

Understanding the key proteins that regulate morphallaxis mechanism in earthworm, *Eudrilus eugeniae*

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

By

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APRIL - 2024



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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of VIJAYALAKSHMI S (42101014) who carried out Project entitled "Understanding the key proteins that regulate morphallaxis mechanism in earthworm, *Eudrilus eugeniae*" under the supervision from September 2023 to April 2024.

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DECLARATION

I, **VIJAYALAKSHMI S (42101014)** hereby declare that the Project Report entitled "**Understanding the key proteins that regulate morphallaxis mechanism in earthworm, *Eudrilus eugeniae***" done by me under the guidance of **Dr. S. Jackson Durairaj** 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 Degree in Medical Biotechnology and Clinical Research.

DATE: 18/04/2024

PLACE: Chennai

A handwritten signature in blue ink, appearing to read 'Vijayalakshmi S.', is written over a circular blue stamp.

SIGNATURE OF THE CANDIDATE

ACKNOWLEDGEMENT

I am pleased to acknowledge my sincere thanks to **The Board of Management of Sathyabama Institute of Science and Technology**, Chennai, for their kind encouragement in doing this project and for completing it successfully. I am grateful to them.

I convey my thanks to **Dr. A. DAYANANDAN Professor (Research)**, Head of the Department, Centre for Molecular and Nanomedical sciences for providing me necessary support and details at the right time during the progressive reviews. I would also like to express my sincere and deep sense of gratitude for his constant support and encouragement during the advancement of my project work.

I am extremely grateful to **Dr S. JACKSON DURAIRAJ, Ph.D.**, my project guide for his valuable guidance, suggestions, and constant encouragement paved the way for the successful completion of my project work.

I am extremely thankful to **Dr. JOHNSON RETNARAJ SAMUEL Associate Professor (Research)**, **Dr. MADAN KUMAR Associate Professor (Research)** and **Dr. BERYL VEDHA Assistant Professor (Research)** for their valuable suggestions and support for the successful completion of my project work.

I wish to express my sincere thanks to all Research Scholars Teaching and Non-teaching staff members of the Centre for Molecular and Nanomedical sciences who were helpful in many ways for the completion of the project.

I thank my parents and family members for their continuous love and support for the completion of my project and master degree.

Vijayalakshmi S

ABSTRACT

Regeneration is a complex fascinating phenomenon and it involves either epimorphosis, morphallaxis or either both. The key regenerative proteins and their role are explored in regeneration mechanisms but not understood in morphallaxis pattern of regeneration. The present studies aim to block epimorphosis using 2mM thymidine and to understand the key proteins that are in morphallaxis. Initially, the worms are injection are with 2mM thymidine and observed for the regeneration ability. On 3rd and 7th day of post amputation, the regeneration is 1/3rd hindered in thymidine injected worms which represents the earthworm follows both epimorphosis and morphallaxis pattern of regeneration. The comparative histological sections confirm, in control worm the blastema cells are extended to form segments, but in thymidine injected worms, the proliferative mass of blastema is not extended to form the segments. The stemness markers like Wnt-3a and Oct-4 are overexpressed in morphallaxis mode of regeneration but another stemness marker c-Myc is notability downregulated. COX2, a key protein that modulate apoptosis is overexpressed in control regenerated worm but which is notability downregulated in morphallaxis. p53 a key protein involved in self-renewal and differentiation is notability upregulated upon morphallaxis. MEK1 plays a crucial role in cell death, proliferation, and differentiation. Our findings reveal that MEK1 is expressed in the epimorphosis block sample, suggesting its involvement in the regulation of cell death and differentiation, which is vital to successful morphallaxis. Thorough findings help us grasp the complex functions of morphallaix in earthworm *E. eugeniae*, which will contribute to advancements in regenerative medicine.

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ABBREVIATIONS

1. 1XPBS	1X Phosphate Buffer Saline
2. BCIP/NBT	Nitro-blue tetrazolium and 5-bromo-4-chloro-3'indolyphosphate
3. β -ME	Beta Mercapto Ethanol
4. SDS	Sodium Dodecyl Sulfide
5. 1X TBST	1X Tris Buffered Saline with Tween
6. ECL	Enhanced Chemiluminescence
7. IgG	Immunoglobulin G
8. IPA	Iso-Propyl Alcohol
9. RPM	Revolutions Per Minute
11. DPX	Distyrene Plasticizer Xylene
12. PVDF	Polyvinylidene fluoride or polyvinylidene difluoride

UNITS

1. **mm** - milli meter
2. **μg** - microgram
3. **μM** – micro-molar
4. **g** – grams
5. **m²** - meter square
6. **ml** – milliliter
7. **l** – liter
8. **V** – volts
9. **kDa** - kilodalton

CHAPTER – 1

INTRODUCTION

1.1 OVERVIEW OF EARTHWORM *EUDRILUS EUGENIAE*

Eudrilus eugeniae is one of the most abundant earthworm species in West African soils. The "African nightcrawler" is the popular name for this worm. It is also found in other regions of the world (Blakemore, R.J., 2015). This species is also earning more interest as a potential waste decomposer and protein source. More fundamental research into its general biology is required to properly confirm this species' significance as a waste decomposer (Reinecke, A. J., & Viljoen, S. A. 1988). Earthworms are the most prevalent soil invertebrates and can be found in many parts of the world, including temperate and tropical zones (Byambas *et.al*, 2019). *Eudrilus eugeniae* is a clitellum-dependent earthworm that requires intact clitellum segments for its survival and regeneration. The worm is an excellent model for cell and molecular biology research. It also has an exceptional ability to regenerate, the worm can be used to study the complicated cell and molecular biological events of regeneration. To survive and regenerate, the clitellum-dependent earthworm requires undamaged clitellum segments. It is an excellent source of protein and has great pharmaceutical potential. This species of earthworm is endemic to tropical West Africa, but it is now common in warm places because of vermicomposting.

<i>Eudrilus eugeniae</i> scientific taxonomy	
Kingdom	Animalia – Animal
Subkingdom	Bilateria
Infrakingdom	Protostomia
Superphyllum	Lophozoa
Phylum	Annelida - annelids,
Class	Clitellate Superorder
Superorder	Metagynophora

Order	Opisthopora
Suborder	Crassiclitellata
Family	Eudrilidae
Genus	Eudrilus
Species	<i>Eudrilus eugeniae</i> - African night crawler, African red
Geographic division	Africa, Australia, Caribbean, America, and Southern Asia

Table 1.1: Scientific taxonomy of the earthworm *Eudrilus eugeniae*

1.1.1 Description of an earthworm *Eudrilus eugenia* (Blakemore, R.J., 2015)

a) External morphological characteristics

- ❖ Body length: Complete matures, 90–185 mm, or up to 250–400 mm under excellent cultivation conditions; posterior tapering, becoming thinly flattened in terminal 'zone of growth'.
- ❖ Width: Approximately 4–8 mm in width.
- ❖ Mass: Average adult mass is around 1.0 grams, with an ideal maximum of 5.0– 6.0 grams.
- ❖ Color: Red-brown dorsum fading posteriorly; anterior with dazzling blue/green iridescent sheen from cuticle diffraction, ventrum beige, clitellum darker (occasionally lighter) than surroundings;
- ❖ Prostomium: A small, open epilogues prostomium.
- ❖ Clitellum: present in the 13, 14, 15–18th segments, and ventrally interrupted.

b) Internal morphological characteristics

- ❖ **Septa:** thickened from 4th to 5th segments; and from 6,7,8,9 and 14,15th segments.
- ❖ **Dorsal blood vessel:** Single, truncated before anterior hearts in the 7th

segment; connect to paired supra-oesophageal in 7–14th segment and paired extra-oesophageal medial to the hearts.

❖ **Hearts:** 7th segment is lateral, 8–11th segment is later-oesophageal, all distended with blood in certain specimens, the anterior hearts were undistended.

❖ **Gizzard:** Weakly muscular in 5th segment present just behind the pharyngeal bulk. ❖ **Intestine:** Origination in 14th or 15th or close to it. Caeca and typhlosole are not present. Small supra-intestinal glands seen in the 8th – 42nd segment of sections may aid digestion and/or play a role in worm immunity.

❖ **Nephridia:** Each segment from 4 has paired, big coiled holo nephridia that are not visibly vesiculate.

❖ **Prostates:** A big pair of digitiform prostates with a white muscular sheen from 18 to 23; sharply muscular enlargements of loops of paired sperm ducts that join to the apex of the copulatory chamber mound in the center. Although 3 it consists of two such glands, a smaller blind duct - the Y-shaped gland - joins to the base of the mound mesially.

❖ **Reproductive System:** Earthworms have two sexes. As a result, each individual has both male and female reproductive systems.

❖ **The male reproductive system:** It is made up of two pairs of testes (10-11 segments), a vasa deferentia (until the 18th segment), and two pairs of accessory glands (17th and 19th segments). A pair of male genital holes allow access to the prostate and spermatic ducts (18th segment). The spermatozoa are held in four pairs of spermathecae (6-9 segments).

❖ **The female reproductive system:** It is made up of a single set of ovaries and an oviduct. The ovaries open into an ovarian funnel that runs beneath them, joins the oviduct, and opens at the female genital aperture (14th segment).

1.2 REGENERATION

1.2.1 Regeneration and stem cell activities

Regeneration is the process of renewing or reviving damaged or amputated bodily parts of certain organisms (Shin SY et al., 2015). Regeneration can either return the entire organism or just the amputated body parts to their pre-amputation state (Santos Jr AR et al., 2014). The majority of this process will be carried out with the assistance of stem cells. It only happens in an organism with a simple structure and specialized cells. These specialized cells may drastically divide nature and produce a big number of cells in a short period of time. Every living body has the tendency to revive and nourish its tissues and organs as part of natural processes (Kolios G & Moodley Y., 2013). Earthworms have a strong capacity for regeneration. Unfortunately, mammals' regenerative abilities are limited, consisting of thick scar development, new skin and hair regeneration, bone fracture repair, and certain important organ regeneration. While stem cells (i.e pluripotent or tissue-specific stem cells) can help in regeneration, regeneration can also happen without them. Cell revival, restoration of actual differentiated cells, activation of native adult stem cells, and conversion of one cell type to another are the four components of 4 regeneration. Tissues or organs that are regenerating need one or more of the components listed above to restore their structure (Stocum DL., 2012). The regeneration mechanism includes a number of signaling components. Factors that alter cell cycle via various signaling transduction pathways, allow cell viability, apoptosis, or cellular destruction to be determined.

1.2.2 MORPHALLAXIS

Neural morphallaxis is a regenerative process characterized by wide-spread anatomical and physiological changes in an adult nervous system. During segmental regeneration of the annelid worm, *Lumbriculus variegatus*, neural morphallaxis involved a reorganization of sensory, interneuronal, and motor systems as posterior fragments gained a more anterior body position (Eduardo E. Zattara, Corey W. Rennolds) Functional recovery of neural networks after injury requires a series of signaling events similar to the embryonic processes that governed initial network construction. Neural morphallaxis, a form of

nervous system regeneration, involves reorganization of adult neural connectivity patterns. Neural morphallaxis in the worm, *Lumbriculus variegatus*, occurs during asexual reproduction and segmental regeneration, as body fragments acquire new positional identities along the anterior-posterior axis(Jia wang,Haopeng Yu.,)

1.2.3 TCTP

While it has been shown that TCTP is a multi-functional protein that regulates cell proliferation, differentiation, apoptosis, anti-apoptosis, stem cell maintenance, and immune responses, but only a few studies associated to regeneration have been reported. To investigate the multi-functional role of TCTP in regeneration, the earthworm *Perionyx excavatus* was chosen(kamarajan rajagopalan,Jackson durairaj.,)

1.2.4 OCT4

Topological-associated domains (TADs) are thought to be relatively stable across cell types, although some TAD reorganization has been observed during cellular differentiation. However, little is known about the mechanisms through which TAD reorganization affects cell fate or how master transcription factors affect TAD structures during cell fate transitions. Here, we show extensive TAD reorganization during somatic cell reprogramming, which is correlated with gene transcription and changes in cellular identity. Manipulating TAD reorganization promotes reprogramming, and the dynamics of concentrated chromatin loops in OCT4 phase separated condensates contribute to TAD reorganization. Disrupting OCT4 phase separation attenuates TAD reorganization and reprogramming, which can be rescued by fusing an intrinsically disordered region (IDR) to OCT4. We developed an approach termed TAD reorganization-based multiomics analysis (TADMAN), which identified reprogramming regulators. Together, these findings elucidate a role and mechanism of TAD reorganization, regulated by OCT4 phase separation, in cellular reprogramming (Zhenhui zhong,Yafei wang.,)

1.2.5 Epimorphosis

Hydra occurs through the reinitiating of embryonic growth and patterning, in contrast regeneration in vertebrates which occurs through a process of epimorphosis(BM sharga, MY Hliudzyk-Shemot.,)

1.3 Thymidine

Thymidine is a nucleoside, a molecule composed of the nucleobase thymine linked to the sugar deoxyribose. It's one of the four nucleosides found in DNA, along with adenine, guanine, and cytosine. Thymidine plays a crucial role in DNA replication, where it pairs with adenine through hydrogen bonding to form the A-T base pair, thus ensuring the accurate duplication of genetic information during cell division.

1. **Epimorphosis:** This process involves the regeneration of lost or damaged material through the growth and differentiation of undifferentiated cells, usually from a large number of cells called blastema. Thymidine has been used in experimental studies to control cell proliferation during ectoblast regeneration. By controlling the presence of thymidine, scientists can influence the rate of cell division and subsequent tissue growth.
2. **Morphallaxis:** Morphallaxis is a regenerative process in which existing tissue is rearranged or replaced to regain lost structure without forming blastema. Thymidine may also play a role in the morphological axis by inhibiting cell proliferation and differentiation during tissue transformation. By affecting cell division and growth, it can affect the regeneration process by controlling the growth and differentiation of cells involved in tissue repair and renewal. However, the specific mechanisms by which thymidine affects epimorphosis and morphological axes may differ between organisms and replication contexts.

In both cases, thymidine's role is linked to its function as a precursor in DNA synthesis. By influencing cell division and proliferation, it can impact the regeneration process by regulating the growth and differentiation of cells involved in tissue repair and restoration. However, the specific mechanisms through which thymidine affects epimorphosis and morphallaxis may vary depending on the organism and the context of regeneration.

CHAPTER – 2

REVIEW OF LITERATURE

2.1 Case Histories on Earthworms

Perrier (1872), Horst (1886), Ude (1894), and Beddard (1886a, 1886b, 1887, and 1888) investigated the reproductive organs of *Eudrilus euginae*, and they correctly pointed out that they differ from those of the other Oligochaeta. In his last series of papers, Beddard (1888) talks about the male and female reproductive organ specifications and their morphology. Gates (1942) made a number of observations based on his dissections. Later on, Vidya Vati updated the above studies in 1947 and made some interesting observations about the morphology and arrangement of the earthworm's reproductive organs (Vidya vati., 1942). According to A.J. Reinecke, researchers focused more on earthworms for decomposition (Reinecke, A.J. et al., 1992). According to several experts, the earthworm species *Eudrilus eugeniae*, *Perionyx excavatus*, and *Eisenia fetida* are potentially suitable for decomposing organic waste (Hink, W.F., 1979, Hartenstein, R. et al., 1979, Haimi, j. and Huhta, V., 1986). Worms failed to survive at temperatures below 12 degrees Celsius, according to S.A Viljoen and A.J Reinecke. They further claim that high growth rates and biomass production were recorded at high temperatures up to 29°C, while high maturation rates and cocoon creation were observed at an ideal temperature of 22 to 25°C. As a result, they both agreed that earthworms are extremely sensitive to temperatures below 12°C and can survive up to 30°C (S.A Viljoen and A.J Reinecke., 1992).

2.2 Regeneration in Earthworms

Stem cells are responsible for the development of regeneration and play a critical role in it (Thorgeirsson S.S., 1996). The Suthira Owlarn assumption describes how regeneration begins in amputated tissue conditions. According to their assumption 1, every time a wound heals, it is referred to as an H- wound,

and every time a wound regenerates, it is referred to as an R- wound. The indications of H- wound and R- wound are predominantly used in the healing and regeneration mechanisms. Assumption 2 states that only when the amputated tissue contexts have been repaired might help the regeneration process to begin; without the missing tissue contexts, just the wound-healing process will be performed (Owlarn s. *et al.*, 2017). As per Kalidas R.M. and colleagues, Oct-4 is highly expressed within the regeneration blastema. Lamin A was no longer detectable in regeneration blastema, confirming the immunohistochemical findings. The prostate tissue pattern, on the other hand, revealed that lamin A had a content material. Finally, they discovered that differentiated cells express lamin A while undifferentiated cell does not. Lmn A1 and A2, which are unique antibodies to lamin A, are effective tools for observing regeneration and stem cells (Kalidas R.M *et al.*, 2015). Animal adaptability and organogenesis are important events in regeneration, according to Subramanian *et al.*, (2016). The regeneration of the mouth and anus in *E. eugeniae* was investigated. For five days, the earthworm was detected in a specific starving behavior; hunger causes the gut microbial flora to synthesize riboflavin. *Bacillus endophyticus*, one of the green riboflavins-producing bacteria, has been seen in 16S rRNA sequencing to be in the intestine to maintain homeostasis throughout the starving phase of early regeneration.

2.3 Role of epimorphosis and morphallaxis in regeneration

The mechanisms of regenerative tissue remodeling in planarians, particularly in the context of morphallaxis. His work delves into cellular dynamics, signaling pathways, and extracellular matrix remodeling during this process. Investigating spatial and temporal regulation and conducting comparative studies with other regenerative models are also key aspects of his research. Overall, Pellettieri's work sheds light on the intricate processes underlying morphallaxis, contributing to our understanding of regenerative biology and its potential applications in medicine (Jason Pellettieri). the transcriptome of anterior regeneration in the earthworm *Eudrilus eugeniae* and its relationship with the processes of epimorphosis and morphallaxis. Epimorphosis involves the regeneration of lost body parts through the proliferation and differentiation of

undifferentiated cells, while morphallaxis refers to tissue remodeling without the formation of a blastema. By analyzing the transcriptome, researchers can identify the genes and molecular pathways involved in both epimorphosis and morphallaxis during anterior regeneration in *E. eugeniae*. Understanding these mechanisms is essential for deciphering the intricate processes underlying regeneration in earthworms and other regenerative organisms.(Sayan Paul, Subburathinam Balakrishnan.,)

2.4 Role of thymidine in regeneration

The multifunctional role of Translationally Controlled Tumor Protein (TCTP) in the regeneration process of the earthworm *Perionyx excavatus*. TCTP is known to play diverse roles in cellular processes, including cell growth, cell cycle progression, apoptosis, and immune responses. In the context of regeneration, TCTP may be involved in coordinating cellular events such as cell proliferation, differentiation, and tissue remodeling. By investigating the specific functions of TCTP during regeneration in *P. excavatus*, researchers aim to elucidate its molecular mechanisms and its significance in the regenerative capacity of earthworms. This research could provide valuable insights into the fundamental processes underlying regeneration and may have implications for regenerative medicine and biotechnology. (Kamarajan Rajagopalan *et.al*)

CHAPTER – 3

AIM AND OBJECTIVE

3.1 Aim of the study

To inhibit epimorphosis mechanism and to reveal the key proteins that regulate morphallaxis modes of regeneration.

3.2 Objective:

- To standardize the dosage of 2mM Thymidine injection in earthworm, *Eudrilus eugeniae* to suppress the epimorphosis mechanism.
- To analyze the expression pattern of key regenerative proteins Wnt3a, Oct4, YAP and H3.
- To evaluate the cell fate determinant proteins TCTP, p53 and COX2 following the inhibition of epimorphosis.
- To determine the oncogene expression c-Myc and MEK1 upon morphallaxis modes of regeneration.

CHAPTER – 4

MATERIALS AND METHODS

4.1 Earthworm culture and maintenance

Earthworms were collected from the International Research Centre of Sathyabama Institute of Science and Technology's Regeneration and Stem Cell Biology Lab. The worms were kept in a plastic container with leaf litter, cow dung, and soil. A diet with an equal ratio of cow dung and jaggery is provided three times per week.

4.2 Injecting thymidine

- The matured set of worms were selected and 2mm thymidine was injected.
- The thymidine was injected according to the weight of the worms.
- The thymidine was injected using an insulin syringe in 30-37th segment.

4.3 Dissection

Worm selection

For dissection of the bud, matured earthworm species of *E. eugeniae* worms were chosen.

Dissection

- ❖ A sterile scalpel blade was used to dissect the head along with the bud in the dorsal side of a worm.
- ❖ The cut was made along the worm's axis on the dorsal side, using the sterile scalpel.
- ❖ The 7th day bud is dissected out along with some body segments using a scalpel and finer forceps to perform histology.

Washing:

- ❖ The bud were properly cleaned with ice-cold distilled water after dissection

to remove all dust and debris

❖ The glands were then washed twice mostly for 4 to 5 minutes in ice-cold 1X PBS.

4.4 Protein isolation

For weakening protein disulfide bonds, β -Mercaptoethanol (β -ME-reducing agent) is utilized. 5% β -Mercaptoethanol (reducing agent) was added to 2X sample buffer (stock 1). At 4°C, the samples were transferred into a new 1.5ml vial containing an equal amount of sample buffer. Manual homogenization was performed using a homogenizer, following which the samples were transferred to a dry bath at 100°C for 5 minutes, centrifuged at 10000 rpm for 5 minutes, and the supernatant was transferred to a fresh 1.5ml vial and stored at -20°C.

4.5 SDS-PAGE

4.5.1 Principle

SDS PAGE is a method of separating proteins by their molecular weight. Predominantly The study of the movement of charge molecules in an electric field is known as electrophoresis. The strength of the field, net charge, size and shape of the molecules, as well as the ionic strength, viscosity, and temperature of the medium in which the molecules are traveling, all influence their rate of movement. Larger molecules, such as proteins, are commonly separated using polyacrylamide gels. Proteins are sorted based on their resistance to the gel matrix, with smaller proteins having less resistance and larger proteins having more. When proteins are electrophoresed in the presence of a detergent sodium dodecyl sulfate and a reducing agent β -Mercaptoethanol, the detergent can bind to the protein backbone and the denaturing effect of SDS is very strong, disrupting the secondary, tertiary, and quaternary structure of the protein, resulting in a linear polypeptide chain coated with negatively charged SDS molecules. Mercaptoethanol aids in the denaturation of proteins by decreasing all disulfide linkages (both inter-disulfide and intra-disulfide bonds).

Polyacrylamide forms a mesh-like matrix that aids in protein separation. During PAGE electrophoresis, the proteins' linear or primary structure was stacked or placed in the appropriate order; as the proteins reached the separating gel, they began to separate based on their size.

4.5.2 Materials

- ❖ Eppendorf;
- ❖ Micro-pipettes;
- ❖ surgical blade;
- ❖ syringe;
- ❖ Petri-dishes;
- ❖ Micro-tips;
- ❖ Centrifuge;
- ❖ Water bath;
- ❖ Vortex
- ❖ Distilled water.

4.5.3 Chemical preparation of SDS-PAGE

30% Acrylamide and 0.3% Bis acrylamide

Acrylamide - 30 g
NN' Methylene Bis-acrylamide - 0.3 g

Make up into 100 ml 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

Make up into 100 ml with distilled water.

4X Stacking Buffer

Tris HCl - 6.05
Distilled water - 40 ml
pH was adjusted to 6.8 with 10N NaOH
Sodium Dodecyl Sulphate (SDS) - 0.4 g
Make up 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
Make up 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
Makeup into 1 ml with distilled water.

1X Running Buffer:

Tris HCl - 3.94 g
Glycine - 15.014 g
SDS - 1 g
Make up into 1L with distilled water.

Staining Solution:

Coomassie Brilliant Blue R-250 - 200 mg
Methanol - 50 ml
Glacial Acetic acid - 7 ml

Makeup into 100 ml with distilled water.

The above mixture was filtered using Whatman No 1 filter paper.

De-staining Solution:

Methanol - 30 ml

Glacial Acetic acid - 7 ml

Makeup into 100 ml with distilled water.

Sl no	Stock Solution	5%	6%	7%	7.5%	8%	9%	10%	12%	13%	15%
1	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
2	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
3	Distilled water (ml)	17.5	16.5	15.5	15.0	14.5	13.5	12.5	10.5	9.5	7.5
4	10% APS (μl)	200	200	200	200	200	200	200	200	200	200
5	TEMED (μl)	20	20	20	20	20	20	20	20	20	20

Table 4.1: Separating gel concentration

Sl no	Stock Solution	5ml	10ml	15ml	20ml
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 (μ l)	100	100	100	100
5	TEMED (μ l)	10	10	10	10

Table 4.2: Stacking gel concentration

4.5.4 Gel preparation

The glass plates were cleaned with ethanol and assembled manually. Glass plates were attached in the appropriate uniform manner with each corner covered by spacers so that they could be utilized as a primary baseline on the gel to aid in the agarose sealing process or as a second level baseline system. 1% agarose was prepared and boiled until liquid then corners were sealed in both surface and bottom area of the spacer. Separating gel was hand produced and carefully put onto the gel. Isopropyl alcohol was used to cover the gel and keep the air out. After removing the IPA and washing it with distilled water, drain stacking gel was produced and poured, and the comb was immediately fastened to the stacking gel. The gel was allowed to solidify for a few minutes before the comb was carefully removed and the wells were clearly washed with water to remove any bubbles. Finally, the bottom of the spacer was carefully removed and the setup was fixed in the bottom chamber, which contained a 1X running buffer, as well as the upper chamber.

4.5.5 Sample Loading and electrophoresis

The wells were loaded with a 60 μ g protein sample and the relevant molecular markers. The equipment was properly polarized and 5 to 6 v/cm was applied to the power pack. The glass plate assembly was removed from the buffer chamber after electrophoresis. The spacer was gently twisted away from the gel, causing the upper glass plate to pull apart.

4.5.6 Staining and De-staining Procedure

The stacking gel was carefully removed from the glass plate and the gel was carefully removed from the glass plate using a cleaver. By carefully gripping the two corners of the gel, the separating gel was extracted and placed in the container containing the Coomassie brilliant blue R-250 (Stainer solution). By gently agitating the gel on a Rocker for 30 minutes, it should be entirely submerged in the staining solution. It was then rinsed with distilled water and transferred to a de-staining solution until the protein bands were clearly visible. The first destainer wash usually takes approximately 30 minutes, and the second wash takes roughly overnight.

4.6 Western blotting

4.6.1 Running the gel

- ❖ Prepared 12% gel, and then placed it in the SDS page unit.
- ❖ Add 1X running buffer, and then add protein ladder at the first lane of the gel for molecular weight determination.
- ❖ Then add a protein sample for 60µg concentration in each well. Set the gel to 80V until the protein run successfully.

4.6.2 Buffer preparation

❖ 10X Transfer Buffer (Stock)

Glycine - 72.1g

Tris-base - 15.2g

Distiller water- 500ml

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,

❖ **1X Transfer buffer:**

100ml - 10X Transfer Buffer

200ml - Methanol

700ml - Distilled water

4.6.3 Transfer to membrane

❖ Cut the PVDF membrane by the same as the gel size. Mark which side is smooth (smooth side only we should place the gel to transfer protein).

❖ Soaked the PVDF membrane in methanol for 1-2 minutes (for activation of membrane). Then placed the PVDF membrane into 1X transfer buffer until prepare the sandwich.

❖ Then prepared the blotting apparatus, sponge, and filter paper to arrange the sandwich setup. Before arranging soak the apparatus, sponge, and filter paper in the 1Xtransfer buffer.

❖ Placed the blotting setup in the blotting unit, and added transfer buffer and 2 gel packs in the unit to maintain it at 4°C until the full transfer.

❖ Transfer the gel at 85V for 65 minutes.

4.6.4 Blocking

A blocking buffer was used to block all proteins which would bind with targetable and non-targetable proteins. 5% of BSA with 20ml of 1X TBST buffer was prepared for Blocking.

4.6.5 Primary Antibody

❖ Primary antibody MEK1 (69kDa), Cox (69kDa), p53 (53kDa),C Myc (49kDa),OCT4 (45kDa),Wnt3a (39kDa),TCTP (19kDa),H3 (15kDa) and incubated for 2 hours. It binds only to the specific protein.

- ❖ Washed 5 times with PBST for 30 minutes.

4.6.6 Secondary Antibody

- ❖ Secondary antibody (Abcam - ab150077, Anti Rabbit IgG produced in goat) (1:1000 dilutions) was added in the 5% BSA and incubated it overnight. It binds with the primary antibody.

- ❖ Washed 5 times with PBST for 30 minutes

4.6.7 Chemi-Doc imaging of the membrane:

The advanced Bio-Rad Chemi-Doc TM XRS+ imaging equipment was used to perform gel imaging for faster and more accurate western blot quantification. It is the only stain-free equipment capable of chemiluminescent, multiplex fluorescence, and routine gel imaging, and it is used to quantify total protein after transfer to the membrane for normalization purposes. avoids the mistakes that can occur when utilizing housekeeping proteins for normalization. After a 2-hour incubation with a secondary antibody, the PVDF membrane was washed 5 times with 1X PBST every 5 minutes. After that, an ECL substrate solution was made by mixing equal amounts of ECL solution A and ECL solution B in a (1:1) ratio, and the blot was washed more than 5 times in the same box before being transferred to a new one with only a few washes to avoid patchy areas on the blot. Then, connect the BioRad ChemiDocTM to the PC by turning it on. After wiping the place specified for putting the membrane with tissue paper, the membrane is kept within the precise position. Apply the prepared ECL substrate to the membrane by drops or sprays, ensuring that all portions of the membrane are covered. Select New protocol → Application → select → blots → chemo, in imaging area → select gel type → Bio-Rad criterion gel in the BioRad image Lab 6.1 software. Set the exposure time to 150 seconds in the Image exposure area and choose Run protocol. The results will be shown in clear bands on the monitor based on the protein luminescence at various exposure durations. File → export → export for publication → set the resolution → save was used to document the results. The complete procedure was performed in complete darkness.

4.7 Histology

Thymidine-treated 7th day regenerated worms, 5th day Nutlin-3a treated samples and their respective control samples were subjected to histological sectioning. Both the regeneration blastema of the control and treated worms were cut using sterile scalpels along with the adjacent two segments and fixed in 10% formaldehyde (Cat.F0080; Rankem, Mumbai, India) for 24 hours. To remove formaldehyde, tissues were gently washed with distilled water and dehydrated with isopropanol and acetone after the tissue was cleared using xylene. Each step was performed for one hour at 50°C. Following the xylene removal step, tissue impregnation was incubated in paraffin wax, and the 5µm sections were made using a microtome (Subramanian et al. 2017). Eosin and hematoxylin stains were used to distinguish the internal arrangements and were observed in Euromex bScope Epi-Fluorescence HBO Microscope (Catalogue Number: BS.3153-PLFi).

CHAPTER – 5

RESULT AND DISCUSSION

5.1 Earthworm Morphology:

- Earthworm have cylindrical shaped and reddish brown segmented body.
- Earthworm are smooth skinned. With a body made of many small segments
- Each segment has stiff little hairs that help the worm move.
- It has a central and peripheral nervous system
- A Glandular tissue called clitellum is present in segments 14-16 of a matured earthworm
- They have no skeleton or other rigid structures to interfere with their movement

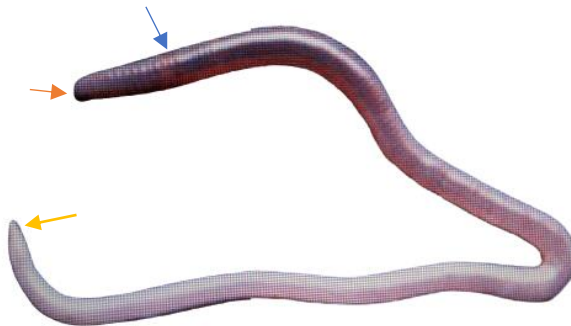


Fig:5.1: MORPHOLOGY OF EARTHWORM Eudrilus eugeniae

5.2 Regeneration in Earthworm:

- Earthworm have remarkable power of regeneration.
- In earthworm, regeneration capability varies among species
- Clitellum plays the vital role in regeneration
- Earthworm regeneration starts with the production of blastemas at the site of amputation, then cells proliferate and differentiate into head or tail buds.

5.3 Injecting thymidine and analyse morphallaxis modes of regeneration

Eudrillus eugeniae regenerates through epimorphosis (cellular proliferation) and morphallaxis (trans-differentiation), as confirmed by 2 mM thymidine injection. There is currently no report available in the academic literature that comprehensively covers the morphallaxis mechanism, specifically concerning trans-differentiation, within the context of TCTP. We administered a combination of 2mM Thymidine(an epimorphosis blocker)

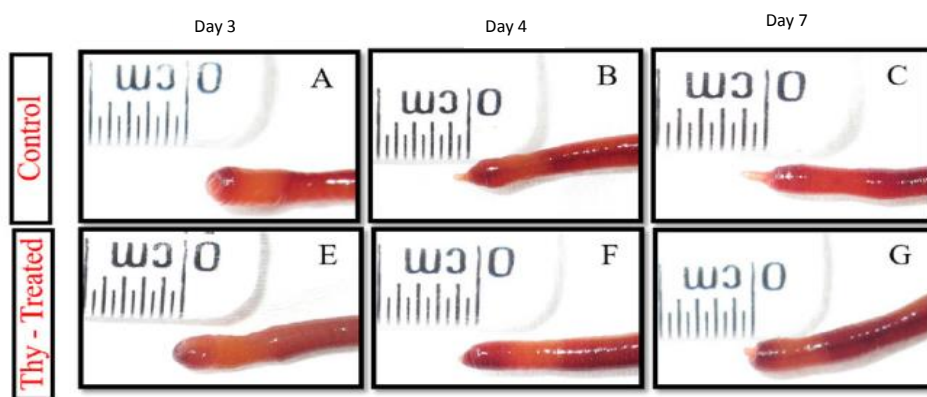


Fig 5.2 Regeneration following 2 mM Thymidine (cell cycle inhibitor) injection.

5.4 Histology

Histology of the 4rd day control blastemal tissue shows well differentiated prostomium, mouth, septum and elongated segments. histology of 2 mM Thymidine injected 8th day regenerative blastema with less developed structures lacking prostomium, mouth, and with truncated regenerative body segments.

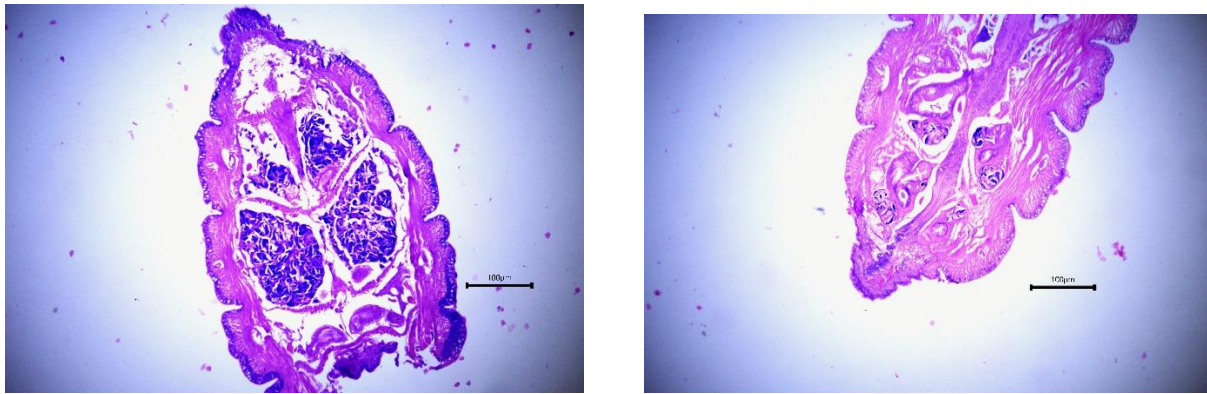


Fig:5.3: 4th day control and treated

Histology of 4th day control blastemal tissue shows well-differentiated prostomium, mouth, septum, and elongated segments. histology of 2 mM Thymidine injected 8th day regenerative blastema with less developed structures lacking prostomium, mouth, and with truncated regenerative body segments. Represents the dorsal side of the earthworm, and the ventral surface of the earthworm

5.5 Western Blotting of MEK1,Cox, p53,C Myc, OCT4, Wnt3a,TCTP and H3 expression

The earthworm's regenerated bud along with some segments was amputated and the different stages of regeneration were examined to investigate **MEK1,Cox, p53,C Myc, OCT4, Wnt3a,TCTP and H3 expression** in *E. eugeniae* during regeneration. On the seventh day, the regeneration was complete so the sample was collected from there. The Key Regenerative proteins are been observed such as Cox is a key mediator of the inflammatory response to tissue damage and other stimuli whereas C Myc serves as a "master regulator" of cellular metabolism and proliferation. The translationally controlled tumor protein (TCTP) is a highly conserved protein that is regulated due to a high number of extracellular stimuli. TCTP has an important role for cell cycle and normal development. MEK1 activity regulates various cell functions through phosphorylation and subsequent activation of ERK1. OCT4 can be a pluripotency and germ cell marker and be used to distinguish from non-CSCs, and participate in determining the biological function of CSCs where as Wnt3a mediates activation of Wnt/beta-catenin signaling and promotes tumorigenesis and tumor growth in experimental glioblastoma. Activated p53 may induce a variety of signaling pathways

such as DNA repair, apoptosis, senescence and others that contribute to the elimination of faulty cells in order to prevent the drifting from regeneration to malignancy.

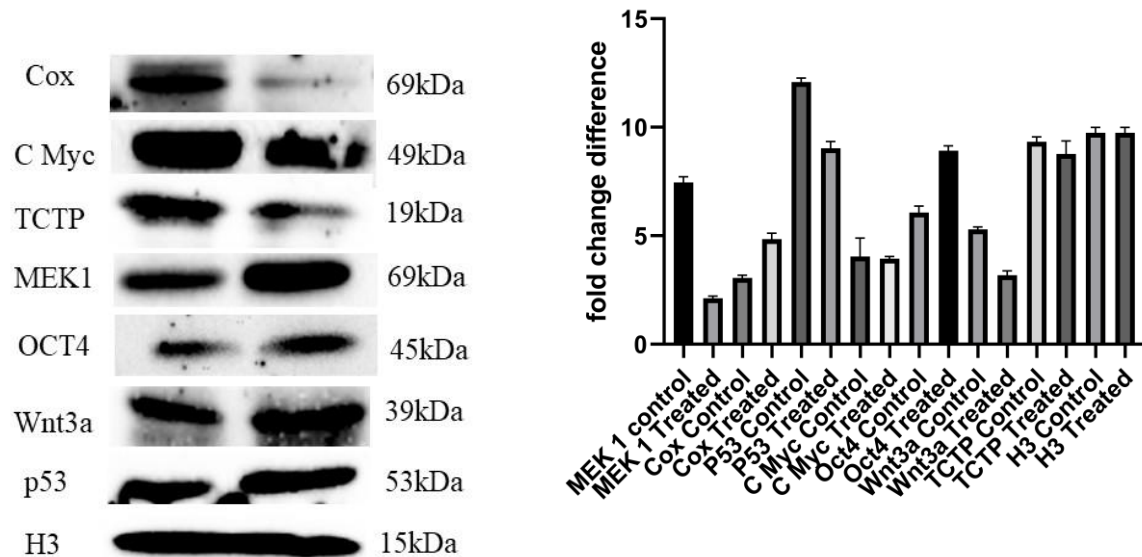


Fig :5.4:According to the results, epimorphosis block reversely activate the key regenerative protein expression pattern. In detail, yamanaka factors of C-myc, OCT4 protein expression was positively increased in epimorphosis block samples. Meanwhile, Cox and TCTP expression was reduced in the 2mM thymidine treated samples. Ste cells activation and MEK1 protein positively activated in the treated samples. TCTP and p53 expression pattern was in the reciprocal relationship in the context

CHAPTER – 6

SUMMARY AND CONCLUSION

- The earthworm *Eudrilus eugeniae* has a remarkable ability to regenerate, and it can be utilized to examine the complex cell and molecular biological processes that occur during regeneration. The current study examines the effects of thymidine induced treatment scenarios on Understanding the key proteins that regulate morphallaxis mechanism in earthworm of this species. The earthworm was treated with 2Mm thymidine. Blocking epimorphosis through thymidine reveals morpholaxis mechanism. Many proteins involved in regeneration mechanism in general in that key protein involved in morpholaxis are revealed in this study such as MEK1 (69kDa)(regulates various cell functions), Cox (69kDa)(key mediator of the inflammatory response), p53 (53kDa)(DNA repair, apoptosis),C Myc (49kDa)(master regulator),OCT4 (45kDa)(cell marker),Wnt3a (39kDa)(activation of Wnt/beta-catenin),TCTP (19kDa)(cell cycle),H3 (15kDa).Oct 4, wnt 3a, c myc are stemness related protein. Oct 4 wnt 3a are upregulated in morphallaxis but c myc is downregulated whereas Tctp cell fate determinant protein is downregulated in morphallaxis. Mek 1 a key protein for cell division, cell maturation and cellular death is upregulated in morphallaxis. Since cell proliferation is blocked using thymidine, in present studies it may be involved only in cell maturation and cell death which are more vital for regeneration.

CHAPTER – 7

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