was built by the scientists working on nervous system organoids in 2022 [18], [21]. The initial transition of organoid research from 2D culture to 3D culture was performed by Dr. Eiraku et al., and the system was called “three-dimensional aggregation culture (SFEBq culture).” Then, in 2009, a milestone study showing an intestinal organoid generation in commercially available ECM (Matrigel) was published. In this study, single leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5)-positive adult stem cells (ASCs) were self-organized and differentiated into crypt-villus structures, which are very similar to the freshly isolated small intestine structures, and it was the first reported study showing organoid generation from ASCs. Additionally, this study provides a foundation for generating organoids in other organoid systems, such as brain organoids [22], [23], [24].

It was reported that organoid research has sharply increased after 2011 because the organoid models provide a field to study histopathology, drug development, and screening studies. Organoids meet the needs of all kinds of research because they offer advantages over traditional 2D culture and animal models [24]. As summarized in Figure 1.1.2, recapitulation of the human tissue environment containing various cell types with a specialized niche is achieved by organoids only because pathological responses of a single cell line and multiple cell lines grown in the same environment are not parallel. Furthermore, species-specific pathological response is not possible to obtain with animal models. For example, neurofibrillary degeneration is one of the primarily seen pathologies in human Alzheimer's disease; however, the pathology is seen in animal models sparsely [24], [25], [26]. In addition to the physiological advantages of organoids, ethical issues about animal models make organoids the greatest model for research [27].

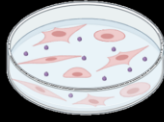
	2D CULTURE	ORGANOIDS	ANIMAL MODELS
			
Diverse cell types	Absent	Present	Present
Multiple cell-cell interaction	Limited	Present	Present
Modelling human physiology	Feasible	Feasible	Limited
High-throughput screening	Applicable	Applicable	Not applicable
Vascularization	Limited	Limited	Feasible
Genetic manipulation	Feasible	Feasible	Limited
Human specific genes	Conserved	Conserved	Absent
Reproducibility	High	Moderate	Low
Neurodegenerative disease modelling	Low	Moderate	Moderate
Cost	Low	Moderate	High

Figure 1.1.2 Comparison of 2D culture, organoids and animal models [3], [18], [19], [20]

1.1.3.1 Brain organoids

The human central nervous system is derived from neuroectoderm in the developmental process. The human brain, as a part of the central nervous system, is mainly composed of three main regions: the forebrain, midbrain, and hindbrain. The main components of the regions are neurons and glial cells. Earlier studies in 1966 hypothesized the self-organizing capacity of these cells. Researchers observed that neural progenitors from early developing chick brains clustered radially by themselves in a dissociation-reaggregation study [22], [28]. Similarly, it was shown that neural progenitor cells form aggregates and neurospheres in suspended culture conditions, and they differentiate into neurons and astrocytes by epidermal growth factor in 1992 [29]. The neural aggregates can also be derived from embryoid bodies, formed from pluripotent stem cells. Then, neural rosettes were discovered. They are a kind of stem cell stage in the transition from embryonic stem cells to differentiated neurons. It was found that they express both stem cell (Sox2) and neural cell markers (PLZF and Dach1), and they are organized around a central lumen that mimics the neural tube. The rosettes can also be further differentiated into diverse mature neuron and glia types [22], [30]. Nonetheless, all these models are considered two-dimensional culture systems or simple cell aggregates that lack the structural and functional complexity that is required for proper brain models [22].

The studies that laid the foundations for brain organoids of different brain regions were conducted by Watanabe and colleagues in the 2000s. They initially created embryoid bodies generated from mouse or human pluripotent stem cells that were cultured in 2D culture conditions to induce forebrain identity. Upon transfer to 3D suspension culture, neuroepithelial cells self-organized into more complex structures resembling the dorsal forebrain [23], [31], [32]. The protocol was subsequently optimized to promote the formation of layered cortical-like structures that could be maintained in culture for over 100 days, mimicking early cortical development. Additionally, region-specific brain identities can be established from embryonic stem cells through modulation of key signaling pathways, including Hedgehog, fibroblast growth factor (FGF), bone morphogenetic protein (BMP), and Wnt [22].

In 2013, Lancaster and colleagues conducted studies that established the foundation for today's brain organoids, which contain more than one brain region within a single structure. Their technique was built based on the previous work published by Watanabe and colleagues, which was carried out by embedding embryoid bodies in Matrigel to support polarization and extension of neuroepithelial buds. Lancaster and her colleagues cultured organoids in a spinning bioreactor, which allowed organoids to grow up to a few millimeters in diameter and develop various brain regions, such as the choroid plexus, retina, and ventral forebrain [22], [33]. Further advancement provides the opportunity to generate other brain region-specific organoids, such as hippocampal organoids and cerebellar organoids [34], [35]. The generation of different brain regions has different purposes. For example, forebrain from iPSCs was generated using a mini spinning bioreactor to clearly demonstrate the outer subventricular zone (OSVZ) and outer radial glia (oRG) cell population to study human cortical development [36]. In another example, cortex from iPSCs was generated to obtain electrophysiologically mature neurons surrounded by astrocytes [37]. Since off-target germ layers are induced during the organoid generation, the pallium was successfully generated from iPSCs and ESCs to obtain diverse interneuron subtypes, preventing off-target mesoderm differentiation, and the pallium was used to model Timothy syndrome [38], [39]. Additionally, advancements in 3D printing technology allow for the creation of mini-spinning bioreactors that facilitate cost-effective organoid production [36]. Commonly used bioprinting techniques are inkjet-based and extrusion-based 3D bioprinting methods, and brain organoids were generated using these methods. For example, in the

inkjet-based method, cells (10^6 - 10^7 cells/ml) and biomaterials are mixed in a “bio-ink” to be ejected on a target surface with a defined pattern. The ejected neuronal structures with proper electrophysiological function are placed in a rat brain for targeted differentiation [40], [41]. In the extrusion-based method, high-viscosity biomaterials are printable, and high-density cell loading (10^8 - 10^9 cells/ml) of the bio-ink with micrometer-scale resolution is possible. The printed constructs can still retain pluripotency characteristics, and when they are incubated in neural induction medium, they differentiate into diverse neuron types. Although three-dimensional (3D) bioprinting represents a pivotal advancement in the precise fabrication of organoid architectures, it continues to encounter several technical limitations. For instance, extrusion-based bioprinting exhibits relatively low throughput, which may hinder its scalability for large-scale applications. Furthermore, the spatial resolution achieved by extrusion printing is generally lower than inkjet-based bioprinting [41], [42].

Considering the application of brain organoids, they have emerged as a valuable model system for neurodegenerative diseases. The diverse tissue type content, long-term culture potential supporting the continuous tissue expansion, and 3D cytoarchitecture relevance to the human establish them as a powerful platform for various biological and clinical applications. Notably, organoids have been extensively utilized in developmental and disease modeling, precision medicine, toxicology testing, and regenerative therapies (Figure 1.1.3) [20], [22]. In 2016, there was no proper model to study the neural development impairment caused by the Zika virus, which leads to microcephaly in fetuses. Qian and colleagues developed forebrain-specific organoids in a miniaturized multi-well spinning bioreactor and generated a model showing progenitor zone organization and a human-specific outer radial glia cell layer. After they induced their organoids with different strains of Zika virus, decreased neuronal cell layer volume resembling microcephaly was observed, showing disease modelling with organoids [22], [36]. Then, in 2017, the fusion of regionally specified organoids was achieved. The fused structures called assembloids allow the interaction and cell migration between different regions to mimic the *in vivo* physiology. Therefore, tangential migration from ventral to dorsal areas and thalamic projections to the cortex become possible [41], [43], [44], [45]. In 2018, vascularized cerebral organoids were generated. The vascularization has been achieved by either transplantation [46], induction [47], or co-culturing methods [48]. However, the scalability of the vascularized organoids has not been overcome yet;

therefore, the protocols must be optimized to be replaced with standard brain organoid culture [45]. Then, in 2022, researchers successfully transplanted a human cortical organoid to the cerebral cortex of early-postnatal rats. The transplanted organoids were successfully integrated into the neural circuits of the rat, enabling the generation of a new platform for neurodevelopmental research [41], [49]. More recently, the cellular composition-related disadvantages of the brain organoid have become prominent because most of the pioneer protocols aimed to generate brain organoids with only neuroectodermal-derived cell populations. Since the organoids do not have a mesoderm-derived cell population, such as microglia, meninges, and blood vessels, they do not fully recapitulate the human central nervous system [50]. The co-culture of the organoids with immune cells has been highlighted recently to better understand various neurologic conditions, immune interactions, and in vivo neuronal microenvironments. In a study, Dr. Geresimova and her colleagues introduced a co-culture model of T cells with midbrain organoids, addressing neurodegenerative conditions such as Parkinson's disease. Their study emphasizes that, despite its novelty, the model lacks critical features like the blood-brain barrier (BBB), suggesting future incorporation of vascularized brain organoids to enhance the study of immune cell penetration and neuroinflammation. Nevertheless, demonstration of infiltration of peripheral T cells to the CNS in a brain organoid model would be a pioneering study for immune-related neurodegenerative diseases like multiple sclerosis and Parkinson's disease [51]. In another study, Dr. Xu and his colleagues co-cultured primitive macrophage progenitors and neural progenitor cells to understand the effect of microglia as brain-resident immune cells on brain development and brain development-associated pathologies. They also showed how Zika virus infection causes an inflammation and how microglia eliminates synaptic components upon infection [52].

Apart from the improvements of human brain organoid generation processes, shortages of 2D cell culture models and animal models must be considered to understand the requirement of disease modeling with brain organoids. In this context, rodent models of Alzheimer's disease (AD) and Parkinson's disease (PD) are known to fail in replicating the pathophysiology of these diseases, while brain organoids provide a good model for recapitulating their early phases [20], [53]. Researchers study the early onset of AD and PD in pluripotent stem cell-derived brain organoids, despite the fact that most neurodegenerative diseases occur in adulthood. For example, SNCA-A53T and LRRK2-G2019S mutations are associated with PD, and induced pluripotent stem cell (iPSC)-

derived organoids carrying these mutations were used to model PD. It was shown that the organoids exhibited synaptic defects and dopaminergic neuronal loss to mimic PD [20], [54]. Since organoid generation is a process ranging from stem cells to embryoid bodies to mature organoids, transient and stable genetic manipulation is possible at these stages. For example, TALEN-inducible knock-in to create a sonic hedgehog (SHH) protein gradient was achieved on H9 and H9 derivatives to study the role of cholesterol metabolism in sonic SHH signaling [55], [56]. In another example, a GFP construct with a Sleeping Beauty transposon was transfected into cerebral organoids by electroporation. The preparation of the organoids was performed to culture them with an air-liquid interface to improve axonal outgrowth (Fischer et al., 2019; Giandomenico et al., 2019).

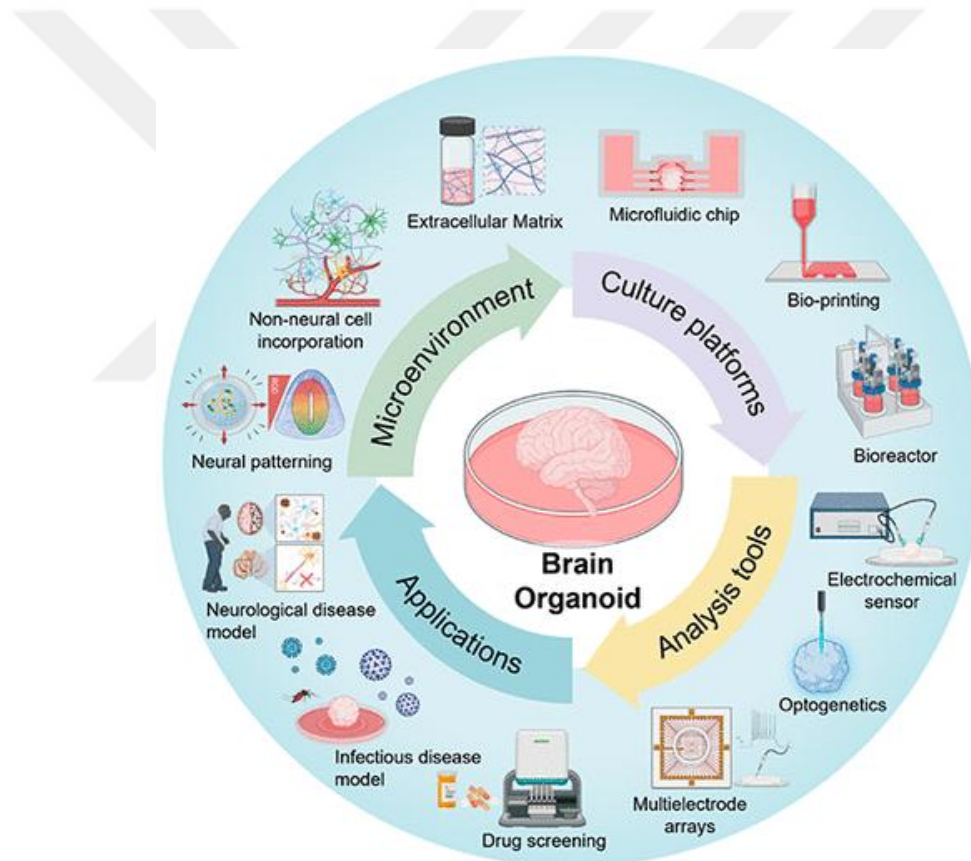


Figure 1.1.3 Application of brain organoids [58]

Immune cells and neurons provide brain organoids to model inflammation-mediated neurodegenerative diseases in addition to genetic-modification-based diseases. It was reported that mature or immature microglia incorporation into a brain organoid resulted in secretion of cytokines and chemokines upon stimuli [59], [60]. It was also

reported that when the heparin concentration at the neural induction step is low, brain organoids develop microglia without performing additional incorporation steps [61], [62]. Also, neurodegenerative diseases, such as Pelizaeus-Merzbacher disease, resulting in reduced myelination, can be modeled with brain organoids [63]. The literature also reports the generation of myelinating 3D culture models, known as myelinoids, from iPSCs and human embryonic stem cells [64], [65]. More recently, a crucial development reported by Dr. Fagiani and her colleagues highlights a glia-enriched 3D model of the human brain that successfully mimics the glial-immune neurodegenerative phenotypes associated with MS. This study employed a short ectopic single transcription factor (SOX10) expression to promote oligodendrocyte differentiation from human iPSC-derived neural precursor cells, generating self-organizing forebrain organoids that integrated neurons, astrocytes, oligodendrocytes, and microglia. This multi-cell type composition allows for the examination of the interplay between neural cells and inflammatory processes, which is pivotal for understanding MS pathology [66]. Also, at least two different research groups integrate the microglia in a region-specific brain organoid to replicate the brain microenvironment and understand how immune cells influence neuronal health. Both studies showed how the integration of microglia affects the brain response to the inflammatory challenges; however, they did not recapitulate inflammation-caused demyelination pathology [67], [68]. Therefore, even though there are several organoid models of central nervous system diseases, no studies have reported demyelination occurring upon inflammation induction, as is observed in multiple sclerosis pathology.

1.1.2 Multiple Sclerosis

Multiple sclerosis is an immune-mediated neurodegenerative disease characterized by demyelination in the central nervous system [69]. A German pathologist, Friedrich von Frerich, made one of the early clinical and pathological descriptions of MS, and he called it “Hirnsklerose [brain sclerosis]” because of the sclerotic lesions spread through the nervous system. He was the first to diagnose the disease of living patients; however, his colleagues mostly rejected his disease description. Simultaneously, Edméé Vulpian in Paris and Ernst Leyden in Berlin examined some patients, and they were the first to claim heredity. [Metin girmek için buraya tıklayın veya dokunun.](#) However, the first well-defined description as ‘la sclérose en plaques’ was made by Jean-Martin Charcot,

the father of neurology, in 1868. He defined three main symptoms of the disease, called Charcot's triad: intention tremor, nystagmus, and scanning speech. Also made a distinct separation between multiple sclerosis and paralysis agitans (now it is called "Parkinson's disease") [70], [71]. Then, Dr. James Dawson conducted a detailed microscopic analysis of post-mortem brain tissues of MS patients utilizing advanced histological techniques in 1916. Then he could write a comprehensive characterization of inflammation around the blood vessel and myelin damage [70]. Today, it is still accepted that MS is an inflammatory central nervous system disease affecting the myelin sheath, even though some factors, including inflammatory demyelination and autoimmunity, underlie it, and its initiation has not been fully revealed yet [72].

1.1.2.1 Etiology

The precise causes of the disease are not fully understood yet. MS is widely considered to arise from multidimensional interaction between genetic susceptibility and environmental factors, leading to an aberrant autoimmune response. In the context of genetic factors, the polymorphism of some genes listed below is considered a factor associated with high susceptibility to MS.

- Immune-related genes: HLA-DR, CD24, IL2RA, CD58, IL4, IL6, IL12B, IL17R, IRF5, EVI5 [73], TNF α , IL-18 [74], [75]

- Vitamin D metabolism: VDR and CYP27B1 [73], [76]

- Certain genes in mitochondrial DNA: ND3, ND5, Cyt5, ATP6 [73], [77]

- CNS function and repair: ApoE and DPP6 [73], [76]

Among the factors, the highest susceptibility is caused by the HLA-DRB1*15:01 allele within the major histocompatibility complex (MHC) class II region. The gene encodes the β -chain of HLA-DR, presenting extracellular-derived antigens to CD4⁺ T helper cells. In MS patients, the CNS myelin antigens are presented to the T cells, allowing the autoreactive T cells to cross the BBB, recognize myelin protein, and enhance neuroinflammation and demyelination [78], [79].

In the context of environmental factors, smoking, vitamin D, sunlight exposure, and Epstein–Barr virus (EBV) infection are the well-established ones correlating with MS

susceptibility. Night shift work, exposure to organic solvents, coffee and alcohol consumption, and obesity are also considered as increased/decreased risk factors [80], [81]. Even though multiple infectious agents are considered as an increased risk factor in MS, EBV, which was first hypothesized by the student of Charcot, stands out as the most consistently implicated and supported by the literature [81], [82]. A region of EBV transcription factor EBV nuclear antigen 1 (EBNA1), central nervous system protein glial cell adhesion molecule (GlialCAM), and myelin basic protein (MBP) is structurally shared by three molecules; therefore, it results in the cross-reactivity of the immune system upon the infection. The mimicry of three molecules causes an attack of the immune system against both EBV antigen and auto-antigens, leading to MS [83], [84]. Vitamin D and sunlight exposure are also among the studied environmental factors affecting MS because deficiency in vitamin D affects both T and B lymphocytes, inhibiting their proliferation and maturation. However, since Vitamin D and ultraviolet radiation (UVR) are connected to each other in terms of the conversion of Vitamin D to an active metabolite, it is difficult to consider the two factors independently. It was also reported that the role of vitamin D is associated with the genetic background because polymorphism in CYP27B1, which is closely related to vitamin D metabolism, is linked with MS [14], [81].

1.1.2.2 Immunopathology

In MS, two complementary paradigms—the "outside-in" and "inside-out" models—are central to current discussions on the origin of multiple sclerosis. The outside-in model suggests that MS is initiated by an autoimmune response originating in the peripheral immune system, targeting myelin within the CNS. In contrast, the inside-out model proposes that the disease begins with a primary degenerative process within the CNS, which subsequently triggers a secondary immune response against released myelin antigens [85]. In the outside-in hypothesis, inflammatory autoimmune responses contribute to the pathological mechanisms underlying neurodegeneration and lesion formation in multiple sclerosis. Although the triggering event is not understood, the inflammatory autoimmune response is initiated by autoreactive T cells, which further react with self-antigens in the CNS. When autoreactive T cells start to recirculate from the lymph node to the CNS, they have crossed through the blood-brain barrier (BBB) and are activated by microglia and astrocytes, which causes the release of cytokines and

chemokines to increase the permeability of the BBB. The increase in permeability functions to recruit antigen-presenting cells such as B cells, monocytes, and macrophages. The processes result in myelin destruction, causing the axonal loss, cognitive impairment, and demyelinated plaques (Figure 1.1.4) [14], [72], [85], [86].

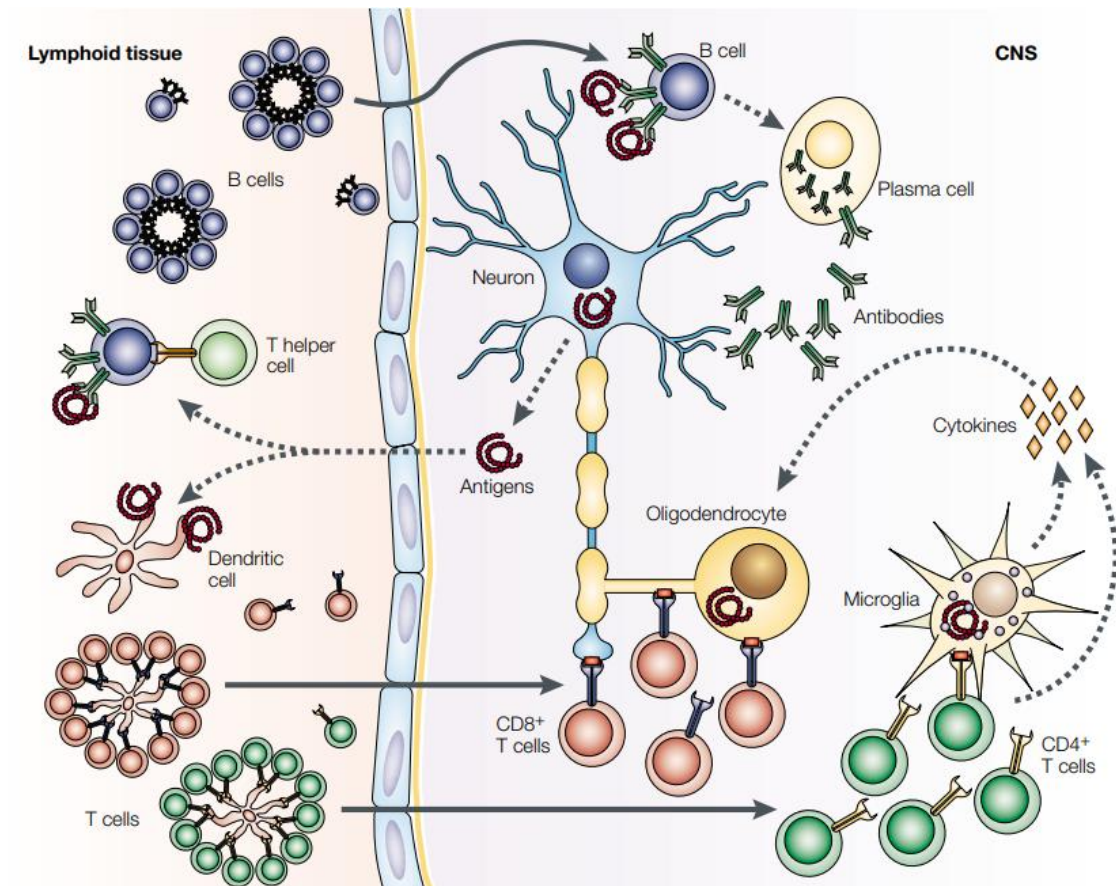


Figure 1.1.4 Inflammatory response mediated neurodegeneration in multiple sclerosis pathophysiology [86].

In the inflammation-mediated site of the MS, numerous studies have demonstrated that the adaptive response is triggered by the interaction between antigen-presenting cells (APC) and T lymphocytes. Inadequately regulated and/or aberrantly activated effector T cells, especially CD4⁺ T helper (Th) and CD8⁺ Th cells, play a vital role in the initiation and the progression of the disease. Two possible reasons for the aberrant behavior of Th cells are the insufficient function of regulatory T cells (T_{reg}) and resistance of effector T cells against T_{reg} [87], [88]. Also, regarding the APC and T cell interaction, pathogen-associated molecular patterns (PAMPs) simultaneously activate the toll-like receptors (TLRs) on APCs, causing the production of cytokines and interleukins such as IL-12, -

23, and -4. The production drives the differentiation of naïve CD4⁺ T cells into IFN γ -secreting CD4⁺ T cells (called Th1) and IL-17-expressing CD4⁺ T cells (called Th17), which also release specific cytokines [87], [88]. The important cytokines produced by Th1 cells are interferon-gamma and tumor necrosis factor, which sustain innate and adaptive immunity. Additionally, these cytokines inhibit Th2 differentiation, which promotes inflammation since Th2 cells secrete anti-inflammatory cytokines like IL-4 and IL-13. In addition to Th1, Th17 is another subset of CD4⁺ T cells and contributes to the inflammation through the production of cytokines such as IL-17, -21, and -22. The increase in cytokine secretion is associated with MS pathogenesis. Besides the T cells and their role in MS, B lymphocytes contribute to the disease through cytokine secretion. The cells are responsible for producing lymphotoxin (also called tumor necrosis factor-beta, TNF- β) and tumor necrosis factor alpha (TNF α). On the contrary, it has also been studied that B cells are responsible for the production of interleukin-10 (IL-10), which has anti-inflammatory properties. Therefore, it is accepted that B cells exhibit a dual role in MS, showing pro-inflammatory and anti-inflammatory effects [87], [89], [90], [91]. Also, B cells identify even lower concentrations of antigens compared to APC, and they induce their differentiation to memory cells and antibody-producing memory cells. Therefore, they act as an APC to introduce antigens to T cells for T cell activation. In addition to functioning as antigen-presenting cells (APCs), they also serve as memory cells. The first evidence of this role of B cells was the presence of oligoclonal bands (OCB) in the cerebrospinal fluid (CSF) of many MS patients. It was reported that the OCB includes autoantibodies against myelin basic protein (MBP), contactin-2, myelin-associated lipids, and myelin oligodendrocyte glycoprotein (MOG). The recognition of the proteins by autoantibodies causes the pathophysiology of MS [92], [93]. In addition to their direct role in immunity, upon activation, immune cells upregulate the expression of some surface molecules. For example, chemokine receptors (CCR)-2, -6, and -5 and cell surface glycoprotein MUC18 (also known as MCAM) upregulation function in the infiltration of immune cells [94], [95].

Regarding the CNS site of the MS, the conventional understanding of T cell-mediated relapses in MS has evolved to contain bidirectional interactions among various immune cell populations. These cell populations include T lymphocytes, B lymphocytes, and myeloid cells in the peripheral immune compartment, as well as CNS-resident cells such as microglia and astrocytes [95]. At the molecular level, upon infiltration of immune

cells into the CNS, they secrete cytokines and shape the glia responses in the brain. The activated cells have higher energy consumption, which causes local hypoxia, acidosis, and excitation/inhibition patterns of neurons. The affected glia cells also secrete neurotoxic factors, such as saturated lipids, which contribute to neurodegeneration. The physiological changes lead neurons to produce toxic lipid peroxidases and reactive oxygen species. The injured neurons start to activate neighboring neurons by secreting glutamate and damage-associated molecular patterns. Then they maintain toxic lipid production, causing the demyelination [96]. At the cellular level, inflammation-caused demyelination may undergo remyelination by peripherally derived macrophages, which induce oligodendrocyte precursor cells (OPCs). Also, CNS resident immune cells, microglia, provide a microenvironment for myelin regeneration by expressing “platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-1), CXCL10, CXCL3, TGF- β 1, MMP12, and MMP14,” which promote the survival and maturation of OPC and support oligodendrocyte remyelination through the expression of semaphorin-3F (SEMA3F) to recruit OPC. Even though the myelin debris inhibits OPC differentiation, microglia provide a proper microenvironment by clearing myelin debris. Proliferating OPCs differentiated into mature oligodendrocytes to form the myelin sheath. However, the newly formed myelin sheath is histologically different from the previous healthy myelin sheath in terms of thickness [91], [97], [98]. Astrocytes, which are activated by microglia through TNF and Interleukin-1 β (IL-1 β) secretion, also contribute to tissue repair following demyelination by producing endogenous neurotrophic factors like brain-derived neurotrophic factors (BDNF). The factors support neuronal and axonal survival. Also, leukemia inhibitory factor (LIF) secretion by astrocytes enhances the OPC maturation [98]. In contrast, microglia-stimulated astrocytes produce CC-chemokine ligand 2 (CCL) and granulocyte-macrophage colony-stimulating factor (GM-CSF) to recruit more microglia. Therefore, remyelination is inhibited via the inhibition of OPC maturation by astrocytes [91], [99].

1.1.2.3 Current therapies

Current MS therapies are mostly focused on reducing relapse frequency, ameliorating symptoms, and delaying progression. The drugs in the therapy approach are called disease-modifying therapies (DMTs) [100]. According to the MS International

Federation report, more than 20 types of DMTs are used to treat MS [101]. Interferon beta (IFN β), as a first DMT and type I interferon, is used in four different forms; however, all forms shift the T cell profile from pro-inflammatory cells (Th1/Th17) to anti-inflammatory cells (Th2). Also, it reduced the T cell adhesion and penetration to the BBB [88]. Glatiramer acetate is an FDA-approved immunomodulator used in MS treatment. It is a peptide containing four amino acids: glutamic acid, lysine, alanine, and tyrosine as an analog of myelin basic protein (MBP), which is one of the major autoantigens in MS. It basically binds to major histocompatibility complex (MHC) II by simulating MBP to decrease the reactivity against MBP [102], [103]. Natalizumab is a monoclonal antibody, and it is the α 4 integrin antagonist. It is the first targeted therapy and targets lymphocyte entry to the CNS, preventing the α 4 β 1 interaction between vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells of the BBB, thereby preventing neuroinflammation [104], [105]. Alemtuzumab is a humanized monoclonal antibody that targets CD52, a surface protein highly expressed on mature lymphocytes, including both T and B cells. After binding to CD52, alemtuzumab induces antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), which result in the destruction of T and B cells. This is followed by a slow and selective immune reconstitution process over months to years [106]. Teriflunomide is an oral immunomodulatory DMT. It inhibits dihydro-orotate dehydrogenase (DHODH), a mitochondrial enzyme essential for de novo pyrimidine synthesis. Activated T and B lymphocytes rely heavily on this pathway to proliferate rapidly during an immune response. By inhibiting DHODH, teriflunomide selectively reduces the proliferation of activated lymphocytes, while sparing resting cells that can use salvage pathways for nucleotide synthesis. Dimethyl fumarate is an oral DMT. It is derived from fumaric acid esters and was originally used in the treatment of psoriasis. In MS, DMF exerts its therapeutic effects primarily through anti-inflammatory and cytoprotective (cell-protecting) mechanisms. The key mechanism of action of dimethyl fumarate involves activation of the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway, which plays a central role in the cellular defense against oxidative stress. Activation of Nrf2 leads to increased transcription of antioxidant response-related genes. These genes encode detoxifying enzymes and antioxidant proteins that protect neurons and glial cells from inflammation-induced damage and oxidative injury, which are both major contributors to demyelination and neurodegeneration in MS. This enhanced cellular resilience contributes to the neuroprotective effects of the drug [107], [108].

Fingolimod (Gilenya®, FTY720 [Novartis Pharma AG]), a structural analog of sphingosine, is the first Food and Drug Administration (FDA)-approved drug, and it is the first generation of sphingosine-1-phosphate receptor (S1PR) modulators [91], [109], [110]. Fingolimod (2-amino-2[2-(4-octylphenyl) ethyl]-1, 3-propanediol) was first discovered in 1992 by Yoshitomi Pharmaceutical Industries. It is a derivative of a fungus (*Isaria sinclairii*) metabolite, myriocin (ISP-I), and mainly acts as an immunosuppressor. It binds to sphingosine-1-phosphate receptor (S1PR) competitively and in a functional antagonistic manner and induces the receptor internalization and degradation via ubiquitination-dependent pathways. The decreased number of S1PR on the cell membrane inhibits egress of autoreactive lymphocytes from lymph nodes, leading to a reduction in the frequency of relapses in MS [111], [112]. In addition to its role in immune system cells, it also has a direct neuroprotective effect on the CNS by passing through the blood-brain barrier and restricting pro-inflammatory cytokine secretion of astrocytes [91].

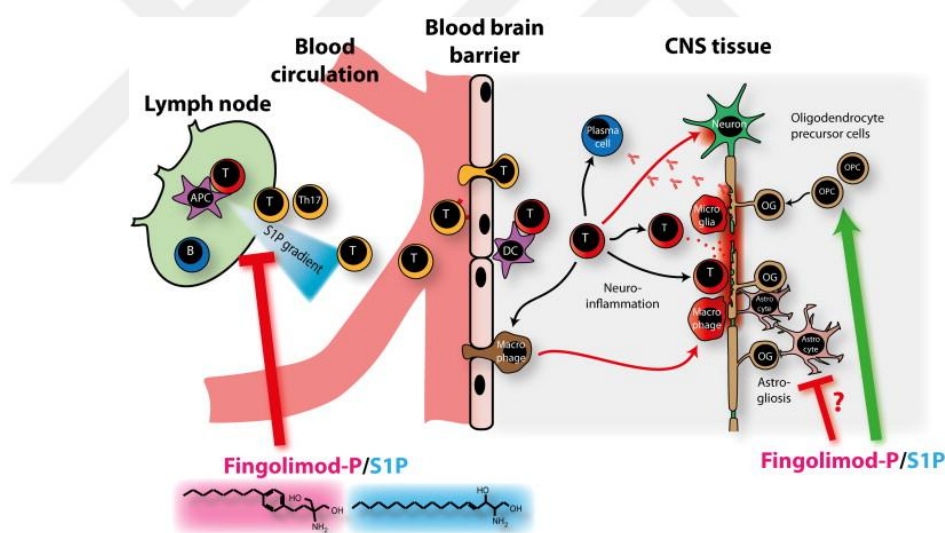


Figure 1.1.5 Action mechanism of fingolimod [113].

Beyond the S1PR, which is one of the G-protein-coupled receptors (GPCR), targeting another GPCR, the histamine receptor, is another disease-modifying target strategy. Since histamine is involved in the regulation of oligodendrocyte progenitor differentiation, demyelination, and remyelination processes, its effects on MS have been researched [114]. A study revealed a lower serum histamine level in RRMS patients compared to healthy individuals [115]. In another study, targeting histamine receptors in an inverse agonist manner suppresses the LPS-induced pro-inflammatory cytokine (*IL*-

1β, *IL-6*, and *TNFα*) expression in microglia [116], [117]. Ciproxifan (cyclopropyl 4-(3-(1*H*-imidazol-4-yl)propyloxy)phenyl methanone) is one of the well-characterized histamine receptor 3 inverse agonists/antagonists [118].Metin girmek için buraya tıklayın veya dokununuz. Since ciproxifan treatment showed memory impairment [119], learning alleviation [120], [121] and improvement of depression-like behavior (Kumar et al., 2019) in addition to its immunomodulatory properties [117], it is a promising immune-mediated neurodegenerative disease-targeted disease-modifying drug because cognitive impairment is one of the most significant challenges in these diseases [117], [119], [123], [124].



1.2. Materials and Method

The solutions used in the experiments are listed in Table 1.2.1, and the chemicals were mentioned in Sections 1.2.1-1.2.13.

Table 1.2. 1 Cell culture medium, solutions, and their chemical content.

Cell Culture Medium and Solutions	Chemical Content
Complete medium for neuroblastoma	DMEM/F12 10% Heat-inactivated FBS 1% penicillin/streptomycin
Complete medium for human oligodendroglioma	High-glucose DMEM 10% FBS 1% penicillin/streptomycin
Complete medium for human embryonic kidney	DMEM 10% FBS 1% penicillin/streptomycin
1xPBS	137 mM NaCl 2.7 mM KCl 10 mM Na ₂ HPO ₄ 1.8 mM KH ₂ PO ₄ 1 M HCl
Crystal violet solution	0.5% crystal violet 10% ethanol
Sodium citrate solution	0.1 M sodium citrate 50% ethanol
MEF medium	High-glucose DMEM 55 µM β-mercaptoethanol 1% NEAA 10% FBS 1% penicillin/streptomycin
Mitomycin C solution	High-glucose DMEM 10 µg/mL Mitomycin C 10% FBS
hESC medium	DMEM/F12 2.5 mM L-glutamine 20% KSR 55 µM β-mercaptoethanol 1% NEAA 4 ng/mL bFGF (freshly added) 10 µM ROCKi (freshly added)
Low bFGF medium	DMEM/F12 20% KSR 3% stem-cell quality FBS 1% NEAA 1% GlutaMAX 4 ng/mL bFGF (freshly added) 50 µM ROCKi (freshly added)
Neural induction medium	DMEM/F12

	1% N-2 supplement 1% GlutaMAX 1% NEAA 1 µg/mL heparin sodium salt from porcine
Differentiation medium	50% DMEM/F12 50% Neurobasal medium 0.5% N-2 supplement 1% GlutaMAX 0.5% NEAA 1% penicillin/streptomycin 50 nM β-mercaptoethanol 1% B27 (with or without vitamin A)
Fixation solution	1xPBS 4% paraformaldehyde
Permeabilization solution	1xPBS 0.25% Triton-X
Blocking solution	1xPBS 0.25% Triton-X 3% bovine serum albumin
NTMT solution	100 mM NaCl 100 mM Tris-HCl, pH 9.5. 50 mM MgCl ₂ 1% Tween-20
NBT/BCIP solution	18.75 mg/mL nitro blue tetrazolium chloride 9.4 mg/mL 5-bromo-4-chloro-3-indolyl-phosphate, toluidine-salt 67% (DMSO) (v/v)

1.2.1 Two-Dimensional Cell Culture Studies

Neuroblastoma (SH-SY5Y), human oligodendroglioma (HOG), and human embryonic kidney (HEK-293) cells were cultured in 10% fetal bovine serum and 1% penicillin/streptomycin supplemented DMEM/F12, high-glucose DMEM, and DMEM, respectively. All applications related to the cells were performed under a clean laminar flow cabinet. When the confluency of SH-SY5Y cells was about 70-80%, they were gently washed with sterile 1X phosphate buffered saline (PBS) and detached from the culture surface with 20 µL/cm² of 0.05% trypsin/EDTA (Sigma, 25300054) at 37°C for 2-3 minutes. The cells were collected by the addition of 60 µL/cm² complete medium. The cells were centrifuged at 200xg for 6 minutes, and the cell pellet was dissolved in 2 mL of complete medium. 8000 cells/cm² were seeded on a culture flask. When the confluency of HOG cells was about 70-80%, they were washed with sterile 1xPBS and detached from the culture surface with 40 µL/cm² of 0.05% trypsin/EDTA at 37°C for 5

minutes. The cells were collected by the addition of 120 $\mu\text{L}/\text{cm}^2$ complete medium. The cells were centrifuged at 630xg for 4 minutes, and the cell pellet was dissolved in 2 mL of complete medium. 8000 cells/ cm^2 were seeded on a culture flask. When the confluency of HEK-293 cells was about 70-80%, they were gently washed with sterile PBS and detached from the culture surface with 20 $\mu\text{L}/\text{cm}^2$ of 0.25% trypsin/EDTA at 37°C for 2-3 minutes. The cells were collected by the addition of 60 $\mu\text{L}/\text{cm}^2$ complete medium. The cells were centrifuged at 200xg for 5 minutes, and the cell pellet was dissolved in 2 mL of complete medium. 8000 cells/ cm^2 were seeded on a culture flask.

1.2.2 Cell Viability Assay

The cytotoxic effects of 17 butterfly derivative compounds (also called fingolimod-derivative or multi-targeted compounds), which were synthesized by Prof. Dr. Holger Stark (University of Göttingen, Germany), were investigated on SH-SY5Y and HOG cells. 1×10^4 cells were seeded in a well of a 96-well plate. After 16-24 hours, 0-400 μM of each compound dissolved in dimethyl sulfoxide (DMSO) was added to each well to be incubated for 24 hours. When the incubation was completed, the medium was removed by sharply inverting the plate onto the sink. 100 μL of crystal violet solution was gently added onto each well, and the plate was incubated in the dark for 10 minutes at room temperature. Later, the solution in the wells was removed by sharply inverting the plate onto the sink, and the wells were gently washed under tap water until all excess solution was removed. 100 μL of sodium citrate solution was added into each well to be incubated for 15 minutes on a shaker (100 RPM) at room temperature. The absorbance was measured at 630 nm using a spectrophotometer (ThermoScientific, Varioskan LUX Multimode Microplate Reader), and percentage cell viability and IC₀₈ for each compound were calculated and compared to only DMSO-treated wells.

1.2.3 Effect of the compounds on the cellular cAMP level

Cyclic adenosine monophosphate (cAMP) change after the compound treatment was measured in HEK-293 cells after they were transfected with pGL4.29 [luc2P/CRE/Hygro] plasmid. Firstly, 1×10^4 HEK-293 cells were seeded on a black 96-well plate with a clear flat bottom, and they were incubated for 24 hours at 37°C. A transfection solution was prepared by mixing 0.15 μg DNA/well, 0.6 μL FuGENE® HD

(Promega, E2311) transfection reagent/well, and basal medium to complete the total volume up to 10 μ L/well. The mixture was incubated at room temperature for 20 minutes. Simultaneously, the medium on the cells was removed, and 90 μ L/well of basal medium was added to the cells. After the incubation was completed, 10 μ L of transfection solution was also added onto the cells to be incubated for 24 hours at 37°C. Next, the cells were incubated for 24 hours at 37°C with the average IC₀₈ dose of each compound specific to each cell line, along with 1 μ M fingolimod as a positive control. Later, luciferase activity-dependent change in cAMP level was measured using the Dual-Glo® Luciferase Assay System (Promega) by following the instructor's protocol. In the protocol, the medium in the cells was replaced with 75 μ L of basal medium and 75 μ L of Dual-Glo Luciferase reagent. The cells were incubated for 1 hour at room temperature in the dark, and firefly luminescence was measured using a Synergy HTX luminometer (BioTek).

1.2.4 Maintenance of human embryonic stem cells

Human embryonic stem cells (hESCs, H9) were purchased from WiCell Research Institute in Madison, Wisconsin (WA009). They were maintained following the feeder-dependent and feeder-independent stem cell culturing protocol defined by WiCell. Firstly, the hESCs were cultured on mouse embryonic fibroblast cells (MEFs). The MEFs were cultured on 0.1% gelatin-coated (Sigma, G1890) 6-well plates. After the preparation of 0.1% gelatin in 1xPBS, it was sterilized by autoclaving it at 121°C for 30 minutes. 2 mL/well of sterile gelatin solution was incubated for at least 30 minutes; the wells were ready to use. MEF cells were thawed by incubating the cryovial in water vapor until small ice crystals remained, and the cell suspension was collected with 5-6 mL of pre-warmed, freshly prepared MEF medium. The cell suspension was centrifuged at 200xg for 5 minutes, and the cell pellet was dissolved in 2 mL of MEF medium to be seeded in a well of a 6-well plate. The cells were incubated at 37°C, 5% CO₂ under standard conditions. When the well confluence was about 80%, the cells were mitotically inactivated (called iMEF) by 10 μ g/mL mitomycin C solution for at least 2.5 hours. Then the cells were washed with 1xPBS three times and incubated with 1 mL of MEF medium to confirm the growth inhibition. The next day, the cells were washed with 1xPBS three times, and 1 mL of hESC medium was added to prepare the well for hESC thawing. H9 cells were thawed by incubating the cryovial in water vapor until small ice crystals remained, and the cell suspension was collected with 8-10 mL of pre-warmed, freshly prepared hESC medium.

The cell suspension was centrifuged at 270xg for 5 minutes, and the cell pellet was gently resuspended in 1 mL of hESC medium to be seeded on iMEF cells. The cells were incubated with 5% CO₂ at 37°C, and the medium was replaced every day until a bunch of colonies were observed under a microscope, which takes 5-7 days. The culture was then maintained in a feeder-independent manner. 1 mg of growth factor reduced Matrigel (Corning, 356230) was thawed on ice and mixed with 12 mL of cold DMEM/F12 with cold micropipette tips. Then, 1 mL of solution was transferred to a well of a 6-well plate to coat 12 wells, and the plates were incubated for 2-3 hours at 37°C. After the coating, hESCs on iMEFs were passaged in a 1:3 to 1:6 ratio. During the passage, the medium on the cells was removed, the cells were washed with 1 mL of D-PBS (Ca²⁺ and Mg²⁺ free), and they were incubated with 600 µL 0.5 mM EDTA for 4 minutes at room temperature. Then, EDTA was gently removed, and the cells were detached from the plate surface by spraying 1 mL of the mTeSR™1 (Stem Cell Technologies) medium. The attached cells were scraped using the thick end of a sterile 1000 µL micropipette tip. The cell suspension was transferred to Matrigel-coated wells, and they were cultured with 5% CO₂ at 37°C. The half medium was replaced every day.

1.2.5 Human brain organoid (hBO) generation

When the morphology of the edges of the hESC colonies started to change and the center of the colonies became dark, they were either passaged with 0.5 mM EDTA or prepared for organoid generation. Two main protocols were mainly followed with minor modifications [65, 125], and the whole process was summarized in Figure 1.2.1. At the beginning of the organoid generation processes, the colonies were incubated with 20 µM of Rho-associated protein kinase inhibitors (ROCKi) for an hour, washed with 1x D-PBS (Ca²⁺ and Mg²⁺ free), and incubated with 600 µL of 0.5 mM EDTA at 37°C for 4 minutes. After the EDTA solution was removed, the cells were incubated with 1 mL of Accutase (Millipore, SCR005) at 37°C, and they were detached from the surface by spraying 1 mL of mTeSR™1. The cell number was determined by a hemocytometer, and they were centrifuged at 200xg for 5 minutes. The supernatant was removed, and the pellet was resuspended with low bFGF medium containing freshly added 4 ng/µL bFGF and 50 µM ROCKi to separate 9000 cells/150 µL/well into the Nunclon Sphera-treated U-shaped-bottom ultra-low attachment 96-well plate (ThermoScientific, 174925). After the cell seeding, the plate was briefly centrifuged to collect all the cells at the bottom, and they

were incubated with 5% CO₂ at 37°C. The medium was half-replaced every other day until the diameter of the stem cell aggregates (called embryoid bodies (EB)) reached about 500-600 µm. The properly grown embryoid body was transferred by sterile cut 200 µL pipette tips into a well of low-attachment 24-well plates and cultured in neural induction medium for 4-5 days. The medium was refreshed every other day until the brighter edges of EBs showed radial organization, forming neuroepithelial tissue. In the next step, they were embedded into Matrigel (Corning, 356234) droplets. Before the process, dimples were formed on sterile parafilm using a 200 µL tip box tray and sterile gloves, and each dimple was cut with sterile scissors. The dimples were placed on a 100 mm sterile petri dish and UV-sterilized for at least 30 minutes. On the transfer day, each neuroepithelial tissue was transferred to each dimple by a sterile cut 200 µL micropipette tip, and the excess medium around the tissue was carefully removed. The Matrigel was thawed on ice, and 28–30 µL of it was put on the tissue using cold and sterile tips. The tissue was replaced in the center of Matrigel with the help of a 10 µL micropipette tip, and then they were incubated for 20-30 minutes at 37°C for Matrigel polymerization. Simultaneously, 500 µL of differentiation medium without vitamin A was added to each well of a 24-well plate. After the polymerization was completed, each dimple was turned to the opposite side with sterile gloves, and the dimple was immersed in the medium to allow the sliding of tissue containing Matrigel into the medium. The tissues were cultured for 2 days at 37°C under static conditions, and then the medium was refreshed for 2 more days. Later, the medium was replaced with differentiation medium with vitamin A, and the culture was carried out on an orbital shaker at 70 RPM until the maturation was completed. Also, the medium was changed every 3-4 days until the end of the maturation. After the dynamic culture was started and tissues were incubated with only differentiation medium for 3–4 days, 20 ng/mL neurotrophin-3 (NT-3) (Merck, GF308) and 20 ng/mL brain-derived neurotrophic factor (BDNF) (Merck, GF301) were supplemented with the culture medium for 10 days. Later, the culture medium was supplemented with 10 ng/mL platelet-derived growth factor-AA (PDGF-AA) (Merck, GF142) and 10 ng/mL insulin-like growth factor I (IGF-1) (Merck, GF306) for 10 days. Later, the culture medium was supplemented with 4 ng/mL 3,3',5-triiodo-L-thyronine (T3) (Sigma, T2877) for 3–4 days and 4 ng/mL T3 and 1 µM ketoconazole (Sigma, K1003) for 6–7 days.

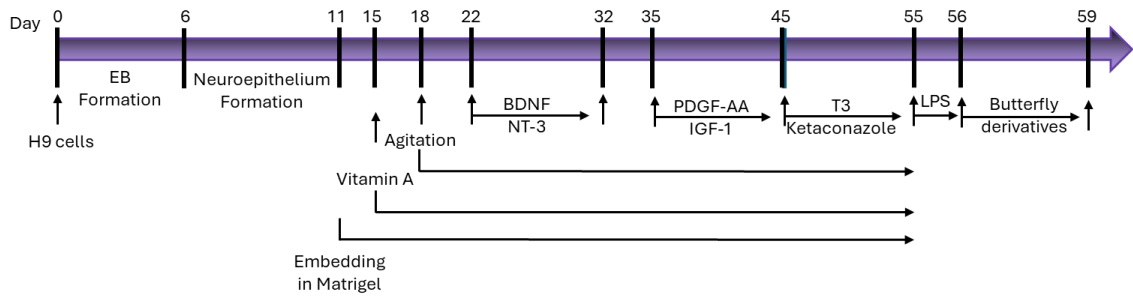


Figure 1.2.1 Timeline of human brain organoid generation process

1.2.6 Inflammation-like response induction on hBOs

After the maturation was completed with the addition of directed growth factors, the inflammation-like response was induced using lipopolysaccharides (LPS) to mimic MS. The organoids were treated with 100 ng/mL LPS (Sigma, L2630) for 24 hours on an orbital shaker at 37°C.

1.2.7 Treatment of inflammation modelled hBOs

After the inflammation-like response was induced by LPS, hBOs were treated with multi-targeted (or called fingolimod derivatives) compounds. After the effect of the compounds on the cellular cAMP level was found, the ones showing higher activity compared to Fingolimod were selected. The hBOs were incubated with 2.3 times higher doses of the IC₀₈ for each selected compound for 60 hours on an orbital shaker at 37°C.

1.2.8 Immunofluorescence staining

The hBOs sets (the ones before LPS induction, after LPS induction, and after compound treatment) were washed with 1xPBS three times and incubated in 30% sucrose solution at +4°C until they were no longer floating in the solution. Then, each hBO was transferred into a microcentrifuge tube with a cut 200 µL pipette tip, and optimum cutting temperature (OCT) compound was added onto it to be stored at -80°C until the sectioning. The 20 µm cryosections were taken using a cryostat (MERLAB, Abdullah Gül University) on lysine-coated slides, and the slides were stored at -80°C until the staining day. On the staining day, the slides were incubated at 56°C for 30-120 minutes and then incubated with fixation solution for 15-20 minutes at +4°C. After the fixation, they were

washed with 1xPBS three times and incubated with permeabilization solution for 10 minutes and then with blocking solution for 1 hour at room temperature. Later, they were incubated with properly diluted primary antibodies (Table 1.2.2) in a humidified chamber for 16 hours at +4°C. When the incubation was complete, they were gently washed with 1xPBS three times and counterstained with 300 nM DAPI for 2–3 minutes at room temperature. The sections were covered with mounting medium (Sigma, F6182) and imaged under a Leica DM6 B microscope. 10 sections were imaged for each set, and 7 regions for each section were analyzed using ImageJ (Fiji, v1.54p). The fluorescent intensity of each protein was normalized to DAPI, and relative fluorescent intensity was calculated by comparing the LPS-induced groups and the compound-treated groups.

Table 1.2.2 Primary antibodies used in immunofluorescent staining.

Antibody	Brand	Catalog No	Dilution
Anti-GFAP	Santa Cruz	sc-33673 AF594	1:100
Anti-Iba1	Santa Cruz	sc-32725 AF594	1:150
Anti-MBP	Santa Cruz	sc-271524 AF488	1:200
Anti-TAU	Santa Cruz	sc-390476 AF488	1:100
Anti-SOX2	Santa Cruz	sc-365823	1:100
Anti-SATB2	Santa Cruz	sc-518006	1:100

1.2.9 Quantitative real-time polymerase chain reaction (qRT-PCR)

The hBOs sets (the ones before LPS induction, after LPS induction, and after compound treatment) were washed with 1xPBS and incubated with 8-11 hBOs/1mL TRIzol (Sigma, T9424) for 1 hour at +4°C. Later, the manufacturer's instructions were followed to obtain total RNA. In the process, the hBOs were homogenized by pipetting and incubated for 5 minutes at room temperature. Then, 0.2 mL chloroform/1 mL TRIzol was added, vigorously shaken for 15 seconds, and incubated for 15 minutes at room temperature. They were centrifuged at 12,000xg for 15 minutes at +4°C, and the upper phase was carefully transferred to a nuclease-free microcentrifuge tube. 0.5 mL isopropanol/1 mL TRIzol was added to the phase; they were incubated for 10 minutes at room temperature and precipitated by centrifugation at 12,000xg for 10 minutes at +4°C. The supernatant was removed, the pellet was washed with 1 mL 75% ethanol/1 mL

TRIzol by vortexing, and it was centrifuged at 7,500xg for 5 minutes. The pellet was air-dried for 5 minutes, dissolved in 30–50 μ L of RNase-free water, and then quantified using a spectrophotometer (ThermoScientific NanoDropTM 2000/2000c Spectrophotometer). The total RNA was used to synthesize cDNA following the manufacturer's instructions (abm, G236). In the protocol, each reaction was set by mixing 2000 ng RNA, 1 μ L of 10 mM dNTP, 1 μ L of 10 μ M oligo (dT), 0.2 μ L of 10 μ M random primer, and nuclease-free water up to 14 μ L on ice. The mixture was incubated for 5 minutes at 65°C and then 1 minute on ice. 1 μ L of OneScript[®] Plus RTase and 5 μ L of 5X RT Buffer were added to the mixture to be incubated for 55 minutes at 55°C and then for 5 minutes at 85°C. The cDNA was diluted 1:2.5, and qRT-PCR protocols were applied following the manufacturer's instructions (Promega, A6002). In the process, 0.25 μ L of 10 pmol forward primer, 0.25 μ L of 10 pmol reverse primer, 5 μ L of GoTaq[®] qPCR 2X master mix, and nuclease-free water up to 6 μ L for each reaction were mixed in a tube of 8-strip tubes. Then 4 μ L of diluted cDNA was added in the tube. The tube was shortly mixed by vortexing and spinning down. The reaction (see Table 1.2.3) was run on the CFX Connect Real-Time PCR System, BioRad. The annealing temperatures for the primers were listed in Table 1.2.4.

Table 1.2.3 Reaction settings for qRT-PCR.

Temperature	Duration	
95 °C	2 minutes	40 cycles
95 °C	15 seconds	
X°C	1 minute	

Table 1.2.4 Gene-specific primer sequences and their annealing (T_m) temperatures.

Genes	Forward Primer	Reverse Primer	T _m (°C)
GFAP	GTGTCAGAAGGCCACCTCAA	TCAGGTCTGGGGAAATGTGC	62
CSF-1	ATGCTCCAGCCAAGATGTGG	ACTGCTAGGGATGGCTTTGG	62
IL-6	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTTCAGGTTG	59
IL-10	TCTCCGAGATGCCTTCAGCAGA	TCAGACAAGGCTTGGCAACCCA	51
NF- κ B	TCGCGCTGAGTATAAAAGCC	GGCAAAGTTTCGTGGATGCG	61
TNF α	TGGGATCATTGCCCTGTGAG	GGTGTCTGAAGGAGGGGGTA	62
β -actin	GCCGCCAGCTCACCAT	GATGCCTCTCTTGCTCTGGG	59

1.2.10 Enzyme-linked immunosorbent assay (ELISA)

The culture medium was collected after each treatment. The human alpha-synuclein (SNCa) SimpleStep ELISA® Kit (abcam, AB260052), human TAU SimpleStep ELISA® Kit (abcam, AB210972), human liver-FABP ELISA Kit (abcam, AB218261), and human TNF alpha ELISA Kit (RayBio®, ELH-TNFa) protocols were applied following the manual supplied by the manufacturer, and the protein concentration for each treatment was calculated.

1.2.11 Alkaline phosphatase assay

In the feeder-dependent culture of hESC, when the hESC confluency was about 60%, they were washed with 1xPBS and then fixed with 4% PFA for 20 minutes at room temperature. The cells were then incubated three times for 10 minutes each with NTMT solution on a shaker in the dark. After the incubation was completed, NBT/BCIP (11681451001, Roche) was mixed with NTMT solution at a 1:50 ratio, and the cells were incubated with the diluted NBT/BCIP solution on a shaker until the colorful products were observed.

1.2.12 Immunohistochemistry staining

For the IHC analysis, the excess Matrigel around each organoid was removed carefully and washed with PBS once. Later, the organoids were incubated with 4% paraformaldehyde (PFA) for 15 minutes at room temperature. After the PFA was aspirated, the organoids were washed with PBS twice, and they were embedded into optimum cutting temperature (OCT) compound to be stored at -80 °C for further analysis. Initially, each organoid was cryosectioned using a Thermo Scientific HM525 NX cryostat to obtain 20 µm slices. The slices were placed on UltraPlus slides for IHC staining. During IHC, the "Triple Stain IHC" kit was used, and the kit's protocol was followed. As indicated in the protocol, the slides were incubated with 3% H₂O₂ for 10 minutes at first and washed with PBS-T (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 0.1% Tween-20) three times for 2 minutes. Later the slides were washed with distilled water twice and TBS-T (20 mM Tris, 150 mM NaCl, 0.1% Tween-20) three times. Properly diluted first primary antibody, mouse monoclonal, and second primary

antibody, rabbit monoclonal antibody, were added to the slides to be incubated in a humidified chamber for 45 minutes. The slides were washed with TBS-T three times for 2 minutes when the incubation time was completed. A 1:1 mixed mouse AP polymer and rabbit HRP polymer were added onto the slides to be incubated for 30 minutes. Then, the DAB chromogen:DAB substrate (1:1000) mixture was added to the slides and incubated for 5 minutes. After the slides were washed with TBS-T three times for 2 minutes, the mix of red activator solution, red substrate, and red working solution (2:10:1) was added onto the slides and incubated for 10 minutes. The slides were washed with distilled water and incubated at 65 °C with a 1X antibody blocker for 10 minutes. After the slides were cooled down for 5 seconds, they were washed with TBS-T three times for 2 minutes. Then two drops of blocker A were dropped onto the slides and incubated for 30 minutes in a humidified chamber. The washed slides with TBS-T were incubated three times with 2 drops of Blocker B for 5 minutes, and an adequately diluted third primary antibody, a mouse monoclonal antibody, was added onto the slides to be incubated for 45 minutes. When the incubation period was over, the slides were washed with TBS-T three times for 2 minutes, and they were incubated with HRP polymer for 15 minutes. Later, a drop of hematoxylin was added to the slides for 5 seconds for counterstaining. When the hematoxylin was removed quickly, the slides were washed with tap water for 1 minute, PBS for 10 seconds, and TBS-T for 2 minutes three times. 1–5 drops of emerald chromogen were also added to the slides and incubated for 5 minutes in a humidified chamber. The slides were quickly washed with tap water and distilled water. Later, they were incubated with a few drops of 85% ethanol, 95% ethanol, 100% ethanol, 100% ethanol, 100% ethanol, and xylene for 20 seconds. Lastly, the slides were covered with mounting medium and a coverslip to be observed under a Zeiss Axio Vert. A1 microscope.

1.2.13 Statistical Analysis

All data were represented as mean $\pm$ standard deviation. At least 3 independent batches were used for analysis. Quantification of fluorescent images was performed by normalizing the fluorescent intensity to DAPI and comparing it to control groups (LPS-uninduced for induction confirmation and LPS-induced for treatment confirmation). Quantification of qRT-PCR was performed by normalizing the internal control (β -actin) and comparing the control groups. All graphs were visualized using GraphPad Prism 9

for Windows, and one-way ANOVA was used to confirm the statistical significance of treatment groups.



1.3. Results and Discussion

1.3.1 Cell Viability Assays

The cytotoxic effects of 17 butterfly derivative compounds were tested on human oligodendroglioma and neuroblastoma cells using the crystal violet assay. After the compounds were dissolved in DMSO and different concentrations of them were prepared in their medium, the two different cell lines were incubated for 24 hours with the compounds. The cell viability was measured using the crystal violet assay protocol, and % cell viability was calculated using the absorbance values of the compound-treated wells compared to the absorbance values of DMSO-treated cells. The graphs depicting the percentage of cell viability in Figures 3.1 and 3.2 were constructed, and the maximum doses corresponding to 92% cell survival were determined as the inhibitory concentration (IC_{08}) (Table 1.3.1). The average IC_{08} dose for further 2D culture studies and a 2.3 times higher average IC_{08} dose for further 3D culture studies were used.

Table.1.3.1 IC_{08} values of 17 butterfly derivative compounds for neuroblastoma and human oligodendroglioma cell lines. Additionally, average IC_{08} doses for neuroblastoma and human oligodendroglioma cell lines were presented alongside the calculated dose for 3D applications.

Compound name	IC_{08} (μ M) SHSY-5Y	IC_{08} (μ M) HOG	Average (μ M)	Dose for organoids (μ M)
ST-1711	8.22	8.22	8.22	18.90
ST-1894	4.48	2.09	3.285	7.56
ST-1893	5.12	4.25	4.685	10.78
ST-1374	48.40	44.74	46.57	107.11
ST-1375	38.87	49.32	44.095	101.42
ST-971	39.74	43.36	41.55	95.57
ST-1058	3.81	3.12	3.465	7.97
ST-1070	22.12	16.36	19.24	44.252
ST-1072	12.18	7.69	9.935	22.85
ST-1211	40.92	43.03	41.975	96.54
ST-1360	59.61	65.52	62.565	143.90
ST-1480	52.32	49.88	51.1	117.53
ST-1895	60.01	33.66	46.835	107.72
ST-967	32.22	23.37	27.795	63.93
ST-1060	38.15	30.08	34.115	78.46
ST-1767	46.03	48.87	47.45	109.14
ST-1801	50.73	47.62	49.175	113.10

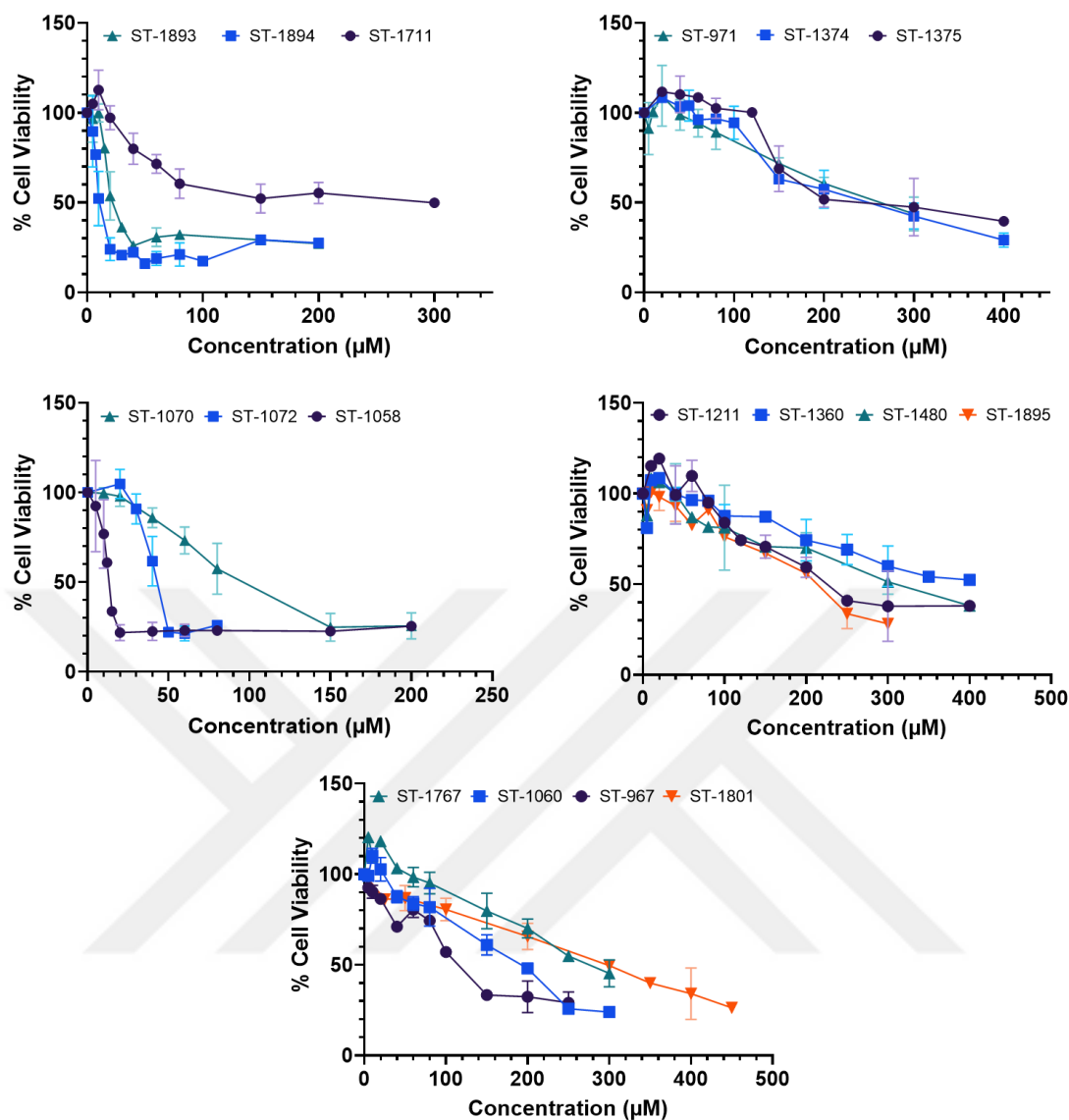


Figure 1.3.1 Effects of 17 butterfly derivative compounds on the cell viability of human oligodendrogloma cells. GraphPad Prism 9.0 visualized the data.

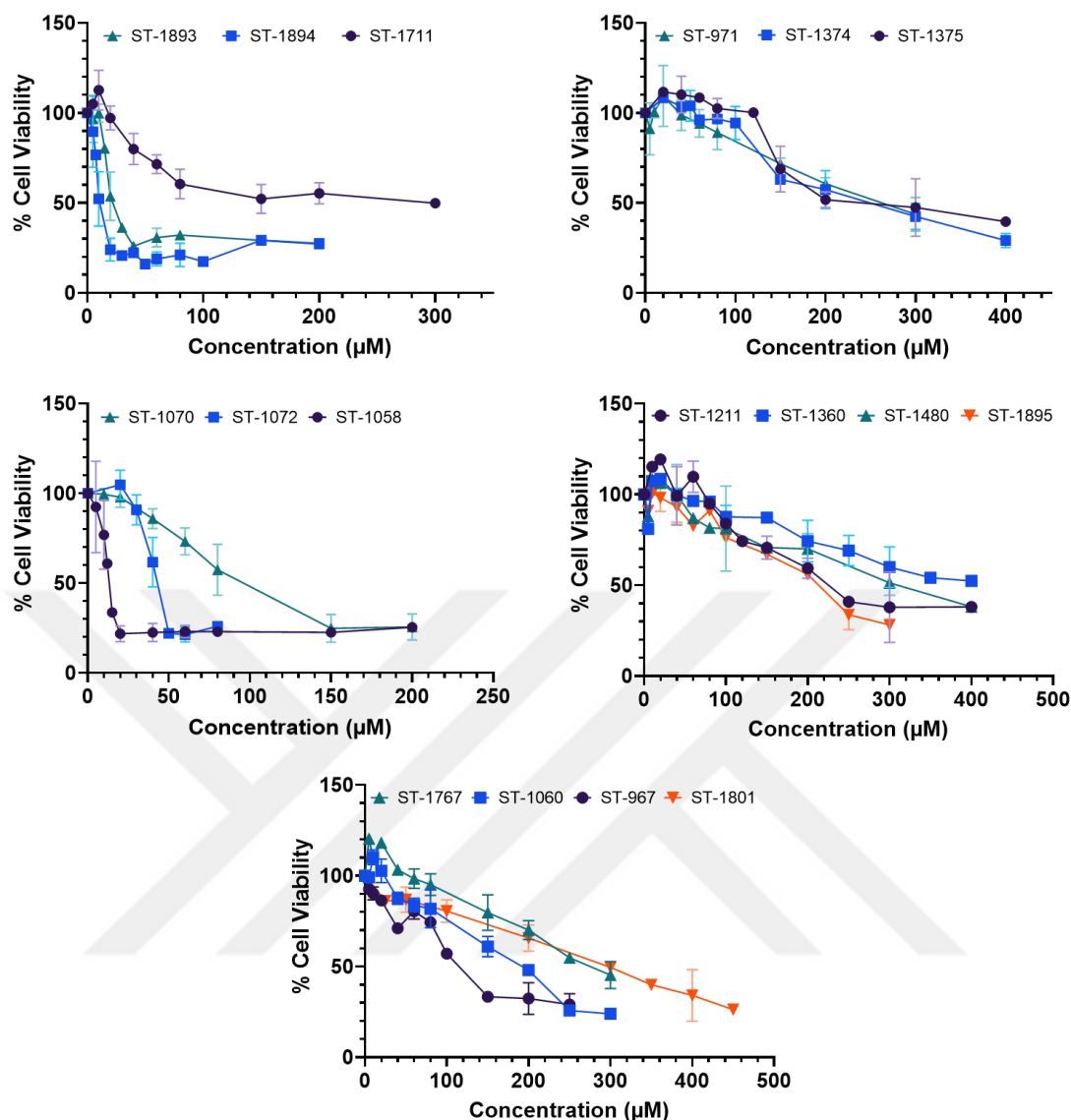


Figure 1.3.2 Effects of 17 butterfly derivative compounds on the cell viability of neuroblastoma cells. GraphPad Prism 9.0 visualized the data.

1.3.2 Effect of the compounds on the cellular cAMP level

After the cytotoxic effects of the compounds were calculated, their indirect effect on cAMP was investigated. Since the butterfly derivative compounds are fingolimod analogues [126], [127], [128], [129], they were expected to bind S1PRs, which are a type of GPCR. To measure cAMP-response element (CRE)-dependent luciferase activity upon binding to the butterfly derivative compounds, a construct was transfected into Hek-2293 cells, considering the activation of cAMP-dependent pathways via ligand binding to GPCRs. [130]. Figure 1.3.3 shows that only ST-1374 treatment causes significantly higher luciferase activity compared to fingolimod. However, since the average luciferase

activity of fingolimod, ST-1894, and ST-971 was close to each other among the other fourteen compounds, they (ST-1374, ST-1894, and ST-971) were selected for further tests.

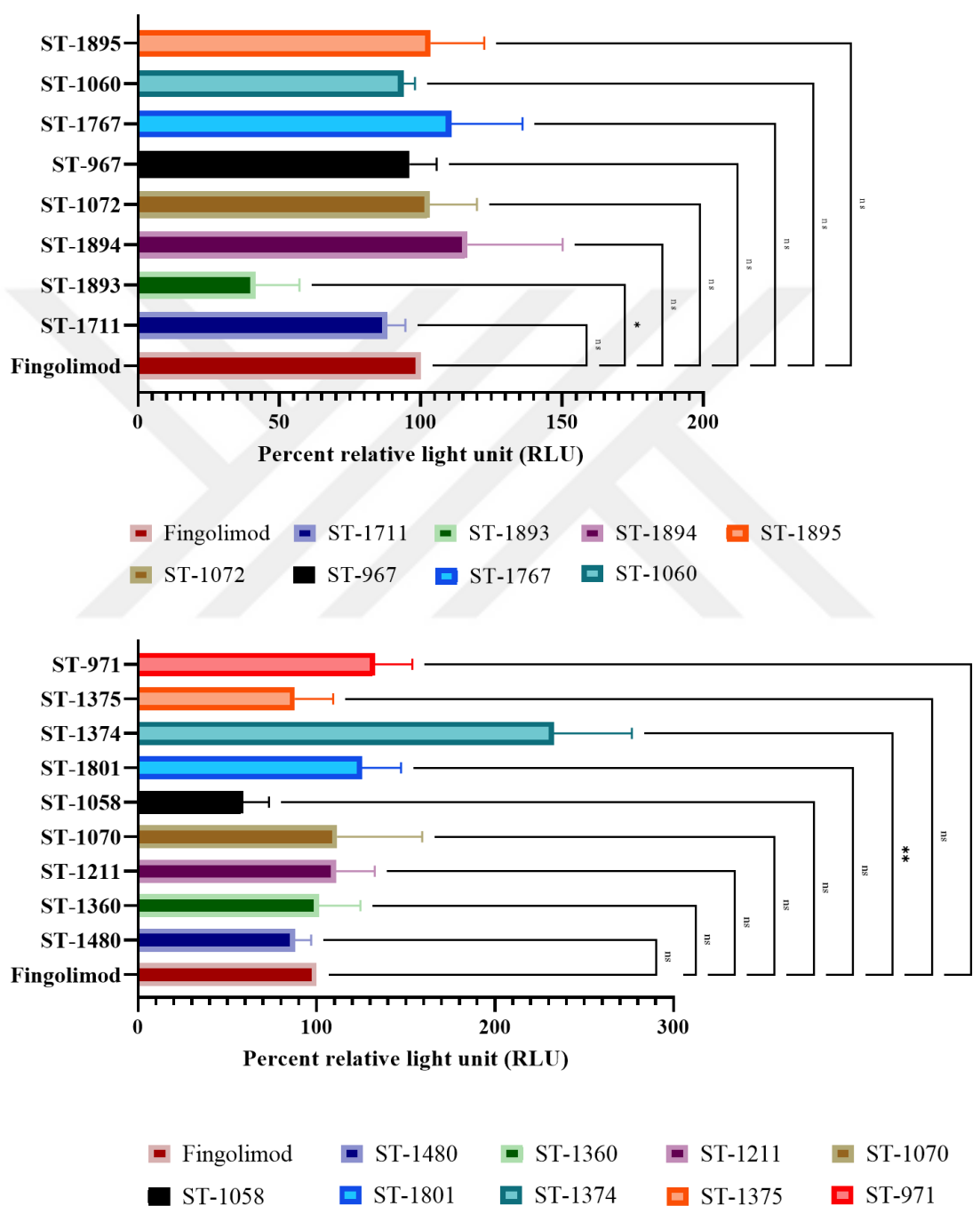


Figure 1.3.3 Effects of the seventeen butterfly derivative compounds on cAMP level. The average percent relative light unit (RLU) representing the CRE-responsive luciferase activity was illustrated for each compound. The data were visualized by GraphPad Prism 9.0; RLU for each treatment was normalized and compared to fingolimod. The data were

statistically analyzed by one-way ANOVA (ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

1.3.3 Human embryonic stem cell culture and optimization of brain organoid generation

After human embryonic stem cells (H9, WiCell) were thawed, they were cultured in a feeder-independent manner at first. In the processes, they were maintained in mTeSR™1 only and passaged with 0.5 mM EDTA. As shown in Figure 1.3.4, H9 cells were grown in a colony. When the centers of the colonies became darker and the distance between them became shorter, they were passed. Figure 1.34A displays the two healthy colonies following the passage.

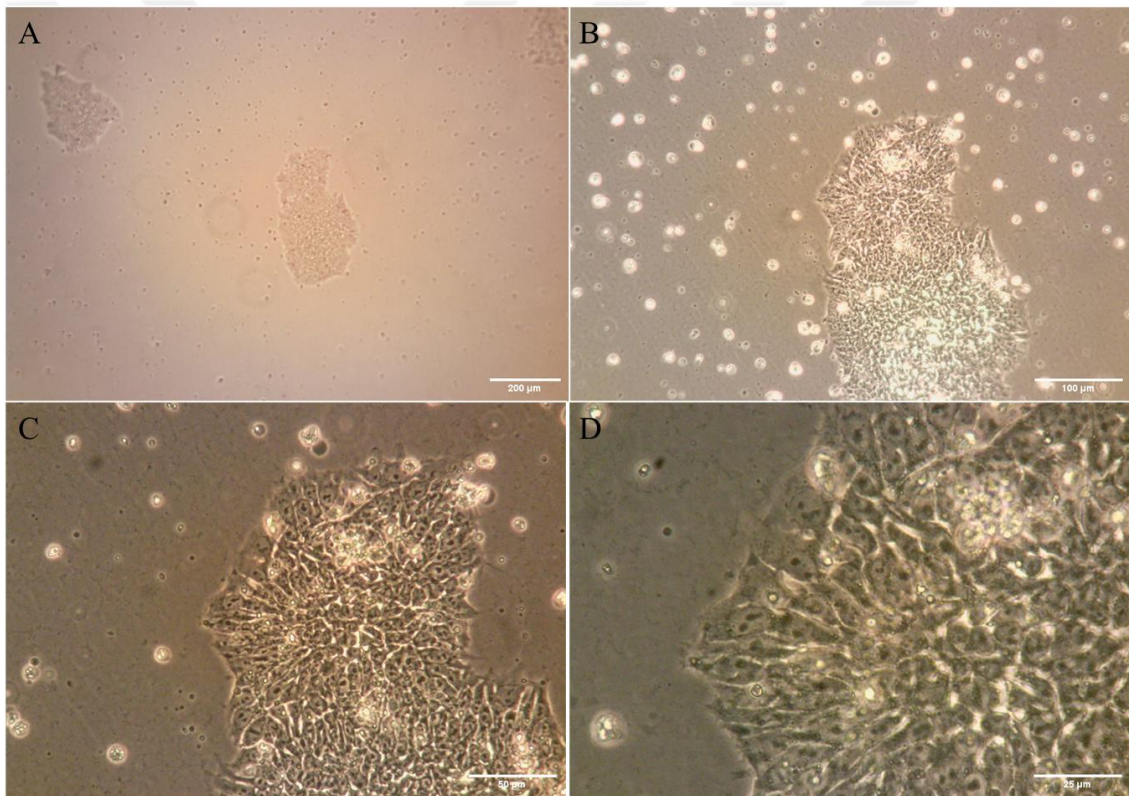


Figure 1.3.4 Healthy H9 colonies a day after the passage. The different magnification rates were used to show the colony and cell morphology. The images were taken using (A) 4X objective lens, (B) 10X objective lens, (C) 20X objective lens, (D) 40X objective lens. The images were recorded using a Zeiss PrimoVert Invert Microscope and an external camera with DinoCapture 2.0.

After the cells in Figure 1.3.4 were maintained for 2–3 weeks, they were cryopreserved in a stem cell freezing medium, and the rest of them were prepared for organoid generation. The first round of organoid generation was performed to test the

reproducibility of the system. The morphology of the organoids was recorded by a phone camera regularly to confirm the morphologies with the reference articles mentioned in the method part (Figure 1.3.5).

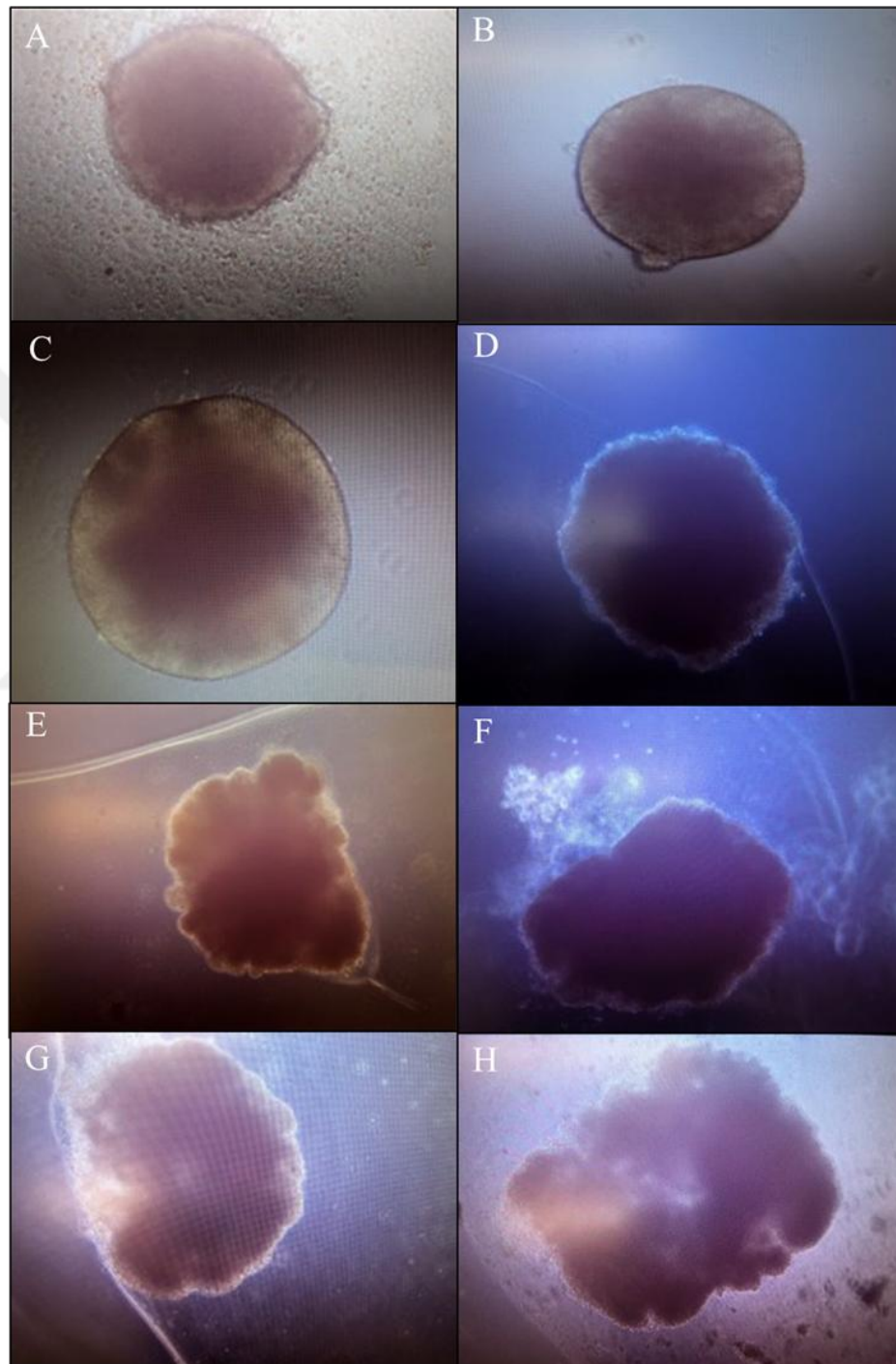


Figure 1.3.5 Development of the first round of brain organoids starting from human embryonic stem cells. (A) Embryoid body on day 5, (B, C) Neuroepithelial structure showing radially organized structure during incubation in neural induction medium on day 7 and 9, (D) Developing neuroepithelial structures in a medium without vitamin A in

a static culture day 12, (E) Developing neuroepithelial structures in a medium with vitamin A on an orbital shaker on day 14, (F, G, H) Developing organoids on day 45, 57, and 70. Images were taken under a Zeiss Primo Vert phase-contrast light microscope and recorded by a phone camera.

In the first round of brain organoid generation, Dr. Lancaster's protocol was followed to test the system; however, many unforeseen problems arose. First of all, the debris around the embryoid body was more than expected (Figure 1.3.5A). Therefore, for further organoid generation rounds, the H9 cells were incubated with ROCKi for 1 hour before the experiment. This incubation prevents cells from the dissociation-induced apoptosis. The ROCKi is called a ROCK inhibitor or Rho kinase inhibitor. The Rho kinases are responsible for the enhancement of actin-myosin contraction and caspase signaling to induce apoptosis [131]. Therefore, ROCKi was used to inhibit the apoptosis of H9 cells. Also, the U-bottom 96-well plate was quickly centrifuged (until the machine reached 100xg) just after the 9000 cells/150 μ L cell seeding to increase the involvement of all cells in embryoid body generation. Secondly, as indicated in the protocol, the seeded cells were incubated for 48 hours, and on the second day, half of the old medium was replaced with fresh media. This process was intended to continue for almost 7 days until the size of the embryoid bodies reached about 600 μ m; however, all embryoid bodies exceeded 600 μ m by day 5. Therefore, the ones with smooth edges and lower disaggregated structures around the central part were transferred into a 24-well plate in a neural induction medium. On day 7, the ectodermally induced structures in the neural induction medium appeared, as in Figure 1.3.5B-C. Since some burst-like structures around the developing neuroectodermal structures prevent tissue integrity, heterogeneous development was foreseen; however, the process was not interrupted. When the radially organized structures with smooth and bright edges were formed, and the size of the structures was increased, they were transferred into a Matrigel droplet in vitamin A-free differentiation medium. Another issue with the process was the difficulty in handling the tissue during its transfer to the Matrigel. The transfer process was difficult because some tissue-like structures were hard to locate in the middle of the Matrigel for proper polymerization. The handling problem could cause one side of the tissue to be covered with Matrigel, but the other side to directly face the differentiation medium or other tissues. Since Dr. Lancaster's protocol was directly applied in the first round, 3-4 tissues in Matrigel were incubated in 60 mm cell culture dishes. However, some of the organoids were fused in time. Therefore, each organoid was incubated in a well of a 24-well plate for the following round. The neural induction process, which was performed in a well of a low-

adhesion 24-well plate for every two embryoid bodies, also resulted in the fusion of some tissues. Therefore, each embryoid body was incubated in a well of a 24-well plate for the following rounds. One of the most important processes related to handling problems was sterility during the transfer of the polymerized Matrigel/tissue complex to the medium. The spraying was not perfectly done because some of the tissue and Matrigel structure were disrupted. Therefore, the dimple was reversed with the help of gloved fingers, and it was submerged in a medium containing a 24-well plate. In this way, the Matrigel/tissue complex was dropped from the Parafilm dimple. Since the gloves were directly interacting with the Matrigel, the contamination was the major concern. Therefore, the two sides of the gloves were sterilized with 70% ethanol and then exposed to 30 minutes of UV light before starting the experiment. After successfully transferring a portion of the tissues, a light microscope recorded their morphologies (Figure 1.3.5E-H). The presence of budding structures around the organoids indicated successful generation; however, the ratio of successfully generated organoids to the initial number was low, prompting the application of the aforementioned changes in subsequent rounds. In addition to morphological tracking, the organoids were cryosectioned and stained with IHC techniques to confirm cellular content (Figure 1.3.6). The neural stem cell population with SOX2, excitatory neurons in the surface layer with SATB2, and neural cells with TUB3 were stained. The staining revealed disrupted sections and their images because the sections were not all taken at one plane. Based on IHC staining, it can be concluded that there is no homogeneous pattern between different sections, and there are some differentiated and undifferentiated parts containing organoids. For example, Figure 1.3.6B showed a dominant brown color showing SATB2 expression, although a mixture of red and brown colors indicates that SOX2 and SATB2 are dominant in Figure 1.3.6C. Figure 1.3.6A and Figure 1.3.6E show the disrupted sections; therefore, staining was not successfully completed. Also, layer-by-layer organization was not observed compared to the reference protocols. Since spatially organized neural differentiation was not observed, the sections were not analyzed for the expression of myelination-related proteins. In addition to the first-round staining of the first-round organoids, the sections from the first round were stained again within several months because it was realized that the kit had expired. Figure 1.3.7 shows the staining with the expired product, and it could be concluded that it did not work properly. Therefore, immunofluorescent antibodies (Table 1.2.2) were ordered for the following rounds of organoid staining.

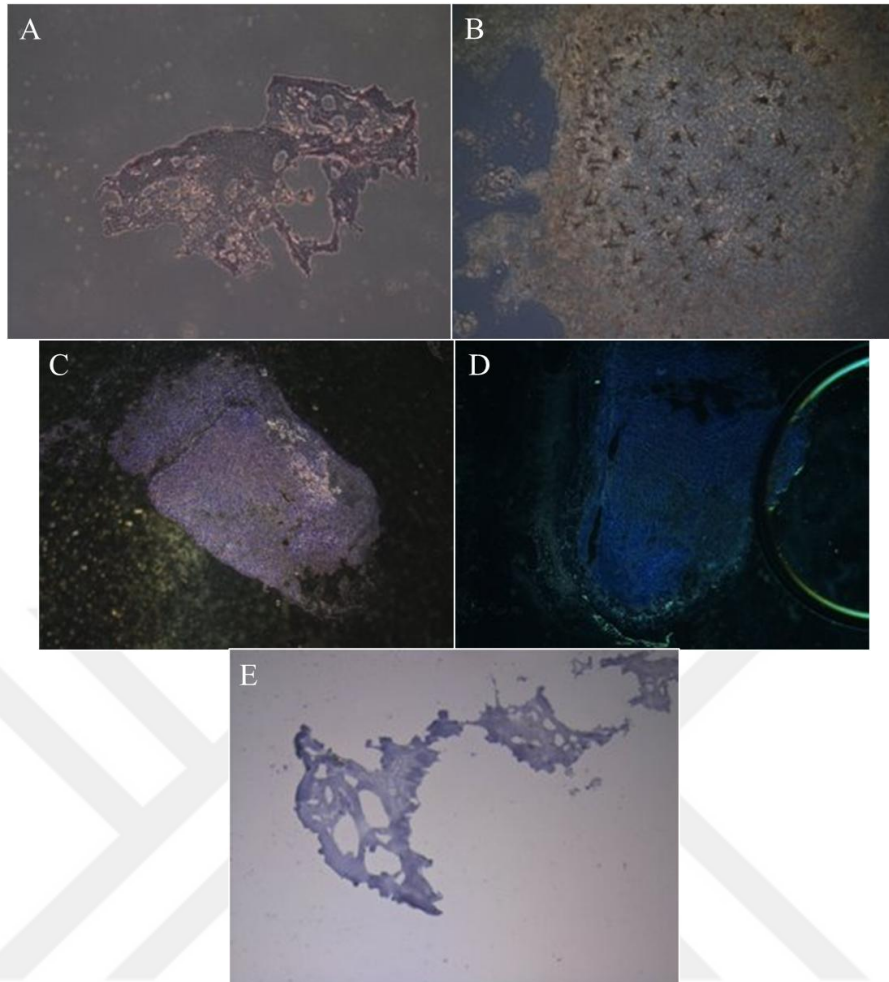


Figure 1.3.6 Immunohistochemical staining of cryosectioned brain organoids treated with 4 μ M ketoconazole at day 70. From A to E, different cryosections were stained with brown DAB chromogen for SATB2, red chromogen for SOX2, green Emerald chromogen for TUB3, and blue Hoechst for counterstaining. Images were taken under a Zeiss Axio Vert. A1 microscope.

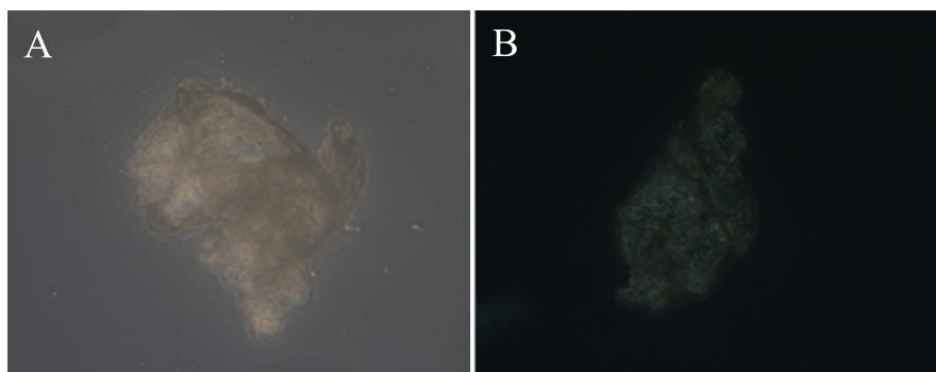


Figure 1.3.7 Immunohistochemical staining of cryosectioned brain organoids with expired kit. A and B show different cryosections, stained with brown DAB chromogen for SATB2, red chromogen for SOX2, green Emerald chromogen for TUB3, and blue Hoechst for counterstaining. Images were taken under a Zeiss Axio Vert. A1 microscope.

As mentioned, the cells in Figure 1.3.4 were maintained for 2-3 weeks, and some of them were cryopreserved in stem cell freezing medium. However, when they were thawed again for further biological replicates of the study, the cells failed to attach and form colonies (Figure 1.3.8). The parameters, like the size of the seeding area and the number of thawed vials, were changed in time; however, any colony formation could not be observed within approximately 9 months. In fact, the colors of the medium for each trial were changed in time, and it was a sign of growth; however, no colony could be recorded.

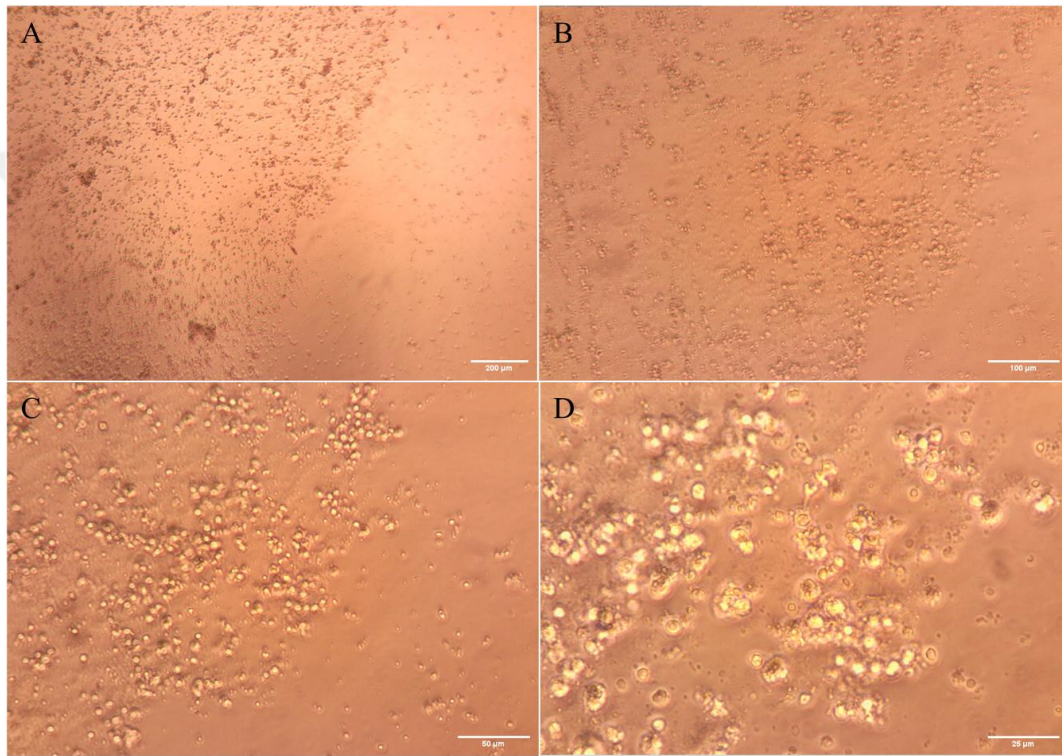


Figure 1.3.8 Unhealthy H9 colonies a day after the passage. Different magnification rates were used to show detached cell morphology. The images were taken using (A) 4X objective lens, (B) 10X objective lens, (C) 20X objective lens, (D) 40X objective lens. The images were recorded using a Zeiss PrimoVert Invert Microscope and an external camera with DinoCapture 2.0.

Later, MEF cells as feeder cells and iPSC from Erdal Lab, Izmir Biomedicine and Genome Center, and MEF cells from Ozcan Lab, Gevher Nesibe Graduate School of Genome and Stem Cell, were obtained independently. After the MEF cells were maintained for 2-3 weeks (Figure 1.3.9), half of them were cryopreserved, and half of them were mitotically inactivated (called iMEFs) by Mitomycin C. At least one day, iMEFs were incubated at 37°C to confirm that they were not growing. Then, iPSCs were seeded on MEF cells (Figure 1.3.10).

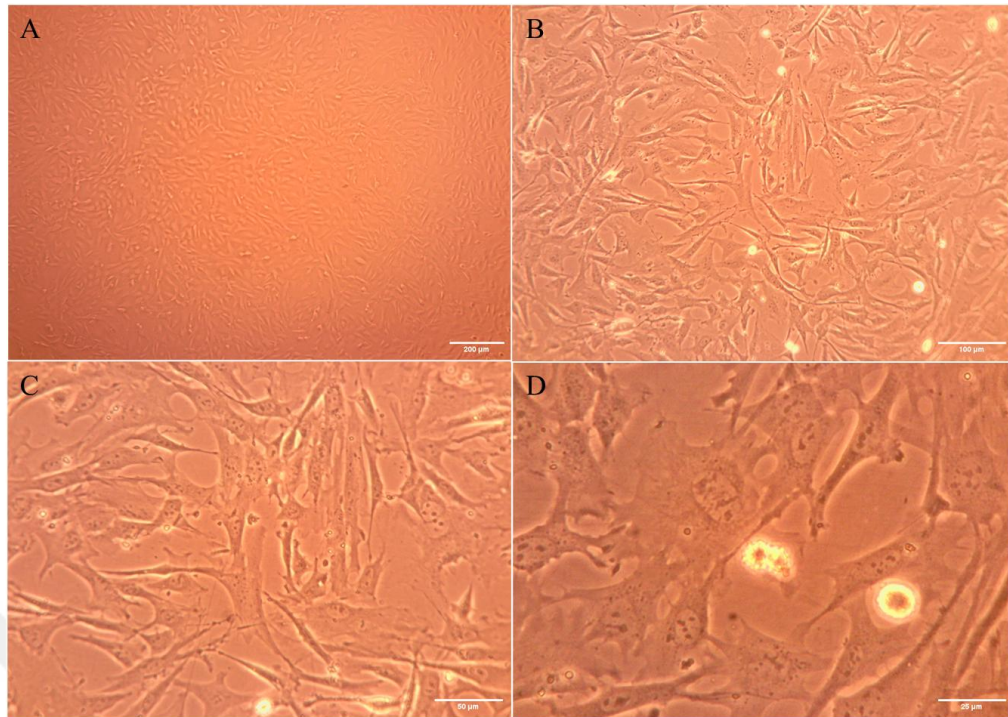


Figure 1.3.9 MEF cells as feeder cells. Different magnification rates were used to show healthy cell morphology. The images were taken using (A) 4X objective lens, (B) 10X objective lens, (C) 20X objective lens, (D) 40X objective lens. The images were recorded using a Zeiss PrimoVert Invert Microscope and an external camera with DinoCapture 2.0.

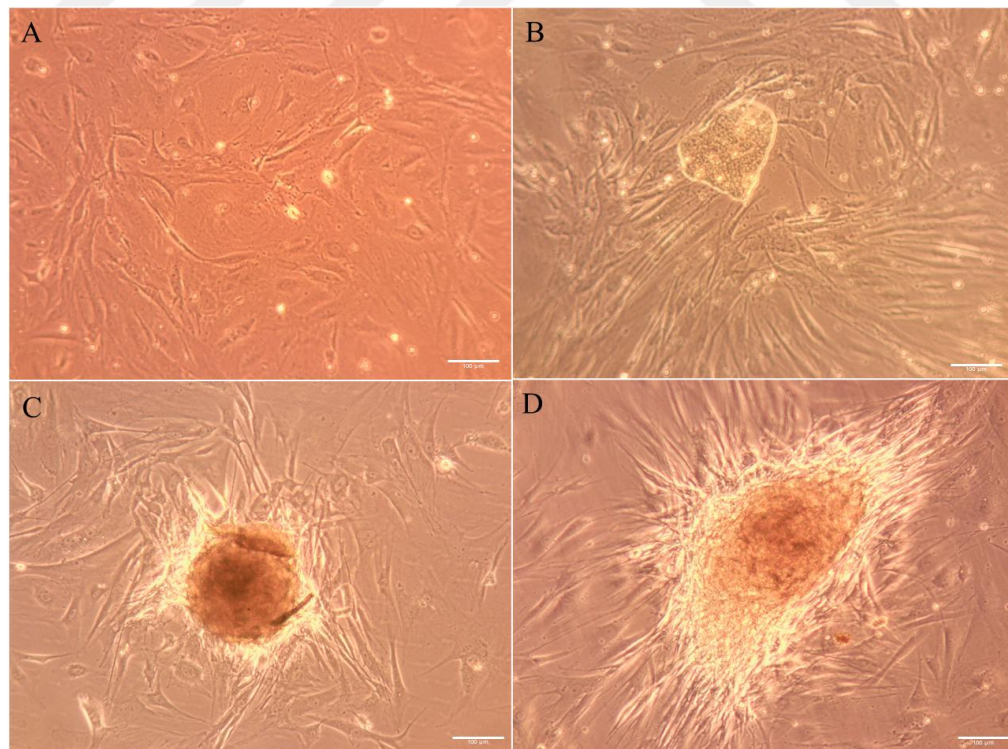


Figure 1.3.10 iPSC culture on feeder cells. (A) MEF cells before the iPSC's thawing. Morphology of the iPSCs (B) 3 days, (C) 12 days, and (D) 19 days after the seeding. The images were recorded using a Zeiss PrimoVert Invert Microscope and an external camera with DinoCapture 2.0

The morphology of the iPSC in Figure 1.3.10 clearly indicates that the iPSC culture was unsuccessful. After the MEF cells were prepared by culturing and incubating with mitomycin C, the iPSC cell line from the Erdal lab was thawed on MEF cells, and the medium was refreshed every day. Even though a colony was observed on day 3 (Figure 1.3.10B), it was not enlarged in size. Even though it was passaged once, the MEF cell aggregate was observed on the culture plate for a while (Figure 1.3.10C-D). The culture took 25 days to have a chance to grow a colony; however, no growing iPSC colony was observed. Later, it was decided to culture hESCs on MEF cells using a feeder-dependent hESC culture method. After MEF cells were thawed and properly maintained as before, hESCs were cultured on MEFs (Figure 1.3.11). The colonies were visible under a microscope within 6-7 days after the seeding, and the medium of the cells was refreshed every day with hESC medium (Table 1.2.1).

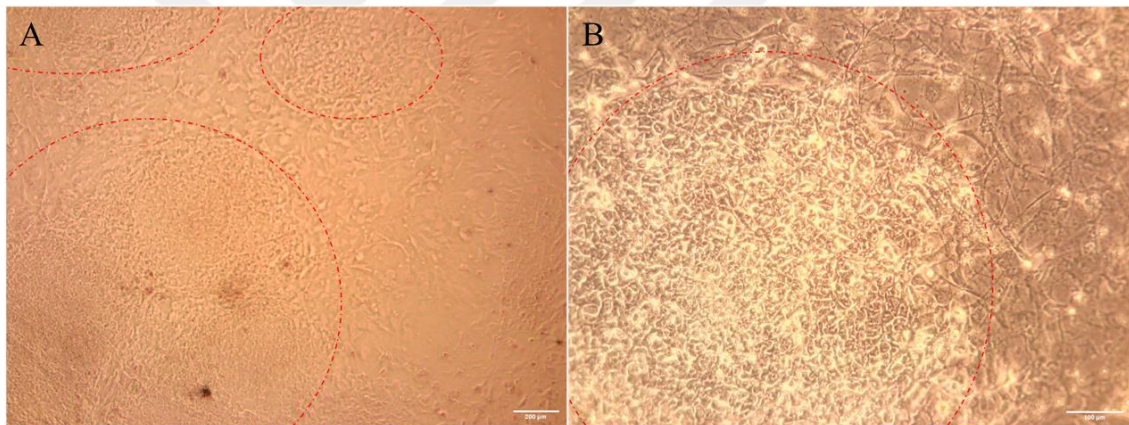


Figure 1.3.11 hESC colonies on MEF cells. The colonies are indicated in red circles. Different magnification rates were used to show healthy cell morphology. The images were taken using (A) a 4X objective lens and (B) a 10X objective lens. The images were recorded using a Zeiss PrimoVert Invert Microscope and an external camera with DinoCapture 2.0.

Since iMEF cells were able to attach to the plate surface for a maximum of 10 days, the hESC colonies were transferred to growth factor-reduced Matrigel-coated plates as soon as possible. Among the enzymatic (by collagenase for feeder-dependent cells and Dispase for feeder-free cells) and non-enzymatic (by EDTA) passaging protocols, non-enzymatic protocols were followed to remove differentiated and unhealthy colonies from the environment, considering the cell survival efficiency [132]. In Figure 1.3.12A-B, the change in cell morphology in the colony was observed after EDTA incubation. The cells

were partially dissociated and ready to be detached from the surface by spraying with medium.

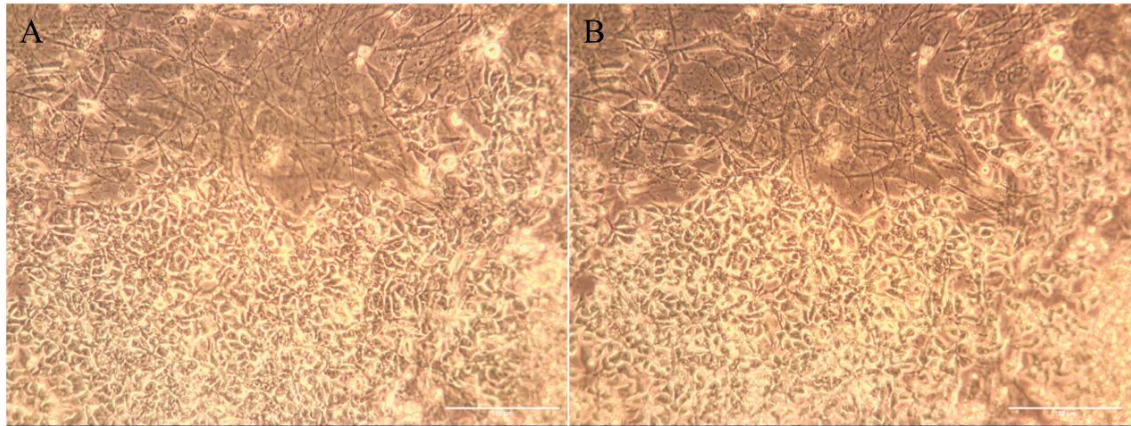


Figure 1.3.12 The EDTA-mediated passaging of hESC colonies on MEF cells. (A) Before EDTA incubation, (B) After EDTA incubation. The images were recorded using a Zeiss PrimoVert Invert Microscope and an external camera with DinoCapture 2.0. Scale bar 100 μm .

Subsequent to the passage of hESC to the Matrigel-coated plates, they were maintained and cryopreserved. During the maintenance procedure, the cells were passaged with EDTA to avoid individualizing colonies, thereby keeping them grouped together (Figure 1.3.13).

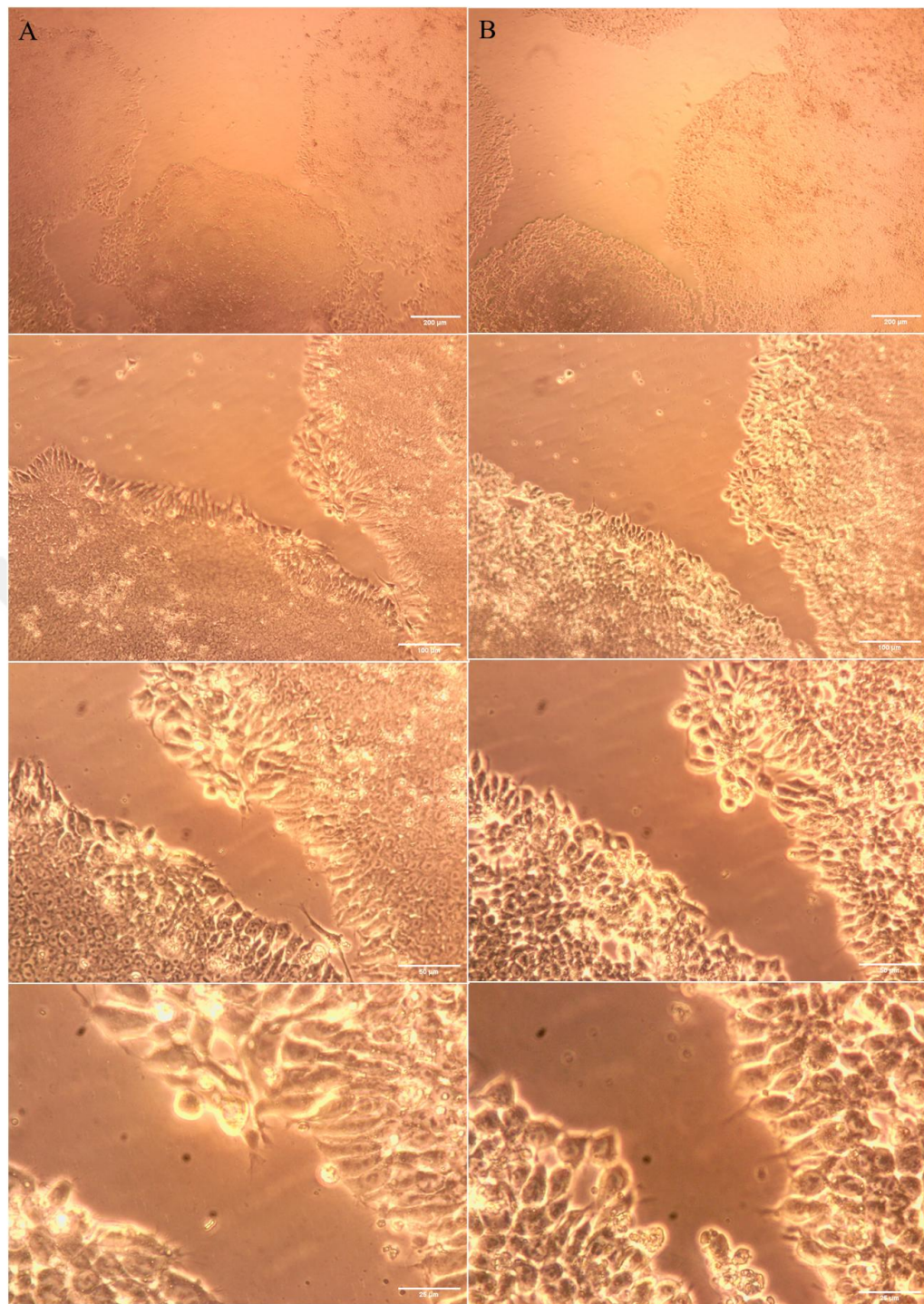


Figure 1.3.13 The EDTA-mediated passaging of hESC colonies on Matrigel. (A) Before EDTA incubation, (B) After EDTA incubation. From top to bottom, objective lens magnification is increased from 4X to 10X, 20X, and 40X. The images were recorded using a Zeiss PrimoVert Invert Microscope and an external camera with DinoCapture 2.0.

After the feeder-dependent and feeder-independent cultures of hESCs were maintained for a while, the stem cell characteristics of the cells were confirmed with an alkaline phosphatase assay. A high level of alkaline phosphatase is one of the markers

that indicate the pluripotency in stem cells. As shown in Figure 1.3.14, the darker purple parts are hESC colonies, and the rest are MEF cells, indicating the pluripotency characteristics of the cells because high levels of alkaline phosphatase activity convert BCIP to an intermediate product, releasing hydrogen, and the hydrogen ions cause a reduction of NBT, a chromogenic substrate, into a purple NBT diformazon [133]Metin girmek için buraya tıklayın veya dokununuz..

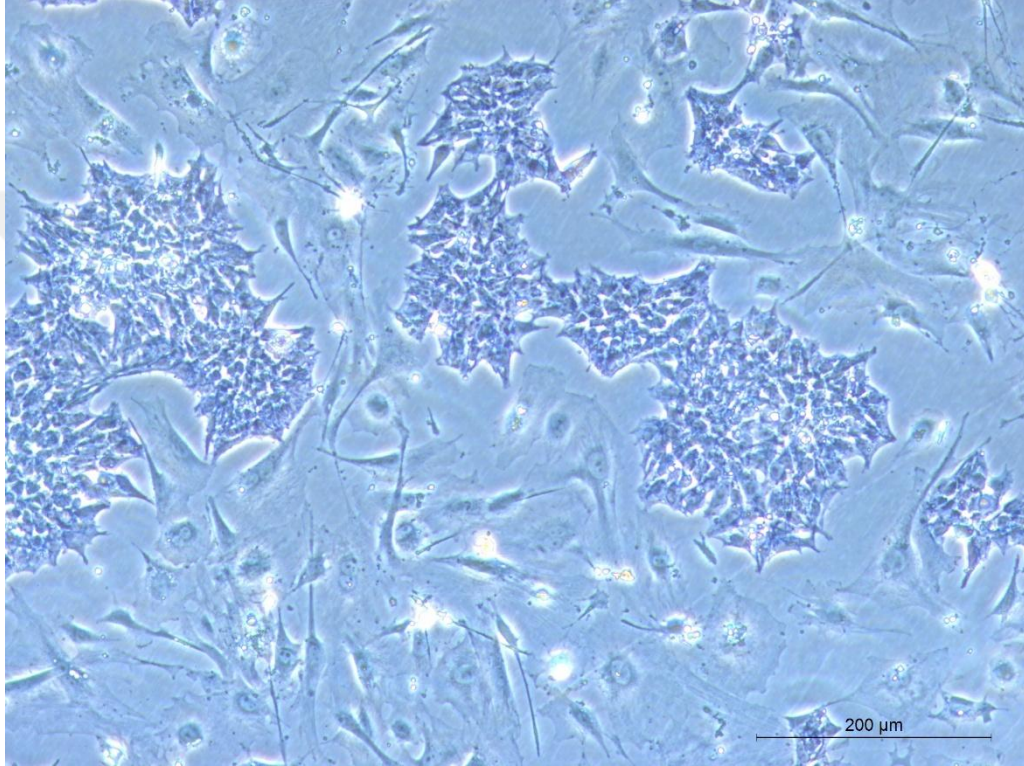


Figure 1.3.14 Alkaline phosphatase assay for MEF and hESCs. The image was taken by Leica DFC450.

1.3.5 Human brain organoid generation

After a well of a 6-well plate containing hESC colonies was confluent, they were ready for the organoid generation. The cell number from a well was enough for approximately 96 organoids. The cells were incubated with 20 μM ROCKi one hour prior to the organoid generation process, and then 9000 cells/150 μL were seeded into a low-attachment U-bottom 96-well plate by Accutase and EDTA-mediated cell passaging. The morphological changes of the cells from day 0 to day 60, indicating the transition from embryoid bodies to mature brain organoids, are represented in Figure 1.3.15.

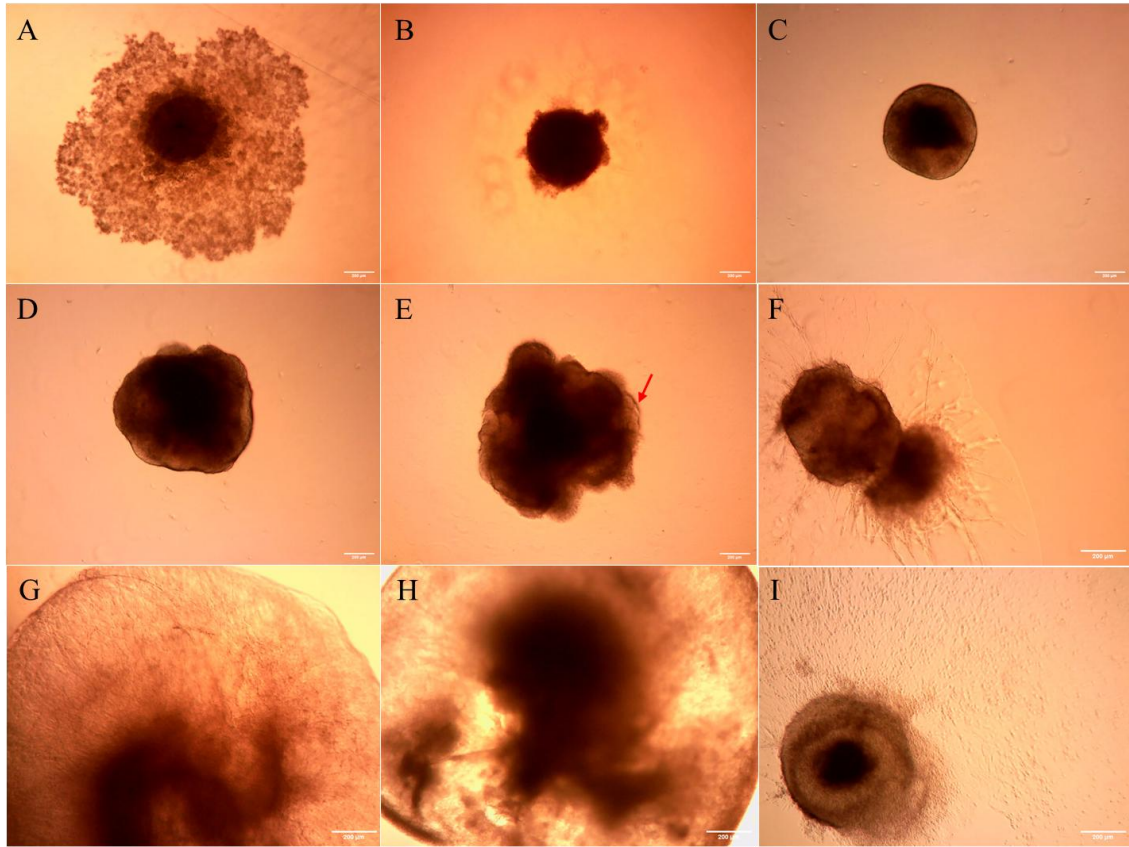


Figure 1.3.15 Morphological changes from the stem cell stage to the organoid stage. (A) Embryoid body on day 1, (B) Embryoid body on day 2 after the first medium change was performed, (C) Embryoid body to be transferred to neural induction medium on day 4, (D) Neuroepithelial tissue to be embedded in Matrigel on day 8, (E) Neuroepithelial tissue forming neural buds (shown with arrow) in Matrigel on day 9, (F) Differentiating organoid on day 12, (G) Differentiating organoid on day 33, (H) Mature organoid on day 55, (I) Failed organoids, showing extended cell processes. The images were recorded using a Zeiss PrimoVert Invert Microscope and an external camera with DinoCapture 2.0. Scale bar, 200 μm .

The first step of the organoid generation was to form EBs (Figure 1.3.15A). They were stem cell aggregates and cultured with low-dose bFGF and high-dose ROCKi to increase cell survival [125]. Also, in this study, after seeding the cells into a well of a U-bottom 96-well plate, the plate was spun quickly to increase the number of cells in the EB. Nevertheless, the debris around the central EB was observed until the first medium change. When half-medium replacement was performed on Day 2, the debris disappeared, and optical clearing around the EB was observed in the following days. These days, the size of EB was regularly monitored, and when the size was about 500-600 μm , they were transferred to a low-attachment 24-well plate in neural induction medium. At this step, keeping the EBs in suspension promotes uniform ectodermal differentiation across the surface of each EB. In this way, the non-neuronal inner part of the EB does not grow

further [125]. When the EBs were incubated in neural induction medium, they were radially organized, forming neuroepithelial tissue, neuroepithelia. The structures are directed to various brain structures depending on the medium formulation during maturation [125], [134]. In the thesis, BDNF and NT-3 from day 16 to day 27 for neural differentiation, PFGF-AA and IGF-1 from day 34 to day 44 for oligocortical structures and expansion of oligodendrocyte progenitor cells, and ketoconazole and T3 from day 44 to day 54 for myelination induction were used to direct neuronal structures [65], [135], [136], [137] (Figure 1.2.1). Also, one of the key factors in the hBO generation protocol was maintenance of agitation. Even though the growth factors function in directed differentiation, they were not efficiently diffused to each cell in the organoids. Therefore, stationary culture conditions were not preferable for 3D culture systems because of the decreased cell survival of the center of the organoid. While orbital shakers were used in this study to maintain the agitation, and spinning bioreactors were used in the literature, the center of the organoids became darker in time, indicating the necrosis [125] (Figure 1.3.15G-H).

After confirming the morphology of the brain organoid using bright field microscopy, we verified the neuronal content through immunofluorescent staining with confocal microscopy (Figure 1.3.16). Figure 1.3.16A demonstrates the spatial expression profiles of SOX2 and SATB2, marking a critical neurodevelopmental transition. Specifically, neural stem/progenitor cells residing within the ventricular zone (adjacent to the brain's inner surface) initiate differentiation into post-mitotic neurons, which subsequently migrate radially toward the developing cortical plate. This process exemplifies the canonical inside-out laminar organization of the cerebral cortex. Figure 1.3.16B displays TAU-immunolabelled axonal projections in close association with glial fibrillary acidic protein (GFAP)-positive astrocytes, suggesting the early formation of neuron–glia interactions. Such spatial arrangements are indicative of the establishment of functional neuroglial networks, essential for synaptic communication and metabolic support within the central nervous system. Lastly, the co-localization of myelin basic protein (MBP), a marker of active oligodendrocyte-mediated myelination, with ionized calcium-binding adaptor molecule-1 (IBA1) clearly indicates the presence of microglial populations. These findings imply simultaneous myelin sheath formation and maintenance of a stable immune microenvironment within the examined region. Taken

together, these observations reflect a structurally organized and neurofunctionally competent tissue architecture, primed to support mature neuronal circuitry.

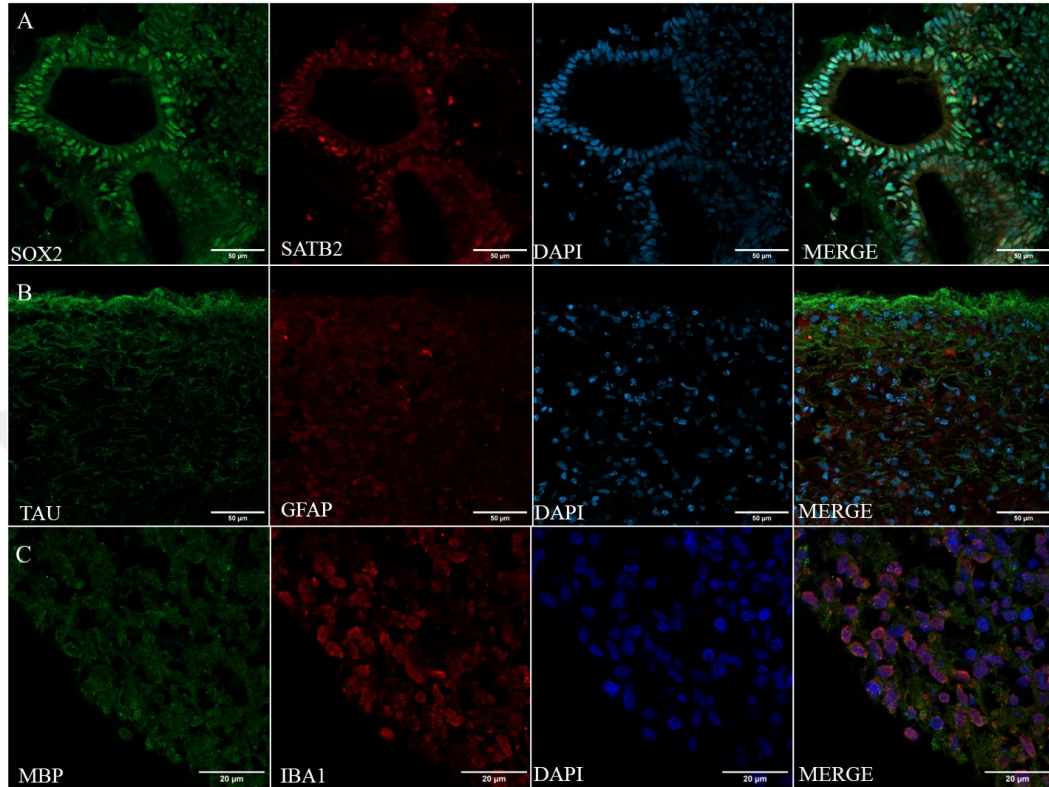


Figure 1.3.16 Confocal microscopy images of cryosectioned brain organoids. (A) anti-SOX2 (green), anti-SATB2 (red), and DAPI (blue) staining; (B) anti-TAU (green), anti-GFAP (red), and DAPI (blue) staining; (C) anti-MBP (green), anti-IBA1, and DAPI (blue) staining. The images were taken with the Zeiss LSM900 confocal microscope with Airyscan 2 and ZEN 3 Blue edition software and ImageJ (Fiji Version 1.8.0).

1.3.6 Inflammation-like response induction on hBOs

When the mature organoids in a dynamic culture were generated, they were incubated with LPS to induce inflammation-like responses. The LPS induction was confirmed with qRT-PCR and IF staining (Figure 1.3.17-19). In the confirmation processes, anti-MBP and anti-2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) antibodies were selected to verify a decrease in myelination after LPS induction because MBP is a marker for compact myelin [138], [139] and CNPase is an oligodendrocyte-specific marker, indicating myelin production [140], [141], [142]. Also, anti-GFAP and anti-IBA1 antibodies were selected to verify an increase in inflammation after LPS induction because GFAP is a marker for inflammation-dependent astrocytes (Giovannoni

& Quintana, 2020)Metin girmek için buraya tıklayın veya dokunun. and IBA1 is a microglia marker, indicating the main immune cells in the brain [143]. In addition to protein level confirmation, the inflammation was also confirmed at the gene expression level by measuring the change in *nuclear factor K-light-chain-enhancer of activated B cells* (*NF-κB*) and *interleukin-6* (*IL-6*) expression. The expression of the genes is responsible for the immune proinflammatory response in the CNS; therefore, their relative increase would show the inflammation induction [144], [145]. Consistent with the literature, the results demonstrate that Iba1+ cells increased by 74%, GFAP-positive cells increased by 60%, and CNPase expression decreased by 61%. However, MBP expression did not significantly change.

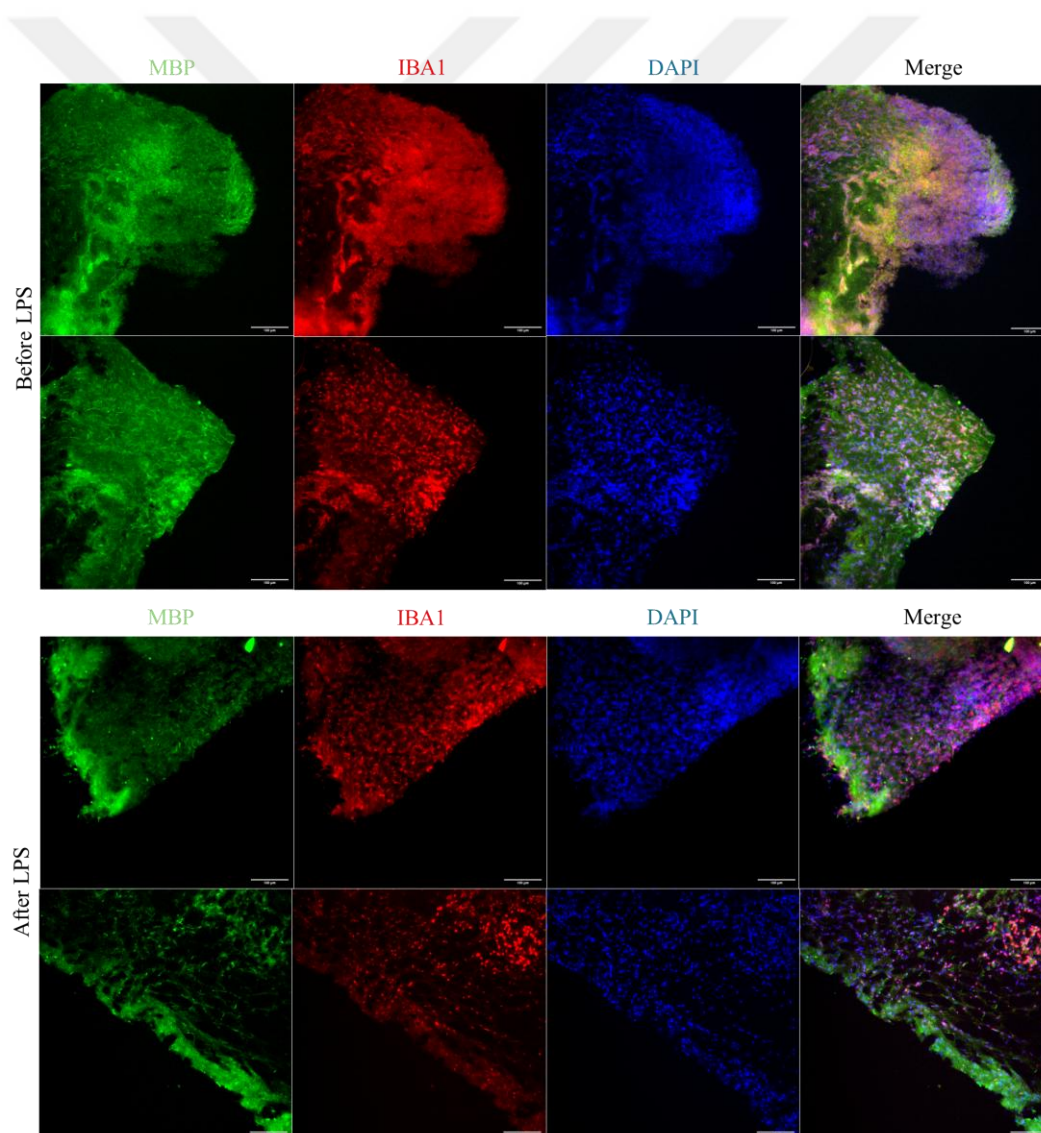


Figure 1.3.17 Representative immunofluorescent images of hBOs stained with anti-MBP and anti-Iba1 before and after LPS induction. The images were taken by Leica DM6 B. Scale bar, 100 μ m.

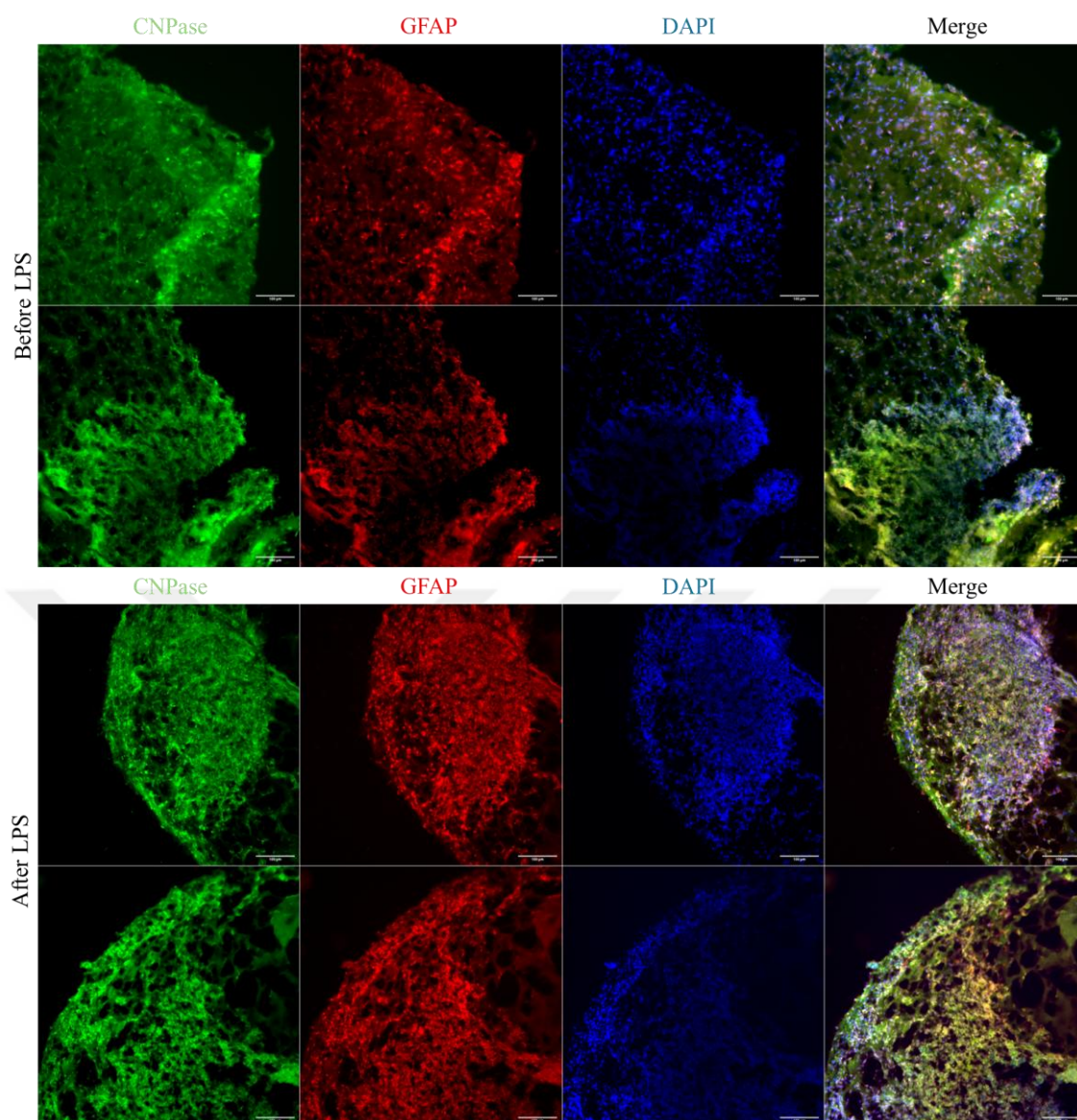


Figure 1.3.18 Representative immunofluorescent images of hBOs stained with anti-CNPase and anti-GFAP before and after LPS induction. The images were taken by Leica DM6 B. Scale bar, 100 μ m.

The level of GFAP protein is responsive to the proinflammatory signals, including LPS and IL-6 [146]. Our results completely support the previous findings because relative *IL-6* expression increased about 6.7-fold, and GFAP expression increased by approximately 60% upon LPS induction. In addition, the relative expression level of *NF- κ B* increased about 3.7-fold. This result was expected because LPS induction activates and translocates NF- κ B, leading to an increase in the expression of inflammatory genes, including TNF α [147], [148]. This occurs because LPS activates the canonical signaling pathway of NF- κ B, which is recognized as a central regulator of LPS [149].

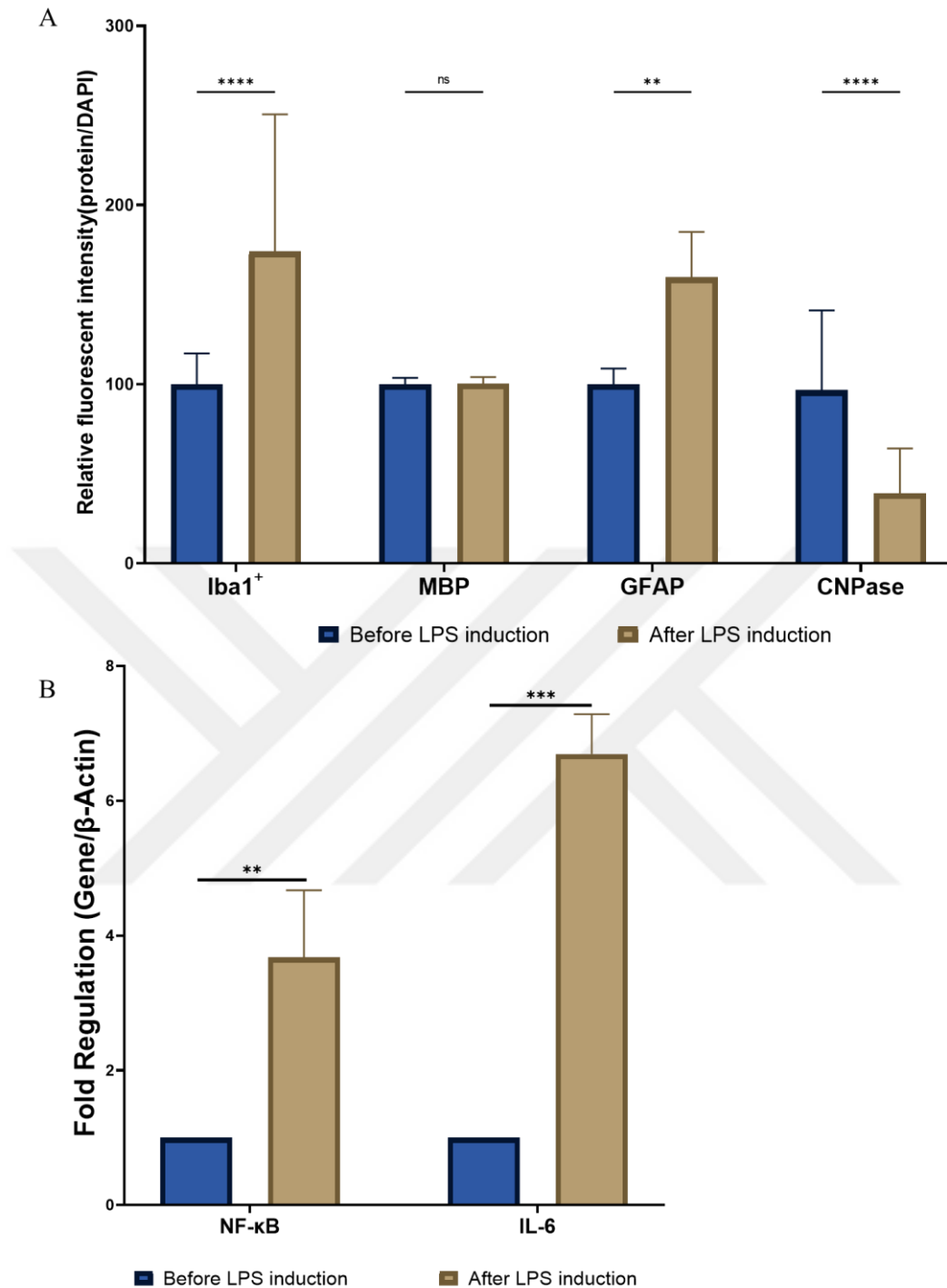


Figure 1.3.19 Quantitative analysis of immunofluorescent images and qRT-PCR. (A) Quantitative analysis of immunofluorescent images of hBOs stained with anti-Iba1, anti-MBP, anti-CNPase, and anti-GFAP (from left to right). Relative fluorescent intensity was measured using ImageJ, normalized to DAPI. (B) Quantitative analysis of qRT-PCR of hBO for expression of NF- κ B and IL-6. The data were normalized to β -actin. Two-way ANOVA with Šidák post hoc test (ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). The graphs were visualized by GraphPad Prism 9.0.

1.3.7 Treatment of hBOs with butterfly derivative compounds

Following the LPS induction confirmation, the hBOs were incubated with selected butterfly derivative compounds. The compounds were selected based on the results indicated in Figure 1.3.3. After the treatment, a part of the treated hBOs—two organoids for each batch—were fixed, cryosectioned, and immunofluorescently stained with fluorophore-conjugated primary antibodies. Only LPS-induced hBOs without further treatment (called LPS) (Figure 1.3.20 and Figure 1.3.26) were considered as the negative control, and LPS-induced Fingolimod-treated hBOs (Figure 1.3.25 and Figure 1.3.31) were considered as the positive control. The LPS-induced and further treated hBOs with ST-971, ST-1374, ST-1505, and ST-1894 were represented in Figures 1.3.21-24 for GFAP and TAU staining and Figures 1.3.27-30 for MBP staining. Figure 1.3.32 displays the quantitative analysis of the GFAP, TAU, and MBP staining.

Also, the rest of the treated hBOs, 8-11 organoids for each batch, were collected for qRT-PCR followed by RNA isolation and cDNA synthesis. The relative gene expression changes of the LPS-induced compound-treated hBOs for *GFAP*, *TNF α* , *colony stimulating factor-1 (CSF-1)*, and *IL-10* were represented in Figure 1.3.33. Only LPS-induced hBOs without further treatment (called LPS) were considered control in the figure.

Regarding the compounds' effects on hBOs, ELISA for secreted protein amount measurement was also performed in addition to changes in cellular protein and cellular transcript level measurement. TAU, fatty acid binding protein (FABP), *TNF α* , and alpha-synuclein amounts after each treatment were illustrated in Figure 1.3.34.

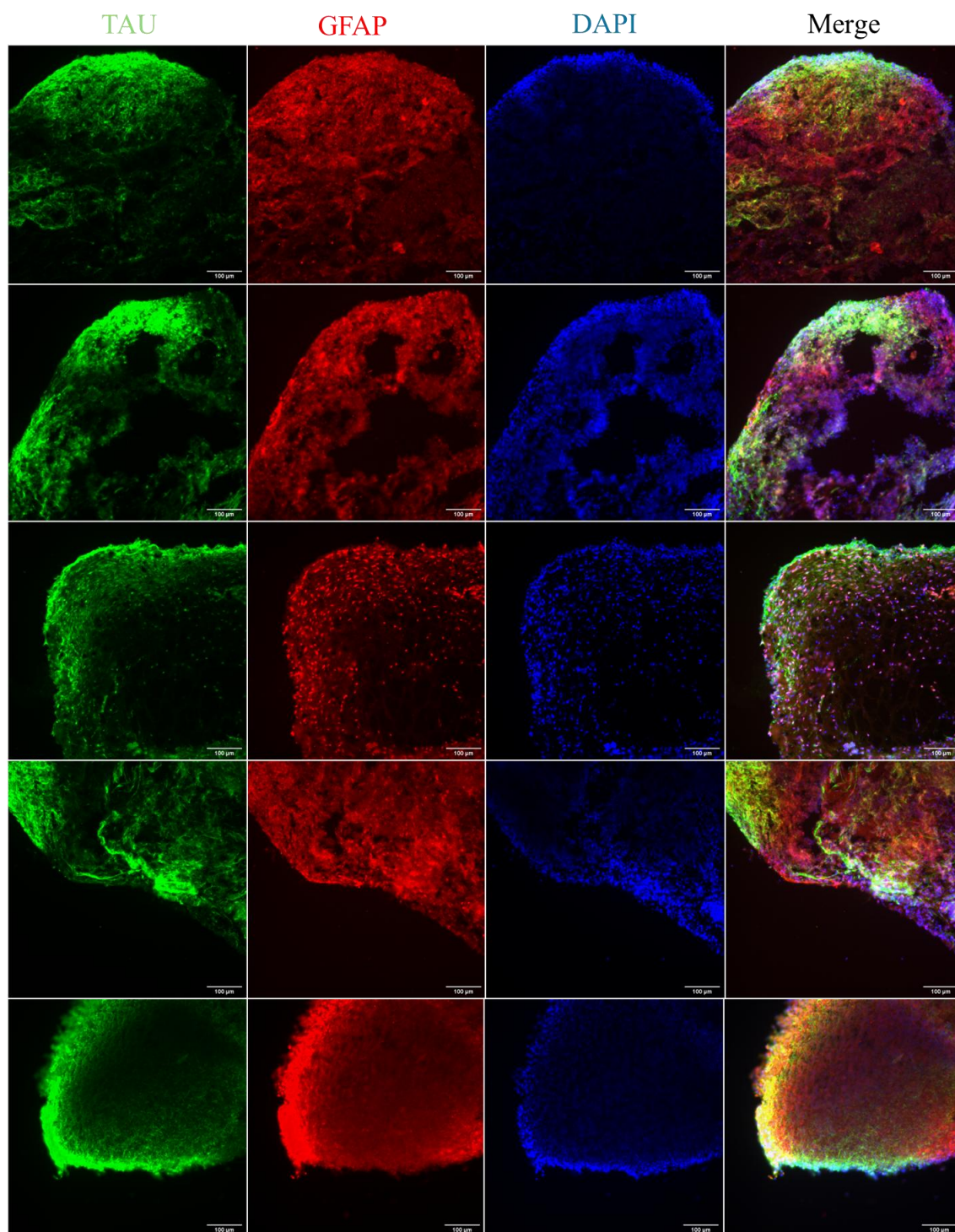


Figure 1.3.20 Representative immunofluorescent images of hBOs stained with anti-TAU (green) and anti-GFAP (red) after LPS induction. The images were taken by Leica DM6 B. Scale bar, 100 μ m.

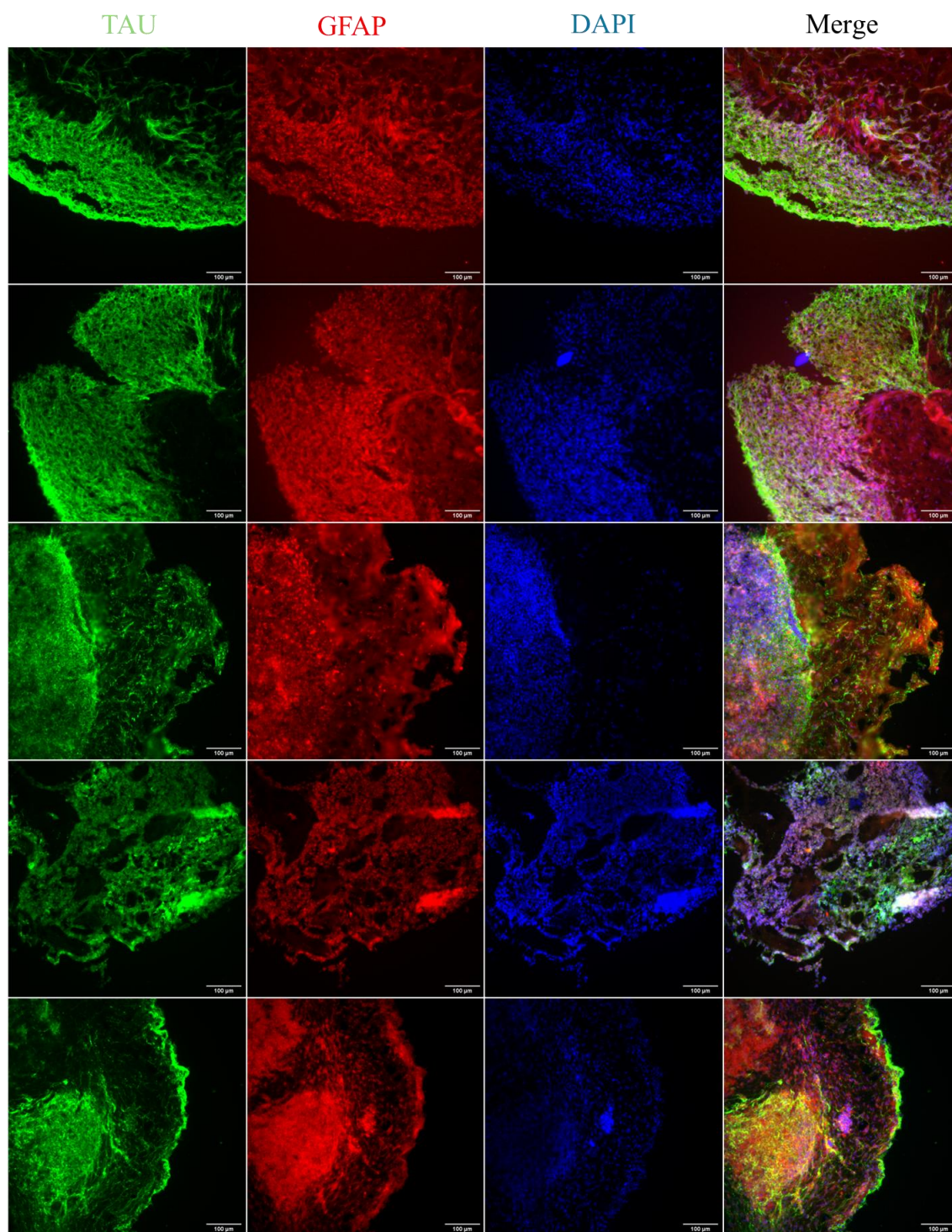


Figure 1.3.21 Representative immunofluorescent images of LPS-induced hBOs stained with anti-TAU (green) and anti-GFAP (red) after ST-971 treatment. The images were taken by Leica DM6 B. Scale bar, 100 μ m.

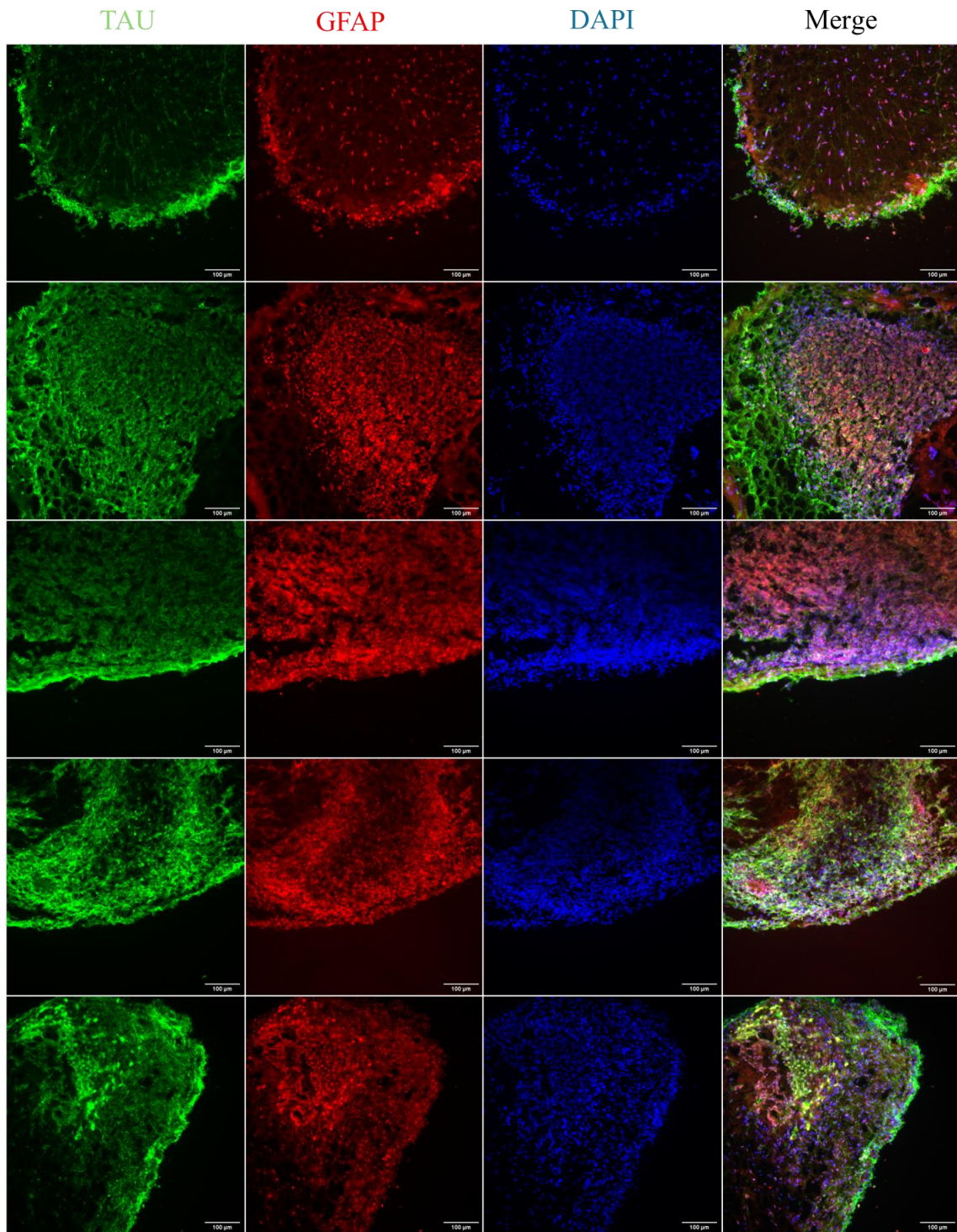


Figure 1.3.22 Representative immunofluorescent images of LPS-induced hBOs stained with anti-TAU (green) and anti-GFAP (red) after ST-1374 treatment. The images were taken by Leica DM6 B. Scale bar, 100 μ m.

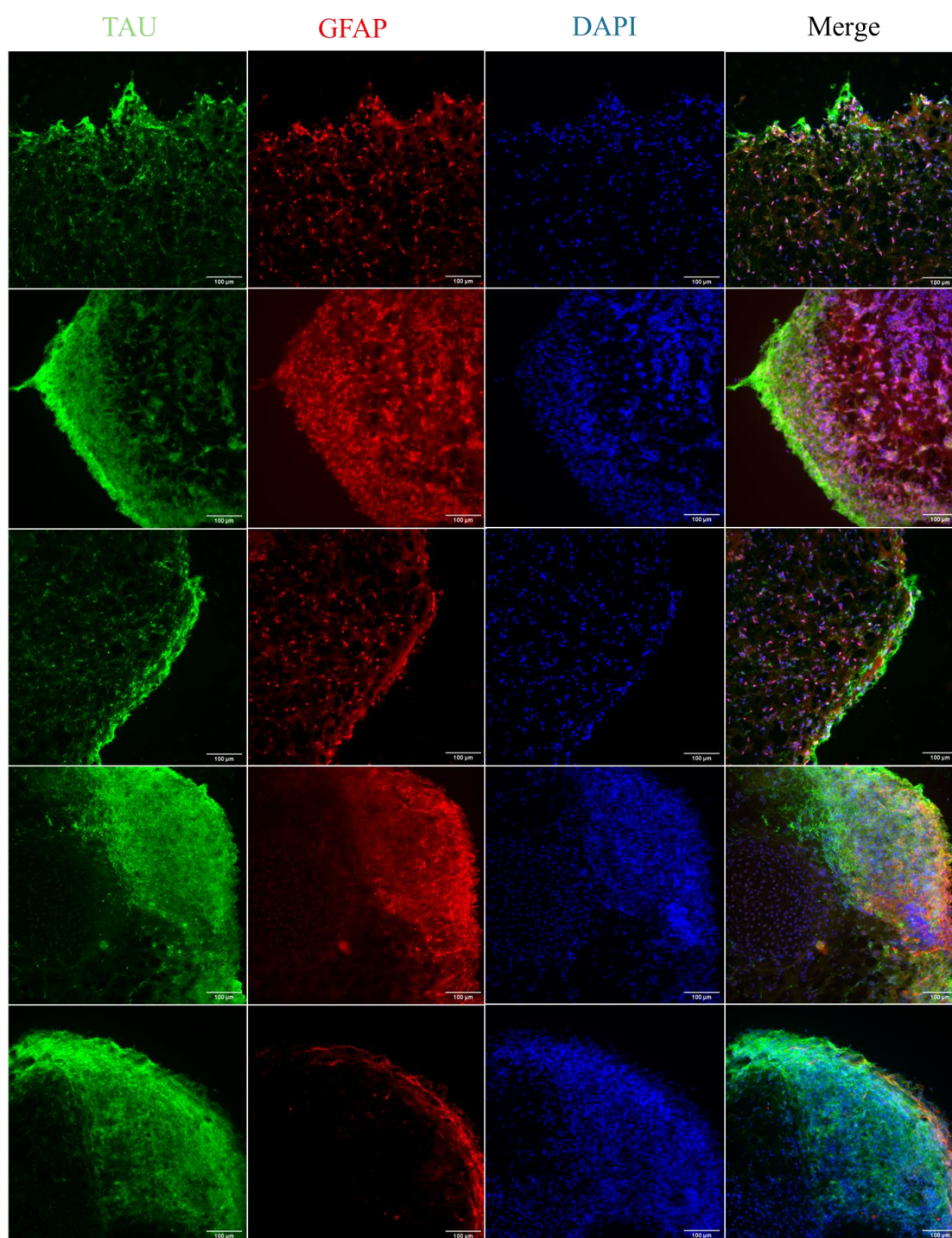


Figure 1.3.23 Representative immunofluorescent images of LPS-induced hBOs stained with anti-TAU (green) and anti-GFAP (red) after ST-1505 treatment. The images were taken by Leica DM6 B. Scale bar, 100 μ m.

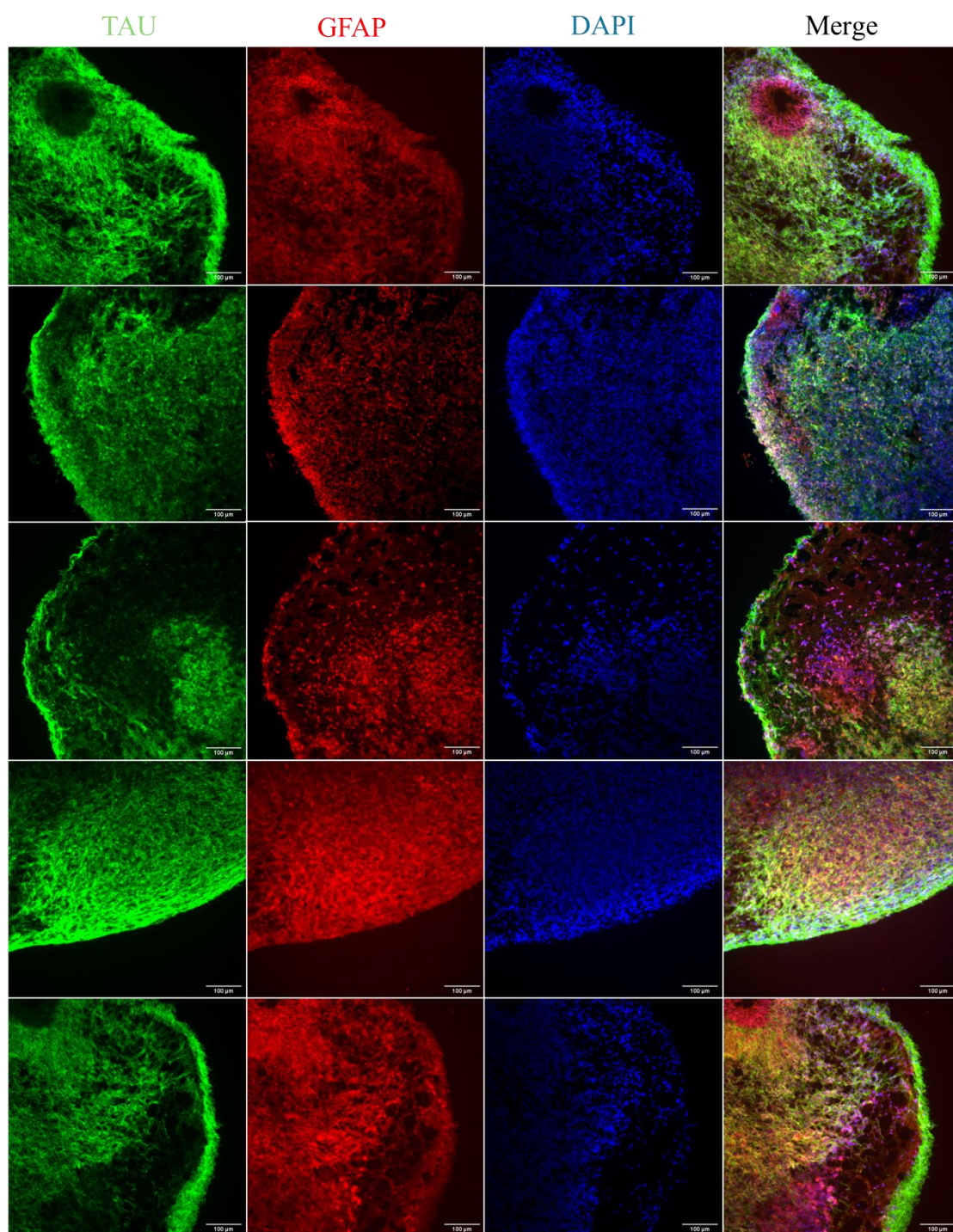


Figure 1.3.24 Representative immunofluorescent images of LPS-induced hBOs stained with anti-TAU (green) and anti-GFAP (red) after ST-1894 treatment. The images were taken by Leica DM6 B. Scale bar, 100 µm.

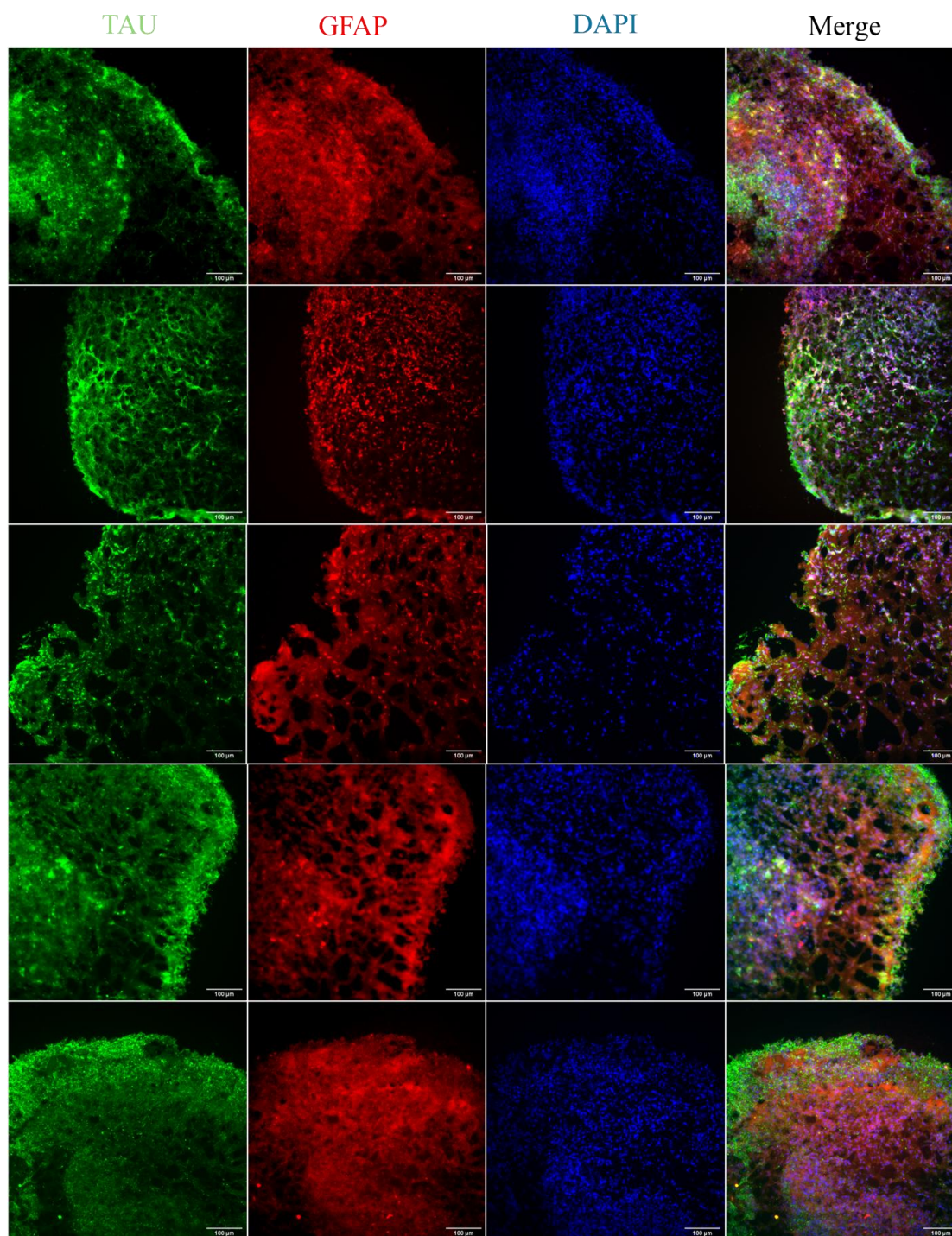


Figure 1.3.25 Representative immunofluorescent images of LPS-induced hBOs stained with anti-TAU (green) and anti-GFAP (red) after fingolimod treatment. The images were taken by Leica DM6 B. Scale bar, 100 μ m.

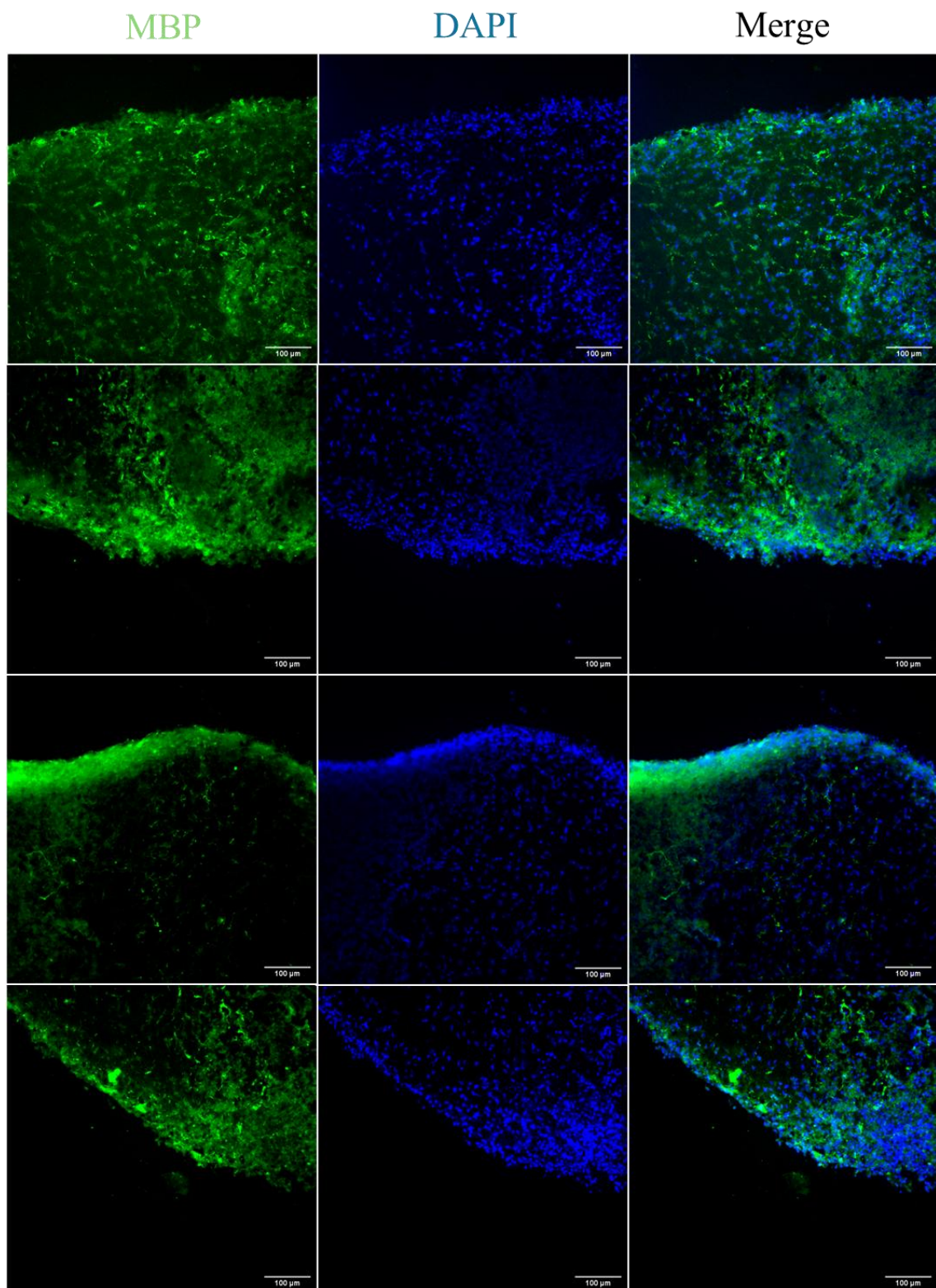


Figure 1.3.26 Representative immunofluorescent images of hBOs stained with anti-MBP (green) after LPS induction. The images were taken by Leica DM6 B. Scale bar, 100 μ m.

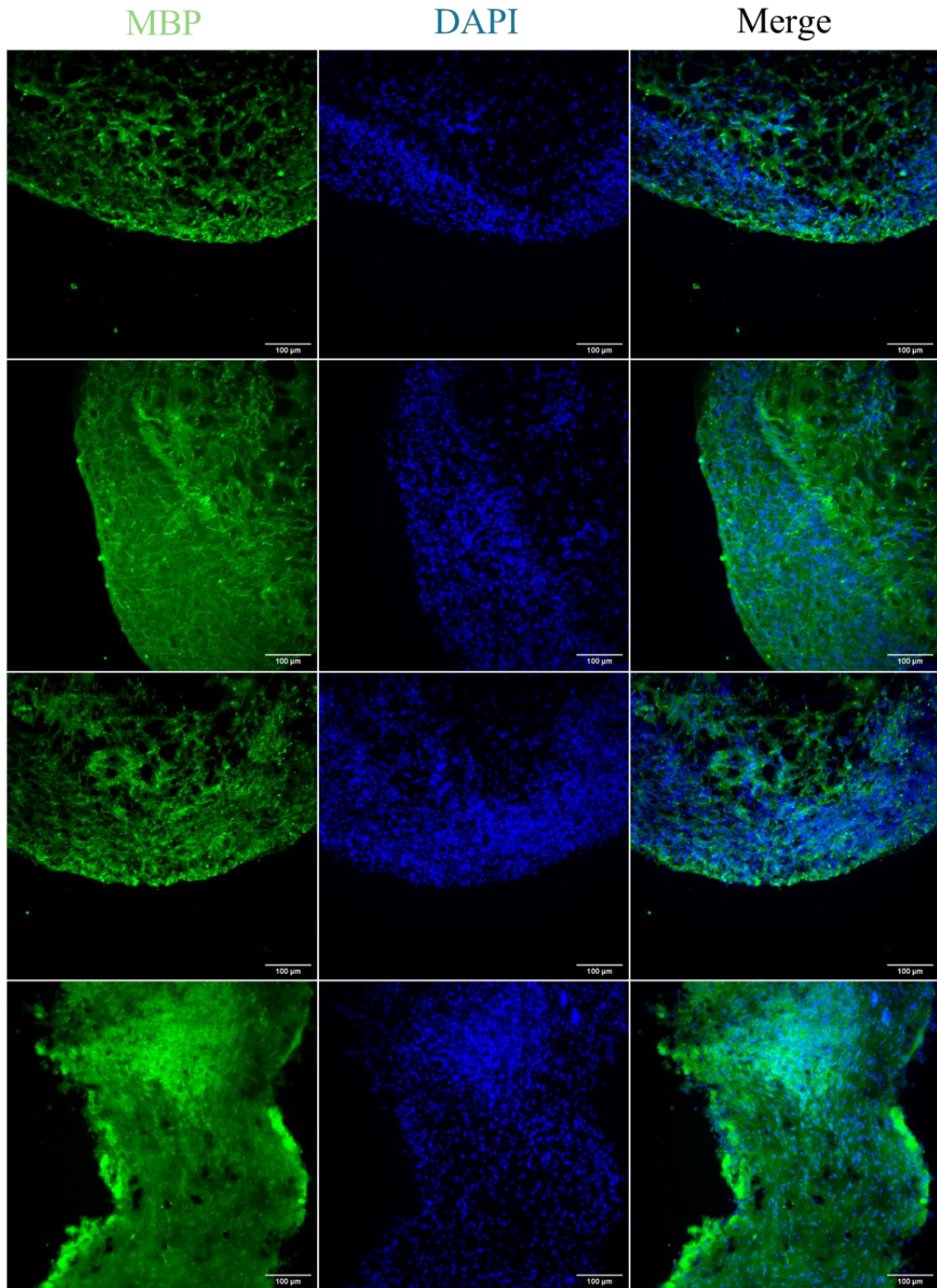


Figure 1.3.27 Representative immunofluorescent images of LPS-induced hBOs stained with anti-MBP (green) after ST-971 treatment. The images were taken by Leica DM6 B. Scale bar, 100 μm .

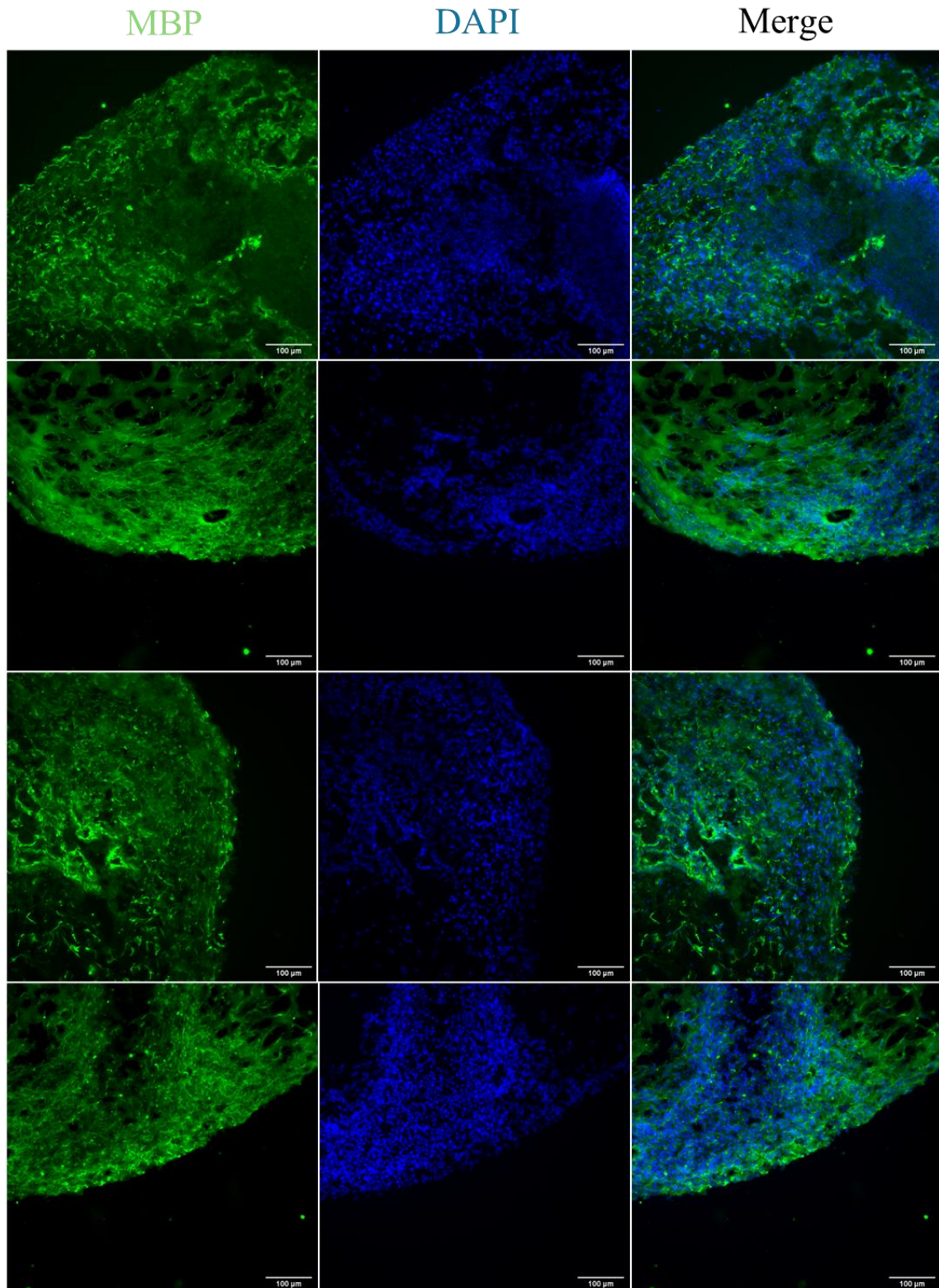


Figure 1.3.28 Representative immunofluorescent images of LPS-induced hBOs stained with anti-MBP (green) after ST-1374 treatment. The images were taken by Leica DM6 B. Scale bar, 100 μ m.

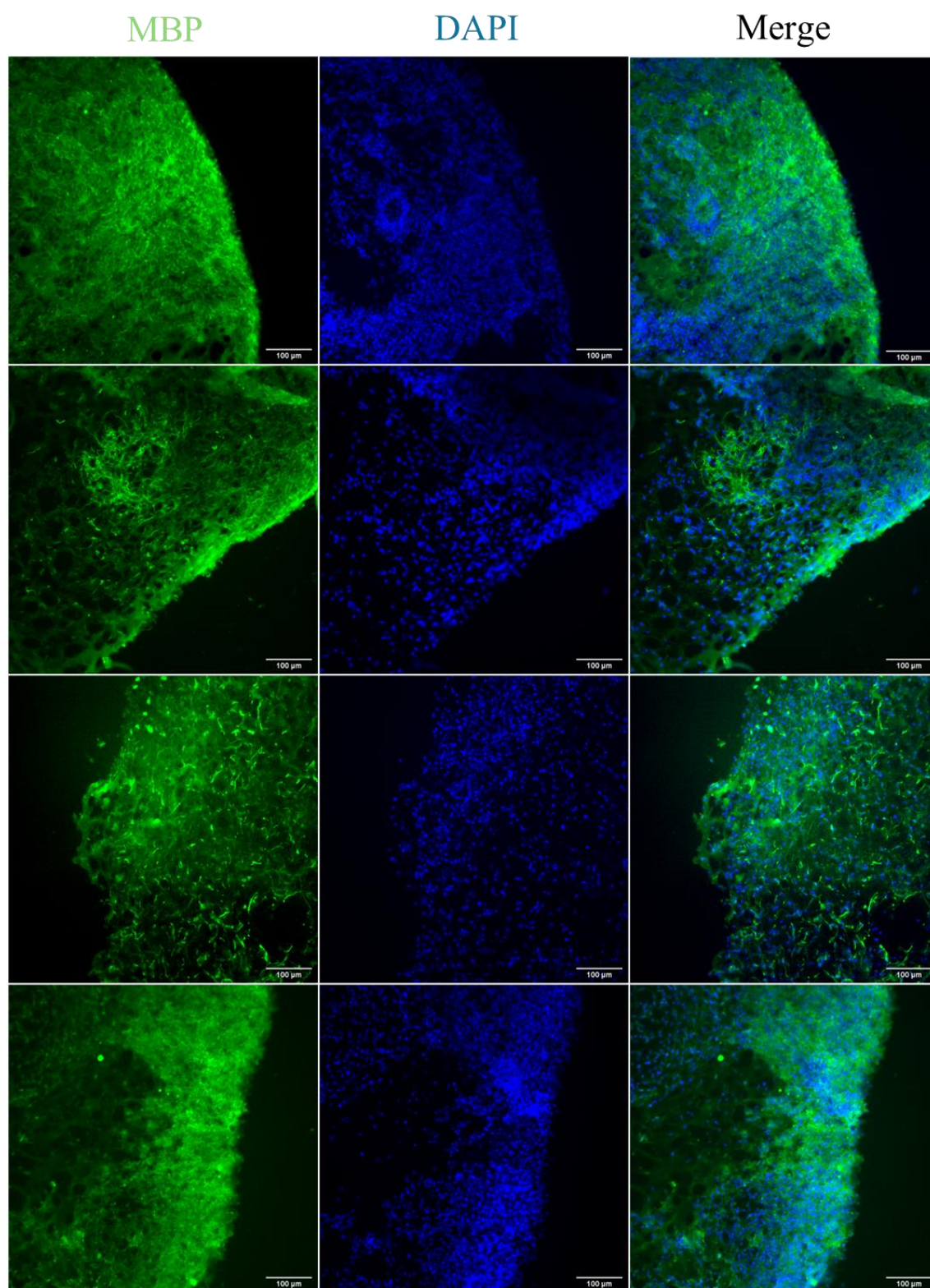


Figure 1.3.29 Representative immunofluorescent images of LPS-induced hBOs stained with anti-MBP (green) after ST-1505 treatment. The images were taken by Leica DM6 B. Scale bar, 100 μm .

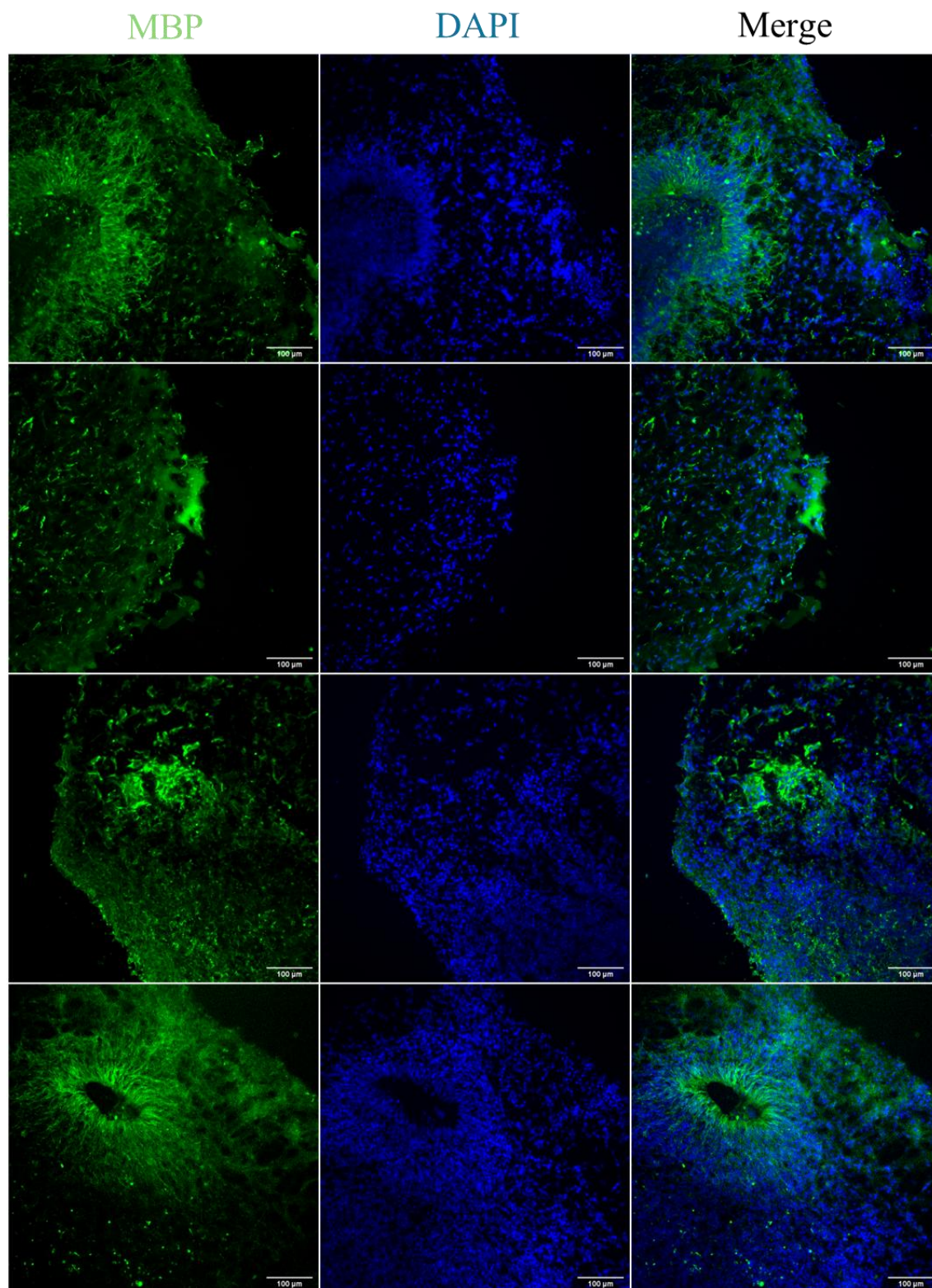


Figure 1.3.30 Representative immunofluorescent images of LPS-induced hBOs stained with anti-MBP (green) after ST-1894 treatment. The images were taken by Leica DM6 B. Scale bar, 100 μ m.

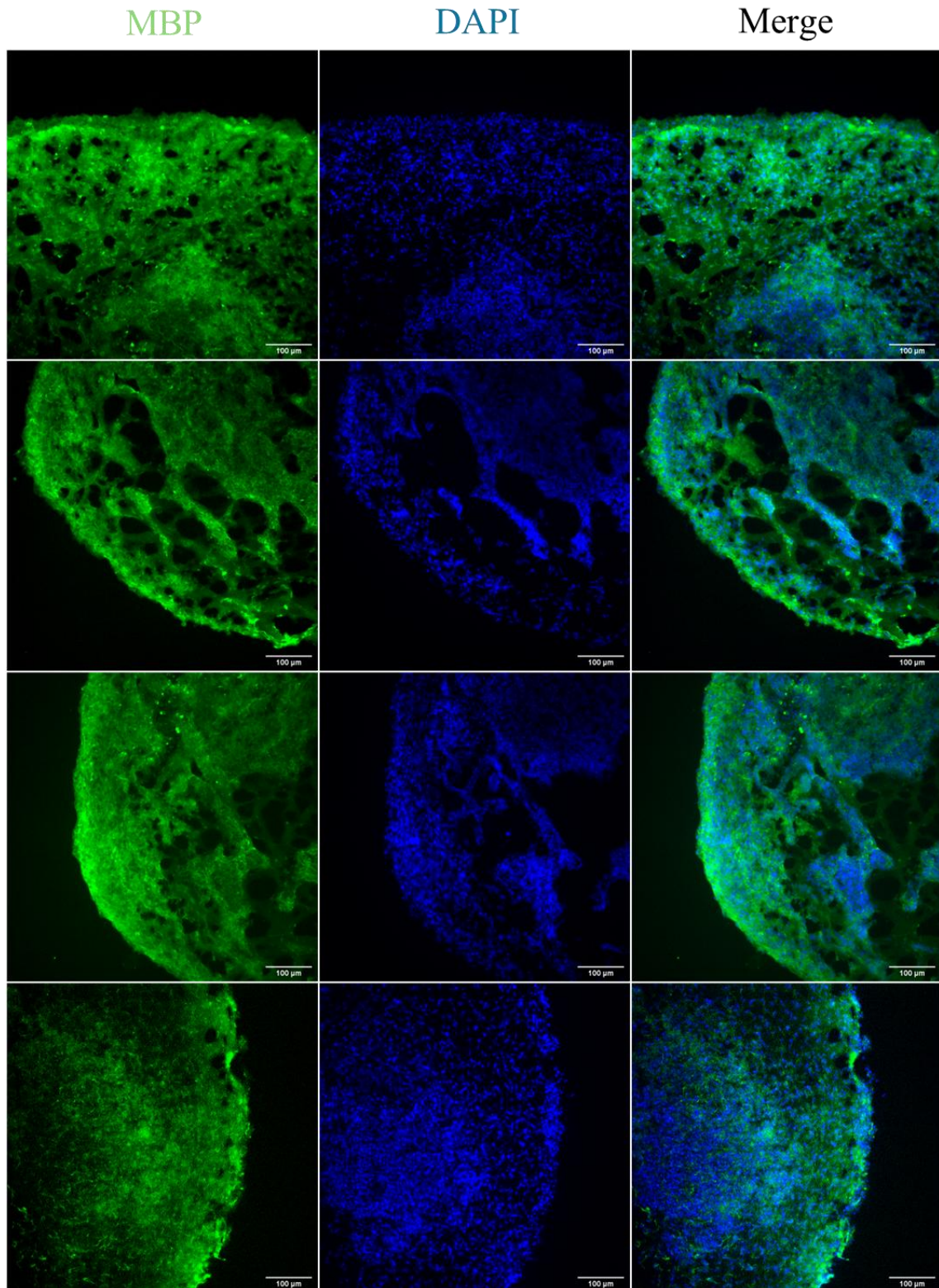


Figure 1.3.31 Representative immunofluorescent images of LPS-induced hBOs stained with anti-MBP (green) after fingolimod treatment. The images were taken by Leica DM6 B. Scale bar, 100 μ m.

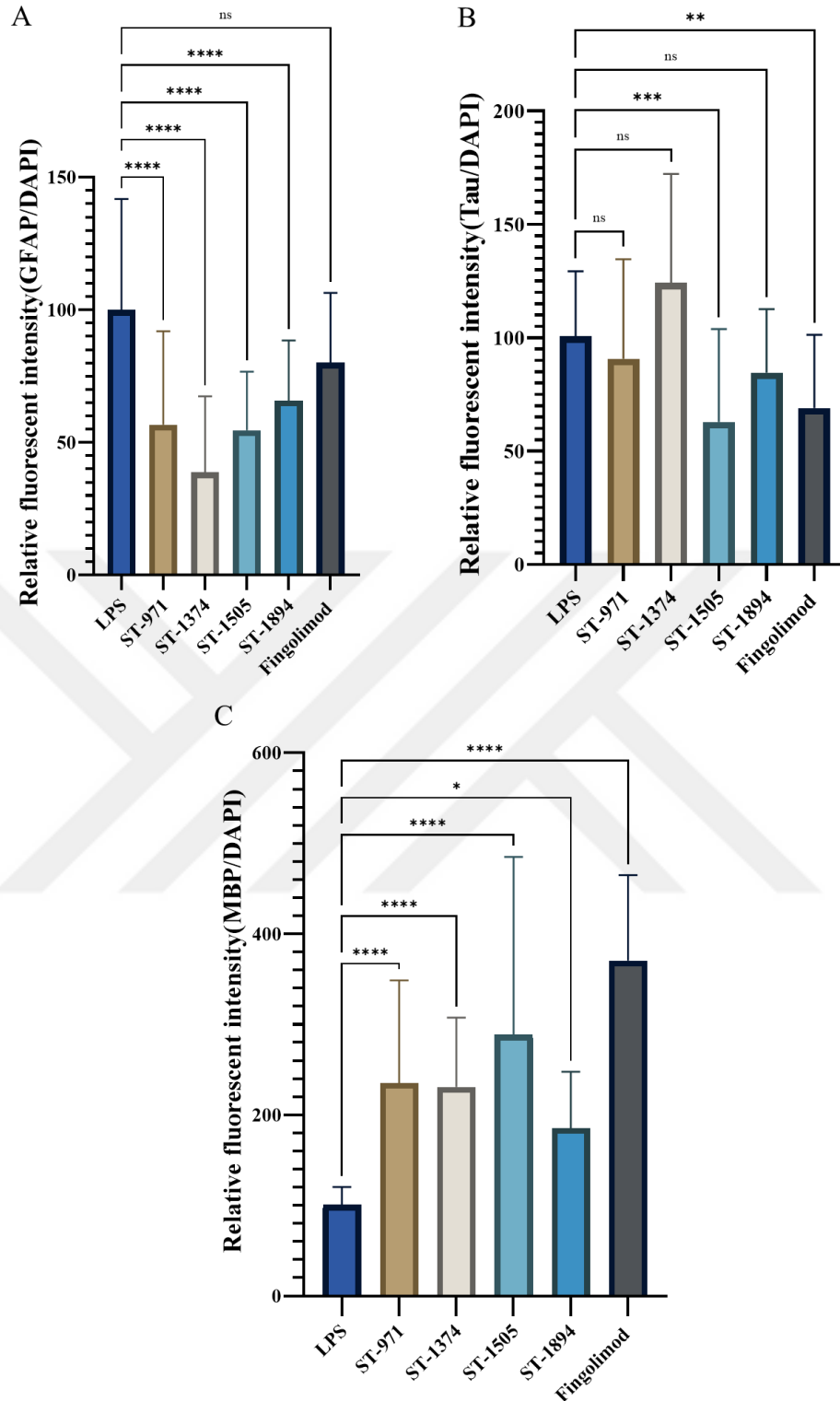


Figure 1.3.32 Quantitative analysis of immunofluorescent staining. The quantitative analysis of immunofluorescent staining was performed on hBOs labeled with (A) anti-TAU, (B) anti-GFAP, and (C) anti-MBP, which were treated with each compound, including LPS as a negative control and fingolimod as a positive control. One-way ANOVA with Dunnett's test was used for analysis (ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). The graphs were visualized by GraphPad Prism 9.0 and represented as Mean $\pm$ SD.

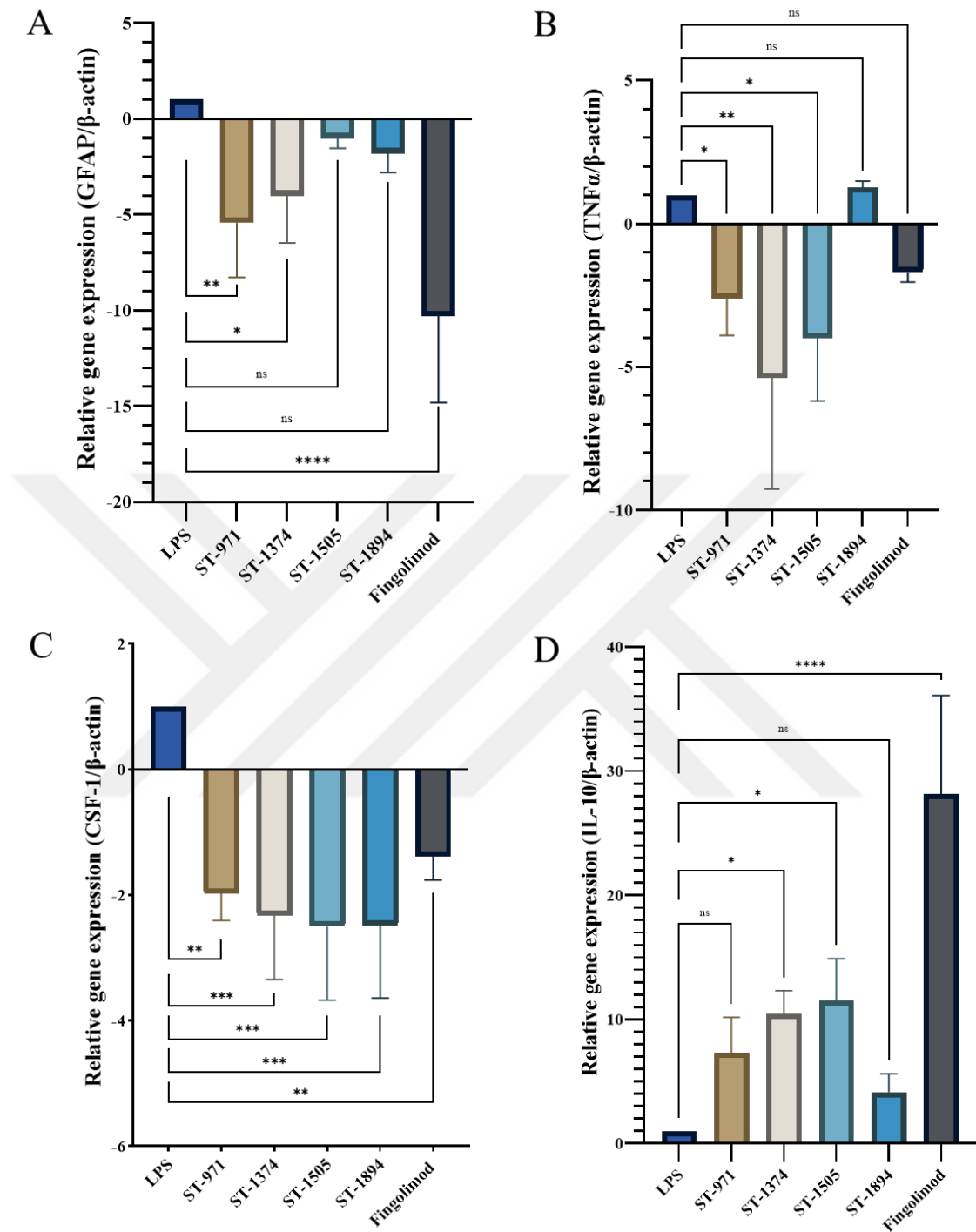


Figure 1.3.33 Quantitative analysis of qRT-PCR. Change in relative gene expression level of LPS-induced compound-treated hBOs for (A) GFAP, (B) TNF α , (C) CSF-1, (D) IL-10. The data from two independent batches were normalized to β -actin, and it was visualized by GraphPad Prism 9.0. The data were statistically analyzed by one-way ANOVA with the Dunnett post hoc test (ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). All the data are presented as the means $\pm$ S.D.

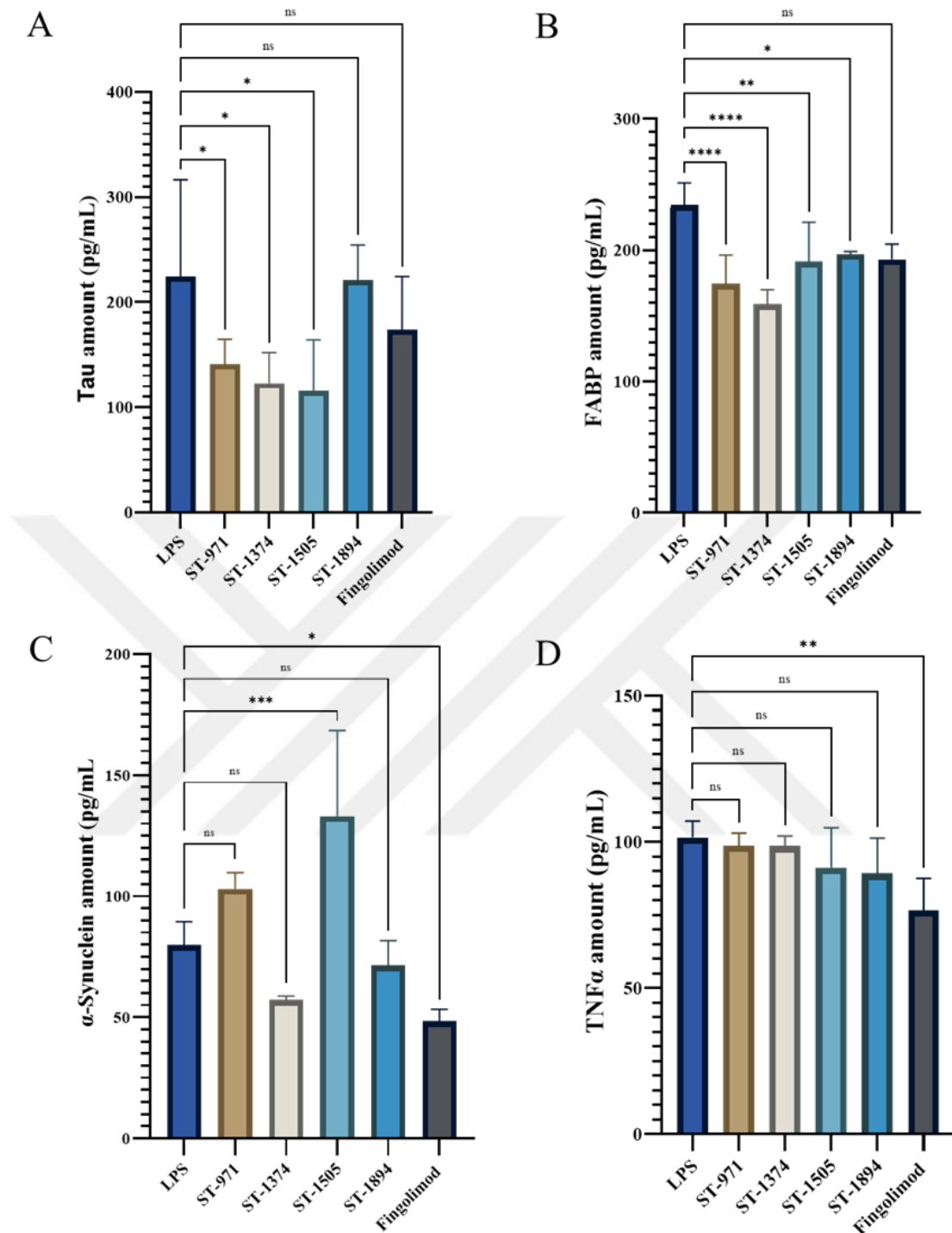


Figure 1.3.34 Secreted protein amount of LPS-induced compound-treated hBOs. Amount and quantitative comparison of (A) TAU, (B) FABP, (C) alpha-synuclein, and (D) TNFα in treated hBOs. The data were visualized with GraphPad Prism 9.0, and each treatment was compared with LPS. The data were statistically analyzed by one-way ANOVA with the Dunnett post hoc test (ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). All the data are presented as the means $\pm$ S.D.

The immunofluorescent staining results revealed that the treatments altered protein expression, which reflected changes in inflammation, cognitive impairment, and myelination (Figure 1.3.32). Fingolimod treatment, as confirmed by the literature, decreases GFAP-positive cells and TAU expression while increasing MBP expression because it reduces astrogliosis, enhances MBP synthesis through remyelination promotion, and inhibits axonal degeneration. [150], [151]. Since TAU accumulation is one of the triggering factors for axonal degeneration, a decrease in TAU amount is an expected outcome upon fingolimod treatment [152]. Similar to fingolimod, all the fingolimod derivative compounds also reduce the GFAP-positive cells significantly. However, although all compounds, except ST-1374, decrease the TAU expression, only ST-1505 causes a significant reduction, approximately 40%. Additionally, all compounds increase the MBP expression, indicating the remyelination of the hBOs. Supporting the immunofluorescent staining results, gene expression analysis revealed that GFAP expression after the treatment of each compound decreases as fingolimod does (Figure 1.3.33). Besides the GFAP, TNF α is also regulated by fingolimod by reducing its secretion from macrophages and microglia [153]. Therefore, 2.6-fold, 5.4-fold, and 4-fold reductions of TNF α expression indicate the decrease in inflammation after treatment with ST-971, ST-1374, and ST-1505, respectively. Given the relationship between CSF-1 abundance and microglial activation mediated by pro-inflammatory cytokines, a decrease in CSF-1 expression was expected following treatment with fingolimod. As shown in Figure 1.3.33C, all treatments, including fingolimod, cause a significant reduction in the expression of CSF-1 [154], [155]. The last gene expression analysis showed that, supporting the literature, the expression level of IL-10 as an anti-inflammatory marker significantly increases in fingolimod-treated hBOs [156] and more than a 10-fold increase upon ST-1374 and ST-1505 treatment. According to the staining and qRT-PCR results, ST-971, ST-1374, and ST-1505 cause an increase in myelination and a decrease in inflammation. Supporting earlier results in the study, the ELISA results reveal that secreted TAU and TNF α amounts are decreased after the treatment. In addition to them, alpha-synuclein and FABP secretion were also measured and compared to the LPS (Figure 1.3.34). Confirming the literature, since an increased amount of alpha-synuclein is associated with the inflammatory side of MS, fingolimod treatment decreases the amount of alpha-synuclein. ST-1374 and ST-1894 also cause a decrease in alpha-synuclein amount, but ST-1505 treatment also causes a significant decrease in alpha-synuclein amount (Figure 1.3.34C). It is also known that a higher level of alpha-synuclein

is associated with cognitive decline; therefore, a significant decrease in the protein amount upon ST-1505 treatment is a promising result for the ST-1505 effect on cognitive impairment [157], [158]. Lastly, it is possible to indicate that the change in amount of FABP, especially FABP3, FABP5, and FABP7, is associated with neurodegeneration and neuroinflammation; therefore, its significant decrease in all treatment groups supports the previous findings (Figure 1.3.34B) [159], [160].



CHAPTER 2

Study Alternative Extracellular Matrix for Brain Organoid Generation

2.1 Introduction

This chapter assesses the exploration of alternative extracellular matrices for brain organoid generation within the context of tissue engineering. After the discussion of tissue engineering components, the importance of natural hydrogels in brain organoid generation will be explained.

2.1.1 Tissue Engineering

Tissue engineering is an interdisciplinary field that integrates principles from biology, materials science, and engineering to develop biological substitutes that restore, maintain, or improve the function of damaged tissues or organs. It emerged in response to the limitations of traditional therapies, such as organ transplantation and prosthetics, which often face challenges including donor shortages, immune rejection, and limited integration with host tissue [161], [162]. The term "tissue engineering" was first formally introduced in the late 1980s, particularly gaining recognition after a National Science Foundation workshop in 1988, where it was defined as “the application of principles and methods of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function.” However, it became more popular with the publication by Dr. Langer and Dr. Vacanti in 1993 [163], [164]. Tissue engineering

concepts have gained importance by leveraging advancements in organoid technology and the clarification of 3D culture terminology in the 2000s, particularly through progress in pluripotent stem cell research. Especially advancements in gene editing technologies such as CRISPR-Cas9, single-cell transcriptomic profiling, and three-dimensional bioprinting have markedly enhanced the functional fidelity, scalability, and reproducibility of organoid systems, thereby broadening their utility in high-throughput pharmacological screening and therapeutic validation efforts since 2021 [165]. Organoids exhibit genetic stability, sustained proliferative capacity, and multilineage differentiation potential, along with an intrinsic ability to self-organize into complex, tissue-like structures. By creating precisely controlled biochemical, biophysical, and mechanical microenvironments, scientists can change how organoids work and how they grow, which makes it possible to recreate important physiological features that are typical of the structure and function of native tissue. Tissue engineering techniques can achieve modulation of functionality and morphogenesis by altering key parameters, which include the main components of tissue engineering such as cells, growth factors, and scaffolds [165], [166], [167].

2.1.1.1 Cells

One of the important factors in tissue engineering is the suitability of cells or tissues in terms of their phenotypic and mechanical characteristics [168]. Most contemporary tissue engineering approaches rely on the isolation and expansion of autologous cells obtained from the patient's own diseased or damaged organ. However, in cases of advanced-stage organ failure, tissue biopsies may yield an insufficient number of viable, functionally competent cells for in vitro expansion and subsequent transplantation. Moreover, certain organs, such as the pancreas, often lack readily expandable primary autologous cells, limiting their therapeutic utility. Under such conditions, stem cells are considered a promising alternative cell source due to their self-renewal capacity and potential to differentiate into multiple tissue-specific lineages. These stem cells may be derived from discarded human embryos (human embryonic stem cells; hESCs), fetal tissues, or adult somatic tissues, such as bone marrow, adipose tissue, or epidermal layers, offering diverse options for regenerative applications [161]. Stem cells differ in their differentiation potential, immunogenicity, proliferative capacity, and interaction with biomaterials, all of which influence their suitability for specific tissue engineering

applications. For example, since stem cells interact with the biophysical and biochemical cues of the scaffold, the choice of stem cell type must match the mechanical stiffness, topography, and ligand composition of the scaffold material to support cell adhesion, migration, and differentiation. The preference of neural stem cells for the ECM-like hydrogel and the preference of MSCs for the stiffer substrates for osteogenic differentiation are two good examples of the importance of cell types [169], [170].

hESCs are characterized by two fundamental properties: prolonged self-renewal capacity while retaining a pluripotent state and the capability to differentiate into a wide array of specialized somatic cell types. These cells are typically derived from the inner cell mass (ICM) of the blastocyst-stage embryo (approximately five days post-fertilization) through microaspiration techniques. Conventionally, hESCs are cultured on feeder layers composed of mitotically inactivated mouse embryonic fibroblasts (MEFs) or human-derived feeder cells to maintain pluripotency and provide essential growth factors. However, advancements in stem cell culture have enabled the development of feeder-free and xeno-free systems, minimizing the risk of cross-species pathogen transmission and immunogenic contamination. hESCs have demonstrated long-term stability in vitro, maintaining an undifferentiated phenotype and normal karyotype over extended passaging (>80 passages) under well-established culture conditions. Critically, these cells can differentiate into cell types representative of all three embryonic germ layers: ¹Ectoderm for generation of neuronal cells and keratinocytes, ²Mesoderm for differentiation into hematopoietic cells, cardiomyocytes, chondrocytes, endothelial cells, and skeletal muscle cells, ³Endoderm for formation of pancreatic islet-like cells and hepatocytes. Moreover, the pluripotency of hESCs is further evidenced by their ability to form embryoid bodies (EBs) in vitro—three-dimensional cell aggregates that spontaneously generate derivatives of all three germ layers—and teratomas when transplanted in immunodeficient animal models, which is a classical in vivo assay for pluripotency. Despite their promising biological potential, the derivation of hESCs involves the destruction of preimplantation embryos, raising substantial ethical, moral, and religious concerns. Therefore, prevailing bioethical regulations in several countries restrict or prohibit the clinical and research use of hESCs [161], [171], [172].

The other stem cell type that can be used in tissue engineering, including organoid generation, is reprogrammed induced pluripotent stem cells derived from somatic cells. In recent decades, significant advancements have been made in the generation of

pluripotent stem cells through genetic reprogramming of differentiated adult somatic cells, a technique that holds immense promise for regenerative medicine. Reprogramming involves the epigenetic and transcriptional resetting of terminally differentiated cells (e.g., fibroblasts) to an iPSC state, thereby generating patient-specific, autologous pluripotent cells without the use of embryos. This approach circumvents the ethical concerns associated with hESCs and minimizes the risk of immune rejection, as iPSCs retain the autologous genetic identity of the donor cell. The groundbreaking work by Takahashi and Yamanaka demonstrated for the first time that mouse embryonic fibroblasts (MEFs) and adult mouse fibroblasts could be reprogrammed into iPSCs via the ectopic expression of four transcription factors: Oct3/4, Sox2, Klf4, and c-Myc, delivered through retroviral vectors. These reprogrammed cells acquired hallmark characteristics of pluripotency, including unlimited self-renewal, expression of embryonic stem cell-specific markers, and the capacity to form embryoid bodies in vitro and teratomas in vivo. Subsequent studies extended the findings to human cells. Yamanaka and colleagues successfully generated human iPSCs (hiPSCs) with similar morphology, proliferation rates, pluripotency-associated gene expression, surface marker profiles, and teratoma-forming ability to those of hESCs. In parallel, Thomson's group achieved comparable results using the transcription factors OCT4, SOX2, NANOG, and LIN28. Despite these advances, comparative analyses have shown that hiPSCs and hESCs, while functionally similar, are not molecularly identical, reflecting subtle but important differences in epigenetic memory and gene expression patterns. Although the molecular mechanisms governing reprogramming are not yet fully elucidated, this technology represents a transformative step toward personalized medicine, disease modeling, and cell-based therapies. Continued optimization of reprogramming methods and comprehensive characterization of iPSCs will be essential for their safe and effective clinical translation. Currently, a substantial proportion of organoids are generated by iPSCs. However, incompletely differentiated iPSCs retain tumorigenic potential, posing a risk for the formation of teratomas upon transplantation. During the directed differentiation protocols, iPSCs can give rise to heterogeneous cellular populations, including undesired lineages such as mesenchymal cells, which can compromise organoid fidelity and functionality. Moreover, iPSC-derived organoids typically exhibit immature phenotypes, closely resembling fetal-stage tissues rather than fully developed adult counterparts, limiting their translational relevance for disease modeling and regenerative therapies [161], [167], [173], [174].

2.1.1.2 Growth factors

The bioengineered microenvironments enhance the structural and spatial fidelity of organoid models while enabling precise control over key biophysical and biochemical parameters, including growth factor gradients, oxygen tension, and matrix stiffness. Such controlled modulation is essential for recapitulating *in vivo* developmental cues and facilitating the maturation and functional specification of organoids [165].

When the proper cell source is determined, the second important factor in tissue engineering is growth factors. Growth factors are orchestrating agents to lead a range of cellular processes, including proliferation, lineage specification, differentiation, and morphogenesis. The spatial and temporal modulation of growth factor signaling is crucial for mimicking *in vivo* developmental programs and for the self-organization of cells into tissue-specific architectures [166], [175], [176]. In the early stages of organoid formation, mitogenic factors such as epidermal growth factor (EGF) and fibroblast growth factors (FGFs) are essential for promoting the proliferation and survival of progenitor cells. For example, FGF2 enhances neural progenitor expansion and maintains stemness during cerebral organoid development, whereas EGF supports epithelial proliferation in intestinal organoids [176], [177], [178]. Differentiation and patterning in tissues and organoids are guided by morphogen gradients, largely mediated by signaling molecules such as WNTs, BMPs (bone morphogenetic proteins), TGF- β (transforming growth factor-beta), and SHH (sonic hedgehog) [176]. For instance, WNT3A and R-spondin-1 synergistically activate the canonical WNT/ β -catenin pathway, promoting crypt-like structure formation in intestinal organoids [179], [180]. Similarly, neuroectodermal induction from human pluripotent stem cells (hPSCs) is typically achieved by inhibition of TGF- β /Activin/Nodal and BMP signaling pathways (commonly referred to as dual SMAD inhibition) using small molecule inhibitors such as SB431542 (TGF β inhibitor) and Noggin (BMP antagonist) or LDN-193189 (BMP inhibitor) [181], [182]. This strategy mimics the *in vivo* dorsal ectodermal environment during early neural plate formation, leading to efficient commitment toward the neural lineage. [183] Additionally, vascular endothelial growth factor (VEGF) and insulin-like growth factors (IGFs) support angiogenesis [184], while neurotrophins such as BDNF further promote tissue-specific morphogenesis and neuronal maturation [185]. Especially in the later stages of development, IGF-1 and BDNF play essential roles in enhancing synaptogenesis, axonal outgrowth, and functional neural network formation, facilitating electrophysiological

activity within organoids [186], [187], [188]. As the organoids mature, another neurotrophic factor, NT-3 in particular, binds to TrkC receptors and plays a pivotal role in the differentiation and maturation of neurons within the developing brain, especially in promoting the survival and axonal growth of corticospinal and sensory neurons [189]. Also, specific cell maturation, for example, glia, is another aspect of the use of growth factors. Platelet-derived growth factor-AA (PDGF-AA), a key mitogen for oligodendrocyte precursor cells (OPCs), binds to PDGFR α receptors to promote the expansion, migration, and early differentiation of OPCs. Its inclusion in later stages of culture facilitates the generation of oligodendrocyte-lineage cells and contributes to the modeling of myelination-related processes within brain organoids [190], [191], [192]. Beyond the variability of growth factors or growth factor cocktails, the controlled delivery of these growth factors, either via media supplementation or through engineered biomaterials with tunable release kinetics, allows for enhanced spatial-temporal control of organoid development. As a result, the combination and timing of growth factor exposure critically determine organoid fidelity, complexity, and physiological relevance [193], [194].

2.1.1.3 Scaffolds

Scaffolds serve as essential structural and biochemical platforms in tissue engineering and organoid research, providing mechanical support and a biomimetic microenvironment that facilitates cell adhesion, proliferation, migration, and differentiation. In tissue engineering, scaffolds are made from biocompatible polymers and are meant to mimic important parts of the extracellular matrix (ECM). They provide both biophysical cues and spatial architecture that are needed for tissue regeneration [195]. Tissue engineering approaches for the scaffolds are broadly categorized into two main strategies: ¹acellular scaffolds, which rely on the host's endogenous regenerative capacity to direct tissue organization, and ²cell-seeded scaffolds, which incorporate exogenous cells to facilitate tissue formation. Acellular scaffolds are typically fabricated either through the de novo synthesis of biomimetic materials or via decellularization protocols that remove cellular constituents from native tissues using mechanical and chemical treatments, resulting in collagen-rich, biologically derived extracellular matrices. In the implantation case, these scaffolds undergo gradual biodegradation and are progressively remodeled and replaced by extracellular matrix (ECM) components

secreted by infiltrating host or transplanted cells [161]. The main determinants of the scaffolds as a polymer, depending on the tissue type, are their biocompatibility, biodegradability, and mechanical properties, along with the manufacturing technology [195]. Biocompatibility of the polymer refers to the ability of the material to perform its intended functions in a biological environment without eliciting any undesirable local or systemic effects. It is essential to ensure that the material supports cell survival, proliferation, and differentiation while minimizing the toxicity and inflammation. In organoid applications, biocompatibility also entails maintaining stem cell pluripotency or lineage specificity without inducing unwanted differentiation or cytotoxicity [196], [197]. Biodegradability refers to the ability of a biopolymer to be broken down into smaller, non-toxic byproducts through biological processes, such as enzymatic action or hydrolysis, within physiological environments. This property is essential in biomedical applications. A biodegradable biopolymer is designed to degrade at a controlled rate, ideally synchronized with tissue regeneration or organoid maturation, so that the material provides support when needed and is safely resorbed by the body or culture system without causing harm or inflammation. In organoid systems, particularly neural and intestinal models, biodegradable scaffolds provide an ECM-like microenvironment that temporarily guides 3D tissue formation, then degrades to allow organoid expansion, vascularization, or remodeling. For example, GelMA (gelatin methacryloyl) is a semi-synthetic hydrogel that undergoes matrix metalloproteinase (MMP)-mediated degradation in a cell-dependent manner, enabling dynamic remodeling during organoid development. However, it must be considered that biodegradability must be balanced with mechanical stability, biocompatibility, and non-cytotoxic degradation products to ensure safe and effective use in both in vivo and in vitro systems [198], [199], [200], [201]. Mechanical properties of biopolymers refer to their ability to withstand physical forces without deformation or failure and to provide structural support appropriate for the biological tissue or application they are intended to mimic. Key mechanical parameters include: ¹Elastic modulus (Young's modulus) indicating stiffness or rigidity, ²Tensile strength indicating the resistance to being pulled apart, ³Compressive strength indicating the resistance to being compressed, ⁴Viscoelasticity indicating the time-dependent deformation under stress, ⁵Toughness and resilience indicating the ability to absorb energy and recover. These properties are crucial when designing scaffolds and biomaterials for tissue engineering, drug delivery, and organoid culture, as they influence cell behavior, including adhesion, differentiation, and migration [202], [203], [204]. For

instance, stem cells are sensitive to substrate stiffness. Neural stem cells preferentially differentiate into neurons on softer substrates (approximately 100-500 Pa), while stiffer substrates (approximately 1-10 kPa) promote glial differentiation. Thus, biopolymers used in brain organoid systems must mimic the soft mechanical properties of the brain ECM to promote accurate tissue development [200].

Even though the determinants should be considered in any type of scaffold application design, the polymers, in terms of their source, are categorized into two main types: ¹synthetic and ² natural [205].

2.1.1.3.1 Synthetic polymer-based scaffolds

Synthetic polymers have emerged as critical components in the field of tissue engineering and regenerative medicine, particularly as biomaterials for scaffold fabrication. These polymers offer tunable mechanical properties, degradation rates, and structural versatility, making them highly adaptable to diverse biological applications. Unlike natural polymers, synthetic polymers can be precisely engineered to meet specific mechanical and biochemical requirements, ensuring reproducibility, stability, and scalability in biomedical applications. Poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(lactic-co-glycolic acid) (PLGA), and polyethylene glycol (PEG) are the commonly used synthetic polymers in tissue engineering. The polymers are precisely fabricated with defined molecular weight, porosity, and surface chemistry, allowing modulation of mechanical strength, biodegradation rate, and cellular interactions. The polymers can also be chemically modified to incorporate bioactive molecules, adhesion ligands such as RGD (Arginine-Glycine-Asparagine) peptides for integrin binding, or growth factors, which facilitate enhanced cell attachment, proliferation, and differentiation. Also, they exhibit minimal batch-to-batch variability compared to natural polymers, ensuring consistency in scaffold performance. Despite their advantages, synthetic polymers can lack bioactivity and cell recognition motifs, which are inherent to natural ECM-derived materials. This necessitates surface modification or copolymerization with natural biomolecules (e.g., collagen, gelatin) to improve cell-material interactions. Furthermore, degradation products of certain synthetic polymers (e.g., PLA and PLGA) may induce local acidosis, which could adversely affect tissue integration [205], [206], [207], [208].

2.1.1.3.2 Natural polymer-based scaffolds

Natural polymers that are used as scaffolds are the organic products of living organisms. The scaffolds can be derived from animals through decellularization and demineralization processes, or they can be derived from plants [205], [209]. Due to their biological origin and structural resemblance to native tissue components, natural polymers are considered promising biomimetic analogs of the extracellular matrix (ECM). They closely replicate the native cellular microenvironment, particularly in skin and other soft tissues. Compared to synthetic counterparts, biopolymers demonstrate several advantageous properties, including enhanced biocompatibility, enzymatic biodegradability, low immunogenicity, and sustainability. These attributes make them ideal candidates for applications in tissue engineering, regenerative medicine, and biomedical device development [205]. When comparing animal-derived and plant-derived scaffolds, researchers conclude that animal-derived scaffolds are less preferable due to ethical concerns [209]. Researchers in basic science are replacing animal-derived products like FBS, extracellular matrix such as Matrigel, and antibodies with alternatives due to ethical concerns about animal welfare [210]. Animal-derived Matrigel is accepted as the “gold standard” natural hydrogel [211]. It is a basement membrane matrix obtained from the “Engelbreth-Holm-Swarm (EHS) mouse tumor.” The tumor took its name from the scientist who discovered it, J. Engelbreth-Holm, and the one who characterized it, Richard Swarm. Matrigel is rich in laminin, type IV collagen, entactin, and heparan sulfate proteoglycan; therefore, it functions as a growth-promoting material. Also, some variants of Matrigel, such as growth factor-reduced Matrigel, are produced by the addition of a 20% NaCl precipitation step [212], [213], [214]. Many tissue engineering applications also utilize the plant-derived scaffold due to its ability to overcome immunogenicity and ethical issues. The cellulose, gelatin, alginate, chitosan, and hyaluronic acids are examples of plant-derived scaffolds (Figure 2.1.1) [205], [209].

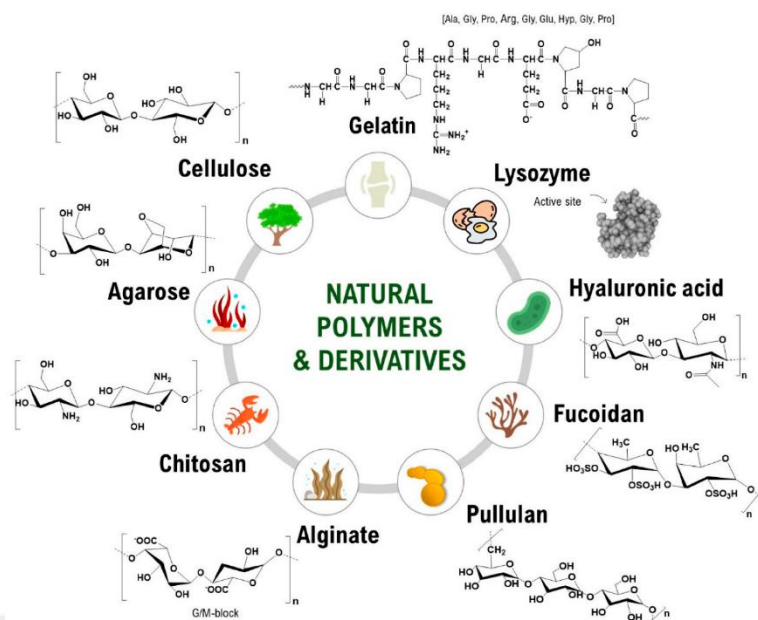


Figure 2.1.1 Natural polymer examples and their structures [215]

Cellulose is one of the most abundant polymers found in plant cells. The intricate pore structure of cellulose makes it a preferable scaffold for skin-related applications. Bacterial cellulose is also used for engineering cartilage tissue [205], [216]. Alginate is also used as a scaffold. It is obtained from brown algae and some bacteria to be transformed into a powder. The monomers of alginate, α -L-glucuronic acid (G) and β -D-mannuronic acid, are differentially distributed depending on the source, and it affects the solubility and viscosity of the alginate. The application areas of alginate scaffolds range from dermal applications, including wound healing, to drug delivery systems [205], [217].

There is also a class of scaffolds called semi-synthetic scaffolds. The scaffolds are a combination product of natural and synthetic polymers with lesser drawbacks [218]. Hydrogels are three-dimensional, hydrophilic polymeric networks with the capacity to absorb and retain substantial amounts of water while maintaining their structural integrity. These biomaterials closely resemble the native ECM of tissue, fulfilling multiple roles such as facilitating nutrient diffusion for resident cell proliferation, providing mechanical support at defect sites, and encapsulating cells and bioactive molecules. Hydrogels are commonly formulated using natural biopolymers, including gelatin, alginate, collagen, hyaluronic acid, and chitosan. Despite their natural polymer content, large-scale hydrogel production often necessitates the incorporation of synthetic and petrochemical-derived monomers, such as acrylic acid and acrylamide, to enable simultaneous polymerization

and cross-linking during fabrication. Gelatin is a natural protein derived from the partial hydrolysis of collagen-rich tissues. It exhibits brittle mechanical properties in its dry state but becomes soft and elastic upon hydration. Due to the presence of cell-adhesive motifs analogous to those found in native collagen, gelatin can function as a cost-effective alternative to collagen in various biomedical applications. Methacrylation of gelatin achieves crosslinking, resulting in the creation of a semi-synthetic scaffold [209]. The product called methacrylated gelatin, or GelMA, owing to its photocrosslinkable nature and ECM mimetic architecture, has been extensively employed in the bioprinting of organoids. These GelMA-based constructs demonstrate improved cell viability, spatial organization, and tissue-specific functionality, making them a promising hydrogel system for organoid engineering and regenerative applications [165].



2.2. MATERIAL and METHOD

2.2.1 Production of methacrylated gelatin (GelMA)

Type A gelatin (300 Bloom, derived from porcine skin) was dissolved in phosphate-buffered saline (PBS, pH 7.4) at 50°C to obtain a 10% (w/v) solution. Subsequently, methacrylic anhydride (MA) was added at a concentration of 4% (v/v) and allowed to react with the gelatin backbone to introduce methacrylate groups, following the protocol described by Irmak et al. (Figure 2.2.1) [219]. The MA was administered at a controlled flow rate of 0.4 mL/min under microwave irradiation (1000 W) to facilitate the methacrylation reaction. Following the reaction, the resulting methacrylated gelatin (GelMA) solution was subjected to dialysis against deionized water to eliminate residual salts and unreacted MA. The purified GelMA was subsequently lyophilized (freeze-dried) and stored at –80°C for long-term preservation.

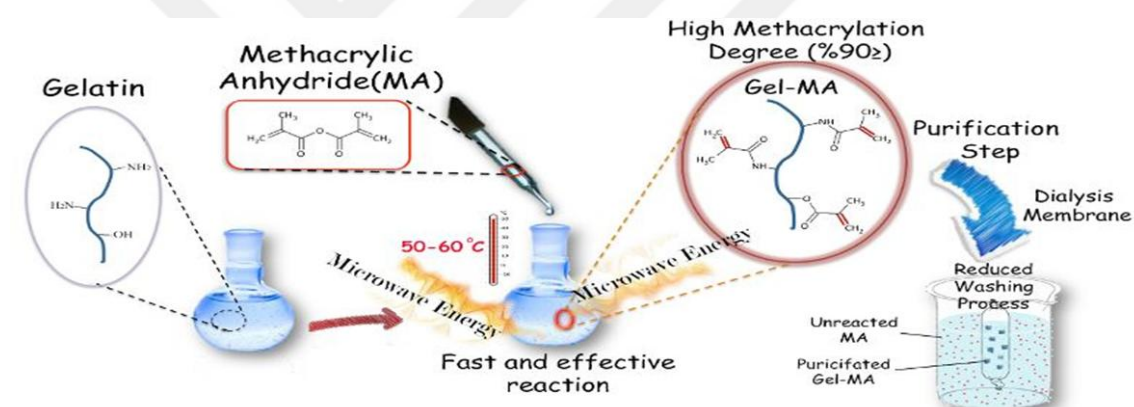


Figure 2.1.2 Synthesis of GelMA via microwave-assisted methacrylation [219]

2.2.1 Human embryonic stem cell (hESC) culture

The H9 human embryonic stem cell (hESC) line (WA09, female; WiCell) was maintained according to the WiCell feeder-dependent culture protocol. Initially, mouse embryonic fibroblasts (MEFs) were seeded onto 0.1% gelatin-coated 6-well plates and subsequently mitotically inactivated using mitomycin C at a final concentration of 10 µg/mL. Once the MEF layer reached approximately 80% confluency and was mitotically inactivated (referred to as iMEF), researchers plated H9 hESCs onto this feeder layer. When plated H9 cells reached a confluency of 70–80%, they were transitioned to a feeder-free culture system by transferring them onto growth factor-reduced Matrigel-coated

(Corning, Cat# 356230) 6-well plates and maintained in mTeSR™1 medium (StemCell Technologies, Cat# 85850). Colonies displaying well-defined, compact borders were passaged weekly using 0.5 mM ethylenediaminetetraacetic acid (EDTA).

2.2.2 Human neuroepithelial tissue formation

Human neuroepithelial tissues (hNETs) were generated based on previously described protocols with minor modifications [65], [125]. H9 human embryonic stem cell colonies plated on iMEF at 70–80% confluency were pretreated with 20 μ M ROCK inhibitor (Y-27632; StemCell Technologies) for 1 hour to enhance cell survival during dissociation. The colonies were then dissociated using a combination of 0.5 mM EDTA and Accutase to generate a single-cell suspension. Following cell counting, the suspension was centrifuged at $270 \times g$ for 5 minutes, and the pellet was resuspended in low bFGF medium consisting of 20% KnockOut Serum Replacement (KSR), 1% GlutaMAX, 1% non-essential amino acids (NEAA), and 4 ng/mL basic fibroblast growth factor (bFGF) in DMEM/F12, supplemented with 50 μ M ROCK inhibitor. The cell suspension was adjusted to a density of 9000 cells per 150 μ L. The cells were seeded into U-bottom 96-well plates to promote the formation of embryoid bodies (EBs) and incubated at 37°C in 5% CO₂. Half of the medium was replaced every other day for 6 days. Upon reaching a diameter of approximately 600 μ m, the EBs were transferred to low-attachment 24-well plates containing neural induction medium composed of 1% N2 supplement, 1% GlutaMAX, 1% NEAA, and 0.01% heparin in DMEM/F12. The neural induction medium was refreshed every other day for five days until hNETs were formed.

2.2.3 Human brain organoid generation

In the process, each hNET was embedded within a hydrogel matrix—either Matrigel (Corning, 356234) or methacrylated gelatin (GelMA)—to facilitate three-dimensional differentiation and maturation. For Matrigel embedding, individual hNETs were transferred and positioned centrally within concave sterile Parafilm dimples, and 30 μ L of Matrigel was applied directly onto each hNET. They were incubated at 37°C for 30 minutes to allow gelation, followed by transfer to 24-well plates containing differentiation medium composed of 50% Neurobasal medium, 0.5% N2 supplement, 1%

B27 (without vitamin A), 50 nM 2-mercaptoethanol, 0.025% insulin, 1% GlutaMAX, and 0.5% non-essential amino acids (NEAA) in DMEM/F12, called NDFM-Vitamin A. For GelMA embedding (Figure 2.3.4), a photoinitiator solution containing 0.3% (w/v) lithium phenyl-2,4,6-trimethylbenzoylphosphine (LAP) in Dulbecco's phosphate-buffered saline (D-PBS) was prepared under sterile conditions. GelMA was incubated in this LAP solution at 37°C for 30 minutes to yield a 0.1% GelMA/LAP hydrogel precursor. Each hNET was placed onto a concave sterile Parafilm dimple and overlaid with 30 μ L of GelMA/LAP solution. The cross-linking of the hydrogel was achieved via ultraviolet (UV) light exposure for 3 intervals of 4–5 seconds. Subsequently, the tissue containing GelMA was transferred to 24-well plates containing NDFM-Vitamin A. As a control to observe the ECM effect on growth, a subset of hNETs was directly transferred to the differentiation medium without embedding. All embedded and control tissues were maintained at 37°C in a humidified incubator with 5% CO₂. The medium was replaced with fresh NDFM-Vitamin A on day 2. Thereafter, medium changes and supplementation with signaling molecules were performed as depicted in Figure 2.2.2, and the differentiation medium was switched to include vitamin A (called NDFM+Vitamin A), with media exchanges every 3–4 days. Also, developing organoids were started to be cultured on an orbital shaker at 70 rpm to promote nutrient diffusion 3–4 days after vitamin A was added to the medium. Then neurotrophic factors were introduced sequentially to direct lineage specification and organoid maturation. 20 ng/mL brain-derived neurotrophic factor (BDNF) and 20 ng/mL neurotrophin-3 (NT-3) were added from day 12 to day 22, 10 ng/mL platelet-derived growth factor-AA (PDGF-AA) and 10 ng/mL insulin-like growth factor-1 (IGF-1) from day 26 to day 36, 40 ng/mL triiodothyronine (T3) from day 36 to day 40, and a combination of 40 ng/mL T3 and 1 μ M ketoconazole from day 30 to day 46.

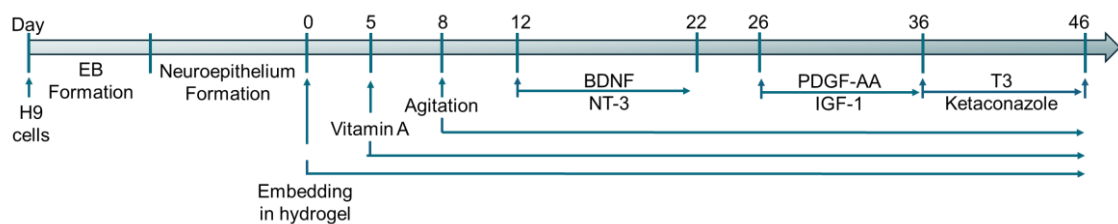


Figure 2.1.3 Timeline of human brain organoid generation process

2.2.4 Scanning electron microscopy (SEM)

GelMA and Matrigel were rinsed three times with 1× phosphate-buffered saline (PBS) and subsequently fixed in 2.5% glutaraldehyde at 4°C for 15 minutes to preserve tissue morphology. Following fixation, the samples were washed three additional times with 1× PBS and stored in PBS at 4°C until further analysis. On the day of SEM analysis, they were subjected to a graded ethanol dehydration series (30%, 50%, 50%, 70%, 90%, and 100%), with each step carried out for 2 minutes to ensure efficient dehydration. After complete removal of ethanol, the samples were treated with 10 µL of hexamethyldisilazane (HMDS) at room temperature for chemical drying. The residual HMDS was evaporated under a fume hood for 1 hour. Dried samples were mounted onto carbon adhesive tape and subsequently sputter-coated with a 5.0 nm gold layer to enhance surface conductivity. All samples were visualized using a Zeiss Gemini SEM300 scanning electron microscope.

2.1.5 Raman spectroscopy

20 µm cryosectioned hBOs and hNTEs were rinsed three times with 1× phosphate-buffered saline (PBS) and subsequently fixed in 4% paraformaldehyde (PFA) at room temperature for 15 minutes to preserve structural integrity. Following fixation, the samples were washed again three times with 1× PBS and stored in PBS at 4°C until further analysis. On the day of analysis, Raman spectral measurements were acquired using a WITec Alpha 300 Raman Microscope equipped with a 785 nm excitation laser. Raw spectra were pre-processed to eliminate background noise through baseline subtraction, and further data processing and analysis were conducted using custom MATLAB algorithms.

2.1.6 Immunofluorescent staining

hBOs were washed three times with 1× PBS and subsequently fixed in 4% paraformaldehyde (PFA) at room temperature for 15 minutes. Following fixation, samples were incubated in 30% sucrose solution at 4°C for up to three days to allow cryoprotection and then embedded in optimal cutting temperature (OCT) compound for cryosectioning. Cryosections approximately 20 µm in thickness were obtained using a

cryostat (MERLAB, Abdullah Gül University). The sections were permeabilized with 0.25% Triton X-100 in PBS for 10 minutes, followed by a blocking step in 3% bovine serum albumin (BSA) in 0.25% Triton X-100/PBS for 1 hour at room temperature. Subsequently, the sections were incubated overnight at 4°C with the following fluorophore-conjugated primary antibodies: Anti-SATB2 Alexa Fluor 647 (sc-518006), Anti-Sox2 AF488 (sc-365823), Anti-GFAP AF594 (sc-33673), and Anti-TAU AF488 (sc-390476). After primary antibody incubation, 4',6-diamidino-2-phenylindole (DAPI) was used for nuclear counterstaining. Imaging was performed using a Leica DM6 B fluorescence microscope and Zeiss LSM900 confocal microscope equipped with Airyscan 2 and processed by ZEN 3 Blue edition software.

2.1.7 Statistical Analysis

All data were represented as mean $\pm$ standard deviation. At least 2 independent batches were used for analysis. Quantification of fluorescent images was performed by normalizing the fluorescent intensity to DAPI. All graphs were visualized using GraphPad Prism 9 for Windows, and one-way ANOVA and two-way ANOVA were used to confirm the statistical significance of treatment groups.

2.3. RESULTS and DISCUSSION

In this part of the study, GelMA, as a natural hydrogel, and Matrigel, as an animal-derived hydrogel, were compared in terms of their usage as an extracellular matrix and their effect on brain organoid generation. Matrigel is a commercially available hydrogel, and it is accepted as the gold standard in organoid generation [220]. GelMA was considered as an alternative extracellular matrix, and it was produced by Dr. Demirtas' research group at Erciyes University, Türkiye [221]. After GelMA was produced, it was stored at 4°C until it was required (Figure 2.3.1). In the GelMA synthesis, methacryloyl groups substitute a part of the amino groups on the side chains of gelatin through a reaction with methacrylic anhydride, resulting in methacrylated gelatin (GelMA) (Figure 2.1.1). Despite this chemical modification, GelMA retains the thermoresponsive behavior of native gelatin, exhibiting a temperature-dependent liquid-gel-solid transition. The introduction of methacryloyl groups imparts photocrosslinkable properties to the material, enabling rapid gelation under ultraviolet (UV) light in the presence of a photoinitiator to form GelMA hydrogels with enhanced thermal stability. Importantly, the bioactive motifs of gelatin, such as the RGD (arginine–glycine–aspartic acid) sequences and matrix metalloproteinase (MMP)-sensitive sites, remain intact, allowing GelMA to support cell adhesion, proliferation, and migration. Moreover, the inherent biocompatibility and biodegradability of gelatin are preserved. The physicochemical properties of GelMA hydrogels, including stiffness, porosity, and degradation rate, can be finely tuned by adjusting the degree of methacrylation and crosslinking conditions, making them highly adaptable for a wide range of biomedical applications [222].

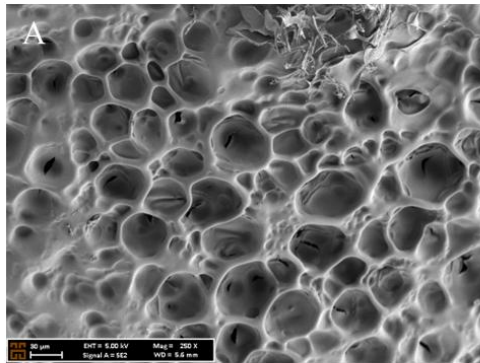


Figure 2.3.1 Methacrylated gelatin (GelMA)

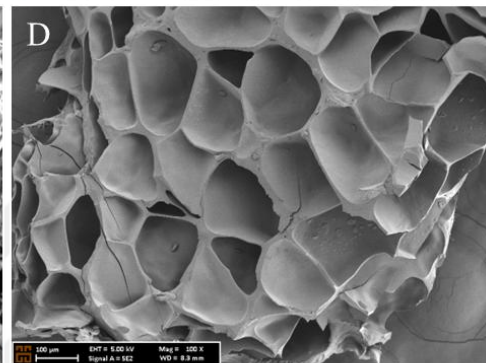
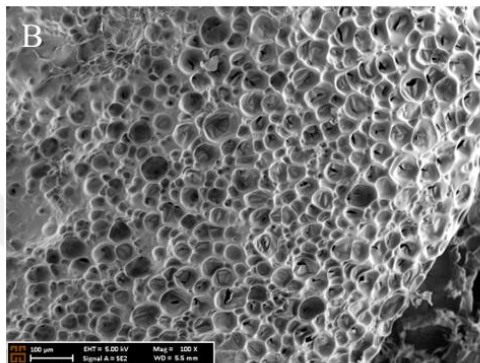
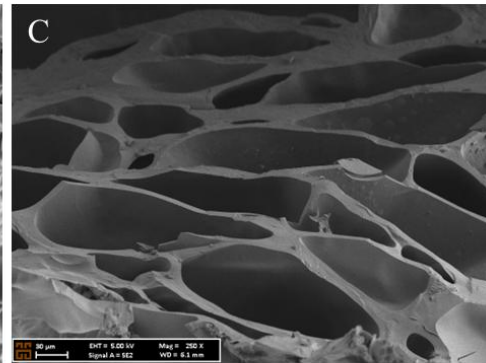
2.3.1 Characterization of GelMA and Matrigel by SEM

The porosity and pore architecture are critical parameters influencing cellular viability, proliferation, and migration within hydrogel scaffolds. High pore interconnectivity facilitates cellular infiltration, neovascularization, and efficient diffusion of nutrients and oxygen, all of which are essential for sustaining cell function, promoting ECM secretion, and enabling the development of functional tissue. Regarding the porosity, it was also known that the average pore diameter has a great impact on the growth of cells and cell penetration into the hydrogel [223]. Therefore, the first characterization of the natural hydrogel was performed with SEM to observe the porous structure [222]. Figure 2.3.2 shows the SEM images of GelMA with different UV exposure times for cross-linking and Matrigel. Additionally, Figure 2.3.3 presents a quantitative analysis that illustrates the differences in pore diameters among the hydrogels. Since UV exposure time directly affects the methacrylation degree, it has an indirect effect on pore size. It was known that the increase in UV exposure time increases the gelation and also decreases the pore size. However, in our study, GelMA, which is formed upon 10 seconds of UV exposure, has the smallest pore size among the other GelMA (Figure 2.3.3A). Also, pore size is directly related to hydrogel concentration and photoinitiator concentration; however, the parameters were not changed in the study [224]. It is promising to indicate that the pore diameters of Matrigel and GelMA-10sec are similar and the average pore diameter is 45 μm , supporting the literature about the GelMA [219]. Regarding Matrigel, since it is not a synthetic hydrogel and is derived from animals, there is batch-to-batch variation among the products. The producer does not specify the pore size, but they focused on the protein content, gelation temperature, and mechanical properties such as elastic modulus/stiffness [225], [226]. The SEM analysis also indicated that GelMA-30sec was more fragile than the other GelMAs. It was an expected mechanical characteristic of the hydrogel because a higher degree of crosslinking is linked to greater brittleness [227]; therefore, the tissue-related experiments were further continued with GelMA-10sec and GelMA-15sec.

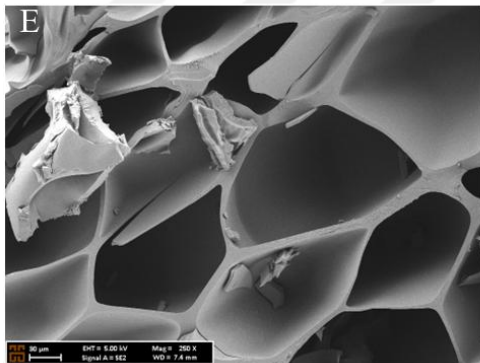
GelMA - 10 seconds



GelMA - 15 seconds



GelMA - 30 seconds



Matrigel

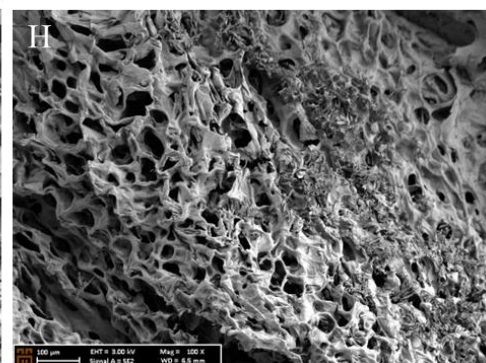
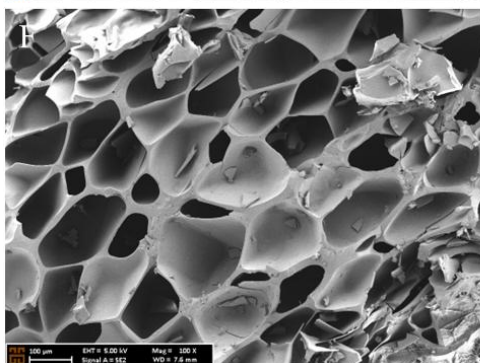
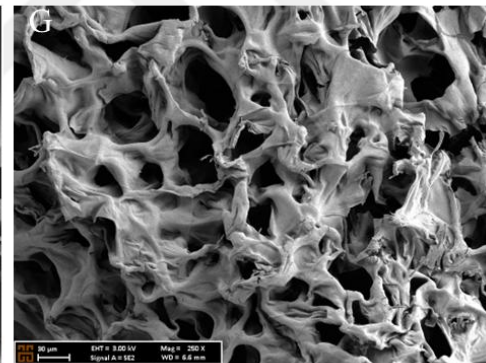


Figure 2.3.2 Representative scanning electron microscopy images of GelMA and Matrigel. (A, B) GelMA, crosslinked with 10-second UV exposure; (C, D) GelMA, crosslinked with 15-second UV exposure; (E, F) GelMA, crosslinked with 30-second UV exposure; (G, H) Matrigel. Scale bar: 30 µm for A, C, E, and G and 100 µm for B, D, F, and H.

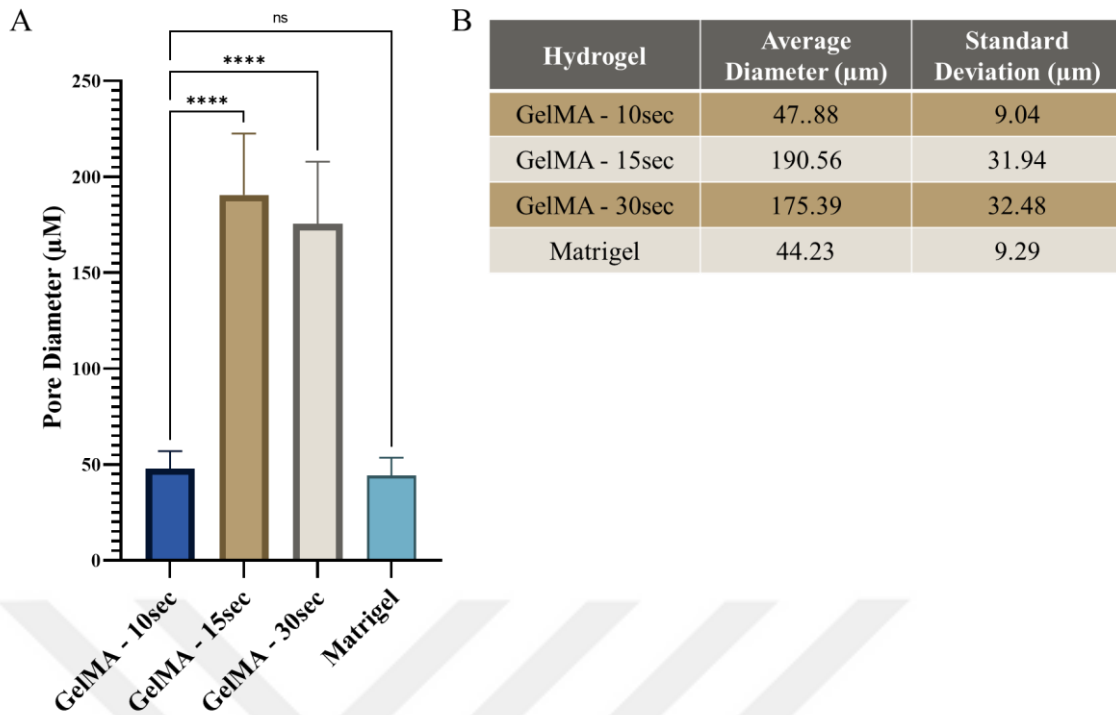


Figure 2.3.3 Average pore size of GelMA and Matrigel. (A) Quantitative analysis of the pore size of GelMA, crosslinked with different UV exposures and Matrigel. (B) Average size and standard deviation of GelMA, crosslinked with different UV exposures and Matrigel. One-way ANOVA with Dunnett's test was used for analysis (ns $p > 0.05$, **** $p \leq 0.0001$). The graphs were visualized by GraphPad Prism 9.0.

2.3.2 Human embryonic stem cell culture

Firstly, the process for generating brain organoids, followed by human embryonic stem cell culture, was optimized as explained in Chapter 1. As explained before, after their arrival, hESCs were cultured with a feeder-independent culture protocol. However, when they were thawed after the first cryopreservation round, the cells did not attach to the culture plate, and they did not form colonies (Figure 1.3.5). Even though the cell seeding area per vial was decreased, no colonies were observed for 9 months. Later, the cells were maintained with a feeder-dependent culture protocol. In the process, MEF cells were obtained from Erdal Lab, Izmir Biomedicine and Genome Center, and Ozcan Lab, Gevher Nesibe Graduate School of Genome and Stem Cell. The culture of MEF cells was represented in Figure 1.3.6. The MEF cells can be mitotically inactivated chemically and physically using mitomycin C and γ -irradiation, respectively. In this study, mitomycin C-mediated inactivation was preferred because its inhibition was equal among the feeder cells on the cell culture plate [228]. Also, the growth was monitored after the mitomycin C incubation to be sure about its effectiveness. The support provided by iMEF cells is

important for the growth of stem cells because the feeder cells produce growth factors, and they provide ECM components and adhesion molecules for stem cell attachment. Since the culture of hESCs on mouse-derived iMEF cells could be the xenocontamination source, the use of human foreskin fibroblasts (hFFs) or adult Fallopian tube epithelial cells could overcome the problem. However, key factors secreted from iMEF cells, such as Activin A and bFGF, that are required for the maintenance of the pluripotency characteristic of the stem cells, are not sufficiently secreted by human cell lines. Therefore, MEF cells support the maintenance of hESCs better [229]. After observation of healthy hESC colonies on iMEF cells (Figure 1.3.7), they were further cultured on growth factor-reduced Matrigel as a part of feeder-independent hESC cultures (Figure 1.3.9). Prior to transferring hESCs to Matrigel, one of the major concerns related to the maintenance of the undifferentiated state of hESCs on MEF cells was tested with an alkaline phosphatase assay (Figure 1.3.10) [230].

2.3.3 Human brain organoid generation with GelMA and Matrigel

The organoid generation process was started by incubating stem cell clusters, or embryonic bodies, in a U-bottom 96-well plate with 4 ng/mL bFGF. The concentration of bFGF is an important factor in maintaining pluripotency and inhibiting nonneuronal differentiation because, in some studies, it was also reported that bFGF functions as a potent proneural factor [231], [232], [233], [234]. Subsequently, the embryoid bodies were ectodermally induced in a medium containing heparin, and morphological changes were observed (Figure 1.3.11D-E) [125]. Heparin is structurally similar to heparan sulfate, and it plays a role in the induction of stem cells to neurons [235], [236]. When the neural induction was completed based on morphological changes, the hNETs were transferred to dimpled Parafilm to be embedded into either Matrigel or GelMA. Figure 2.3.4 shows the technical steps used to illustrate the process. In the process, the tissues should be centered after the Matrigel is added; otherwise, the tissue may protrude from the structure during polymerization. Also, in the GelMA embedding process, no more than 4 tissues should be exposed to UV light simultaneously to keep UV efficiency equal among the tissues. Also, while the tissues were transferred to a 24-well plate, it was important not to disturb the tissue structure. Therefore, the preferred method in the study was to turn down the Parafilm dimple and immerse the Parafilm into a medium containing a well of a 24-well plate.

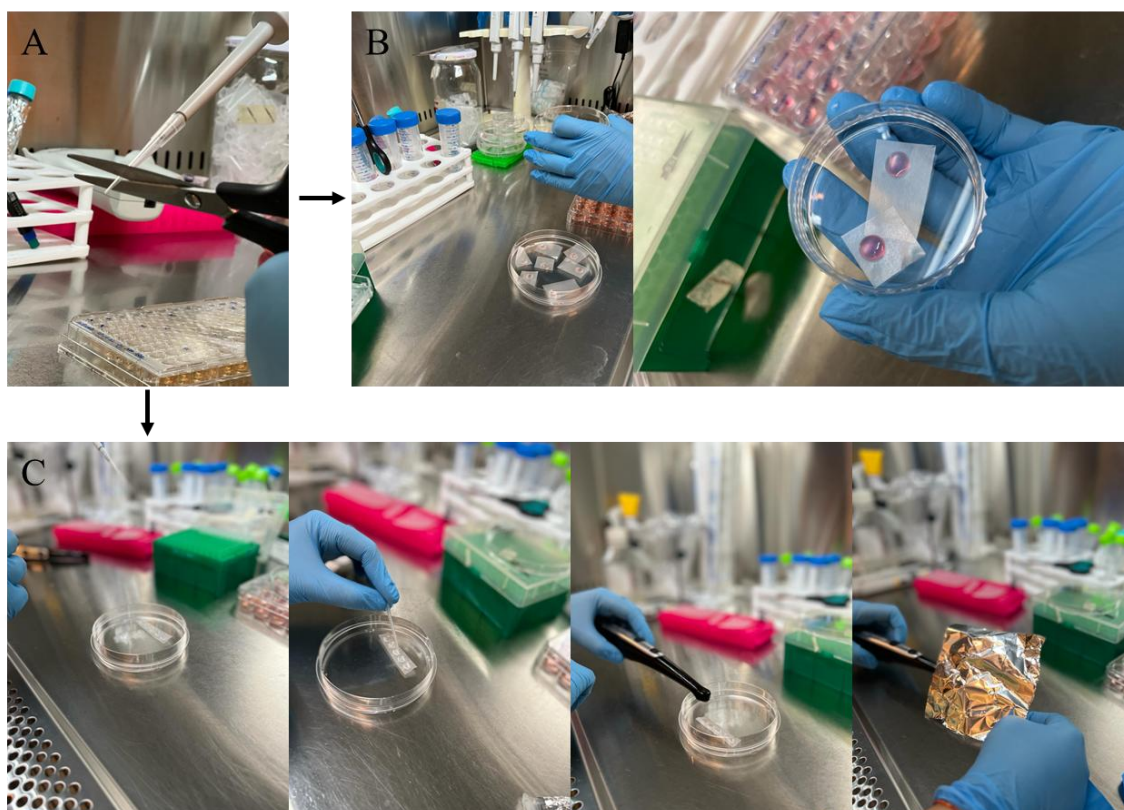


Figure 2.3.4 The neuroepithelial tissue transfers to the Parafilm dimple, and it is embedded in the different extracellular matrix. (A) The tissue transfer preparation by a cut 200 μ L pipette tip, (B) Embedding to Matrigel: each tissue was placed into the center of each dimple on a sterile dish, and 30 μ L of cold Matrigel was added on the tissue to be incubated at 37°C, (C) Embedding to GelMA: each tissue was placed into the center of each dimple, and 30 μ L of LAP/GelMA mixture was added on the tissue to be exposed to UV.

After the tissues were embedded in either Matrigel or GelMA, the differentiation protocol was followed until the end of the maturation (Figure 2.2.2). During the differentiation, the structures were recorded by a light microscope to monitor size and morphological changes. Figure 2.3.5-8 displays the organoids cultured in four different conditions: GelMA crosslinked with 10 seconds of UV exposure, GelMA crosslinked with 15 seconds of UV exposure, Matrigel, and an ECM-free culture environment. During the growth, their diameter was measured, and a representative measurement is shown in Figure 2.3.9. Lastly, the growth was quantitatively analyzed to statistically determine the tissue expansion change (Figure 2.3.10).

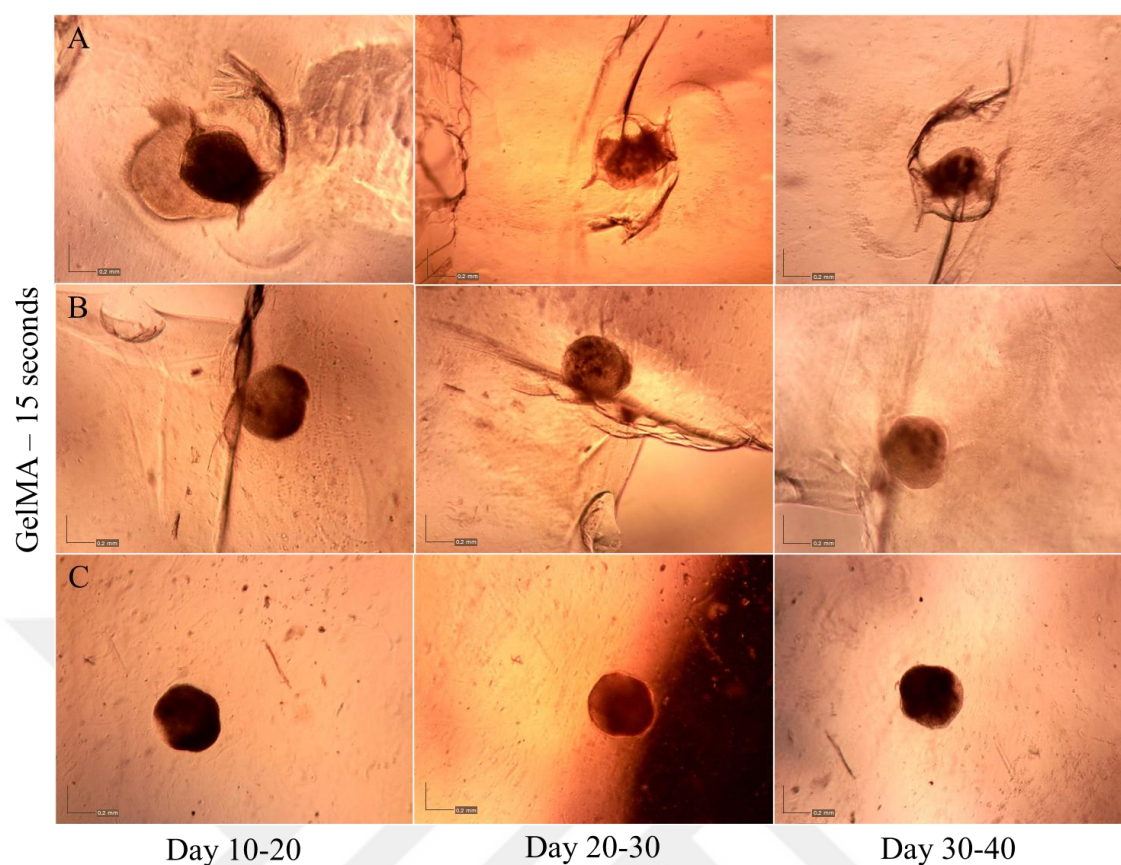


Figure 2.3.5 Morphological changes of organoids embedded in GelMA, crosslinked with 15 seconds of UV light. A, B, and C represent different organoids, and their growth was recorded in time from left to right.

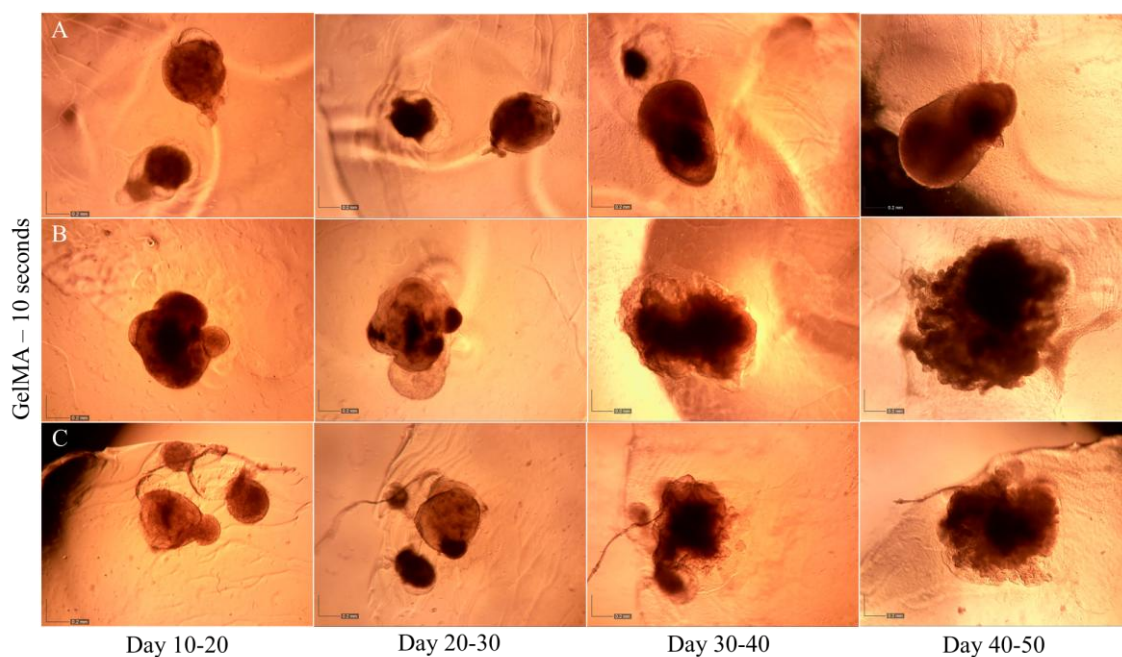


Figure 2.3.6 Morphological changes of organoids embedded in GelMA, crosslinked with 10 seconds of UV light. A, B, and C represent different organoids, and their growth was recorded in time from left to right.

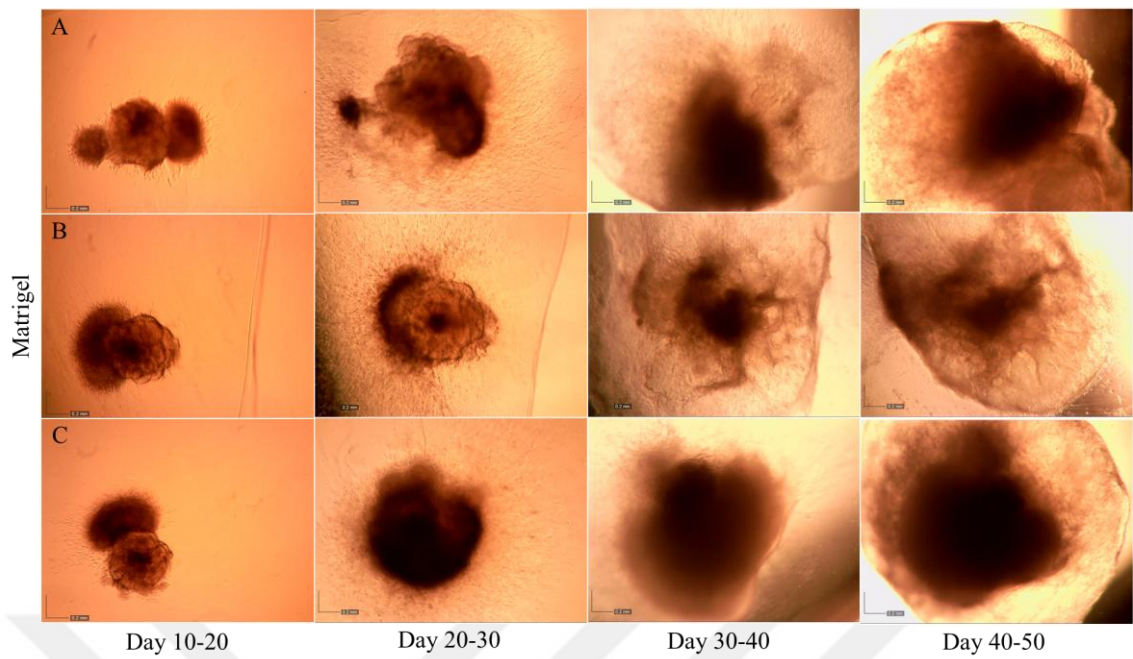


Figure 2.3.7 Morphological changes of organoids embedded in Matrigel. A, B, and C represent different organoids, and their growth was recorded in time from left to right.

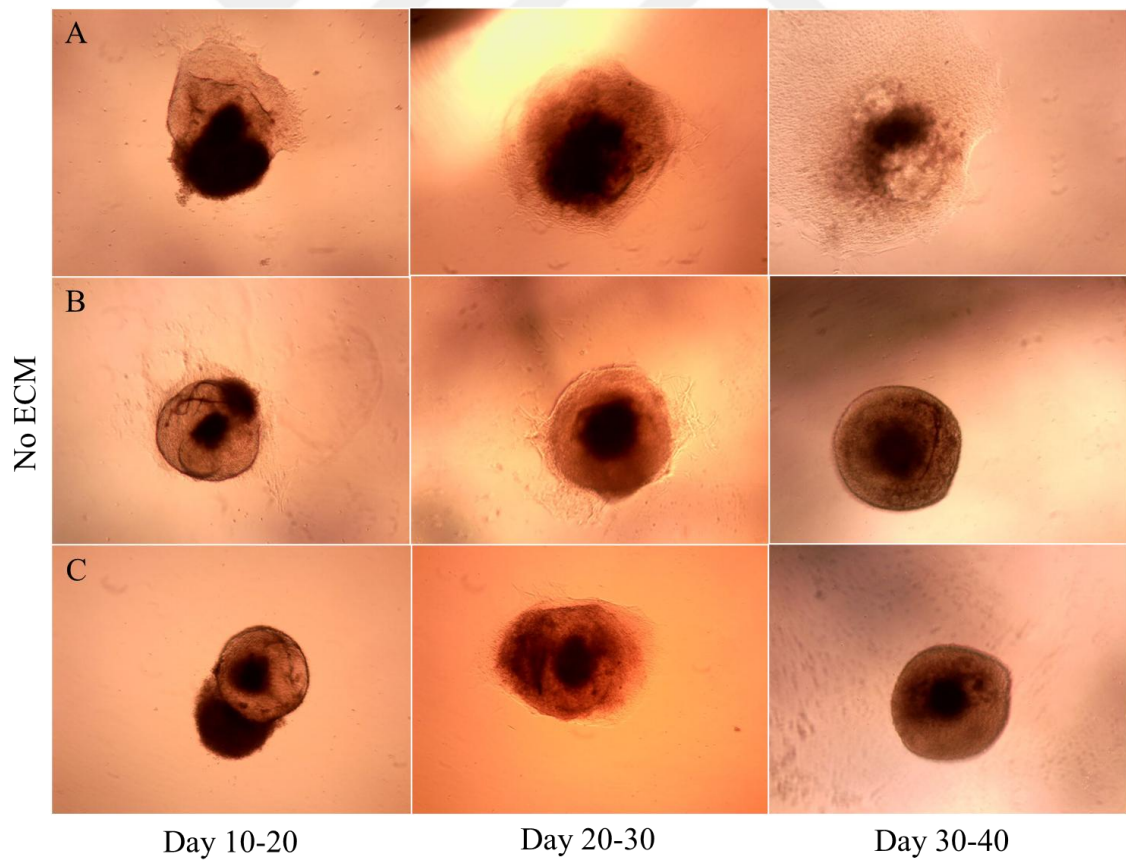


Figure 2.3.8 Morphological changes of organoids that were not embedded in any extracellular matrix. A, B, and C represent different organoids, and their growth was recorded in time from left to right.

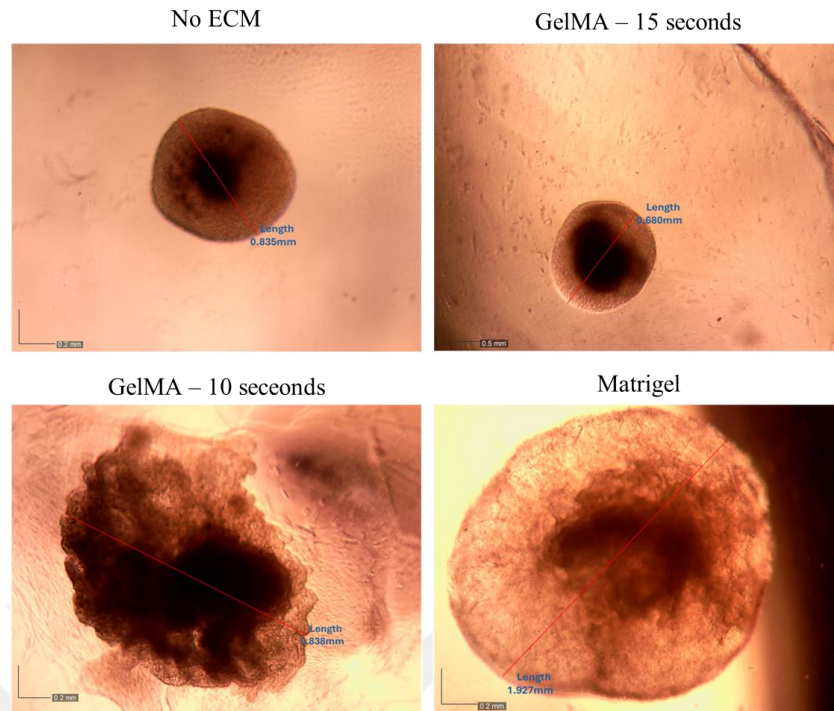


Figure 2.3.9 Representative measurement of organoid diameter. (A) Organoids that are not embedded in any material, (B) hBOs that are embedded in GelMA, crosslinked with 15-second UV exposure, (B) hBOs that are embedded in GelMA, crosslinked with 10-second UV exposure, (C) hBOs that are embedded in Matrigel. The image processing was performed using ImageJ Fiji version 1.54p.

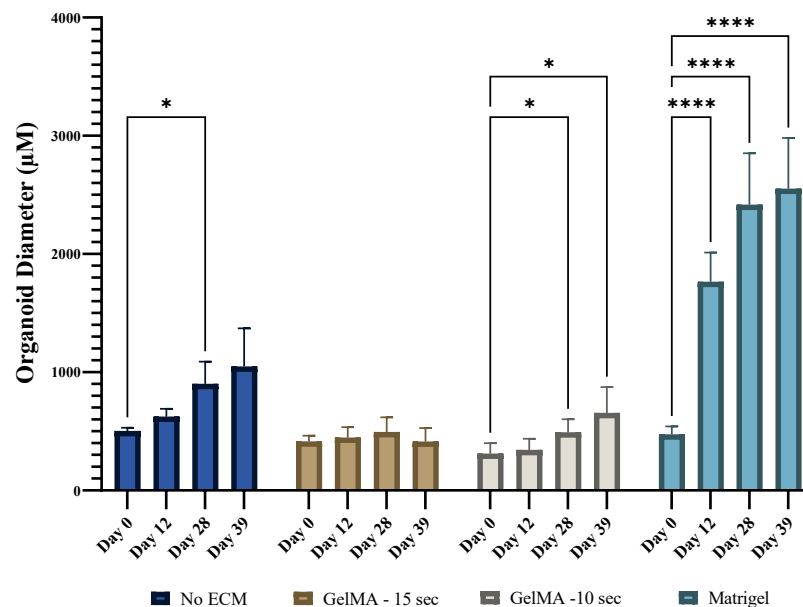


Figure 2.3.10 Quantitative analysis of the organoid size grown in either GelMA or Matrigel. The growth of organoids was monitored under a light microscope, and their diameter was measured on day 0, day 12, day 28, and day 39. The data were statistically analysed by one-way ANOVA with Dunnett post hoc test (ns (not indicated on graph p > 0.05, * p < 0.05, **** p ≤ 0.0001),

As shown in Figure 2.3.5-10, the tissue expansion was not significant in the ECM-free culture environment and GelMA-15 sec. Considering the importance of pore size, the large pore size of GelMA-15 seconds may limit the cell attachment because of the decreased surface area [237]. Also, considering the importance of ECM on tissue growth, even when the tissues were expanded in an ECM-free environment, they lacked neural buds; therefore, brain organoids failed to morphologically generate in the absence of ECM (Figure 2.3.8). The neuroepithelial bud outgrowth is characteristic of the early stage of brain organoids [125]; therefore, ECM-free organoids failed to form brain structures.

2.3.4 Characterization of human brain organoids with immunofluorescent staining

When the brain organoids were confirmed with tissue expansion and morphological changes, immunofluorescent staining was performed to confirm different neuron types that were generated by directed differentiation with growth factors. Figure 2.2.2 shows that BDNF, NT-3, PDGF-AA, and IGF-1 were used to help cells mature. BDNF and NT-3 are neurotrophins that influence neuronal differentiation and synapse formation through their interactions with the tropomyosin receptor kinase B (TrkB) receptor and the p75 neurotrophin receptor (p75NTR), utilizing their mature and immature forms, respectively [238], [239]. The activation of TrkB pathway The activation of the TrkB receptor signaling cascade exerts neuroprotective and pro-regenerative effects on damaged retinal ganglion cells by facilitating their regeneration and subsequent maturation. Moreover, activation of the BDNF/TrkB signaling pathway facilitates neuronal regeneration and encourages neurite outgrowth, encompassing dendritic branch development. This effect is likely mediated through modulation of the Rab5-Rab11 endosomal trafficking system, involving downstream pathways such as the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) and phosphatidylinositol 3-kinase (PI3K) signaling cascades [240]. Following the BDNF and NT-3 incubation of the organoids, they were further incubated with PDGF-AA and IGF-1. The two mitogens are responsible for the oligodendrocyte progenitor cell (OPC) survival and proliferation, and the following T3 treatment is responsible for the differentiation of oligodendrocytes [65]. It has been reported that PGDF-AA promotes oligodendrocyte differentiation through the Erk1/2 pathway and causes subsequent activation of Olig2 as an oligodendrocyte-specific transcription factor, and it also enhances the proliferation of committed oligodendrocytes at later stages of development [241]. In addition to the role of PDGF-AA on

oligodendrocyte maturation and expansion, IGF-1 has a similar function. It enhances oligodendrogenesis, supporting the OPC differentiation and inhibiting the apoptosis of developing oligodendrocytes through the Noggin signaling and PI3K-dependent inhibition of the caspase-3 pathway [242], [243]. At the latest stage of organoid maturation, T3 and ketoconazole were used. T3 is a hormone that increases myelination [244], and ketoconazole is an FDA-approved drug showing the enhancement effect on myelination by compacting the myelin sheath [245], [246].

After all growth factors were incubated with the organoids grown in Matrigel and GelMA, the organoids were prepared for immunofluorescent staining and stained with fluorophore-tagged anti-SOX2, anti-SATB2, anti-GFAP, and anti-TAU antibodies. Figure 2.3.11 and Figure 2.3.14 show the representative confocal microscopy images of organoids. Figure 2.3.12-13 and Figure 2.3.15-16 show the representative images of H9 cells and organoid cryosections stained with the antibodies.

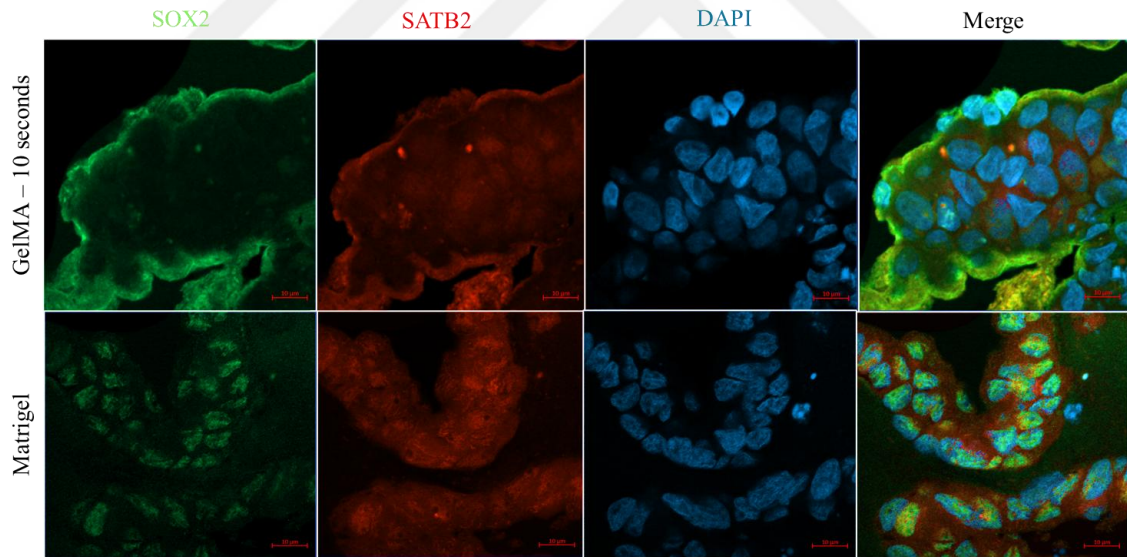


Figure 2.3.11 Representative immunofluorescent images of organoids embedded in Matrigel and GelMA crosslinked with 10 seconds of UV exposure. The cryosections were stained with anti-SOX2 (green) and anti-SATB2 (red) antibodies. Nuclei were counterstained with DAPI (blue). The images were taken by a Zeiss LSM900 confocal microscope equipped with Airyscan 2 and controlled by ZEN 3 Blue edition software. Scale bar, 10 μ m.

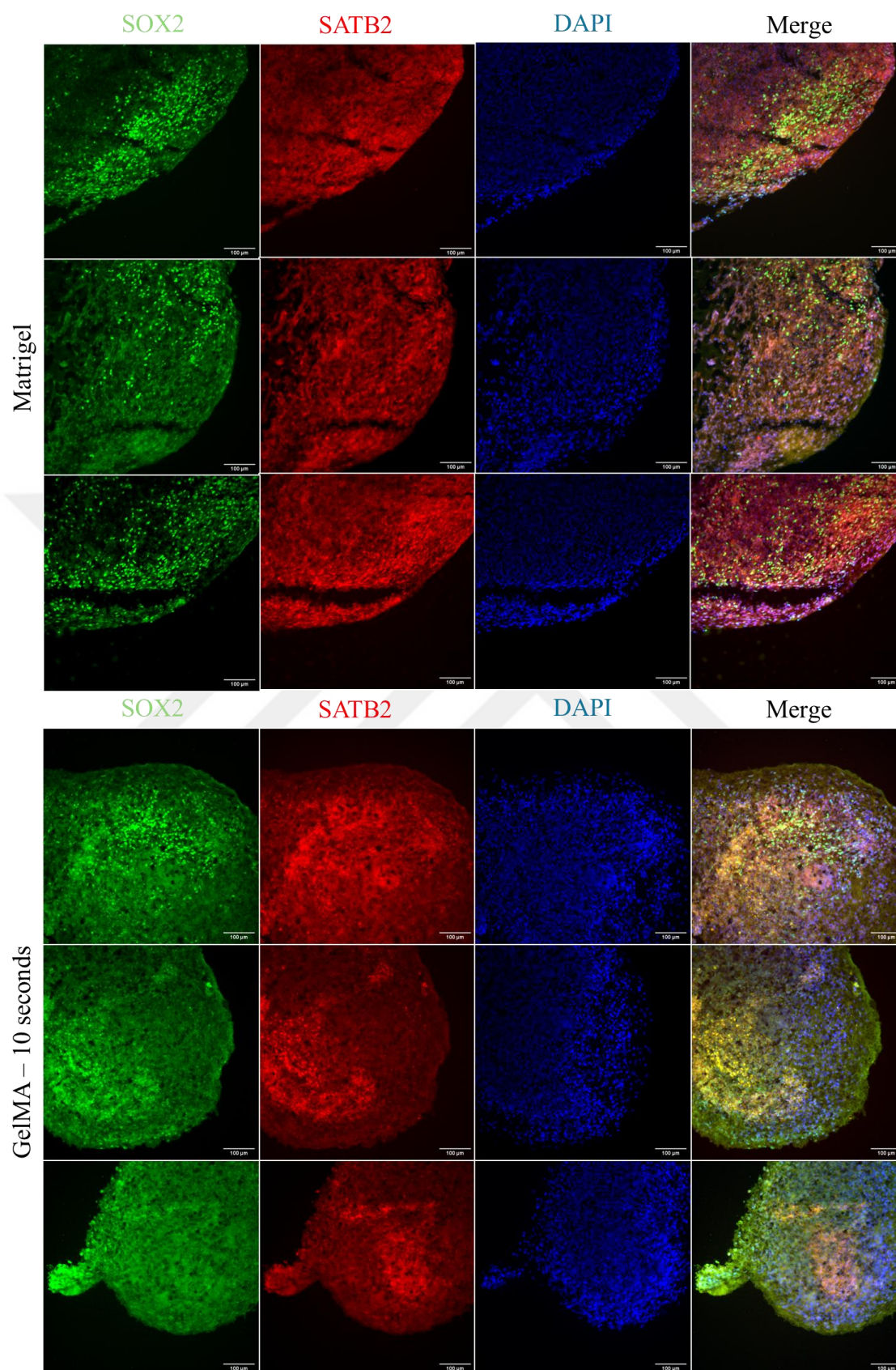


Figure 2.3.12 Representative immunofluorescent images of organoids embedded in Matrigel and GelMA (crosslinked with 10-second UV exposure), stained with anti-SOX2 (green) and anti-SATB2 (red) antibodies. Nuclei were counterstained with DAPI (blue). The images were taken by Leica DM6 B. Scale bar, 100 µm.

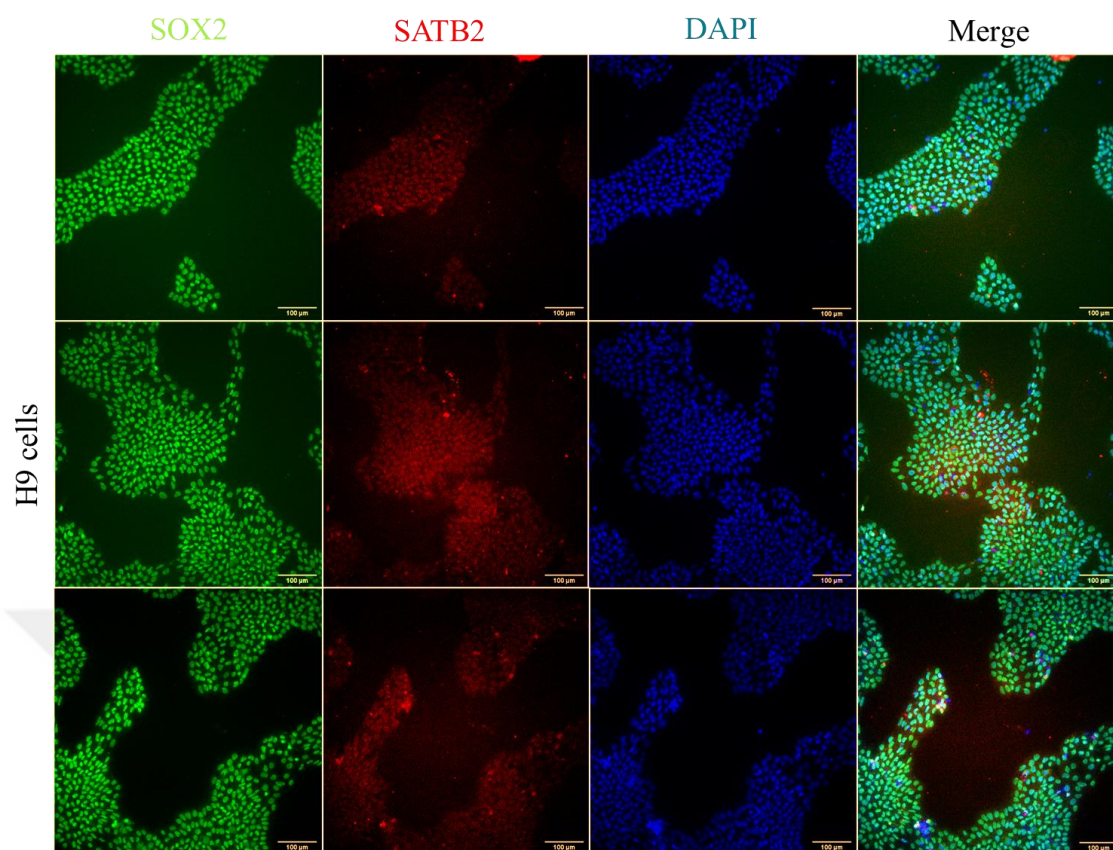


Figure 2.3.13 Representative immunofluorescent images of human embryonic stem cells stained with anti-SOX2 (green) and anti-SATB2 (red) antibodies. Nuclei were counterstained with DAPI (blue). The images were taken by Leica DM6 B. Scale bar, 100 μm .

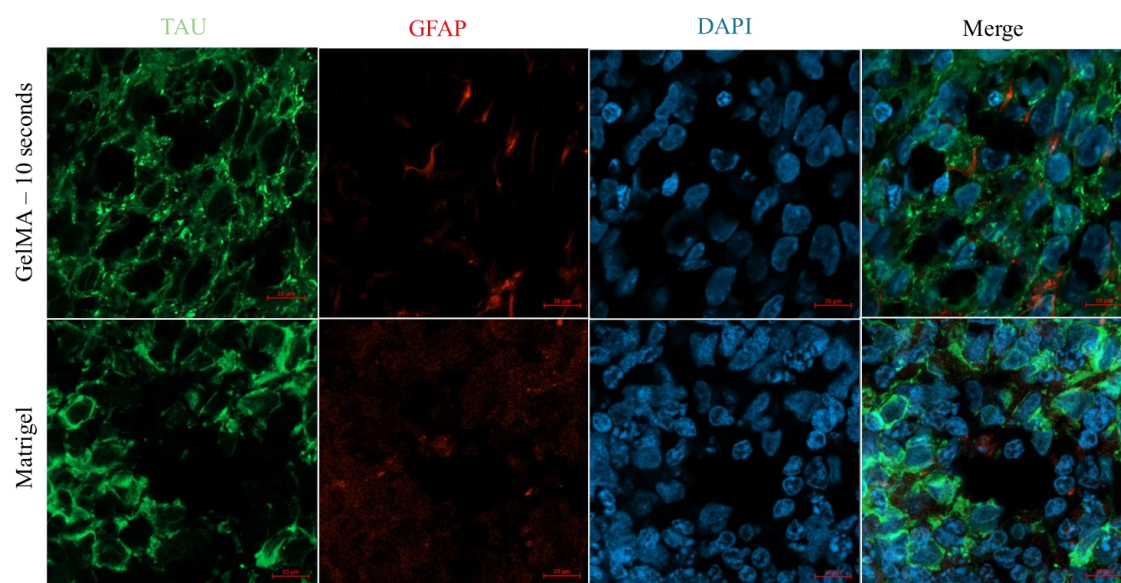


Figure 2.3.14 Representative immunofluorescent images of organoids embedded in Matrigel and GelMA crosslinked with 10 seconds of UV exposure. The cryosections were stained with anti-TAU (green) and anti-GFAP (red) antibodies. Nuclei were counterstained with DAPI (blue). The images were taken by a Zeiss LSM900 confocal microscope equipped with Airyscan 2 and controlled by ZEN 3 Blue edition software. Scale bar, 10 μm .

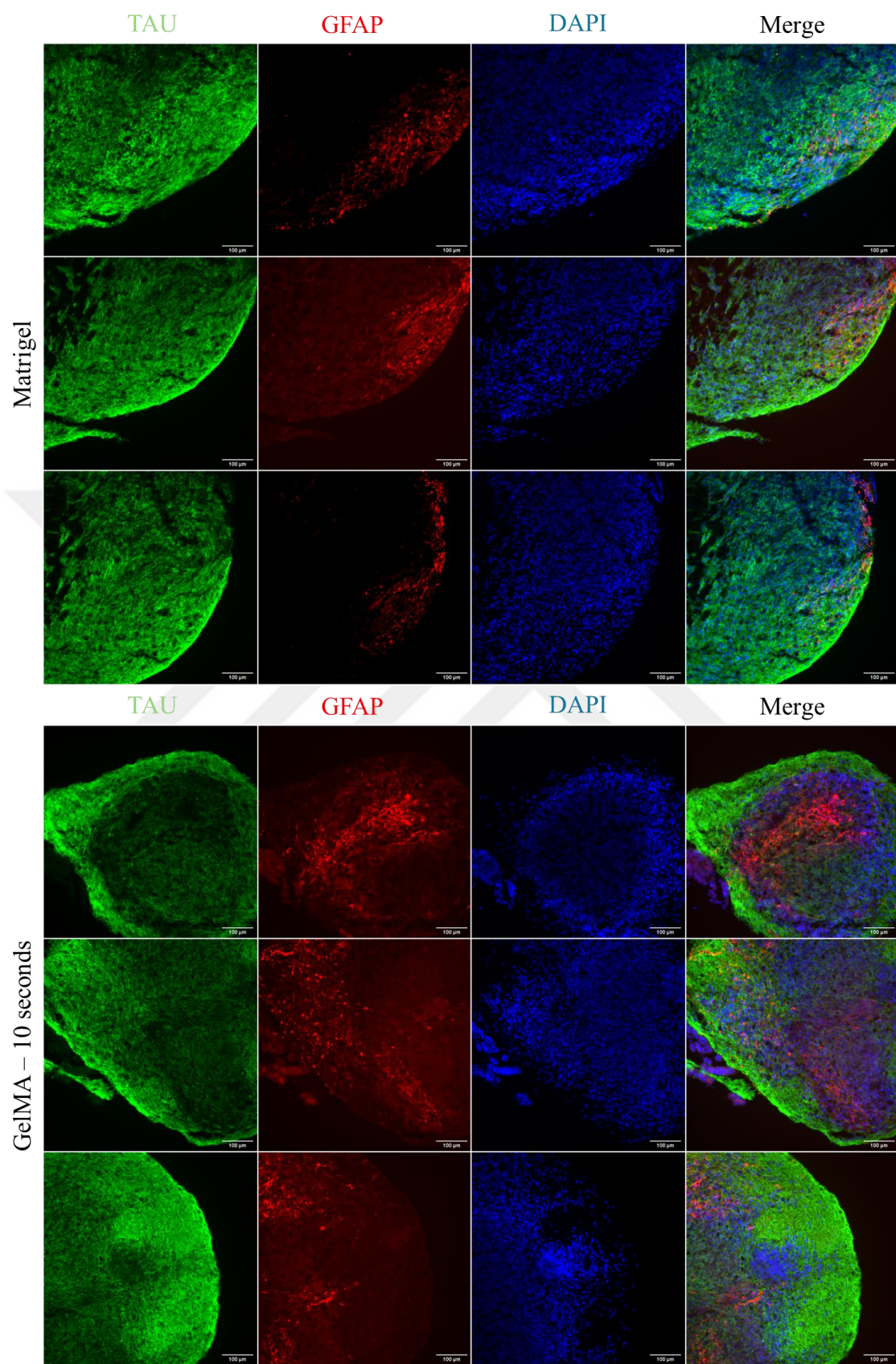


Figure 2.3.15 Representative immunofluorescent images of organoids embedded in Matrigel GelMA (crosslinked with 10-second UV exposure), stained with anti-TAU (green) and anti-GFAP (red) antibodies. Nuclei were counterstained with DAPI (blue). The images were taken by Leica DM6 B. Scale bar, 100 µm.

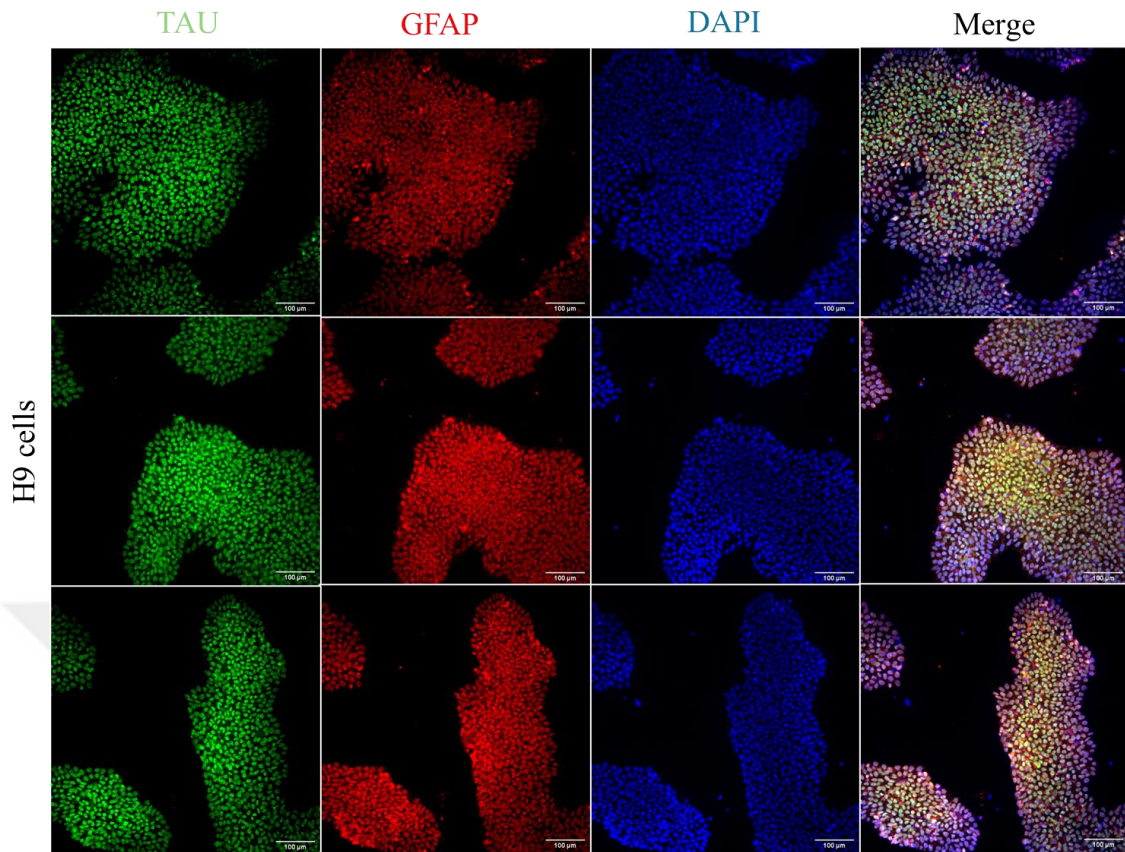


Figure 2.3.16 Representative immunofluorescent images of human embryonic stem cells stained with anti-TAU (green) and anti-GFAP (red) antibodies. Nuclei were counterstained with DAPI (blue). The images were taken by Leica DM6 B. Scale bar, 100 μ m.

As shown in Figure 2.3.11-18, all markers were expressed in all organoids grown in Matrigel and GelMA. The first staining was performed to differentiate the neural progenitor cells from cortical neural excitatory cells using anti-SOX2 and anti-SATB2 antibodies [247], [248], [249]. The SOX2 is also expressed in embryonic stem cells and inhibits nonneuronal cell differentiation through Wnt signaling, therefore confirming the literature. H9 cells did not show SATB2 expression and showed SOX2 expression (Figure 2.3.13) [250]. Since organoid maturation was completed in organoids embedded in Matrigel and GelMA, the SATB2 expression was observed in Figure 2.3.12. However, it is possible to indicate that the spatial position of cell types is different in organoids grown in different ECM. Although all surfaces of section planes included SATB2-positive cells in Matrigel cryosections, only the central region included SATB2-positive cells in GelMA cryosections. On the contrary, all surfaces of section planes included SOX2-positive cells in GelMA cryosections, while the cortical region included SOX2-positive cells in Matrigel cryosections. It indicates that the composition or physical properties of

different ECMs affect the regionalization and patterning during cortical organoid development. GelMA may limit proper cortical layer formation, preventing outward migration of SATB2-positive upper-layer neurons and resulting in a centralized accumulation. In addition to SOX2 and SATB2 staining, TAU and GFAP staining were also performed in embryonic stem cells and organoids grown in either Matrigel or GelMA (Figure 2.3.14-16). GFAP is an intermediate filament type III protein and is accepted as an astrocyte marker, although it could be expressed in stem cells. Even though it is expressed in stem cells, it does not meet the phenotypic criteria; therefore, GFAP expression in H9 was an expected result (Figure 2.3.16) [251], [252]. TAU is a microtubule-associated protein and mature neuron marker indicating the axon, and the TAU expression was clearly observed as an extension around the nucleus in both organoids grown in Matrigel and GelMA (Figure 2.3.14-15) [253]. It was clearly observed that GFAP was differentially expressed in organoids grown in different ECMs. Matrigel supports the GFAP expression in the cortical region, although GelMA supports the GFAP expression in the central region. It again indicates that ECM type profoundly influences the spatial pattern of glial differentiation in brain organoids.

2.3.5 Raman spectroscopy analysis

Raman spectroscopy is a label-free method to obtain information about the chemical bonding of a specimen. Lipid, protein, and nucleic acid-derived signals, or their magnitude, are different among the cells at different developmental stages [254]. Raman spectra of 0-2265 cm^{-1} were measured from the human embryoid body stage to mature organoid stages (Figure 2.3.17). Since the organoids were cultured in different ECMs, the differences among the organoids were observed in signals (Figure 2.3.18).

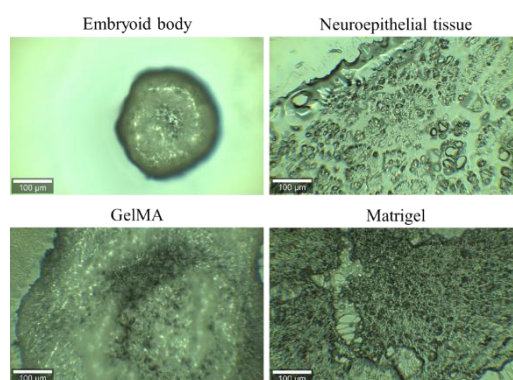


Figure 2.3.17 Representative images of samples for Raman spectroscopy analysis

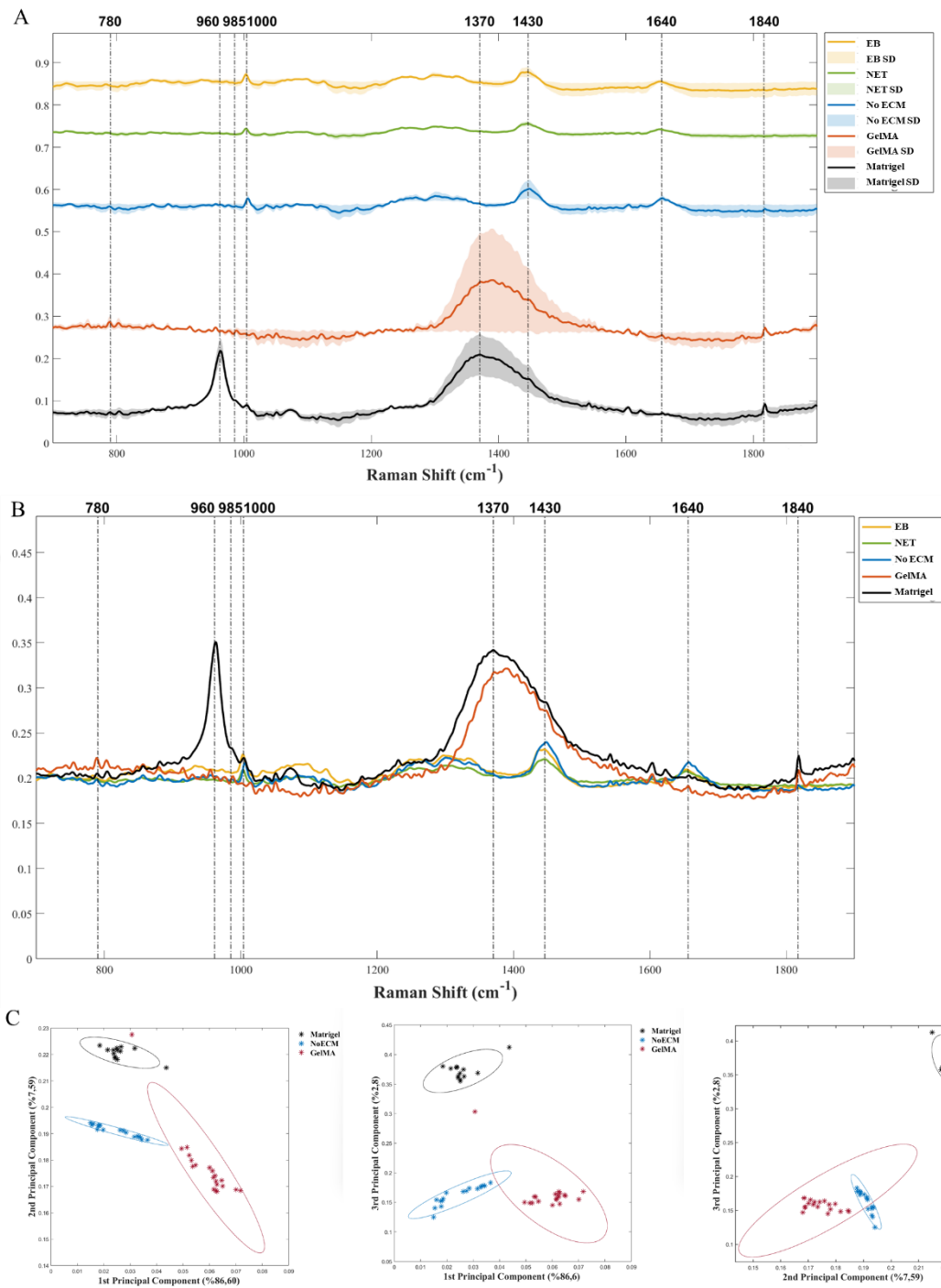


Figure 2.3.18 Raman spectroscopy measurement results. (A, B) Raman spectra of embryoid body (yellow), neuroepithelial tissue (NET) (green), organoids grown in Matrigel (black), organoids grown in GelMA (red), and organoids grown in an ECM-free culture environment, called no ECM (blue). (C) PCA plots of Raman signal intensity of samples. The data represent the mean.

Raman spectrometry has proven to be an essential tool for analyzing cellular differentiation from hESCs towards organoids. This technique offers unique advantages due to its ability to provide molecular-level insights into the biochemical changes

occurring during differentiation, making it particularly useful in the context of studying complex tissues like the brain [255], [256]. One of the fundamental benefits of Raman spectrometry is its label-free, non-destructive nature, which allows for the real-time monitoring of live cells without perturbing their native state or function. Brain organoids consist of multiple cell types, including neural stem cells, neurons, and glial cells, which undergo intricate changes during differentiation. The ability of Raman spectroscopy to capture these changes in the molecular composition of cells can help researchers detect distinct states of organoid maturation and differentiation [257], [258]. Considering the advantages of Raman spectroscopy, the brain organoids grown in either Matrigel or GelMA were analyzed with the technique to identify specific biomarkers and characterize chemical changes associated with different developmental stages. The results showed that there is a differential peak at 814 cm^{-1} , 860 cm^{-1} , 921 cm^{-1} , 1247 cm^{-1} , 1354 cm^{-1} , 1450 cm^{-1} , 1657 cm^{-1} , 1003 cm^{-1} , and 1032 cm^{-1} . The identified peaks in the spectrum may represent specific vibrations of biochemical compounds that may serve as markers for the differentiation process; therefore, the peaks were compared with those in previous studies. Confirming the hypothesis, some of the peaks are correlated with the literature, showing the neural differentiation. In the literature, it was shown that the axonal peak was around 1650 cm^{-1} and the myelin peak was around 2950 cm^{-1} . As a result, the axonal peak was observed around 1657 cm^{-1} , supporting the literature [259]. However, the 2950 cm^{-1} was not observed because of the spectral range of the study. If the range was broad during the measurement, the peak may not be measured. Even though Raman spectroscopy has great potential to detect the spatial distribution of biomolecules, the signals could be weak for some biomolecules, and this is one of the disadvantages of the method [260]. The problem is overcome with the signal enhancement methods using gold nanoparticles [261]. Besides the axon peak, it was also known that Raman scattering at 1445 cm^{-1} , indicating the CH_2 bending of proteins and lipids, was different between myelinating and unmyelinating axons [259]. The peak was also different among the embryoid bodies and organoids. (Figure 2.3.18A). One of the biggest remarkable peaks in Figure 2.3.18B was around 1354 cm^{-1} . The peak is more pronounced in organoids grown in Matrigel and GelMA compared to those grown in other conditions. The literature shows that the peak around $1335\text{-}1345\text{ cm}^{-1}$ differs in different organoids, which were induced by different growth factors, and the source of the peak is collagen. The information is directly correlated with our results because both hydrogels in the experiment are collagen-derived sources [262]. The neural differentiation was also detected by the peak at 1440 cm^{-1} ,

which indicates the lipids. Since lipids and DNA/RNA are the changed parameters in the cell cycle, cell cycle-arrested cells like neurons and stem cells show different patterns in the Raman spectra. Our result also has a peak at 1450 cm^{-1} ; therefore, the different neuronal stages of the samples are different from each other [263].



CHAPTER 3

Conclusion and Future Prospects

3.1 Conclusion

This initial section of the research examined the cytotoxic effects of the previously synthesized multitargeted drug candidates on human neuroblastoma and oligodendroglioma cell lines. Later, their GPCRs' binding capability was measured through the cAMP response element-driven luciferase activity. The compounds that have higher luciferase activity compared to fingolimod were selected. Simultaneously, the protocol for generating brain organoids was optimized to produce organoids containing oligodendrocytes, astrocytes, microglia, and mature neurons. After the organoids were successfully generated, multiple sclerosis was modeled by inducing an inflammation-like response with LPS. The model was confirmed at the protein and transcript levels. Later, the effects of the selected compounds were investigated on MS-modelled organoids. Following the analysis at the transcript level by qRT-PCR and at the protein level by immunofluorescent staining and ELISA, it was concluded that ST-1505 would be a promising drug candidate because of its positive effect on myelination and negative effect on inflammation in MS-modelled brain organoids.

In the second part of the study, the previously synthesized collagen-based methacrylated gelatin, called GelMA, was investigated as an alternative extracellular matrix for brain organoid generation. The protocol for generating brain organoids with diverse neuron types was optimized in the first part of the study using mouse sarcoma-derived Matrigel as a commercially available extracellular matrix. Following the gelation optimization processes of GelMA, human embryonic stem cells were subjected to the

differentiation protocol using either GelMA or Matrigel. The mature brain organoids were compared in terms of their size by light microscopy, neural progenitor and excitatory neuron profile, abundance of astrocytes and mature neurons by immunofluorescent staining, and differences in biochemical signals coming from lipid content and nucleic acid content by Raman spectroscopy. The results revealed that GelMA supports the differentiation of diverse neuron types, just as Matrigel does, and it would be an alternative extracellular matrix for brain organoid generation.

3.2 Societal Impact and Contribution to Global Sustainability

The development of a brain organoid model to test multi-targeted pharmaceutical agents for multiple sclerosis and cognitive impairment, and the study of alternative extracellular matrix for brain organoid generation hold an important potential for societal impact and contribution to global sustainability. Multiple sclerosis is a neurodegenerative disease affecting more than 2.9 million people worldwide, and there is no exact cure for the disease. A part of the study in the thesis investigated the effect of multi-targeted pharmaceutical agents on multiple sclerosis, and comparative studies with an already used MS drug called fingolimod were conducted. Therefore, the study aimed to improve human health and quality of life. Also, this part of the study aimed to advance scientific knowledge that can enhance public awareness in health-related fields. In the other part of the study, innovative approaches were used. This part of the study addresses a critical bottleneck in neuroscience research and disease modeling by replacing Matrigel, a biologically variable and animal tumor-derived extracellular matrix, with collagen-based methacrylated gelatin (GelMA), a more defined, tunable, and ethically sound alternative. By improving the reproducibility, safety, and scalability of brain organoid generation, this work enhances the reliability of human brain models used in the study of neurodevelopmental and neurodegenerative diseases. Ultimately, it contributes to the advancement of precision medicine, drug screening, and personalized therapies—leading to better outcomes for patients and reducing the societal burden of brain disorders. Overall, both parts of the study have employed brain organoid technology rather than animal models, thereby promoting ethical and sustainable scientific practices.

In the context of the United Nations Sustainable Development Goals (SDGs), this study aligns with SDG 3 (“Good Health and Well-Being”) by promoting the development of reliable brain disease models that can improve healthcare outcomes. It helps SDG 9 (“Industry, Innovation, and Infrastructure”) by making new, scalable biomaterials like GelMA. Additionally, it contributes to SDG 12 (“Responsible Consumption and Production”) by reducing dependence on animal-derived matrices such as Matrigel, thereby encouraging more ethical and sustainable research practices. Collectively, these contributions highlight the study’s broader impact on health, innovation, and sustainability at a global level.

3.3 Future Prospects

The future prospects of this research lie in the interdisciplinary integration of therapeutic development and advanced disease modeling technologies. Building upon the exploration of fingolimod-derived multi-targeted compounds, future work can focus on designing novel analogues with enhanced specificity for S1P receptor subtypes, reduced systemic side effects, and additional neuroprotective or anti-inflammatory properties. These compounds may offer broader therapeutic benefits not only for multiple sclerosis (MS) but also for other neurodegenerative or neuroinflammatory disorders. Parallel to this pharmacological approach, the use of methacrylated gelatin (GelMA) as a defined, tunable extracellular matrix substitute presents a powerful opportunity to establish reproducible and ethically sound 3D brain organoid models. These models can be used to recapitulate key features of MS pathophysiology, such as demyelination and neuroinflammation, through exposure to pro-inflammatory cytokines or immune cells. By integrating these organoids with patient-derived induced pluripotent stem cells, it becomes possible to investigate individual-specific disease mechanisms and therapeutic responses in a controlled in vitro setting. This also paves the way for the creation of a high-throughput, human-relevant screening platform to assess the effectiveness of fingolimod analogues and combination therapies. Additionally, GelMA’s mechanical properties can be fine-tuned to study how biophysical cues influence immune-CNS interactions and oligodendrocyte behavior, offering more profound insights into the mechanobiology of MS. Beyond scientific advancement, this work contributes to

sustainable biomedical research by reducing reliance on animal-derived materials like Matrigel and aligning with ethical and translational goals. Overall, this research direction aims to bridge drug discovery, disease modeling, and sustainable innovation in the pursuit of more effective and personalized treatments for MS and related conditions.



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APPENDIX

Table A.1 License number of the figures used in this document.

Figures	License Number
Figure 1.1.1	6070190591654
Figure 1.1.3	6133651258283
Figure 1.1.4	6133651476424
Figure 1.1.5	6040670118521
Figure 2.2.1	6133660398274

CURRICULUM VITAE

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SELECTED PUBLICATIONS AND PRESENTATIONS

J1) Acar B, Pepe NA, Zivkovic A, Stark H, Sen A. Neuroinflammatory Human Brain Organoids Enable Comprehensive Drug Screening Studies: Fingolimod and its Analogues in Focus. *Curr Med Chem.* 2025 Oct 8. doi: 10.2174/0109298673435364251002112630. Epub ahead of print. PMID: 41088935.

J2) Aktas Pepe, N., **Acar, B.**, Erturk Zararsiz, G., Ayaz Guner, S., & Sen, A. (2025). Role of Long Non-Coding RNA X-Inactive-Specific Transcript (*XIST*) in Neuroinflammation and Myelination: Insights from Cerebral Organoids and Implications for Multiple Sclerosis. *Non-Coding RNA*, 11(3), 31. <https://doi.org/10.3390/ncrna11030031>

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C1) Acar B, Kaya M, Pepe NA, Demirtas, TT, Sen, A (2025) Microwave-assisted gelatin methacryloyl hydrogel (GelMA): a reliable extracellular matrix for brain organoid generation. FEBS OPEN BIO, Volume 15, Page 454-454, Supplement 2, P-42-008

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C3) Demirkol NM, Ayten MN, Acar B, Pepe NA, Sen, A (2025) Co-culture spheroid model for neurodevelopment and disease modeling. FEBS OPEN BIO, Volume 15, Page 464-465, Supplement 2, P-42-040

C4) Ayten MN, Demirkol NM, Pepe NA, Acar B, Sen, A (2025) Therapeutic potential of pyrvinium pamoate in multiple sclerosis applying brain organoids. FEBS OPEN BIO, Volume 15, Page 464-464, Supplement 2, P-42-039

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C6) Aktaş Pepe, N., Atalay, T. N., Acar, B., Şenol, H., & Şen, A., (2023). Investigating the anti-inflammatory activities of the new hybrid compound 4-((1-(3- nitrobenzyl)-4,5-dihydro-1H-1,2,3-triazol-4-yl)methoxy)benzohydrazide . FEBS (p. 103). Tours, France

C7) Acar, B., Stark, H., & Şen, A., (2023). Investigating multitargeting effect of fingolimod/ciproxifan butterfly derivatives for multiple sclerosis on brain organoids . FEBS (p. 193). Tours, France

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