

EVALUATING THE PROLIFERATIVE POTENTIAL OF AROMATIC TURMERONE IN NEURAL STEM CELL-LIKE CELLS ISOLATED FROM ZEBRAFISH EMBRYOS

*Submitted in partial fulfillment of the requirements for the award of Master of
Science in Medical Biotechnology and Clinical Research*

By

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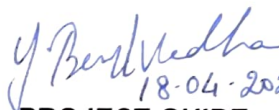
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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Ms. SHARMILA A (42101013)** who carried out Project entitled "**EVALUATING THE PROLIFERATIVE POTENTIAL OF AROMATIC TURMERONE IN NEURAL STEM CELL-LIKE CELLS ISOLATED FROM ZEBRAFISH EMBRYOS**" under the supervision of **Dr. Y. BERYL VEDHA**, Assistant Professor (Research) from September 2023 to April 2024.


18-04-2024
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DECLARATION

I, **Ms. SHARMILA A (42101013)** hereby declare that the Project Report entitled **"EVALUATING THE PROLIFERATIVE POTENTIAL OF AROMATIC TURMERONE IN NEURAL STEM CELL-LIKE CELLS ISOLATED FROM ZEBRAFISH EMBRYOS"** done by me under the guidance of **Dr. Y. BERYL VEDHA, Assistant Professor (Research)** at the Centre for Molecular and Nanomedical Sciences, Sathyabama Institute of Science and Technology, Chennai, is submitted in partial fulfilment of the requirements for the award of Master of Science in Medical Biotechnology and Clinical Research.

DATE: 18/04/2024

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ABSTRACT

The zebrafish (*Danio rerio*) is a prominent model organism in biomedical research, valued for its genetic, developmental, and physiological parallels with humans. Native to South Asia, this species has proven crucial for studies in vertebrate development, genetics, pharmacology, and disease modeling due to its transparent embryos and rapid development. The use of zebrafish in primary cell cultures, particularly those derived from embryos, allows for detailed in vitro investigations of cellular processes and drug screening, maintaining many in vivo characteristics such as specific cell markers and behaviors. The primary cell culture helps in culturing NSCs. This study focuses on the derivation and maintenance of a novel zebrafish Neural Stem Cell-like (NSC-like) line, derived from blastomeres without the use of feeder cells but only in 0.1% gelatin layer. These NSC-like cells are cultured in Dulbecco's modified Eagle's medium enriched with fetal bovine serum, showcasing a variety of morphological characteristics, predominantly forming tightly packed cell clumps. Immunostaining techniques confirmed the expression of *SOX2*, a pluripotency marker well-known in mammalian embryonic stem cells, and *TUJ1*, a neuronal marker, indicating the maintenance of essential stem cell properties in these cells. Furthermore, the impact of aromatic turmerone on cell proliferation was investigated. Both quantitative RT-PCR and immunoblotting methods demonstrated that aromatic turmerone significantly enhances the proliferation of these NSC-like cells. Collectively, our findings confirm that the cells we have isolated and cultured are indeed a Neural Stem Cell-like line and highlight the potential of aromatic turmerone as a promoter of proliferation in this novel cell line, offering promising implications for both basic research and therapeutic applications in regenerative medicine

Key words: Primary cell culture, Neural stem cell-like cells Isolation, Aromatic turmerone, Neuronal and stem cell marker, Gene expression

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ABBREVIATIONS

ALS	Amyotrophic Lateral Sclerosis
Ar-turmerone	Aromatic Turmerone
CaCl ₂	Calcium Chloride
cDNA	Complementary DNA
DAPI	4,6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribo Nucleic Acid
ESCs	Embryonic Stem Cells
FACS	Flurescence Activated Cell Sorting
FBS	Fetal Bovine Serum
GFAP	Glial Fibrillary Acidic Protein
HSCs	Hematopoietic Stem Cells
IPA	Iso propyl alcohol
iPSCs	Induced Pluripotent Stem Cells
KCL	Potassium Chloride
MACS	Magnetic Activated Cell Sorting

MgCl ₂	Magnesium Chloride
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NRPS	Neuronal Progenitor Cells
NSCs	Neural Stem Cells
OECD	Organisation for Economic Co-operation and Development
PBS	Phosphate Buffered Solution
PBST	Phosphate Buffered Saline with Tween 20
PVDF	Polyvinylidene Difluoride
RNA	Ribo Nucleic Acid
RNase	Ribonuclease
RPM	Revolutions per minute
RT-qPCR	Real Time Quantitative Polymerase Chain Reaction
SOX ₂	Sex determining region Y-box 2
SDS PAGE	Sodium Dodecyl Suphate Polyacrylamide Gel Electrophoresis
TEMED	N,N,N',N'-Tetra methyl ethylene diamine
TUJ	β III-Tubulin Antibody

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CHAPTER 1

INTRODUCTION

1.1 ZEBRAFISH (*Danio rerio*)

Danio rerio, widely known as the zebrafish, is a small tropical freshwater fish that has become a staple in biomedical research due to its genetic, developmental, and physiological similarities to humans. They habitat from the Ganges region in South Asia, including India, Nepal, Bangladesh, and Myanmar, the zebrafish have adapted to a variety of aquatic environments, from slow-moving streams to rice paddies (Engeszer *et al.*, 2007). Zebrafish have been established as an important model for studying vertebrate development and genetics. The optical transparency of embryos and the rapid development of zebrafish facilitate *in vivo* studies of cellular processes and genetic mutations affecting development (Kimmel *et al.*, 1995). This journey covers multiple decades, during which significant moments have led to the establishment of zebrafish as a fundamental resource for developmental biology, genetics, pharmacology, and disease modelling. The evolution of zebrafish in research highlights the efficacy of model organisms in enhancing our comprehension of intricate biological processes.

1.1.1 COMMENCEMENT AND ADOPTION AS A MODEL ORGANISM

The scientific use of zebrafish began in the 1960s when George Streisinger at the University of Oregon recognized its potential as a model organism for studying vertebrate development and genetics. Streisinger was drawn to the zebrafish for several reasons, including its small size, ease of care, and, most importantly, the optical transparency of its embryos, which allows for direct observation of developmental processes (Streisinger *et al.*, 1981). This pioneering work laid the foundation for zebrafish research, establishing techniques for breeding, genetic manipulation, and observation of embryonic development.

1.1.2 GENETIC SCREENING AND THE EXPANSION OF ZEBRAFISH RESEARCH

A significant leap in zebrafish research occurred in the 1990s with the advent of large-scale genetic screening, notably by Christiane Nüsslein-Volhard and Wolfgang Driever in Germany and Mark Fishman in the United States. These

screenings were designed to systematically identify mutations affecting various aspects of zebrafish development, leading to the discovery of numerous genes essential for vertebrate development (Driever *et al.*, 1996; Nüsslein-Volhard, 1994). The success of these screening demonstrated the utility of zebrafish in genetic research and led to an explosion of interest in the organism across the scientific community.

Zebrafish has become one of the important model organisms for biomedical research, offering a unique combination of advantages that facilitate understanding of biological processes and disease mechanisms. Their popularity in research has surged due to several key features: they are small and inexpensive to maintain, they have a high fecundity with hundreds of eggs produced per mating, and they develop externally and rapidly, with most of their organs formed within 24 hours post-fertilization. Furthermore, their embryos are transparent, allowing for direct observation of developmental processes in living organisms.

The genetic makeup of zebrafish shares significant similarity with humans, with approximately 70% of human genes having at least one zebrafish counterpart. This genetic homology extends to disease genes, making zebrafish an invaluable model for human diseases, including cancer, heart disease, and neurological disorders (Howe *et al.*, 2013; Kettleborough *et al.*, 2013). Advances in genetic engineering techniques, such as CRISPR/Cas9, have further enhanced the utility of zebrafish, enabling precise modifications of the zebrafish genome to study gene function and disease mechanisms (Hwang *et al.*, 2013)

The development of a wide range of genetic and genomics tools, such as detailed genome sequences and diverse mutant and transgenic lines, have reinforced the zebrafish's importance as a model organism. (Postlethwait *et al.*, 2000; Amsterdam *et al.*, 1999). Along with research communities and databases that offer support, these resources are crucial for conducting zebrafish research.

1.1.3 ETHICAL AND PRACTICAL ADVANTAGE

The small size and ease of care of zebrafish make them suitable for laboratory research. They require relatively simple aquatic systems for maintenance, can be housed in large numbers, and their care and use in research are subject to ethical regulations that ensure treatment. These factors, combined with the ethical

advantage of using a lower vertebrate model before mammalian models, contribute to the zebrafish's prominence in research.

1.2 TAXONOMY

- Kingdom: Animalia
- Phylum: Chordata
- Class: Actinopterygii (ray-finned fishes)
- Order: Cypriniformes
- Family: Cyprinidae
- Genus: *Danio*
- Species: *rerio*

This classification highlights the zebrafish's position within the vertebrate lineage, sharing a common ancestry with other fish in the Cyprinidae family, as well as with more distant terrestrial vertebrates.

1.3 NOMENCLATURE

The scientific name "*Danio rerio*" was officially given by Francis Hamilton (formerly Buchanan) in 1822, based on specimens collected from India. The genus name "*Danio*" is derived from the Bengali name "Dhani," meaning "of the rice field," which reflects the natural habitat of these fish in the rice paddies of South Asia. The species name "*rerio*" is thought to be derived from a local indigenous name for the fish.

Danio rerio has undergone various taxonomic revisions. It was originally described under the genus *Cyprinus*. Later, it was moved to the genus *Brachydanio*, a name that was widely used until the late 20th century when taxonomic studies based on more detailed morphological and molecular analyses reclassified it back to the genus *Danio* (Fang, 2003).

1.4 GENETIC INSIGHTS

The zebrafish genome was fully sequenced and published in 2013, revealing approximately 26,000 protein-coding genes within its 1.4 billion base pairs, underscoring the genetic complexity and applicability of this model organism. Remarkably, around 70% of human genes have at least one zebrafish ortholog, and about 84% of genes known to be associated with human disease have a counterpart

in zebrafish, highlighting its relevance in human genetic and disease studies (Howe *et al.*, 2013).

1.5 ECOLOGICAL AND CONSERVATION PERSPECTIVES

Native to the freshwater environments of South Asia, zebrafish prefer habitats such as rice paddies, ponds, and slow-moving streams, which offer abundant vegetation and protection. Understanding the natural ecology of zebrafish is crucial for their conservation and informs ecological and environmental research, emphasizing the necessity of sustainable practices in both wild and laboratory populations (Spence *et al.*, 2008).

1.6 PRIMARY CELL CULTURE

Primary cell cultures are grown as explant cultures or as single cells after being separated by enzyme digestion. They are obtained directly from healthy animal tissue. These cells are a model that is closer to the *in-vivo* state than continuous cell lines because they have only experienced a limited number of cell divisions and have not undergone any transformation (Freshney, 2016). Primary cultures are invaluable for modelling diseases, especially when derived from patient tissues, allowing for the exploration of disease mechanisms and the development of targeted therapies (Pampaloni *et al.*, 2007).

1.6.1 APPLICATIONS OF PRIMARY CELL CULTURE

Drug Discovery and Development: Primary cell cultures are extensively used in pharmacology for screening and testing new drugs. Because they closely mimic the physiological state of *in vivo* cells, they can provide more accurate information on a drug's efficacy and toxicity than immortalized cell lines. This is crucial for predicting the clinical relevance of preclinical findings (Pouton & Haynes, 2007).

- **Regenerative Medicine:** Primary cultures from stem cells or progenitor cells are critical in regenerative medicine, providing insights into the mechanisms of differentiation and tissue regeneration. These cells are used to study disease progression and are also pivotal in developing tissue-engineered products for regenerative therapies (Atala, 2012).
- **Cancer Research:** Culturing primary cells from tumors allows researchers to study cancer cell biology, heterogeneity, and drug resistance in a controlled

environment. This approach is particularly valuable for personalized medicine, as it enables the testing of therapeutic strategies on cancer cells derived from individual patients (Gillet, Varma, & Gottesman, 2013).

- 3D Cell Culture: The advancement of 3D cell culture models that better mimic the natural surroundings of cells. These systems enable primary cells to thrive in structures that mimic the layout of tissues, providing an enhanced understanding of cellular interactions, shape, and function (Edmondson *et al.*, 2014).

1.6.2 ZEBRAFISH IN PRIMARY CELL CULTURE

Zebrafish cell cultures preserve many of the genetic pathways and biochemical responses found *in vivo*, making them suitable for genetic studies, toxicological assays, and drug screening (Lieschke *et al.*, 2007). Researchers can establish primary cell cultures from a variety of zebrafish tissues, including muscle, heart, liver, and neurons. This diversity allows for tissue-specific studies and the investigation of disease models at the cellular level (Jung *et al.*, 2012).

The use of zebrafish embryos in primary cell culture has emerged as a novel and powerful tool for studying cellular processes and drug screening in a physiologically relevant context. This approach leverages the unique advantages of the zebrafish model, including its genetic tractability, rapid development, and the ease with which large numbers of embryos can be obtained and manipulated. Zebrafish embryo-derived primary cell cultures allow for the examination of cell behavior, gene function, and drug effects in a controlled environment while retaining much of the biological complexity of the whole organism. Zebrafish primary cells are utilized to simulate human diseases, offering a better understanding of the cellular processes involved in these diseases and enabling the evaluation of possible treatment options (Ridges *et al.*, 2012). Primary cultures maintain many of the *in vivo* characteristics, including specific cell markers and behaviors, thereby providing a more accurate representation of biological processes than immortalized cell lines.

One of the critical advantages of using zebrafish embryo-derived primary cell cultures is the opportunity to study vertebrate development and physiology using techniques that are difficult or impossible to perform *in vivo*. For instance, high-resolution live imaging, electrophysiological recordings, and single-cell analyses

can be conducted more readily on cells in culture than on cells within the living organism. Moreover, these cultures can be used for high-throughput screening of pharmacological agents and toxicity testing, offering a bridge between *in vivo* studies in whole organisms and *in vitro* assays in cell lines (Zon *et al.*, 2005).

1.7 STEM CELL

Ernst Haeckel, a German scientist, coined the phrase "stem cells" for the first time in 1868 and published it in scholarly journals. According to Haeckel's writings from 1868, "stem cells" refer to both the fertilized egg that gives rise to all other cell types in the body and the unicellular evolutionary genesis of all multicellular animals. The latter concept has given rise to the contemporary understanding of stem cells, which are defined as cells that may divide to self-renew and specialize into different types of cells in tissues and organs (Zhao and Moore, 2018). Stem cells are a crucial part of the biological system because of their special ability to transform into different types of cells and their ability to renew themselves. Stem cells are essential in developmental biology, regenerative medicine, and therapeutic applications due to their unique properties. Understanding stem cell potency is essential in grasping their significance in both natural organism development and clinical applications. Stem cells are categorized based on their ability to differentiate into various cell types (Poliwoda *et al.*, 2022).

1.7.1 TYPES OF STEM CELL

- Totipotent stem cells: These are the most versatile stem cells, capable of developing into any cell type in the body, including the placenta and embryonic tissues. The classic examples of totipotent cells are the zygote and the cells in the early stages of the embryo (up to around four days in humans) (Takahashi and Yamanaka, 2006).
- Pluripotent stem cells: These cells can evolve into almost any cell type in the body but lack the ability to form an entire organism. Embryonic stem cells (ESCs), derived from the inner cell mass of the blastocyst, are a primary example. Induced pluripotent stem cells (iPSCs), reprogrammed from adult somatic cells, also fall into this category (Thomson *et al.*, 1998; Takahashi *et al.*, 2007).

- Multipotent stem cells: Multipotent stem cells can develop into a limited range of cell types related to their tissue of origin. Hematopoietic stem cells (HSCs), which can differentiate into various blood cell types, are an example (Weissman, 2000).
- Oligopotent and Unipotent stem cells: These stem cells have the narrowest differentiation potential. Oligopotent stem cells can differentiate into a few types, while unipotent cells are restricted to a single cell type but can renew themselves, a critical feature for tissue repair and maintenance (Fuchs and Segre, 2000).

1.8 NEURAL STEM CELLS

Neural stem cells (NSCs) are a pivotal component of the central nervous system's developmental and regenerative processes. NSCs possess the dual abilities to self-renew, maintain their stem cell population, and differentiate into the diverse specialized cell types of the nervous system, including neurons, astrocytes, and oligodendrocytes. This balance between proliferation and differentiation is crucial for brain development and for the potential repair mechanisms in neurodegenerative diseases and injuries.

1.8.1 CHARACTERISTICS OF NEURAL STEM CELL

- Self-Renewal: NSCs can proliferate and produce offspring that retain their stem cell identity, a process essential for maintaining the stem cell pool throughout life (Gage, 2000).
- Multipotency: They are multipotent, capable of differentiating into multiple cell types that constitute the nervous system. This multipotency is tightly regulated by the microenvironment or niche in which these cells reside (Temple, 2001).
- Niche Dependency: The behavior of NSCs is highly influenced by their niche, which provides signals that regulate NSC maintenance, proliferation, and differentiation. The niche comprises various cell types, extracellular matrix components, and soluble factors (Morrison *et al.*, 2008).

NSCs are detected by the expression of particular molecular markers. *Nestin*, an intermediate filament protein, *Sox2*, a transcription factor important for stem cell maintenance, and, in some cases, GFAP, glial fibrillary acidic protein, are typical

markers for identifying NSCs. NSCs can be isolated using a variety of approaches, including fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS), depending on whether they express surface markers or transgenic reporters (Doetsch, 2003; Conti *et al.*, 2010). The destiny of NSCs is regulated by a complicated interaction of signaling pathways, such as *Notch*, *Sonic Hedgehog* (Shh), and *Wnt/β-catenin*. These pathways control neural stem cell growth, specialization, and continued existence. Recent research has shown how changes in these pathways can impact brain development and lead to neurological disorders ((Imayoshi *et al.*, 2013).

1.8.2 THERAPEUTIC APPLICATION OF NEURAL STEM CELL

Parkinson's, Alzheimer's, Huntington's, and Amyotrophic Lateral Sclerosis (ALS) are examples of neurodegenerative diseases in which the structure or function of neurons gradually deteriorates. Neural stem cells (NSCs) possess the ability to restore lost or damaged neurons and modify the brain's environment to stop additional neuron loss. For instance, the purpose of NSC transplantation in Parkinson's disease is to replenish the dopaminergic neurons lost in the substantia nigra (Lindvall *et al.*, 2010). Strokes and severe brain injuries can cause considerable cell death and functional impairments. NSC can regenerate damaged parts of the brain by developing into neurons and glial cells that can integrate into existing brain circuits. Furthermore, NSCs can produce neurotrophic substances that aid in brain self-repair processes (Steinbeck *et al.*, 2015). Spinal cord injuries frequently cause irreversible loss of function below the site of injury due to the central nervous system's inability to recover. Transplanting NSCs into the damaged spinal cord allows them to develop into neurons and glial cells, possibly restoring connection and function. NSCs can also influence the injury environment by lowering inflammation and facilitating tissue healing (Tetzlaff *et al.*, 2011). In addition to direct therapeutic uses, NSCs provide a platform for *in vitro* modelling of neurodegenerative disorders. This enables the investigation of disease mechanisms and the screening of possible therapeutic compounds in a controlled environment, expediting the drug discovery and development process (Marchetto *et al.*, 2010).

1.9 AROMATIC TURMERONE

Aromatic turmerone is a bioactive component predominantly found in the essential oil of turmeric (*Curcuma longa*), a spice hailed for its extensive use in culinary, cosmetic, and medicinal practices across various cultures, notably within Asian countries (Gupta *et al.*, 2013). Turmeric's multifaceted applications are largely attributed to its array of compounds, including curcuminoids and turmerones, with aromatic turmerone standing out for its unique properties (Ammon *et al.*, 1991). Turmerone is part of the sesquiterpene group of terpenes and contributes to the distinctive smell of turmeric. Apart from just adding aroma, aromatic turmerone is being recognized in the scientific community for its possible healing properties, which enhance the overall usefulness of turmeric (Liu *et al.*, 2014). The compound exhibits a range of bioactive effects, including anti-inflammatory, antioxidant, and potential anticancer activities. Its anti-inflammatory properties are particularly notable, with studies suggesting that aromatic turmerone can modulate inflammatory pathways and cytokine production, making it a candidate for treating inflammatory conditions (Joe *et al.*, 2004). The antioxidant capacity of aromatic turmerone contributes to its therapeutic potential, offering protection against oxidative stress-related damage. This is significant for chronic diseases where oxidative stress plays a pivotal role in disease progression (Aggarwal *et al.*, 2009). The antimicrobial properties of aromatic turmerone have also been the subject of research, with studies indicating its effectiveness against a variety of microbial strains. This suggests its potential application in developing natural antimicrobial agents, which could be particularly valuable in the face of rising antibiotic resistance (Martins *et al.*, 2009). However, its role in promoting cell proliferation, especially of neural stem cells, represents a promising avenue for applications in neurology and regenerative medicine. This aspect of aromatic turmerone underscores the broader therapeutic implications of turmeric's components beyond curcumin.

1.9.1 PROLIFERATIVE PROPERTY OF AROMATIC TURMERONE

The study by Hucklenbroich *et al.* (2014) represents a foundation in the research on aromatic turmerone's effects on neural stem cells. Their investigation revealed that aromatic turmerone significantly enhances the proliferation of NSCs *in vitro* and *in vivo*. Notably, *in vivo* administration of aromatic turmerone led to an increased

number of newly generated neural cells in the subventricular zone of adult rats, highlighting its potential to stimulate neurogenesis.

CHAPTER 2

LITERATURE SURVEY

2.1 ZEBRAFISH IN PRIMARY CELL CULTURE

A study conducted by Kimmel *et al.* in 1995 established that zebrafish is a leading model for studying vertebrate development and genetics. The embryos' optical transparency and the zebrafish's rapid development enable *in vivo* studies of genetic mutations and cellular processes that affect development. A study conducted by Meade *et al.* in 2019 reported the development of the first *in vitro* primary cell culture of adult zebrafish spinal neurons. The study involved isolating spinal neurons through enzymatic digestion of the dissected intact spinal cord from adult zebrafish. The neurons were then maintained for five days in primary cell culture for subsequent electrophysiological analyses. The findings of the study revealed that the resulting neurons in culture exhibit distinct neuronal morphology compared to other cell types, as confirmed by immunofluorescence labelling. This distinct morphology allows for easy identification of the neurons based on their appearance alone. The cultured neurons display large cell somas and long-extending neurites, indicating their viability and functionality. Moreover, the study demonstrates that these cultured neurons serve as a robust platform for neurophysiological studies, including calcium imaging and whole-cell patch clamp experiments.

A study conducted by Sassen *et al.* in 2017 demonstrated that expression constructs are an efficient way to transfect primary zebrafish cells. This process enables time-lapse imaging, using fluorescent marker proteins to reveal subcellular structures. The study also revealed that zebrafish primary cell culture is a flexible tool that can be used to investigate cell biology issues in addition to the robust *in vivo* model that zebrafish provide. To enable live cell imaging of fully differentiated cells, such as neurons and myocytes, the authors of the study developed a simple methodology for creating primary cell cultures from 2-day post-fertilization (dpf) zebrafish embryos. By using transgenic, cell-type-specific fluorescent reporters, they were able to differentiate between various cell types based on their appearance. The study also showed that cells expressing fluorescent markers could be effectively sorted by flow cytometry, making it possible to create enriched

cultures. Furthermore, the researchers discovered that many fluorescent vital dyes were useful for supporting subcellular imaging of living primary cells.

In 2012, Huang *et al.* conducted a study using transgenic zebrafish lines that were specifically engineered to express fluorescent proteins (Green Fluorescent Proteins and Red Fluorescent Proteins) in different cell types. This allowed for the observation of cell differentiation within primary cultures. The zebrafish lines represented key biological layers such as mesoderm, endoderm, and ectoderm, with cell types including vascular endothelial, hematopoietic, cardiac, skeletal muscle cells, pancreatic beta cells, and dopaminergic neurons. The study involved dissociating embryos into single cells that lacked fluorescence, indicating an undifferentiated state. Throughout 5 to 9 days of culture, certain cells began expressing fluorescence, marking their differentiation. The researchers observed endothelial cells forming networks, hematopoietic cells clustering, and cardiac myocytes demonstrating contraction. Furthermore, the study demonstrated that adding growth factors such as FGF and VEGF could promote specific differentiation, while an inhibitor could modulate this process. This approach provided a comprehensive method for studying cell development, the effects of various substances on differentiation, and potentially aiding in therapeutic drug discovery. Zebrafish primary cell cultures were used as a versatile platform in this regard.

2.2 NEURAL PRIMARY CELL CULTURE

In 1991, Kitani *et al.* established primary cultures of mouse neural precursor cells by enzymatically dissociating embryonic Day 10 fetal heads and negatively selecting non-neural contaminating cells. The enriched neuroepithelial cell suspension was plated on poly-D-lysine-coated dishes to inhibit fibroblastic cell growth. The study found no significant differences in morphology or immunocytochemical staining characteristics between neural precursor cells obtained through enzymatic dissociation and manual dissection of fetal neuroepithelia. The efficiency of this method allowed for the generation of a large number of neural precursor cells, enabling a detailed molecular analysis of regulatory factors that influence proliferation and differentiation during the early development of the mouse central nervous system. In 2004, Lin., *et al.* demonstrated that rat neural stem cells and progenitor cells grown in NASA-designed rotating wall vessel bioreactors inside collagen gels showed an accelerated phase of growth and differentiation starting

from the third week of the study. After six weeks, there was a significant increase in cell counts, including over a tenfold rise in cells expressing *nestin* and *GFAP*, and a twofold increase in *TuJ1* gene expression compared to the static control. The *TuJ1*+ neurons cultivated in the RWV bioreactor displayed extensive neurite outgrowth and a substantially higher number of synapsin I+ pre-synaptic puncta around MAP2+ cell bodies and dendrites. These results indicate that the combined cell-collagen-bioreactor culture system effectively supported long-term growth and differentiation of NSC/NP, and that RWV bioreactors could play a significant role in creating neural tissue-like constructs that have the potential to be used in cell replacement therapies.

In 2020, Goshi *et al.* introduced a new "tri-culture" medium to study neuroinflammation by combining neurons, astrocytes, and microglia. This medium was compared to the traditional "co-culture" medium of only neurons and astrocytes. The tri-culture model successfully maintained a physiologically relevant mixture of cells for 14 days and demonstrated reduced caspase activity, increased secretion of neurotrophic factor IGF-1 (Insulin-like growth factor-1), and reduced CX3CL1 (C-X3-C motif chemokine ligand 1) levels. The tri-culture model was shown to more accurately mimic *in vivo* neuroinflammatory responses than traditional culture systems, offering a valuable tool for studying neuroinflammation with enhanced relevance. In 2016, Kumar and Mallick developed a method for culturing neurons from chicken embryos, which allowed for long-term culture without glial contamination. The researchers monitored neuronal development in real-time and labelled synaptic vesicles for live-cell imaging. This method was cost-effective and simpler than existing approaches and had the potential for growing 3D-multilayered brain structures *in vitro*.

In 2013, Chen *et al.* developed a primary neuron culture system using zebrafish, for studying nerve growth and guidance. This system allowed for the plating of various central nervous system neurons, including fluorescent motor neurons, onto biological and synthetic substrates. Through microscopy, they observed growth cones at the tips of axons extending at a rate of 21.4 ± 1.2 μm per hour. Their functionality tests showed that BDNF (Brain-derived neurotrophic factor) stimulated axon outgrowth and chemoattraction, while myelin-associated glycoprotein inhibited outgrowth. The study proved to be a valuable tool for understanding neuronal

development and axon guidance. In 2015, Welzel *et al.* devised a technique to isolate neuronal cells from zebrafish embryos. They began by breaking down sterilized embryos into individual cells and cultivating them on a laminin substrate in a serum-free medium that was optimized for neural cell growth. Within a week, some cells exhibited neuronal traits. To enhance the purity of the neuronal cells, the researchers employed magnetic-activated cell sorting with anti-PSA-NCAM microbeads, which took advantage of the expression of PSA-NCAM in the central nervous system cells of zebrafish embryos. This approach effectively separated a fraction enriched in neuronal progenitor cells (NRPs), as evidenced by the expression of neuronal markers and the formation of neurite-connected cell aggregates. These enriched cultures exhibited high viability and metabolic activity. Further investigation using immunocytochemical techniques confirmed the enrichment of neuronal cells within the sorted fraction and identified a subset of cells as glial, based on specific markers. This sorting method yielded a significantly homogeneous population of viable NRPs, which were capable of forming interconnected neuronal aggregates that mimicked aspects of neural development. This innovative approach provided a refined method for studying neuronal development in zebrafish, generating enriched cultures of NRPs that not only displayed characteristic neuronal aggregation but also maintained their neuronal identity over time.

In 2018, Russo *et al.*, introduced methodologies for generating primary cell cultures from both zebrafish embryos and adult brains, highlighting the necessity of initiating cultures with a minimum of 100 embryos for consistent outcomes. They stressed the critical need to prevent contamination throughout the embryo's development phase. The team favored using Type 2 collagenase for its efficacy in the enzymatic dissociation process, underlining the importance of precise cell quantification prior to electroporation. A notable challenge identified was maintaining the viability of hard-to-culture cell types, particularly neurons. They discovered that the viability of neuronal cells was notably enhanced by utilizing Leibovitz's L-15 medium enriched with B-27. The researchers viewed their work as laying down a fundamental approach to zebrafish primary cell culture, offering a versatile framework for the culture of specific cell types and for a range of scientific explorations. The study thus presents critical methodologies for the establishment of primary cell cultures from

zebrafish, serving as a valuable resource for subsequent research endeavors in this field.

2.3 AROMATIC TURMERONE PROLIFERATIVE PROPERTY

Aromatic turmerone had been widely examined for its potential to promote the proliferation of neural stem cells (NSCs). This was of great interest because NSCs possess the capability to differentiate into various neural cell types, essential for the development, maintenance, and repair of the nervous system. Enhancing NSC proliferation through aromatic turmerone could offer therapeutic strategies for neurodegenerative diseases or support recovery from brain injuries.

In 2014, Hucklenbroich *et al.* investigated the impact of aromatic turmerone on neural stem cells. Their research demonstrated a significant boost in NSC proliferation, observed both *in vitro* (under laboratory conditions) and *in vivo* (within the brains of adult rats). Specifically, the *in vivo* studies revealed an augmented number of newly formed neural cells in the subventricular zone, a brain region associated with neurogenesis. These findings provided strong evidence for the neurogenic capabilities of aromatic turmerone, setting the stage for subsequent investigations into its therapeutic potential for neurological disorders. The results obtained by Hucklenbroich *et al.*, 2014 indicated that aromatic turmerone might be a valuable natural substance for creating treatments aimed at enhancing neurogenesis and facilitating brain repair. This could hold important implications for managing conditions such as Alzheimer's disease, stroke, and traumatic brain injuries, where neural tissue regeneration could potentially mitigate symptoms or improve recovery outcomes.

CHAPTER 3

AIM AND OBJECTIVES

3.1 AIM

To isolate and characterize neural stem cell-like cells from zebrafish embryos using a primary cell culture.

3.2 OBJECTIVES:

- To establish primary culture cells from the blastula stage embryo of zebrafish
- To isolate blastomeres from the blastula stage embryos
- To initiate zebrafish embryonic stem cell-like cell lines
- To identify and characterize the neural stem cell-like cells using neural stem cell markers
- To evaluate the proliferative potential of aromatic turmerone in isolated stem cells

CHAPTER 4

MATERIALS AND METHODS

4.1 ZEBRAFISH MAINTANANCE

Zebrafish are a cost-effective and easily obtainable option as they are widely available in pet stores and fish stores. We procured wild-type Zebrafish, *Danio rerio*, from a nearby pet store and housed it at the Regeneration Stem Cell Biology lab in the International Research Centre at Sathyabama Institute of Science and Technology in Chennai. The fish was maintained in accordance with the institutional standard operating guidelines and OECD (The Organization for Economic Cooperation and Development) guidelines.

4.2 ZEBRAFISH HOUSING CONDITION

The zebrafish were bred in a well-maintained glass tank containing either dechlorinated or filtered water. The water was maintained at a temperature range of 26-28°C and a pH level of 7.0-7.5 to make sure the fish were in a suitable environment. To avoid overcrowding and maintain water quality, male and female fish were kept in different tanks to prevent breeding.

To ensure the fish's health and safety, the tank was properly cleaned every two days and the water was refilled. During the cleaning, the fish were temporarily transferred to a holding tank containing water from the main tank. After the main tank had been cleaned and refilled, the fish were returned to their original tank.

To guarantee that the fish had enough oxygen, an air pump motor was installed within the tank. The machine continually bubbled air via a tube into the water, aerating the water and providing enough oxygen for the fish to breathe. This contributed to maintaining a healthy and safe environment in which the zebrafish might grow.

4.3 ZEBRAFISH FEEDING

The fish were fed natural feed twice every day. Artemia, a species of planktonic crustacean, is fed to Zebrafish because it is simple to digest owing to its tiny size. It is high in protein and other key components that the fish requires for development and general health. Aside from artemia, bloodworms and frozen daphnia can be

utilized as food. Artemia is kept at -20°C to ensure purity and freshness and is split into little tubes for convenience.

4.4 ZEBRAFISH BREEDING

The fishes were properly fed before breeding. The fishes were then moved to the breeding tank in a ratio of 1:2 or 2:3 (male: female) depending on the requirement for embryos. The breeding tank has a bottom divider with small openings to prevent the embryos from being consumed. It also includes a partition that separates the male and female. The breeding tank was kept in the dark overnight. Zebrafish spawn within a short time after the onset of the light phase of their photoperiod. Fertilized embryos appeared transparent and they settled at the bottom of the tank, while unfertilized embryos appeared opaque. After breeding, the parent fish were removed and placed in a separate resting tank before being put into the usual tank. The bred female fish was left alone for 1-2 weeks. The bottom divider was carefully removed, and the embryos were collected using pasture tubes and stored in a petri plate. To eliminate the debris such as fish waste, the collected embryos were washed thrice with RO water and then thrice with 1X E3 media.

4.5 E3 MEDIA PREPARATION

4.5.1 PREPARATION OF 60X STOCK FOR 1 LITER

- Sodium Chloride (NaCl) – 17.4gm
- Potassium Chloride (KCl) – 0.8gm
- Calcium Chloride (CaCl₂, 2H₂O) – 2.9gm
- Magnesium Chloride (MgCl₂, 6H₂O) – 4.88gm

The above-mentioned salts were added to 800 mL of distilled or deionized water in a large beaker or container. The salts containing the beaker were stirred until they completely dissolved. pH for 7.2 was checked. After all salts had dissolved, the volume of the solution was adjusted to 1 liter by adding distilled or deionized water. The 60x E3 stock solution was autoclaved and stored at room temperature or in a refrigerator. The container was labelled with the concentration and date of preparation.

4.5.2 PREPARATION OF 1X E3 MEDIA

- From the 60X stock media, 16.5ml was taken and added in 983.5ml of distilled water
- 3 to 4µl of 1% methylene blue was added to prevent fungal contamination, and it was stored at 4°C.

Note – Before adding 16.5ml of 60X stock media, 1000ml of distilled water was autoclaved and cooled down.

4.6 PRIMARY CELL CULTURE

The blastula stage embryos were collected, and a primary cell culture was initiated. Before that, the embryos were washed with E3 and then subjected to an antibiotic wash to get rid of any bacteria present on the surface. The cells were then cultured using DMEM media in a 0.1% Gelatin-coated plate.

This method of culturing cells from blastula-stage embryos has been utilized in various research studies to investigate early embryonic development and stem cell differentiation.

4.6.1 DMEM MEDIA PREPARATION

DMEM (Dulbecco's Modified Eagle Medium) is a widely used cell culture media that supports the growth and proliferation of many cell types. It contains various nutrients, vitamins, and minerals that are essential for the survival and growth of cells.

To prepare the media, follow these detailed instructions:

- One liter of Millipore water was taken and sterilized by autoclave
- 17.4g of DMEM medium powder was weighed and added to the sterilized water. The mixture was stirred well until the powder dissolved completely.
- 1g of Streptomycin, 1g of Penicillin, and 1 ml of Amphotericin B were added to the media. These antibiotics were used to help prevent bacterial, fungal, and yeast contamination in the cell culture.
- 3.75g of sodium bicarbonate was added to the media acts as a buffering agent and that helped to maintain the pH of the media

- The pH of the media was checked using a pH meter or pH indicator strips. The pH was adjusted to 7.2 to 7.4 using hydrochloric acid (HCl) or sodium hydroxide (NaOH).
- The media was filtered through a 0.22µm Millipore filter using a filtration unit which helped to remove any remaining bacteria, fungi, or particles that may interfere with the cell culture.
- The media was stored at 4°C for future use.

4.6.2 GELATIN COATING (0.1%)

Gelatin coating is a widely used technique in cell cultures to improve the adhesion, spreading, and growth of cells. This technique is particularly useful for sensitive cell types that are prone to detachment or struggle to adhere to the substrate. Gelatin coating provides a surface that promotes cell adhesion and also offers additional nutrients that can help to support cell growth and proliferation. The coating acts as a bridge between the cells and the culture dish, allowing them to firmly attach and expand.

4.6.2.1 Preparation Of 0.1% Gelatin

To prepare the gelatin solution

- 0.1 gram of gelatin powder was measured using an accurate weighing scale.
- Then, 100 ml of Milli Q water was taken in a clean, sterile container.
- The gelatin powder was slowly added to the water while stirring continuously to avoid clumping. It was ensured that the powder fully dissolved in the water.
- Once a clear and uniform solution was obtained, it was transferred to an autoclave container. The mixture was autoclaved and allowed to cool down to room temperature before being used.

4.6.3 PRIMARY CELL CULTURE PROTOCOL

- Embryos were collected and washed thrice with sterile filtered RO water with (100 mg of streptomycin and penicillin) and thrice with sterile E3 media with (100 mg of streptomycin and penicillin) . After the E3 wash, the embryos were washed three times with sterile filtered E3 and then incubated in an antibiotic solution (100 mg of streptomycin, 100 mg of penicillin, and 100 µl of amphotericin B in 5 ml of sterile E3) for an hour.

- After the antibiotic wash, the embryos were washed in bleach (sodium hypochlorite) solution for 1-2 minutes. Following the bleach wash, the embryos were washed five times with sterile E3.
- Then, the embryos were collected in a 1.5ml Eppendorf tube (centrifuge tube) and crushed using a homogenizer. To the cell homogenate, 1 ml of 15% media was added and centrifuged at 1300 RPM for 5 minutes.
- The supernatant was discarded, and the pellet was dissolved with fresh 1 ml of 15% DMEM media. Using the blunt tips, cells were suspended slowly and then seeded in a 6-well plate with 0.1% gelatin-coated solution and kept in the CO₂ incubator.

4.6.4 SUBCULTURE PROTOCOL

- The old media was removed, and the 6-well plate was washed twice with 1X PBS.
- Then, 2 ml of 0.05% trypsin was added for cell dissociation (Enzymatic technique).
- The 6-well plate was then incubated at 37°C for 1 to 2 minutes in a CO₂ incubator.
- Once the cell structure changed, 2 ml of 15% serum media was added to the plate to reduce the activity of trypsin.
- The media was pipetted out to detach the cells from the bottom of the plate. The cells, along with the media, were transferred into a 15ml tube and centrifuged at 1000 RPM for 5 minutes.
- The supernatant was removed, and 1 ml of fresh media was added to the cell suspension, followed by gentle vortexing until the cells were well mixed.
- Then, the cells were counted with a Trypan blue stain in the Automatic cell counter.
- Finally, the cells were seeded into a new gelatin-coated 6-well plate, which contained 2ml of 15% serum media and kept in CO₂ incubator.

4.7 IMMUNOFLUORESCENCE

Immunofluorescence is a technique that utilizes fluorescent detection to aid in the attachment of antibodies and antigens. Fluorophores are employed in cells and tissues to detect and visualize a variety of molecules, including proteins, glycans, and small biological and non-biological compounds. These substances, when exposed to a specific wavelength of light, emit light. By using specific antibodies that have been tagged with fluorophores, various compounds can be detected in a given tissue or cell sample. Depending on the biological subject under investigation, a confocal or fluorescent microscope is typically employed to image tissue sections. Immunofluorescence is a versatile biological tool with a wide range of applications in patient care and research.

4.7.1 REQUIREMENTS FOR IMMUNOFLUORESCENCE

- PBST Buffer (100 µl of Tween 20 was taken and added in 100 ml of 1X PBS)
- Ice Cold Methanol (or) 4% paraformaldehyde (2 grams of Paraformaldehyde was weighed and added in 50 ml of 1X PBS)
- Permeabilization Buffer (10 µl of Triton X 100 was taken and added in 10 ml of 1X PBS)
- Blocking Buffer (50 micrograms of BSA and 114.25 micrograms of Glycine were measured and added in 5 ml of 1X PBST)
- 1% BSA (50mg of BSA in 5 ml of 1X PBST)
- Primary Antibody (Ab) (Ab and 1% BSA in 1X PBST)
- Secondary Antibody (Ab) (Ab and 1% BSA in 1X PBST)
- 1X PBS
- DAPI
- Mounting Medium (9ml of 80% Glycerol in 1 ml of 1X PBS with pinch of Ascorbic acid)

4.7.2 IMMUNOFLUORESCENCE PROTOCOL

4.7.2.1 Cells Seeding

- Coverslips were placed inside each well of a 6-well plate and coated with 0.1% gelatin to enhance cell adherence.
- After trypsinization, the cells were counted and seeded (1,00,000 cells per well) in the plate kept inside the CO₂ incubator. They were then allowed to adhere and grow until they reached the desired confluence.

4.7.2.2 Fixation

- After seeding and allowing cells to adhere and grow to the desired confluence, the cells were washed thrice with 1X PBST and incubated with 100% ice-cold methanol to preserve cell structure.

4.7.2.3 Permeabilization

- The cells were washed again thrice with 1X PBST for 5 minutes and incubated with Permeabilization Buffer for 10 minutes on ice to improve the penetration of the antibody.

4.7.2.4 Blocking

- The cells were washed again thrice with 1X PBST for 5 minutes and incubated with Blocking Buffer for 30 minutes at room temperature.

4.7.2.5 Immunostaining

- The cells were incubated with primary antibody overnight at 4°C.
- The cells were then washed with 1X PBS thrice for 5 minutes and incubated with a secondary antibody for 1 hour in the dark.
- The cells were washed again with 1X PBS thrice for 5 minutes in the dark.

4.7.2.6 Counterstaining

- The cells were incubated with DAPI for 1 minute and rinsed with 1X PBS once.

4.7.2.7 Mounting and Analyzing

- After staining, the coverslips were mounted on slides with a mounting medium that contains an anti-fading agent.
- The cells were analyzed using a fluorescence microscope.

4.8 CELLOGER AUTOMATED LIVE CELL IMAGING SYSTEM

The Celloger Automated Live Cell Imaging System is a highly advanced tool that is widely used in cell biology research to observe cellular dynamics and behaviors in real time. It combines cutting-edge imaging technology with automated data collection and analysis features, making it possible for scientists to investigate various cellular activities like proliferation, migration, differentiation, and apoptosis over prolonged periods.

4.8.1 PRINCIPLE

The Celloger system works by merging automated microscopy with computer-controlled image acquisition and analysis. It typically consists of a motorized microscope equipped with high-resolution cameras, environmental control units (temperature, humidity, CO₂), and robotic stages for precise positioning of samples.

4.8.2 WORKING

The process of using the Celloger system involved the following steps:

4.8.2.1 Cell Culture:

- Neural stem cell-like cells are seeded in a 6-well culture plate at a density of 5000 cells per well in a complete growth medium (15% DMEM with 2X concentration of Amphotericin B). In the second row in each well aromatic turmerone was added to check the proliferation of ar- turmerone treated cells

were growing compared to non-treated cells. The culture plate is then placed inside the Celloger system's environmental chamber, which is set to 37°C, 5% CO₂, and 95% humidity to maintain optimal cell growth conditions.

4.8.2.2 Imaging Parameters:

- Time Interval: Images are captured every 30 minutes for a total duration of 24 hours.
- Imaging Channels: Phase contrast imaging is used to visualize cell morphology.
- Magnification: A 4x objective lens is used for imaging to capture a sufficient number of cells within the field of view.
- Image Resolution: High-resolution images are acquired to ensure clear visualization of individual cells and accurate analysis.

4.8.2.3 Autofocus Setup:

- Autofocus Algorithm: The Celloger system is equipped with an autofocus algorithm that continuously monitors the focus during imaging. The algorithm adjusts the microscope's focus based on changes in image contrast or sharpness.
- Focus Points: Before starting the experiment, focus points are set at four locations within each well of the culture plate to ensure comprehensive coverage of the sample area.
- Autofocus Interval: Autofocus is performed before capturing each image frame to maintain optimal focus throughout the experiment.

4.8.2.4 Image Analysis:

- After the experiment, the captured images are analyzed using the Celloger's built-in software or custom image analysis algorithms.

4.9 RNA ISOLATION

RNA isolation is a crucial step in molecular biology that needs to be carried out to extract RNA from cells or tissues to analyze gene expression, study RNA-related disorders, and develop diagnostic tools. Because ribonucleases (RNases) are commonly found and highly efficient enzymes, they quickly degrade RNA, which presents a major challenge in RNA separation. Therefore, there is a need for rapid and efficient techniques to break down cells, inactivate RNases, and separate RNA from proteins, DNA, and other biological components to carry out successful RNA isolation procedures.

4.9.1 REQUIREMENTS FOR RNA ISOLATION

- RNAiso Plus (Trizol)
- Chloroform
- Isopropanol
- 75% ethanol (prepared with DEPC-treated water)
- RNase-free water

4.9.2 PROTOCOL

- Media was removed and the cells were washed with 1X PBS.
- 1 ml of RNAiso Plus was added to the plate and the cells were scraped with the help of a cell scraper
- The scraped cells were collected with a pipette and repeatedly agitated until cells were completely resuspended
- The solution was kept at room temperature for 5 mins
- To the solution, 0.2 ml of chloroform per 1 ml of RNAiso Plus used for homogenization was added. The centrifuge tube was capped and the mixture was shaken until the solution became milky.
- The solution was then kept at room temperature for 5 minutes.
- The mixture was centrifuged at 12,000g for 15 minutes at 4°C. After centrifugation, the solution was separated into three layers; the top liquid layer, which contained RNA, a semisolid middle layer, mostly comprising DNA, and a bottom organic solvent layer.

- The top liquid layer was carefully transferred to a new centrifuge tube, ensuring that the middle layer was not disturbed.
- To this new tube, 0.5 - 1 ml of isopropanol per 1 ml of RNAiso Plus used for homogenization was added, and the mixture was stirred well. The mixture was then kept at room temperature for 10 minutes.
- The mixture was centrifuged at 12,000g for 10 minutes at 4°C to precipitate the RNA.
- The supernatant was carefully removed, ensuring that the pellet was not disturbed. It was noted that the presence of residual isopropanol was not problematic.
- An amount of 75% cold ethanol, equivalent to the volume of the removed supernatant, was added.
- The solution was then centrifuged at 7,500g for 5 minutes at 4°C . Following centrifugation, the supernatant was discarded, taking care not to disturb the precipitate.
- The precipitate was dried by leaving the tube open for several minutes. This step was critical to ensure that all residual ethanol evaporated completely.
- Once dry, the precipitate was dissolved in an appropriate amount of RNase-free water. This re-suspension was performed gently to ensure that the RNA was fully dissolved without degradation.

4.10 RNA QUANTIFICATION USING QUBIT FLUOROMETER

- The required number of Qubit tubes for standards and samples was set up. The Qubit RNA BR assay was noted to require 2 standards. It was specified that only thin-wall, clear, 0.5-mL PCR tubes should be used for the Qubit 4 Fluorometer.
- The Qubit working solution was prepared by diluting the Qubit RNA BR Reagent 1:200 in Qubit RNA BR Buffer. A clean plastic tube was used each time the Qubit working solution was prepared.
- Qubit™ working solution was added to individual assay tubes so that the final volume in each tube, after adding the sample, reached 200 µL.
- 10 µL of Qubit™ standard was added to the appropriate tube.
- 1–20 µL of the sample was added to the appropriate tube.
- The contents were vigorously vortexed for 3–5 seconds.

- All tubes were allowed to incubate at room temperature for 2 minutes before proceeding to read standards and samples. Then the samples were quantified using Qubit Fluorometer

4.11 cDNA SYNTHESIS USING BIO-RAD KIT

4.11.1 COMPONENT

- 5x iScript Reaction Mix - 4µl
- iScript Reverse Transcriptase - 1µl
- Nuclease-free water - Variable
- RNA template (100fg – 1ug total RNA) - Variable
- Total Volume - 20µl

4.11.2 REACTION PROTOCOL

The complete reaction mix was incubated in a thermal cycler using the following protocol:

- Priming - 5 min at 25°C
- Reverse transcription - 20 min at 46°C
- RT inactivation - 1 min at 95°C
- Optional step - Hold at 4°C

4.12 QUANTIFICATION OF cDNA USING QUBIT FLUOROMETER

4.12.1 PREPARATION OF STANDARDS AND SAMPLES

- The required number of assay tubes (or tube strips) for standards and samples was set up. It was noted that the Qubit 1X dsDNA HS Assay requires 2 standards.
- 10 µL of each Qubit standard was added to the appropriate tube.
- 1–20 µL of each user sample was added to the appropriate tube.
- The Qubit 1X dsDNA working solution was added to each tube such that the final volume reached 200 µL.
- Each sample was mixed vigorously by vortexing for 3–5 seconds.
- All tubes were allowed to incubate at room temperature for 2 minutes.
- The cDNA was then quantified at the Qubit Fluorometer.

4.13 GENE EXPRESSION USING qPCR

Conventional PCR or Traditional PCR involves analyzing the DNA on an agarose gel after the reaction is finished to identify the amplified product. On the other hand, real-time PCR (RT-PCR) enables the detection and measurement of the amplified product as the reaction is occurring, providing results in real-time.

4.13.1 PRINCIPLE

Real-time PCR allows for the immediate detection of PCR products by using a fluorescent marker that increases in intensity in tandem with DNA concentration. This method utilizes either DNA-binding dyes or sequence-specific primers or probes with attached fluorescent labels. As amplification occurs, fluorescence is tracked using advanced thermal cyclers equipped with modules for detecting fluorescence. The fluorescence level measured in each cycle correlates to the quantity of DNA amplified. A major advantage of real-time PCR over conventional PCR is its precision in quantifying the initial template and its exceptional sensitivity across a wide dynamic range. This technique can yield both quantitative and qualitative data, where quantitative real-time PCR is often called qPCR. In contrast, traditional PCR offers only semi-quantitative analysis. Real-time PCR also simplifies the process by eliminating the need for gel electrophoresis to assess results, thus shortening the experiment duration and enhancing throughput. Moreover, the risk of contamination is minimized due to the use of a closed tube system for both reaction execution and data analysis.

4.13.2 SYBR GREEN DYE

SYBR Green is the most frequently utilized dye for binding to double-stranded DNA in RT-PCR. This dye shows very low fluorescence when unbound in solution; however, its fluorescence can increase by up to a thousand times upon binding to dsDNA. During RT-PCR, the intensity of SYBR Green's fluorescent signal grows in direct relation to the amount of dsDNA produced in the amplification process. The dye's excitation and emission peaks are at 494 nm and 521 nm, respectively. Additionally, the SYBR Green supermix is a premixed 2x reaction cocktail that includes SYBR Green I dye at an optimized concentration, along with Taq DNA polymerase, dNTPs, and buffers, designed to detect a broad spectrum of DNA targets.

4.13.2 MATERIALS REQUIRED FOR SETTING qPCR

- SYBR Green SuperMix
- Template DNA (cDNA)
- Forward and Reverse Primers
- Pipettes, Pipettes tips
- Sterile Microcentrifuge tubes
- Vortex, Microcentrifuge
- qPCR microplates or tubes
- Nuclease-free water

4.13.3 PROTOCOL FOR GENE EXPRESSION ANALYSIS USING qPCR

RT-PCR plays a crucial role in the analysis of gene expression, a key area of focus across various sectors of biomedical research, from basic science to industrial and medical applications. Typically, gene expression analysis involves comparing the expression levels between test and control samples. The process begins with the isolation of RNA from these samples, followed by the conversion of this RNA into cDNA through reverse transcription. The expression levels of both target and reference genes (β -Actin) are then measured in each cDNA sample using a qPCR assay. Subsequent data analysis allows for the calculation of the relative expression of the target genes within the samples.

4.13.4 IMPORTANT POINTS WHILE DESIGNING PRIMERS:

- The designed Primers should have a GC content of 50-60%.
- It is best if the melting temperature (T_m) of the primers is maintained between 50 and 65°C.
- Repeats of G's or C's longer than 3 bases should be avoided, and G's and C's should be placed at the ends of the primer.
- The sequence of forward and reverse primers should be checked to ensure there is no 3' complementarity, to avoid primer-dimer formation.

4.13.5 PRIMER DILUTION:

- The primer tubes were centrifuged at 8000 rpm for 30 seconds.
- The primer was dissolved with the appropriate amount of nuclease-free water as specified in the primer datasheet.
- The tubes were vortexed and allowed to set for 2 minutes at room temperature.
- The tubes were kept in a boiling water bath at 60°C for 10 minutes.
- The tubes were centrifuged again at 8000 rpm for 30 seconds.
- An aliquot was made by taking 10 µl of the primer and 90 µl of nuclease-free water into a sterile centrifuge tube. The tubes were labelled properly and stored at -20°C.

Table 4.1- Primers used for qRT-PCR

S.NO	Oligo name	Oligo sequence	Tm [°C]	GC-Content
1	Nanog FOR	5'-CCAAAAGGCCAAAGATGCAG-3'	57.3	50
2	Nanog REV	5'-GGAACCCCTTCTCGACTGCT-3'	61.4	60
3	Lin28 FOR	5'-TACCCAAAAGAGGCGGTCAA-3'	57.3	50
4	Lin28 REV	5'-CGATTTGCCCTGAGATCCTG-3'	59.3	55
5	Pou5f1 FOR	5'-CCGCCGTCACAATATCACCT-3'	59.3	55
6	Pou5f1 REV	5'-CGGAGACAGAGATGGGGATG-3'	61.4	60

4.13.6 PREPARATION OF REACTION MIXTURE

- The frozen 2X SYBR Green supermix, template DNA, and primers were thawed. The tubes were briefly vortexed and spun to collect the contents at the bottom.
- The components were added into each reaction tube as specified in Table 4.2. Each reaction mix was briefly vortexed, and the tubes were spun at low speed in a microcentrifuge (2000 rpm for 2 minutes).

Table 4.2: Reaction Mix - for 10 μ l

Step	Volume	Final Concentration
Nuclease free water	1 μ l	-
Forward Primer	1 μ l	300 nM (100-500nM)
Reverse Primer	1 μ l	300 nM (100-500nM)
2x SYBR Green super mix	5 μ l	1x
Template DNA	2 μ l	cDNA equivalent to 1pg to 100 ng of total RNA
Total reaction volume	10 μ l	

4.13.7 IMPORTANT NOTES:

- To account for potential pipetting loss, a reaction mix was prepared with a 10% excess.
- Triplicate samples were prepared as a master mix, with the sample template added just once.
- A no-template control (NTC) was included to detect possible contamination or primer-dimer formation.
- The reaction triplicates were loaded into PCR tubes or microplates, which were then properly sealed. To enhance results, the tubes or microplates were briefly spun at low speed in a microcentrifuge to eliminate any bubbles. The sealed reactions were stored on ice or at 4°C until the initiation of the RT-PCR.
- The thermal cycler was programmed according to the recommended RT-PCR protocol, including optional melt curve analysis as specified in Table 4.3.

- The sealed reaction vessels were placed in the thermal cycler block, and the RT-PCR protocol was initiated.

Table 4.3- RT-PCR protocol

Cycling Step	Temperature	Hold Time (min:sec)	No. of cycles
Initial denaturation and enzyme activation	95 ° C	02:00	1
Denaturation	95° C	00:15	40
Annealing/ Extension	55-60° C	00:20	
Meltcurve (Optional)	65-95° C (in 0.5 ° C increments)	00:05	1

4.13.8 qPCR ANALYSIS METHODS

It is important to choose reaction conditions for qPCR that will result in reliable and effective amplification for both relative and absolute measurements. The CT or Cq values obtained during qPCR are used for absolute and relative quantification.

- In absolute quantification, the CT or Cq values of test samples are compared to those of standards of known quantity using a standard curve. This analysis will provide the copy number or concentration of the sample and this method is most widely used for the detection of pathogen or copy number analysis.
- In relative quantification, the CT or Cq values of the test samples are compared to those of the control samples. This analysis will yield the ratio of the relative amount in the fold difference of the target gene in the test vs control samples and it is commonly used for gene expression studies. This analysis can be done using the software also we can calculate manually in an Excel sheet using formulas.

4.14 PROTEIN SAMPLE ISOLATION:

- Beta-mercaptoethanol (β -ME), a reducing agent, is utilized to cleave disulfide bonds within proteins. Prior to use, 2X sample buffers were equilibrated to room temperature.

- Subsequently, 5% Beta-Mercaptoethanol was incorporated into the 2X sample buffer (stock 1). This mixture was then added to the cell culture, and cells were dislodged using a cell scraper. To ensure thorough resuspension, the cell mixture was pipetted repeatedly.
- The cell lysates were then heated in a dry bath at 100°C for 5 minutes, followed by centrifugation at 10,000 rpm for 5 minutes.
- The resulting supernatant was carefully transferred to a new 1.5ml tube and stored at -20°C for further analysis.

4.15 SDS-PAGE ANALYSIS:

SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) serves as a key method for protein separation based on molecular weight. Electrophoresis explores the movement of charged molecules through an electric field, influenced by factors such as field strength, molecule charge, size, shape, and the medium's ionic strength, viscosity, and temperature. In this technique, proteins are separated within a polyacrylamide gel—a matrix that provides variable resistance hindering the mobility of proteins differently based on their size, with smaller proteins moving faster due to less resistance. The molecular weight of proteins is estimated by their mobility under the influence of SDS and β -ME. SDS strongly denatures proteins, unraveling secondary, tertiary, and quaternary structures into linear polypeptides coated with negatively charged SDS molecules. Beta-mercaptoethanol enhances this denaturation by reducing disulfide bonds within and between protein chains. As proteins migrate through the gel, they arrange into their primary structures within the stacking gel and subsequently separate by size upon entering the resolving gel. This separation process allows for the analysis of protein molecular weights.

4.15.1 MATERIALS:

- Eppendorf's
- Micro-pipettes
- Micro-tips
- Centrifuge
- Water bath
- Vortex

- Distilled water
- Scraper

4.15.2 CHEMICAL PREPARATION OF SDS-PAGE:

➤ 30% Acrylamide and 0.3% Bis acrylamide:

- Acrylamide – 30 g N,
- N' Methylene Bisacrylamide - 0.3 g, Made into 100ml with distilled water.

➤ 4X Separating Buffer:

- Tris HCL - 18.2 g
- Distilled water – 60 ml
- pH was adjusted to 8.8 with 10N NaOH
- Sodium Dodecyl Sulphate (SDS) - 0.4 g, Made into 100 ml with distilled water.

➤ 4X Stacking Buffer:

- Tris Hcl - 6.05 g
- Distilled water - 40ml pH was adjusted to 6.8 with 10N NaOH
- Sodium Dodecyl Sulphate (SDS) - 0.4g, Made into 100 ml with distilled water.

➤ 2X Sample Buffer:

- Tris stacking buffer (pH 6.8) – 25 ml
- Sodium Dodecyl Sulphate (SDS) – 4 g
- 20% Glycerol – 20 ml, Made into 100 ml with distilled water. During protein sample preparation, 5% β -Mercaptoethanol and during loading 0.001% (w/v) of Bromo-phenol blue was added.

➤ 10% Ammonium Per Sulphate (APS):

- APS – 100 mg Made into 1 ml with distilled water.

➤ **1X Running Buffer:**

- Tris Hcl - 3.94 g
- Glycine - 15.014 g
- SDS -1 g Made into 1 L with distilled water.

➤ **Staining Solution:**

- Coomassie Brilliant Blue R-250 – 200 mg
- Methanol – 50 ml
- Glacial Acetic acid – 7 ml Made into 100 ml with distilled water. The mixture was filtered using Whatman No 1 filter paper.

➤ **Destaining Solution:**

- Methanol – 30 ml
- Glacial Acetic acid – 7 ml Made into 100 ml with distilled water

Table.4.4- Separating gel concentration

Stock Solution	5%	6%	7%	7.5%	8%	9%	10%	12%	13%	15%
30% Acrylamide + 0.3% Bis-acrylamide (ml)	5.0	6.0	7.0	7.5	8.0	9.0	10.0	12.0	13.0	15.0

4X Tris Separating Buffer (ml) pH 8.8	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
Distilled water (ml)	17.5	16.5	15.5	15.0	14.5	13.5	12.5	10.5	9.5	7.5
10% APS (μl)	200	200	200	200	200	200	200	200	200	200
TEMED (μl)	20	20	20	20	20	20	20	20	20	20

Table.4.5- Stacking gel concentration

S.No	Stock Solution	5 ml	10 ml	15 ml	20 ml
1.	30% Acrylamide + 0.3% Bis-acrylamide (ml)	0.65	2.5	3.75	5.0
2.	4X Tris Stacking Buffer (ml) pH 6.8	1.25	2.5	3.75	5.0
3.	Distilled water (ml)	3.05	5.0	7.5	10.0
4.	10% APS (μl)	100	100	100	100
5.	TEMED (μl)	10	10	10	10

4.15.3 PREPARATION OF GEL:

The glass plates were cleaned by using ethanol and manually assembled. Glass plates were fixed in the proper equalized manner with each corner covered by using spacers so that they are used as a primary baseline on gel which helps in the process of agarose sealing or second level baseline system. 1% agarose was prepared and boiled until liquidy then corners were sealed in both surface and bottom area of the spacer. Separating gel was manually prepared and loaded on the gel carefully. The gel was overlaid with isopropyl alcohol so that it excludes the air. Then IPA was removed and washed with distilled water, after the drain stacking gel was prepared and added, instantly comb was fixed on the stacking gel. The gel was allowed a few minutes to solidify, then the comb was carefully removed and wells manifestly washed with water to eliminate the bubbles the bottom of the spacer was carefully removed and the setup was fixed in the bottom chamber which carried 1X running buffer as well as an upper chamber.

4.15.4 SAMPLE LOADING AND ELECTROPHORESIS:

A protein sample of 80µg and the appropriate molecular markers were loaded into the wells. The apparatus was connected to the power pack with proper polarity and 60V was applied. After electrophoresis, the glass plate assembly was taken from the buffer chamber. The spacer was gently twisted so that the upper glass plate pulled away from the gel.

4.15.5 STAINING AND DESTAINING THE GEL:

The gel was carefully taken out from the glass plate and the stacking gel was removed by using a cleaver. The separating gel was removed by gently grasping the two corners of the gel and placed in the container containing the Coomassie brilliant blue R250 (stainer solution). The gel should be fully submerged in the staining solution for 60 minutes by gentle agitation on a Rocker. Then it was washed with distilled water and transferred to a destaining solution until protein bands are visualized clearly. Commonly, the first destainer wash is about 30 minutes, then the second wash is about overnight.

4.16 IMMUNOBLOTTING

Immunoblotting, also known as Western blotting, is a highly specific biochemical technique that is used to detect specific proteins in a complex mixture of proteins extracted from cells. The principle of immunoblotting is based on the separation of proteins by molecular weight using SDS-PAGE, followed by the transfer of these proteins from the gel to a stable membrane surface, typically nitrocellulose or polyvinylidene fluoride (PVDF). This transfer is often facilitated by electric current, preserving the separation achieved during SDS-PAGE. Once transferred, the membrane is treated with a specific primary antibody that binds to the target protein. A secondary antibody, which recognizes the primary antibody, is then applied. This secondary antibody is conjugated to a detectable marker such as an enzyme or a fluorescent tag. Detection is achieved through the application of a substrate that reacts with the enzyme or tag on the secondary antibody, producing a visual signal such as color change, fluorescence, or luminescence. This signal is then detected and quantified with imaging equipment to confirm the presence and determine the amount of the protein of interest. Immunoblotting leverages the specificity of antigen-antibody interactions and the sensitivity of enzymatic amplification, making it an indispensable tool for molecular biology research to analyze protein expression and modification.

4.16.1 BUFFER PREPARATION

- 10X Transfer Buffer (Stock)
 - Glycine - 72.1 g
 - Tris-base - 15.2 g
 - Distiller water- 500 ml

The 10X transfer buffer was stored at room temperature, and during blotting the 1X transfer buffer was prepared from the 10X in the ratio of preparation viz., For a Total Volume of 1 liter 100 ml of 10X Transfer Buffer, 200 ml of Methanol and 700 ml of Distilled water

4.16.2 RUNNING THE GEL

A 12% SDS gel was prepared and placed in the SDS-PAGE setup. A 1X running buffer, prepared by diluting 100 ml of 10X running buffer with 900 ml

of distilled water, was poured into the setup. A 3 µl protein ladder was loaded into the first lane of the gel for molecular weight determination. Other wells received the prepared protein samples at a concentration of 60 µg per well. Once all samples were loaded, the system was run at 60V until the samples reached the bottom line of the gel.

4.16.3 MEMBRANE TRANSFER

Upon completion of the run, the glass slides were removed from the setup and the stacking gel was cut off. The remaining gel was then placed in the transfer buffer for 5 minutes. Simultaneously, a PVDF membrane was cut to match the size of the gel and activated by soaking in methanol for 1-2 minutes. This membrane was then kept in 1X transfer buffer while preparing the blotting sandwich. The blotting apparatus, along with sponge and filter paper, was arranged into a sandwich setup and soaked in 1X transfer buffer before use.

4.16.4 BLOCKING

A blocking buffer was used to block all proteins which would bind with targetable and non targetable proteins. 5% of Bovine serum albumin (BSA) with 20ml of 1X PBST buffer was prepared for Blocking and let in for 2 hours.

4.16.5 PRIMARY ANTIBODY

For studying protein expression of ar- turmerone, Primary antibodies were used for the experiment and these primary antibodies were added to the 5% of bovine serum albumin (BSA) at (1:5000 dilutions) and incubated overnight in cold conditions.

Actin (produced from mouse polyclonal Abcam, ab8226) - (1:5000 5%BSA).

TUJ1 (produced from rabbit monoclonal Abcam, ab52623) - (1:5000 5%BSA).

After its overnight incubation, the membrane was left to stabilize at room temperature for 30 minutes prior to beginning the wash steps.

4.16.6 SECONDARY ANTIBODY

The secondary antibody used, Anti-Rabbit IgG from goat (Abcam, AF568 – ab175471), was prepared in a 1:1000 dilution with 5% Bovine Serum Albumin (BSA) and incubated on the membrane for one hour at room temperature. All

steps following the addition of the secondary antibody were conducted in darkness to protect the light-sensitive antibodies from denaturing. After this period of dark incubation overnight, the washing process was initiated.

4.16.7 GEL IMAGING

Gel imaging has been carried out using the sophisticated BioRad ChemiDoc™ XRS+ imaging system for faster and more accurate western blot quantification. only stain-free enabled instrument capable of chemiluminescent, multiplex fluorescent, and routine gel imaging and it is used to quantify total protein for normalization purposes after transfer to the membrane.

After the incubation with a secondary antibody for 2 hours has been completed, the PVDF membrane is washed with 1X PBST every 5 minutes for 5 times. The blot was visualized in ChemiDoc system using ECL substrate solution by adding equal amounts of ECL solution A and ECL solution B mixed in a (1:1 Ratio)

4. 17 STATISTICAL ANALYSIS

Statistical significance between the expression levels of Pou5f1, Lin28, and Nanog in aromatic turmerone-treated and untreated neural stem cell-like cells was assessed using Student's t-test, with a p-value < 0.05 indicating significance. Data normalization was performed against β -actin to ensure consistency, and results were derived from three independent experiments to validate reproducibility.

CHAPTER 5

RESULTS & DISCUSSION

5.1 ESTABLISHMENT OF ZEBRAFISH NEURAL STEM CELL-LIKE CELL LINE BY PRIMARY CELL CULTURE

The zebrafish Neural Stem Cell-like cell line was meticulously engineered by isolating blastomeres from blastula-stage embryos, a developmental stage spanning from 128 to 256 cells, as initially described by Kimmel *et al.*, 1995. This developmental stage was specifically targeted due to the presence of undifferentiated cells, rendering it an optimal choice for initiating Embryonic Stem Cell Culture, as affirmed by Sun *et al.* in 1995. Moreover, blastomeres were particularly favored for their inherent capability to generate a homogeneous cell population, a feature highly desirable for studies necessitating ample cellular material, as underscored by Myhre and Pilgrim in 2010.

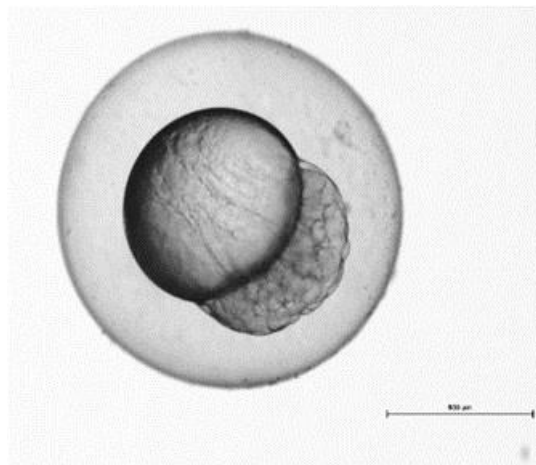


Fig 5.1- Blastula stage zebrafish embryos

In our experimental setup, approximately 15 to 20 blastula-stage (4hpf) embryos (Fig 5.1) were used. To obtain primary cell culture, the embryos were rinsed in antibiotic solution (Streptomycin and Penicillin) and antifungal (Amphotericin B) to eliminate potential contaminants. Following this, the embryos were gently homogenized and strained using a sterile cell strainer (40μm), which were subsequently seeded onto a 60mm petri dish containing 10% DMEM media.

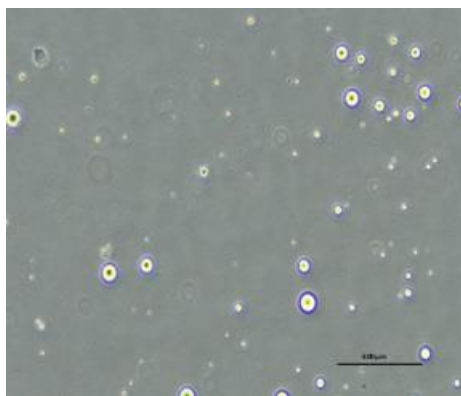


Fig 5.2- Blastomeres from Blastula stage embryos

Initial observations, as shown in Fig 5.2, revealed the emergence of blastomeres within a span of 2 to 3 hours post-seeding. The cells were grown well till 2 to 3 days and it was depicted in Fig 5.3 A and B. Even though a mixture of different cell morphologies like striated cells, and putative neurons were observed respectively, however a drastic depletion of cells were noticed from day five onwards and finally the cells disintegrate which is illustrated in Fig 5.3 C and D.

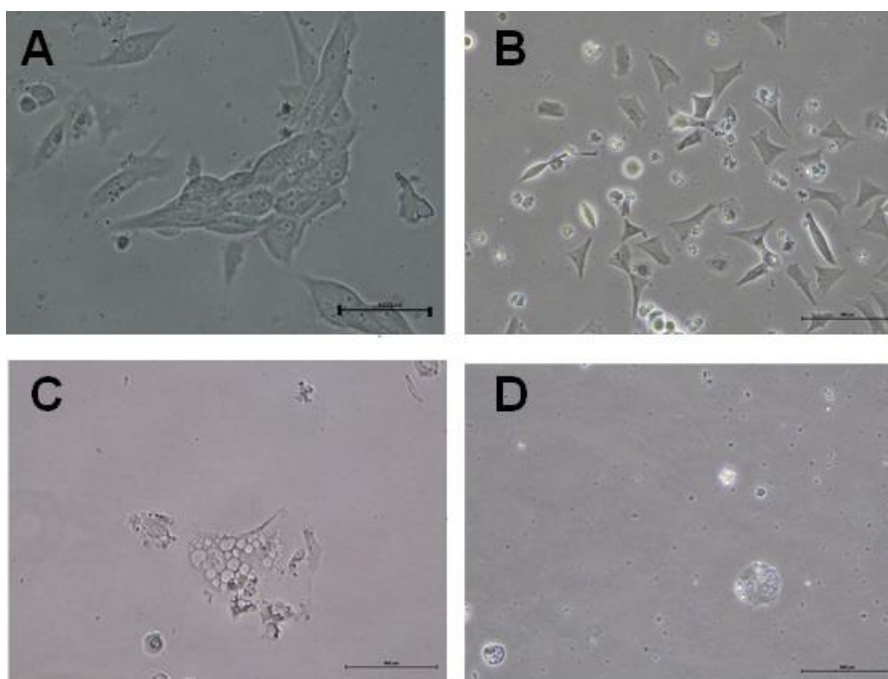


Fig 5.3- Blastula derived primary cell lines from Zebrafish embryos. (A) Primary cells aggregates observed at day 1. (B) Primary cells aggregates observed at day 2. (C, D) Relative number of primary cell aggregate formation drastically decreased.

In response to the unsatisfactory growth of cells, slight changes were made for subsequent attempts. For this experiment, the petri plates (60mm) were coated with FBS and it was kept in incubator for overnight. On the succeeding day the cells were seeded as mentioned before. The results were shown in Fig 5.4. which indicated that cells were attached and started to grow with different morphologies for four days (Fig 5.4 A and B) and again the growth decreased from fifth day onwards. Moreover fungal and bacterial contamination were evident which affected the growth of cells.

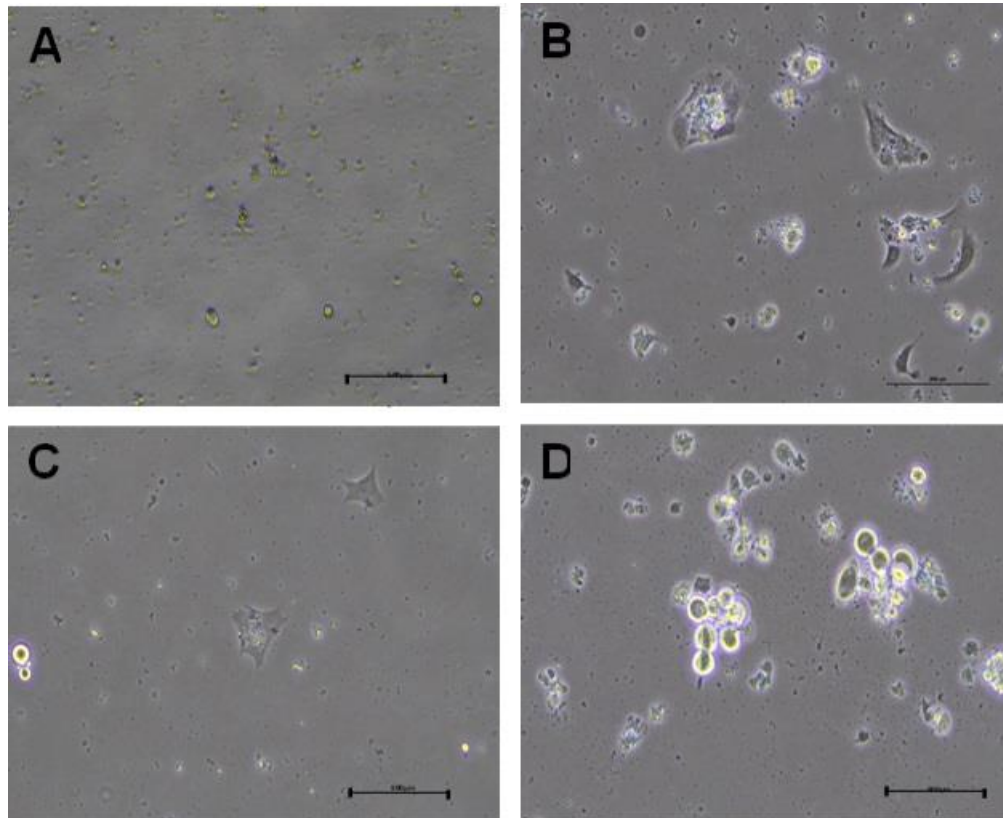


Fig 5.4- Primary Cells Grown in FBS coated plate. (A) The blastomeres isolated from embryos on day 1. (B, C) Cell aggregates formation on day 2 and 3. (D) Depletion of cell aggregates was observed on day 5

To address the contamination issue, the washing steps were increased using 2X concentration of antibiotic solution (Streptomycin and Penicillin) along with anti-fungal (Amphotericin B) agent and by this method the contamination was avoided. Instead of FBS coating, the plates were coated with 0.1% gelatin and after one hour the cells were seeded. Moreover, in this method instead of 10% DMEM medium, 15% medium was used. In addition, a new method was employed to collect the

cells. In the former method, the embryos were homogenized and strained using sterile cell strainer with the mesh size of 40 μ m. In the current method instead of straining, the embryos were homogenized and subjected to centrifugation at 1300 rpm for 5 minutes. The supernatant was discarded and the pellet was carefully suspended in 15% media.

Notably, the centrifugation method, utilizing 100 embryos, resulted in significantly improved cell growth compared to the straining method (Fig 5.5 and Fig 5.6). Numerous cell clumps were observed in the centrifugation method, whereas only one or two were observed in the straining method.

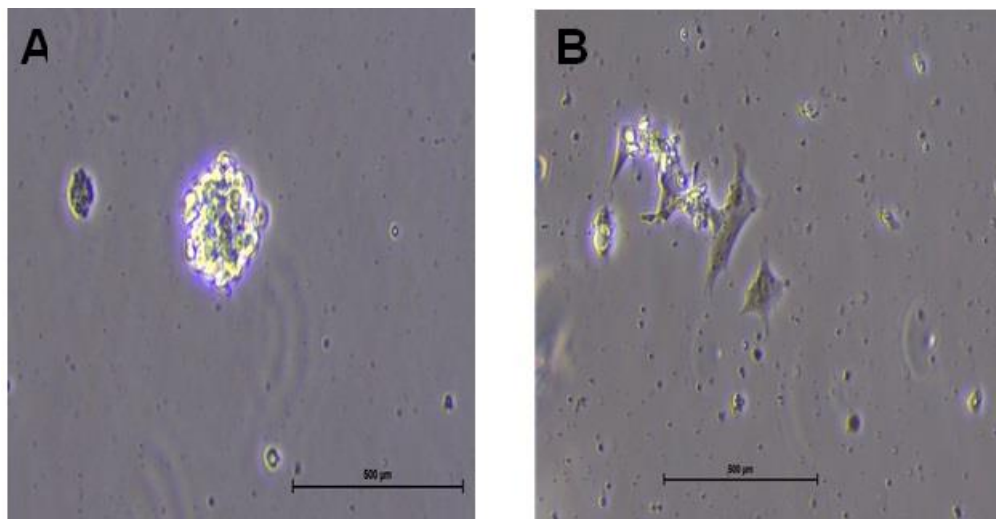


Fig 5.5- Primary Cells cultured in 0.1% Gelatin-Coated plate by Strained method.

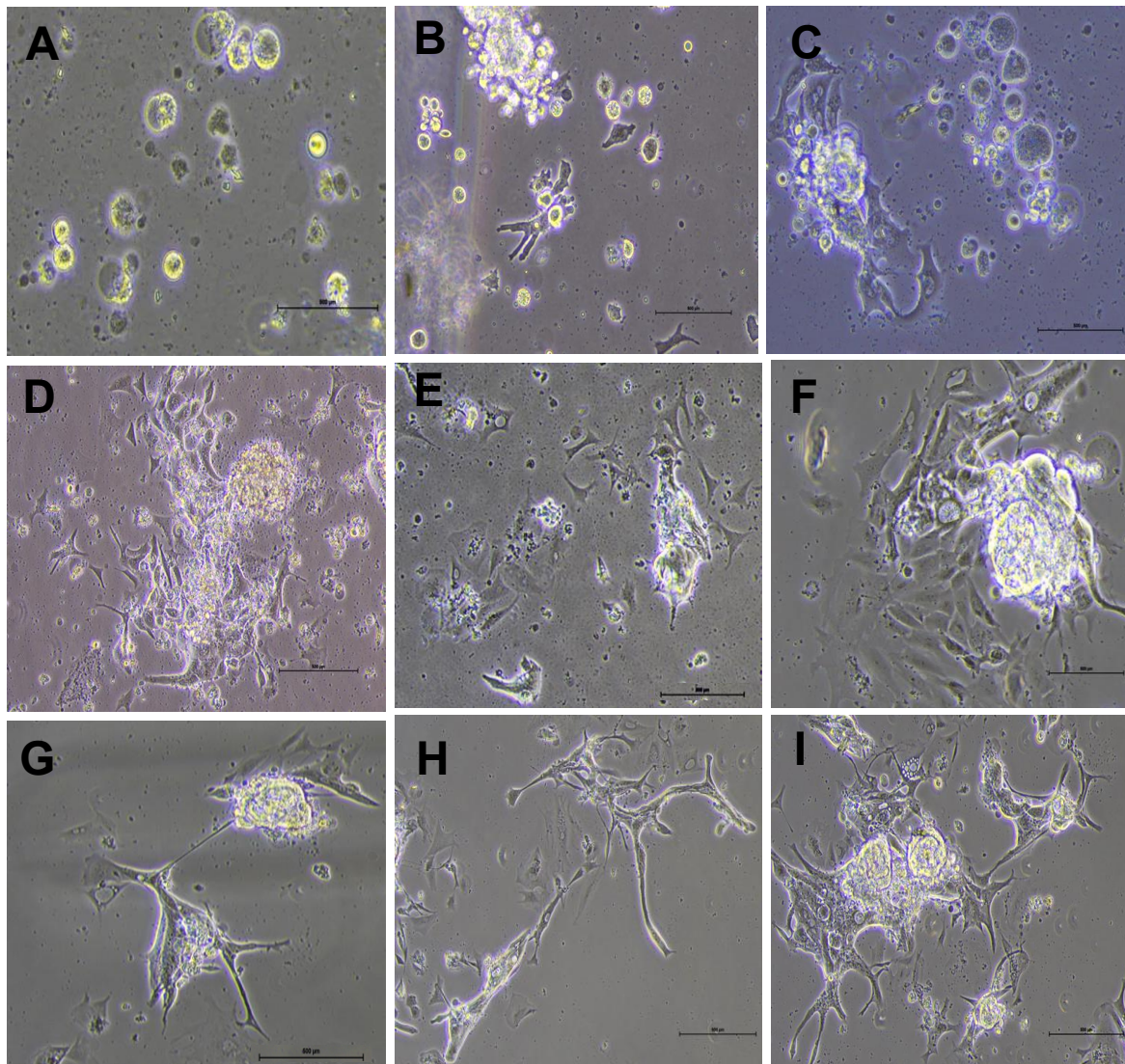


Fig 5.6- Primary Cells Cultured in 0.1% Gelatin-Coated plate by centrifugation method. Here the cells in Figures A, B, and C were observed on the second day after the seeding. D and E were observed on the third day. Fig F-I was observed on the fourth day. In comparison with the previous method, the cell growth was significant in the current method.

Furthermore, diverse cell morphologies were observed in the centrifugation method (Fig 5.7) till the tenth day after which the cells depleted (Fig 5.8).

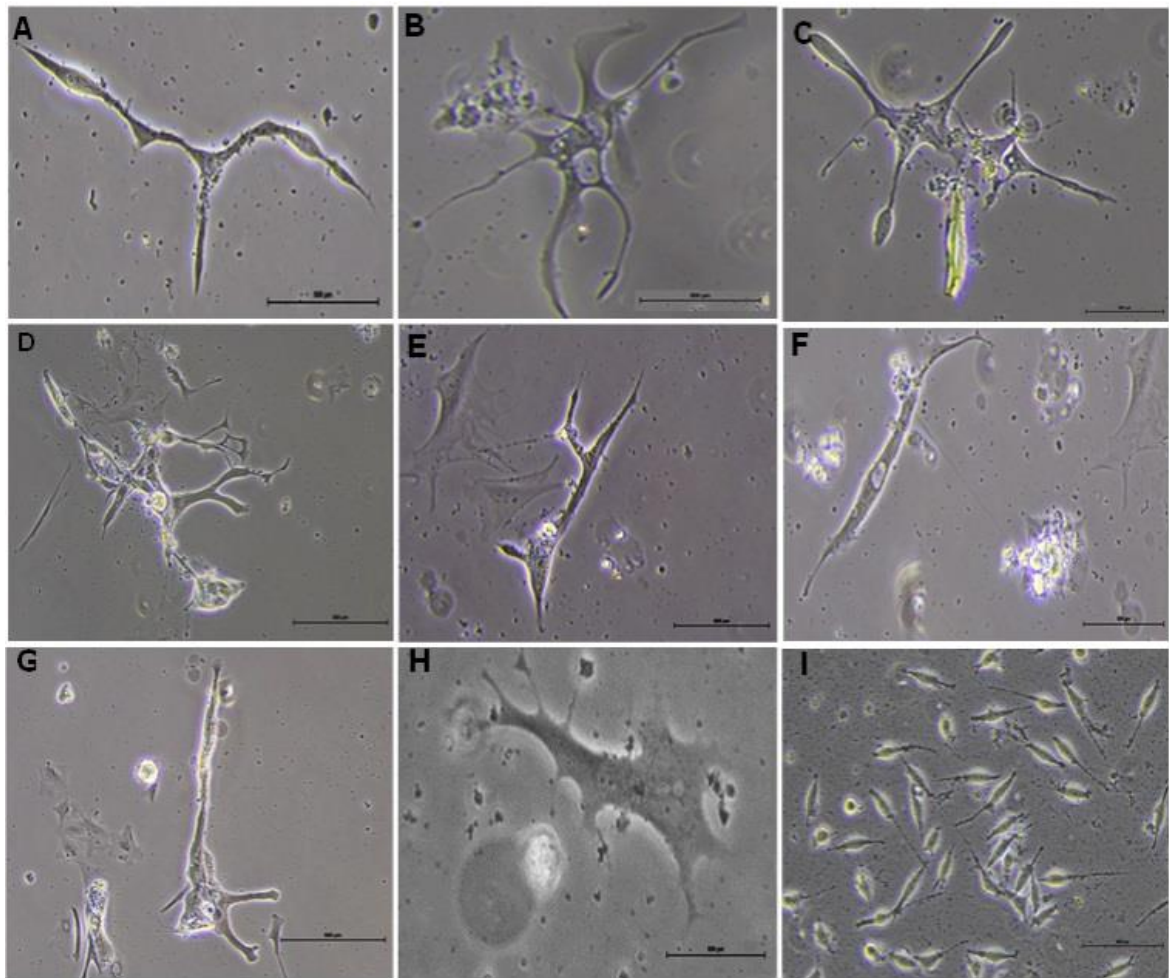


Fig5.7- Different Morphologies of Cells

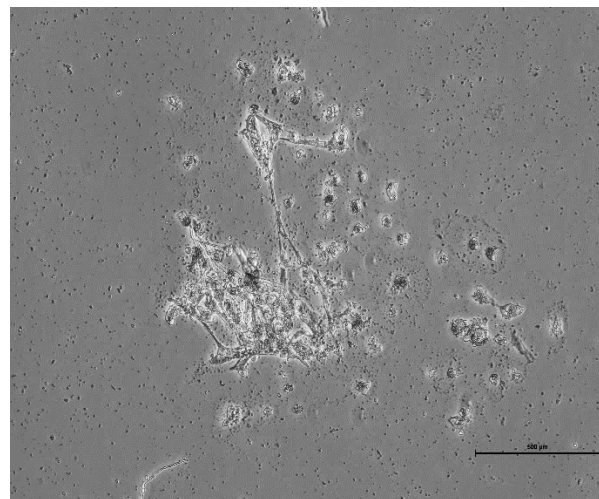


Fig 5.8- Cells Depleted on 11th Day

In comparison with the previous method, optimized protocol resulted in significant improvement in cell growth and viability, enabling the sustained proliferation of cells thereby providing a solid foundation for further experimentation and investigation.

The cultivation and maintenance of zebrafish embryonic stem (ES) cells have traditionally relied on complex feeder layers and specific culture conditions to ensure pluripotency and viability. Fan *et al.* (2004) demonstrated that zebrafish ES cells, when supported by zebrafish embryonic fibroblasts or rainbow trout spleen cells as feeder layers, displayed self-renewal and pluripotent properties. However, these cells experienced a significant loss in the expression of the pluripotency marker *pou2* upon removal from their feeder layer, indicating a dependency on the feeder layer for maintaining their stem cell characteristics.

Similarly, Xing *et al.* (2008) reported that zebrafish ESC-like cells, initially derived using feeder layers, showed reduced pluripotency and tended towards differentiation when cultured in feeder-free media. These findings suggest that the traditional feeder layer-based methods, while effective in some respects, may not fully support the long-term maintenance and pluripotency of ES cells under altered culture conditions. In contrast, our study employs a novel approach by deriving a zebrafish Neural Stem Cell-like (NSC-like) line from blastomeres during the blastula phase, cultured distinctly without the use of feeder cells and using only a 0.1% gelatin layer. This method diverges significantly from the traditional feeder layer-based techniques.

The derivation of stem cell lines from zebrafish embryos has been explored in various studies, with a notable example provided by Ho *et al.* (2014). In their research, zebrafish ESC-like cell lines were successfully derived from blastomeres harvested at the blastula stage. Notably, these cells formed homogenous aggregates within 3 to 5 hours after seeding, and the majority of these aggregates subsequently attached to the culture surface within 24 to 30 hours. This pattern of rapid aggregation and attachment is indicative of the cells' inherent properties for self-renewal and possibly their pluripotency.

Our study bears similarities to the research conducted by Ho *et al.* but with some distinct differences in outcomes and observations. Similar to their findings, our zebrafish neural stem cell-like (NSC-like) cells were isolated from blastomeres at the blastula stage. However, while Ho *et al.* observed the formation of homogenous aggregates shortly after seeding, our study documented distinct morphological features in isolated cells that were evident 24 hours post-isolation. This difference

in cellular behavior and morphology might suggest variations in the intrinsic cellular properties between ESC-like and NSC-like cells, or it could reflect differences in the cellular environment or the methodology applied during the initial culture setup.

Following the isolation of primary cells, we performed immunofluorescence assay. This involved probing for the presence of specific biomarkers, such as *SOX2* and *TUJ1*, which are indicative of neural stem cells and neuronal differentiation, respectively.

5.2 IMMUNOFLUORESCENCE

After isolating cells displaying characteristics suggestive of neural stem cells, a thorough immunofluorescence analysis was performed to confirm their identity. This analysis employed specific markers, *SOX2* and *TUJ1*, along with Alexa Fluor 488 as a secondary antibody, to identify cellular expression patterns.

5.2.1 RESULTS FOR SOX2

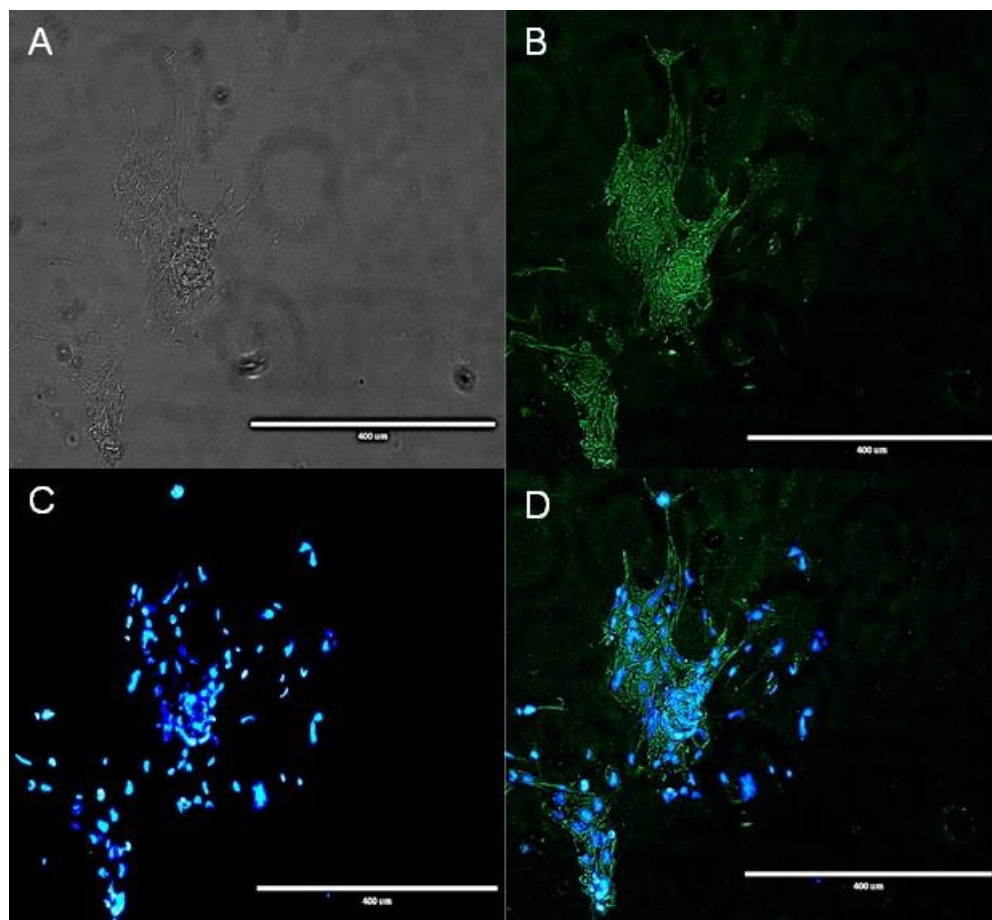


Fig 5.9- SOX2 result

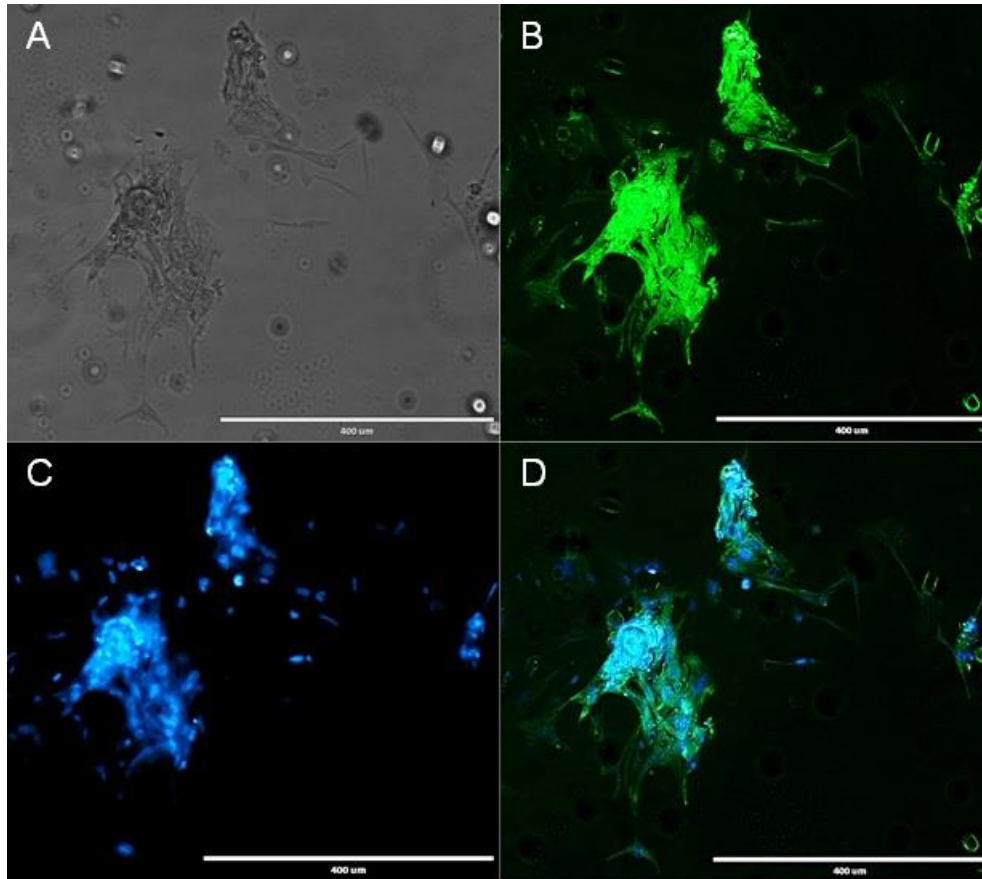


Fig 5.10- SOX2 result

In **Fig 5.9 and 5.10**, **(A)** The transmitted light image provides a clear view of the overall cell morphology and integrity. **(B)** The green channel specifically showed intense fluorescence within the nuclei of several cells, indicating a high expression of SOX2. **(C)** DAPI staining revealed well-defined nuclei in all cells. This uniform staining is crucial for accurately assessing the specificity and localization of the SOX2 signal in the green channel) and **(D)** The green fluorescence of SOX2 overlapped extensively with the blue DAPI signal, confirming that SOX2 expression is predominantly nuclear. The absence of green fluorescence in non-nuclear regions further supports the specificity of the SOX2 antibody and the absence of non-specific binding of the secondary antibody

The initial finding of the analysis revealed distinct green fluorescence signals localized within the cell, indicating the expression of SOX2. This observation clearly indicates the stem cell identity, highlighting their ability to maintain an undifferentiated state.

5.2.2 RESULTS FOR β III-TUBULIN (TUJ1)

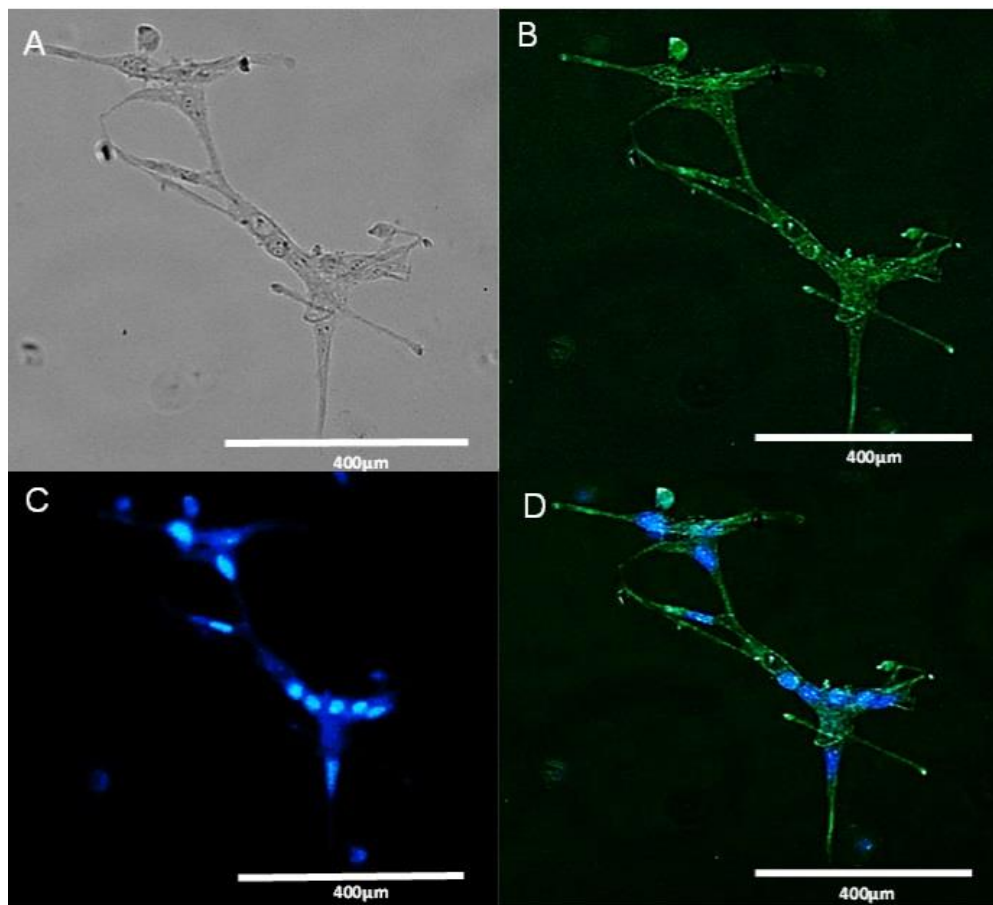


Fig 5.11- β III-TUBULIN result

In **Fig 5.11 and 5.12**, **(A)** The transmitted light image provides a clear view of the overall cell morphology and integrity. **(B)** The green channel specifically showed intense fluorescence within the nuclei of several cells, indicating a high expression of *TUJ1*. **(C)** The DAPI staining in these images reveals the nuclei of all cells present, providing a backdrop against which the specific neuronal staining can be assessed. Well-defined, round-to-oval nuclei are typical for neurons, and these should align with *TUJ1* staining where neuronal differentiation is successful. **(D)** The green fluorescence signals dispersed throughout the cell cytoplasm were observed, indicating *TUJ1* expression.

TUJ1, known as β III-tubulin, a recognized marker of neuronal differentiation. The detection of *TUJ1* expression in the cytoplasm suggested the initiation of neuronal commitment and maturation within the cell population, reinforcing their potential for neural lineage commitment.

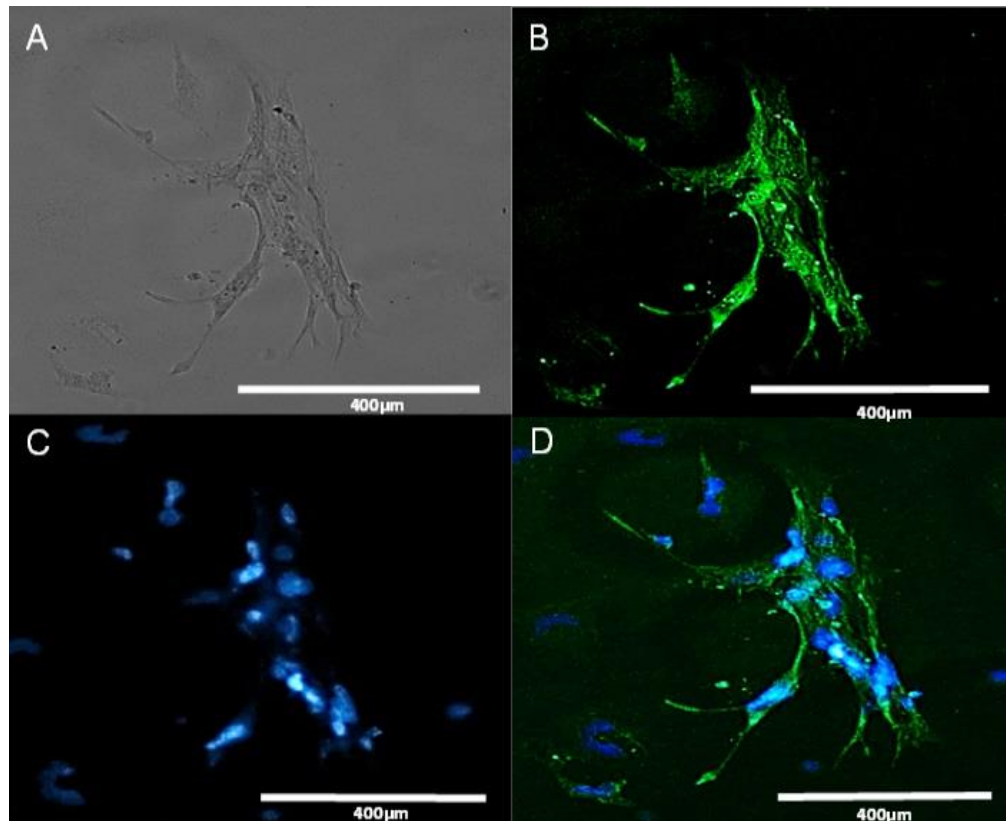


Fig 5.12- β III-TUBULIN result

Our findings on the expression of *SOX2* and *TUJ1* in the NSC-like cells are significant. *SOX2* is a well-documented marker for pluripotency in ESCs and has been shown to play a crucial role in maintaining the self-renewal and undifferentiated state of stem cells in various species (Avilion *et al.*, 2003). The presence of *TUJ1*, a neuronal marker, further highlights the differentiation potential of these NSC-like cells towards neural lineages, which is crucial for their application in neurodegenerative disease models or regenerative therapies. This dual expression aligns with previous findings in the field where neural stem cells derived from other species also exhibit markers indicative of both stemness and lineage-specific differentiation potential (Conti *et al.*, 2005).

5.3 CELLOGER AUTOMATED LIVE CELL IMAGING SYSTEM

In our study, Celloger time-lapse imaging was employed to monitor the dynamics of cell emergence from clumps and the morphological features of isolated neural stem cells and also to check the proliferative potential of ar- turmerone in isolated cells. Initial observations indicated that within the clumps, individual cells began to emerge over the course of several days. These emerging cells displayed a variety of morphological characteristics, consistent with the pluripotent nature of stem cells (Fig 5.13 and 5.14). Notably, cells transitioned from a compact morphology to more extended and differentiated forms, suggesting successful stem cell activation and differentiation.

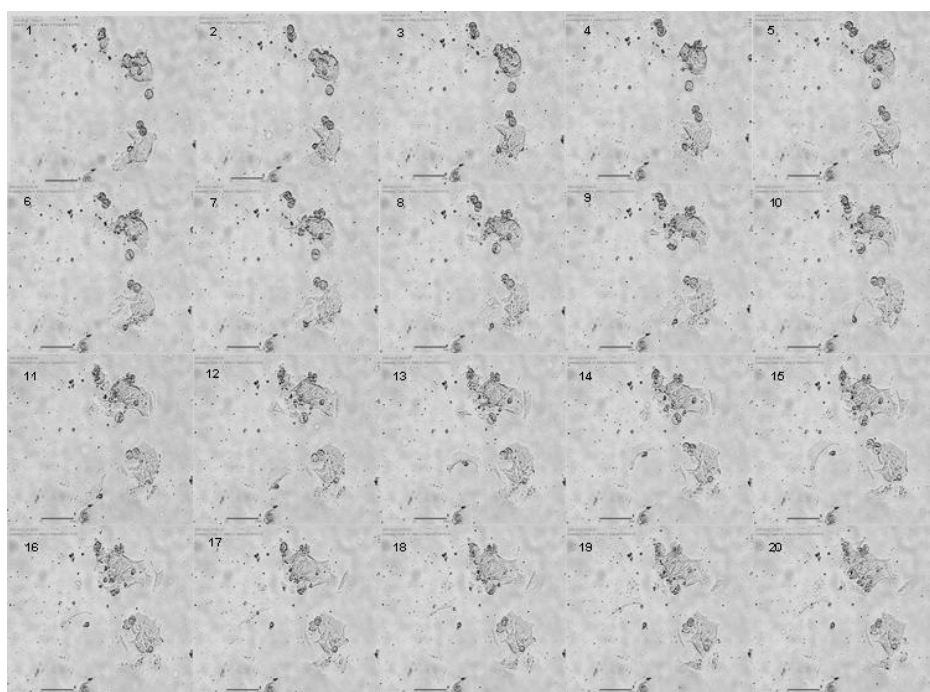


Fig 5.13- Image tile of Control

Figure 5.13 illustrates the emergence of cells from clumps. Various types of cells, each with distinct morphological features, were observed to emerge from the cell clumps. This signifies that the cultured cell clumps possess stem cell properties, as they have the ability to differentiate into various types of cells.

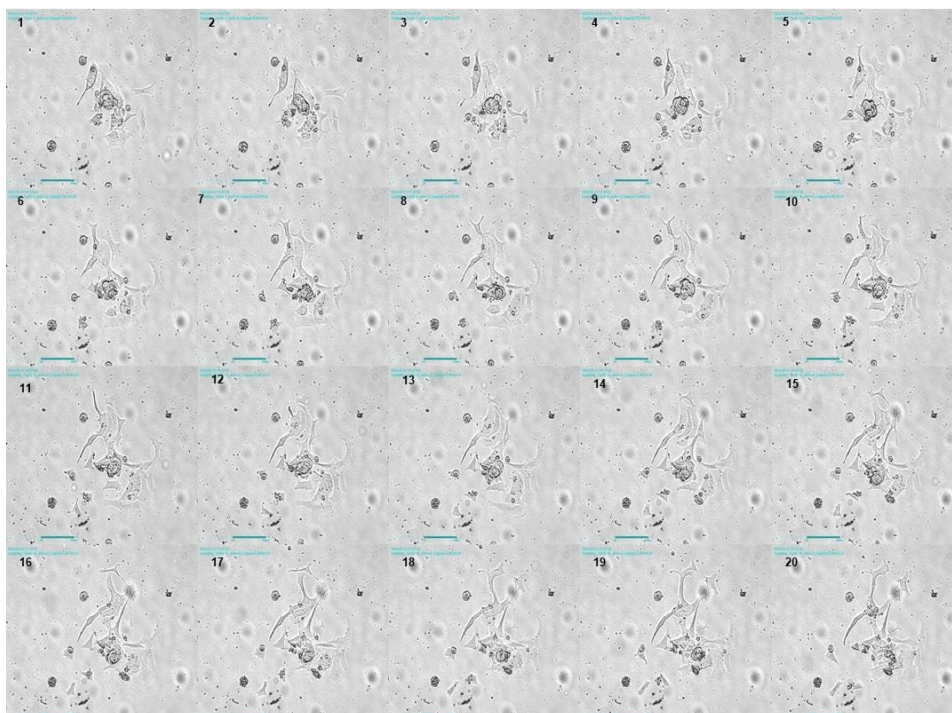


Fig 5.14- Image tile of ar- turmerone treated cells

Compared to control (Fig 5.13) the cells treated with ar- turmerone (Fig 5.14) showed more proliferation. To further understand how ar- turmerone influences neural stem cell-like cells proliferation qPCR was done.

5.4 GENE EXPRESSION

To elucidate the effects of aromatic turmerone on stem cells proliferation, qRT-PCR was employed to assess the expression of key stem cell markers. The genes selected for this study, POU5F1 (also known as OCT4), LIN28, and NANOG, are critically involved in maintaining pluripotency and self-renewal capacities in stem cells. The qPCR experiments were conducted on both aromatic turmerone treated and untreated neural stem cells. RNA was extracted from these cells after treatment and converted to cDNA, which served as the template for qPCR. This methodology aims to quantify the relative changes in gene expression induced by aromatic turmerone treatment, focusing on the selected genes. POU5F1 maintains the undifferentiated state of the stem cells by regulating the expression of genes. POU5F1 expression serves as a validation step to confirm the pluripotent state of stem cells. High expression of POU5F1 indicates the presence of undifferentiated pluripotent cells. (Fig 5.15), The presence of OCT4 at the transcriptional level

suggests that the neural stem cell-like cells retain a pluripotent state. OCT4 is crucial for maintaining an undifferentiated state in stem cells, and its expression indicates that these cells have not yet committed to a specific differentiation path. LIN28 present in various cell types but its expression level vary on development stage. It is particularly abundant in embryonic development and decreases in abundant as cells differentiate into specialized cell types. Its expression implies that the cells are likely maintaining a youthful, undifferentiated state conducive to stem cell proliferation and possibly resisting premature differentiation. Similar to POU5F1, the expression of NANOG is indicative of cells maintaining pluripotency. NANOG supports the cellular framework necessary to sustain self-renewal and pluripotency, further reinforcing the undifferentiated status of your neural stem cell-like cells. (Fig 5.15) shows that the expression of genes (POU5F1, LIN28 and NANOG) was hiked in aromatic turmerone treated isolated neural stem cell-like cells compared to Control.

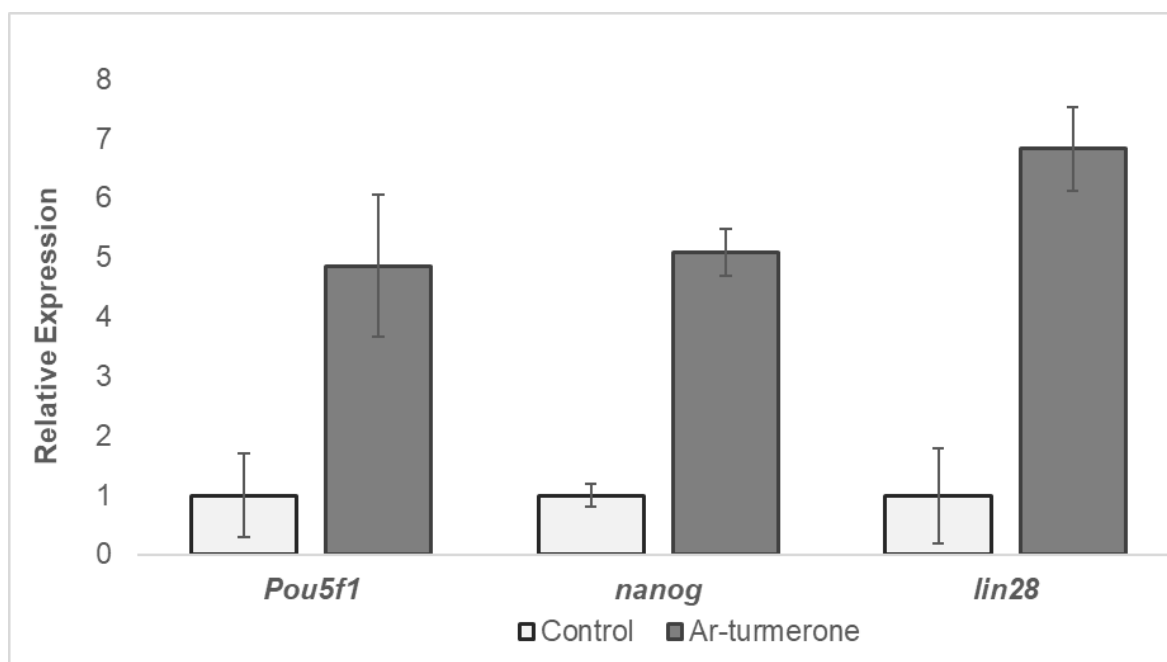


Fig 5.15- Bar graphs illustrate the relative expression levels of *Pou5f1*, *Lin28*, and *Nanog* in cells treated with aromatic turmerone versus those that were untreated, revealing a substantial increase in the treated group.

5.5 IMMUNOBLOTTING

Immunoblotting was performed to evaluate the expression of neuronal markers in isolated neural stem cell-like cells, both untreated and treated with aromatic turmerone. Protein lysates prepared from these cells were probed with antibodies specific to neuronal markers, including *TUJ1*, to determine the effects of aromatic turmerone on the protein expression level.

In the immunoblots, the untreated neural stem cell-like cells (Fig 5.16 LANE 1) displayed a baseline expression level of *TUJ1*, indicated by a distinct band. This band serves as a control, demonstrating the inherent expression of neuronal proteins in the isolated cells, which confirms their neural stem cell-like characteristics. Comparatively, the aromatic turmerone-treated neural stem cell-like cells (Fig 5.16 LANE 2) showed a more intense band for *TUJ1*, indicating an increased expression level of this protein. The enhanced band intensity in the turmerone-treated samples suggests that aromatic turmerone positively influences the proliferation or differentiation of these cells towards neuronal lineages, possibly by promoting protein synthesis or stabilizing the protein against degradation.



Fig 5.16- Blotting result. LANE 1 (Control) and LANE 2(aromatic turmerone)

The positive effect of aromatic turmerone on the proliferation of NSC-like cells observed in our study provides new insights into the possible applications of natural compounds in stem cell biology. Previous studies have demonstrated the role of aromatic turmerone in promoting neural stem cell proliferation in vitro and facilitating recovery in neurodegenerative disease models (Hucklenbroich *et al.*, 2014). The ability of aromatic turmerone to enhance proliferation without compromising the expression of key pluripotency and differentiation markers suggests its potential utility as an adjunct in stem cell culture systems, aimed at increasing cell yields while maintaining cellular integrity and functionality.

CHAPTER 6

SUMMARY AND CONCLUSIONS

This study has successfully detailed the derivation and maintenance of a novel zebrafish Neural Stem Cell-like (NSC-like) line obtained from blastomeres of blastula phase embryos, cultured without the reliance on feeder cells, using only a 0.1% gelatin layer. Cultivated in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum, these cells exhibited diverse morphological characteristics, notably forming tightly packed cell clumps. Through immunostaining, we confirmed the expression of SOX2, a marker of pluripotency in mammalian embryonic stem cells and *TUJ1*, a neuronal marker, thus verifying the retention of critical stem cell properties in these NSC-like cells. Further investigation into the effects of aromatic turmerone revealed its significant role in enhancing cell proliferation, as evidenced by quantitative RT-PCR and immunoblotting. The findings from this study unequivocally establish that the cells we have isolated represent a Neural Stem Cell-like line. Additionally, the pronounced proliferation-promoting effect of aromatic turmerone on this line underscores its potential utility in regenerative medicine, offering valuable insights for both fundamental research and clinical applications. This research paves the way for future explorations into stem cell-based therapies and highlights aromatic turmerone as a promising agent for enhancing stem cell proliferation.

REFERENCES

- Ammon, H.P. and Wahl, M.A., 1991. Pharmacology of Curcuma longa. *Planta medica*, 57(01), pp.1-7.
- Amsterdam, A., Nissen, R.M., Sun, Z., Swindell, E.C., Farrington, S. and Hopkins, N., 2004. Identification of 315 genes essential for early zebrafish development. *Proceedings of the National Academy of Sciences*, 101(35), pp.12792-12797.
- Atala, A., 2012. Regenerative medicine strategies. *Journal of pediatric surgery*, 47(1), pp.17-28.
- Avilion, A.A., Nicolis, S.K., Pevny, L.H., Perez, L., Vivian, N. and Lovell-Badge, R., 2003. Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes & development*, 17(1), pp.126-140.
- Chen, Z., Lee, H., Henle, S.J., Cheever, T.R., Ekker, S.C. and Henley, J.R., 2013. Primary neuron culture for nerve growth and axon guidance studies in zebrafish (*Danio rerio*). *PloS one*, 8(3), p.e57539.
- Conti, L. and Cattaneo, E., 2010. Neural stem cell systems: physiological players or *in vitro* entities?. *Nature Reviews Neuroscience*, 11(3), pp.176-187.
- Doetsch, F., 2003. The glial identity of neural stem cells. *Nature neuroscience*, 6(11), pp.1127-1134.
- Driever, W., Solnica-Krezel, L., Schier, A.F., Neuhauss, S.C.F., Malicki, J., Stemple, D.L., Stainier, D.Y.R., Zwartkruis, F., Abdelilah, S., Rangini, Z. and Belak, J., 1996. A genetic screen for mutations affecting embryogenesis in zebrafish. *Development*, 123(1), pp.37-46.
- Edmondson, R., Broglie, J.J., Adcock, A.F. and Yang, L., 2014. Three-dimensional cell culture systems and their applications in drug discovery and cell-based biosensors. *Assay and drug development technologies*, 12(4), pp.207-218.
- Engeszer, R.E., Patterson, L.B., Rao, A.A. and Parichy, D.M., 2007. Zebrafish in the wild: a review of natural history and new notes from the field. *Zebrafish*, 4(1), pp.21-40.

- Fan, L., Crodian, J., Liu, X., Aleström, A., Aleström, P. and Collodi, P., 2004. Zebrafish embryo cells remain pluripotent and germ-line competent for multiple passages in culture. *Zebrafish*, 1(1), pp.21-26.
- Fang, F., 2003. Phylogenetic analysis of the Asian cyprinid genus *Danio* (Teleostei, Cyprinidae). *Copeia*, 2003(4), pp.714-728.
- Freshney, R.I., 2015. *Culture of animal cells: a manual of basic technique and specialized applications*. John Wiley & Sons.
- Fuchs, E. and Segre, J.A., 2000. Stem cells: a new lease on life. *Cell*, 100(1), pp.143-155.
- Gage, F.H., 2000. Mammalian neural stem cells. *Science*, 287(5457), pp.1433-1438.
- Gillet, J.P., Varma, S. and Gottesman, M.M., 2013. The clinical relevance of cancer cell lines. *Journal of the National Cancer Institute*, 105(7), pp.452-458.
- Gorissen, M., Manuel, R., Pelgrim, T.N., Mes, W., de Wolf, M.J., Zethof, J., Flik, G. and van den Bos, R., 2015. Differences in inhibitory avoidance, cortisol and brain gene expression in TL and AB zebrafish. *Genes, Brain and Behavior*, 14(5), pp.428-438.
- Goshi, N., Morgan, R.K., Lein, P.J. and Seker, E., 2020. A primary neural cell culture model to study neuron, astrocyte, and microglia interactions in neuroinflammation. *Journal of neuroinflammation*, 17, pp.1-16.
- Gupta, S. K., et al. (2013). *Curcuma longa* (Turmeric): A review. **Pharmacognosy Reviews**, 7(13), 1-6.
- Haeckel, E., 1868. *Natürliche Schöpfungsgeschichte*, Georg Reimer. *Berlin, Germany*.
- Hamilton, F., 1822. *An account of the fishes found in the river Ganges and its branches* (Vol. 1). Archibald Constable.
- Ho, S.Y., Goh, C.W.P., Gan, J.Y., Lee, Y.S., Lam, M.K.K., Hong, N., Hong, Y., Chan, W.K. and Shu-Chien, A.C., 2014. Derivation and long-term culture of an

- embryonic stem cell-like line from zebrafish blastomeres under feeder-free condition. *Zebrafish*, 11(5), pp.407-420.
- Howe, K., Clark, M.D., Torroja, C.F., Tarrance, J., Berthelot, C., Muffato, M., Collins, J.E., Humphray, S., McLaren, K., Matthews, L. and McLaren, S., 2013. The zebrafish reference genome sequence and its relationship to the human genome. *Nature*, 496(7446), pp.498-503.
- Huang, H., Lindgren, A., Wu, X., Liu, N.A. and Lin, S., 2012. High-throughput screening for bioactive molecules using primary cell culture of transgenic zebrafish embryos. *Cell reports*, 2(3), pp.695-704.
- Hucklenbroich, J., Klein, R., Neumaier, B., Graf, R., Fink, G.R., Schroeter, M. and Rueger, M.A., 2014. Aromatic-turmerone induces neural stem cell proliferation *in vitro* and in vivo. *Stem cell research & therapy*, 5, pp.1-9.
- Hwang, W.Y., Fu, Y., Reyon, D., Maeder, M.L., Tsai, S.Q., Sander, J.D., Peterson, R.T., Yeh, J.J. and Joung, J.K., 2013. Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nature biotechnology*, 31(3), pp.227-229.
- Imayoshi, I. and Kageyama, R., 2011. The role of Notch signaling in adult neurogenesis. *Molecular neurobiology*, 44, pp.7-12.
- Jung, D.W., Oh, E.S., Park, S.H., Chang, Y.T., Kim, C.H., Choi, S.Y. and Williams, D.R., 2012. A novel zebrafish human tumor xenograft model validated for anti-cancer drug screening. *Molecular BioSystems*, 8(7), pp.1930-1939.
- Kettleborough, R.N., Busch-Nentwich, E.M., Harvey, S.A., Dooley, C.M., De Bruijn, E., Van Eeden, F., Sealy, I., White, R.J., Herd, C., Nijman, I.J. and Fényes, F., 2013. A systematic genome-wide analysis of zebrafish protein-coding gene function. *Nature*, 496(7446), pp.494-497.
- Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B. and Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. *Developmental dynamics*, 203(3), pp.253-310.
- Kitani, H., Shiurba, R., Sakakura, T. and Tomooka, Y., 1991. Isolation and characterization of mouse neural precursor cells in primary culture. *In Vitro Cellular & Developmental Biology-Animal*, 27, pp.615-624..

- Kumar, A. and Mallick, B.N., 2016. Long-term primary culture of neurons taken from chick embryo brain: A model to study neural cell biology, synaptogenesis and its dynamic properties. *Journal of Neuroscience Methods*, 263, pp.123-133.
- Lieschke, G.J. and Currie, P.D., 2007. Animal models of human disease: zebrafish swim into view. *Nature Reviews Genetics*, 8(5), pp.353-367.
- Lindvall, O. and Kokaia, Z., 2010. Stem cells in human neurodegenerative disorders—time for clinical translation?. *The Journal of clinical investigation*, 120(1), pp.29-40.
- Liju, V. B., et al. (2014). Chemical composition and antimicrobial activity of the essential oil of *Curcuma longa* L. *Indian Journal of Pharmaceutical Sciences*, 76(6), 556-560.
- Marchetto, M.C., Winner, B. and Gage, F.H., 2010. Pluripotent stem cells in neurodegenerative and neurodevelopmental diseases. *Human molecular genetics*, 19(R1), pp.R71-R76..
- Martins, C. V. B., et al. (2009). *Curcuma longa* L. essential oil composition and antimicrobial activity. *Pharmaceutical Biology*, 47(11), 1059-1063.
- Meade, M.E., Roginsky, J.E. and Schulz, J.R., 2019. Primary cell culture of adult zebrafish spinal neurons for electrophysiological studies. *Journal of neuroscience methods*, 322, pp.50-57.
- Morrison, S.J. and Spradling, A.C., 2008. Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. *Cell*, 132(4), pp.598-611.
- Myhre, J.L. and Pilgrim, D.B., 2010. Cellular differentiation in primary cell cultures from single zebrafish embryos as a model for the study of myogenesis. *Zebrafish*, 7(3), pp.255-266.
- Nüsslein-Volhard, C., 1994. Of flies and fishes. *Science*, 266(5185), pp.572-574.
- Pampaloni, F., Reynaud, E.G. and Stelzer, E.H., 2007. The third dimension bridges the gap between cell culture and live tissue. *Nature reviews Molecular cell biology*, 8(10), pp.839-845.

- Poliwoda, S., Noor, N., Downs, E., Schaaf, A., Cantwell, A., Ganti, L., Kaye, A.D., Mosel, L.I., Carroll, C.B., Viswanath, O. and Urits, I., 2022. Stem cells: a comprehensive review of origins and emerging clinical roles in medical practice. *Orthopedic reviews*, 14(3).
- Pouton, C.W. and Haynes, J.M., 2007. Embryonic stem cells as a source of models for drug discovery. *Nature Reviews Drug Discovery*, 6(8), pp.605-616.
- Postlethwait, J.H., Woods, I.G., Ngo-Hazelett, P., Yan, Y.L., Kelly, P.D., Chu, F., Huang, H., Hill-Force, A. and Talbot, W.S., 2000. Zebrafish comparative genomics and the origins of vertebrate chromosomes. *Genome research*, 10(12), pp.1890-1902.
- Reynolds, B.A. and Weiss, S., 1992. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *science*, 255(5052), pp.1707-1710.
- Russo, G., Lehne, F., Méndez, S.M.P., Dübel, S., Köster, R.W. and Sassen, W.A., 2018. Culture and transfection of Zebrafish primary cells. *JoVE (Journal of Visualized Experiments)*, (138), p.e57872.
- Sassen, W.A., Lehne, F., Russo, G., Wargenau, S., Dübel, S. and Köster, R.W., 2017. Embryonic zebrafish primary cell culture for transfection and live cellular and subcellular imaging. *Developmental biology*, 430(1), pp.18-31.
- Spence, R., Gerlach, G., Lawrence, C. and Smith, C., 2008. The behaviour and ecology of the zebrafish, *Danio rerio*. *Biological reviews*, 83(1), pp.13-34.
- Steinbeck, J.A. and Studer, L., 2015. Moving stem cells to the clinic: potential and limitations for brain repair. *Neuron*, 86(1), pp.187-206.
- Sun, L., Bradford, C.S., Ghosh, C., Collodi, P. and Barnes, D.W., 1995. ES-like cell cultures derived from early zebrafish embryos. *Molecular marine biology and biotechnology*, 4(3), pp.193-199.
- Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K. and Yamanaka, S., 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *cell*, 131(5), pp.861-872.

- Takahashi, K. and Yamanaka, S., 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *cell*, 126(4), pp.663-676.
- Temple, S., 2001. The development of neural stem cells. *Nature*, 414(6859), pp.112-117.
- Tetzlaff, W., Okon, E.B., Karimi-Abdolrezaee, S., Hill, C.E., Sparling, J.S., Plemel, J.R., Plunet, W.T., Tsai, E.C., Baptiste, D., Smithson, L.J. and Kawaja, M.D., 2011. A systematic review of cellular transplantation therapies for spinal cord injury. *Journal of neurotrauma*, 28(8), pp.1611-1682.
- Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S. and Jones, J.M., 1998. Embryonic stem cell lines derived from human blastocysts. *science*, 282(5391), pp.1145-1147.
- Weissman, I.L., 2000. Stem cells: units of development, units of regeneration, and units in evolution. *cell*, 100(1), pp.157-168.
- Welzel, G., Seitz, D. and Schuster, S., 2015. Magnetic-activated cell sorting (MACS) can be used as a large-scale method for establishing zebrafish neuronal cell cultures. *Scientific reports*, 5(1), p.7959.
- Xing, J.G., Lee, L.E., Fan, L., Collodi, P., Holt, S.E. and Bols, N.C., 2008. Initiation of a zebrafish blastula cell line on rainbow trout stromal cells and subsequent development under feeder-free conditions into a cell line, ZEB2J. *Zebrafish*, 5(1), pp.49-63.
- Zhao, X. and Moore, D.L., 2018. Neural stem cells: developmental mechanisms and disease modeling. *Cell and tissue research*, 371, pp.1-6.
- Zon, L.I. and Peterson, R.T., 2005. In vivo drug discovery in the zebrafish. *Nature reviews Drug discovery*, 4(1), pp.35-44.