

**UNDERSTANDING THE REGULATORY ROLE OF HOMEBOX
PROTEIN HOX-D3 IN DIFFERENT REGENERATION PATTERNS OF
EARTHWORM, *Eudrilus eugeniae***

**Submitted in partial fulfillment of the requirements for the award of
Bachelor of Technology degree in Biotechnology**

by

CHANDINI SENGUPTA (39230009)



**DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIO & CHEMICAL ENGINEERING**

SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

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JEPPIAAR NAGAR, RAJIV GANDHI SALAI, CHENNAI - 600 119

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This is to certify that this Project Report is the bonafide work of **CHANDINI SENGUPTA (39230009)** who carried out the project entitled "**UNDERSTANDING THE REGULATORY ROLE OF HOMEBOX PROTEIN HOX-D3 IN DIFFERENT REGENERATION PATTERNS OF EARTHWORM, *Eudrilus eugeniae***" under our supervision from September 2022 to April 2023


Internal Guide

DR. SUDHA S

PhD



External Guide

DR. S JACKSON DURAIRAJ

PhD



Head of the Department

DR. V RAMESH KUMAR

PhD

Submitted for Viva-voce Examination held on 10/5/2023


Internal Examiner


External Examiner

DECLARATION

I, Chandini Sengupta (39230009) hereby declare that the Project Report entitled **"UNDERSTANDING THE REGULATORY ROLE OF HOMEBOX PROTEIN HOX-D3 IN DIFFERENT REGENERATION PATTERNS OF EARTHWORM, *Eudrilus eugeniae*"** done by me under the guidance of Dr. Sudha S. Ph.D., Department of Biotechnology and Dr. S Jackson Durairaj, Ph.D., International Research Centre at Sathyabama Institute Of Science And Technology (Deemed To Be University) Accredited With A Grade "A" By NAAC, Chennai, is submitted in partial fulfillment of the requirements for the award of Bachelor of Technology degree in Biotechnology.

DATE: 10/5/2023

PLACE: CHENNAI


SIGNATURE OF THE CANDIDATE

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ABSTRACT

Regeneration is a highly complex process that requires the coordination of numerous molecular events, and identifying the key ruler that governs is keen to investigate. HOX-D3 is a multi-functional protein that regulates organ patterning and positioning, stem cell maintenance, and also increasing cancer stemness, but a minimum number of studies are only reported connecting with regeneration. To investigate the multi-functional role of HOX-D3 in relation to Wnt3a upon regeneration, the earthworm *Eudrilus eugeniae* was chosen. By performing amputation, histology, and western blotting the multifunctional role of HOX-D3 in connecting with regeneration is revealed. Studies with asymmetrical amputation at the 10th segment, symmetrical amputation with half of the clitellum amputated, and *in-vitro* regeneration studies show different regeneration patterns which are ideally used to study HOX-D3 functions. Upon asymmetrical regeneration, restoration of anterior segments with the initiation of wound healing, bud formation, pre-blastema formation, and blastema formation on the 1st, 2nd, 4th, and 6th day respectively proving that asymmetrical regeneration is more defined with proper segmental restoration. Also, we observed that the process of asymmetrical regeneration is augmented in juvenile worms due to the presence of more stem cells. While symmetrical regeneration is incomplete with less defined segment restoration. When maintaining the amputated earthworms (30-37th segments) under *in-vitro* conditions for 7 days, it shows abnormal patterning of regeneration representing a loss of segmental memory. Western blotting results with HOX-D3 and Wnt3a represented in asymmetrical elucidates successful regeneration wherein HOX-D3 and Wnt3a are simultaneously expressed. But in symmetrical regeneration where improper regeneration patterns are followed, Wnt3a shows higher expression as a tissue reconstructor measure. While *in-vitro* regeneration shows abnormal regeneration patterns HOX-D3 is highly expressed whereas Wnt3a protein remains suppressed. Overall, our results conclude that for initiating a successful regeneration pattern in earthworms, *Eudrilus eugeniae* HOX-D3 and Wnt3a need to be expressed simultaneously.

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LIST OF ABBREVIATIONS

IPA	Isopropyl alcohol
DAMPs	Damage-Associated Molecular patterns
β -ME	β - Mercaptoethanol
ECM	Extracellular Matrix
DDR	DNA Damage Response
CIT	Cancer Immunotherapy
ATP	Adenosine Triphosphate
HOX-D3	Homeobox protein HOX-D3
IAPs	Inhibitor Of Apoptosis Proteins
EL	Epithelial Layer
CML	Circular Muscle Layer
LU	Lumen
LCL	Longitudinal Cell Layer

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1. INTRODUCTION

Earthworms are soil organisms commonly called 'farmer's friend'. More than 6000 species of earthworms (Crassiclitellata) were identified (Anderson et al., 2017) and exist in all places of the world except Antarctica. Nightcrawlers belong to a sizable, widely distributed population with a long evolutionary history. The majority of earthworm species can be found among decaying logs, river banks, leaf litter, and even along trees at the seaside. Earthworms engineer terrestrial ecology, and they have a significant economic impact. Earthworm compost offers farmers important assistance. They were significant to Aristotle, who referred to them as the "Intestines of the Earth." As a defense and safety measure, some of the bioluminescent worms are releasing light to keep them safe from their predators. About their morphology, they have approximately 120 segments and they are distinguished into four major parts. The pre-clitellum segments are called as head region which contain important vital organs to perform major functions of the earthworm. It includes prostomium, simple brain, 4 pairs of tubular hearts, 2 pairs of testes, seminal vesicle and a part of ovary. The thickened collar like structure next to pre-clitellum segments are called as clitellum which possesses various glands which aid in their reproduction. A pair of prostate glands, an accessory reproductive gland that spans very next to the clitellum segments. The mid to distal region of the worm are called as intestine which are composed of repetitive body segments and it finally ends with a specialized structure called as anus which are unusually opened at a dorsal surface. Earthworms play an important role in the environment by cycling and releasing nutrients in the soil. They are primarily burrowing animals and feed on decaying matter, living bacteria, fungi, rotifers and other microbes.

1.1: EARTHWORM, *EUDRILUS EUGENIAE*

Eudrilus eugeniae is an anatomically advanced earthworm that has the origins in the tropical region of Western Africa in the soils of Savannah desert. The life cycle of the species from the stage of a cocoon to maturity is as short as 47 days (Blakemore, 2015). The common name of the species *Eudrilus eugeniae* is African Nightcrawler and they are epigenic in nature. *Eudrilus eugeniae* is highly known for its wound healing and fast regenerative ability.

1.1.1: Characteristic features of *Eudrilus eugeniae*

Size Range: 7 mm long to 300 mm long when extended.

Colour: Reddish-brown but clitellum appears to a swollen, glandular, and extremely distinctive body region that contains a viscous, sticky sac. It appears as a saddle-like ring on its skin, typically with a light-colored pigment between the thirteenth and eighteenth body segments.

Prostomium: Small, open epiglobus.

Dorsal pores: None

Setae: Eight per segment from which 2 are closely paired

Nephropores: Just behind the anterior furrow of each segment, longitudinal slits or slightly more median.

Male pores: In segment 17 on tips of longitudinally grooved, retracted as lateral slits with wrinkled lips just anterior to 17/18th segment in line with setae.

Female pores: Combined with modified 'spermathecal pores' lateral, presetal in 14 as raised intrasegmental openings just anterior to setae.

It has a higher growth rate as little as 5 weeks to reach maturity (*Nature and Science*, 5(2), 2007, B.O. Oboh, D.O. Akintobi and C. Ejidereonwu, *Morphometric Studies In*, 2007). It can tolerate temperature difference ranges from 15-30°C (Dominguez et al., 2001).

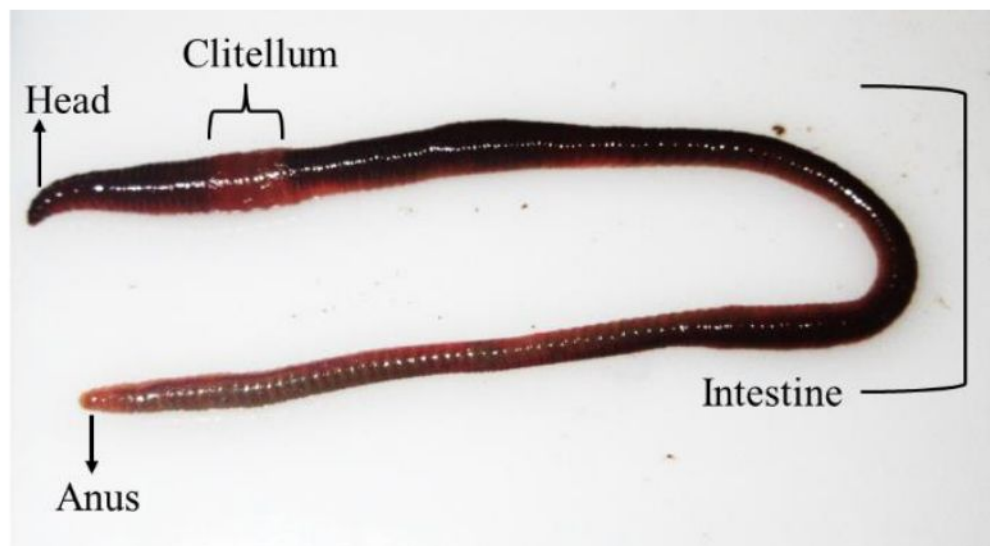


Fig 1.1: *Eudrilus eugeniae*

1.2: REGENERATION STUDIES

Regeneration studies were started from the 19th century onwards Dinsmore, Mittwoch, Batson has an important role in the research of regeneration. In 18th century E B, Wilson discussed the suspension sponge cells in the book of "The cell in development and inheritance" regeneration studies were started from sponges. In cell replacement therapy the mesenchymal stem cells are necessary and they isolated and expanded by an allogeneic approach was developed by Jothi A Kode (Kode et al., 2009) and that could be an effective tool in the regenerative medicine. Generally, scientists are mainly focusing on the planarian worms but it also has only the limited regeneration ability. James discussed the regeneration loss from evolution (Sikes & Newmark, 2013) by using observation of Central Nervous System (CNS) regeneration from the multiple vertebrates including lower vertebrates, the final conclusion is Fish and Salamanders (Lower vertebrates) has highly capable of axon regeneration also the development of neurons after the head injury (Tanaka & Ferretti, 2009). Dane discussion is about the jaw growing improvements, fixation and supportive systems by the tissue regeneration plate portions (Robinson, D.Q., 2001). Dane discussion is about the jaw growing improvements, fixation and supportive systems by the tissue regeneration plate portions (Robinson, D.Q., 2001). Biopolymers play an important role in the process of 3 regeneration in medical fields. Nalinkant and Damani prove that bioresorbable polymers that could be helping in the process of regeneration, using the leasable solvents, drugs, and biopolymers to ameliorate the mechanism of tissue regeneration (Matapurkar, B.G. and Rehan, H.S., 1997). Earthworms has a wonderful regeneration capability, reports suggest that *Eudrilus eugeniae* has prodigious regeneration ability and restore its lost body segments within 24 days ((Johnson Retnaraj Samuel et al., 2012)

In contrast to the current medical approach, which mostly focuses on treating symptoms, regeneration entails restoring tissues and organs that have been damaged by congenital disease or trauma. but through the use of a combination of biology, medicine, genetics, technology, and chemistry, it is now possible to speed up the recovery in some areas of the body or completely replace an organ that has been permanently damaged. The field of regenerative medicine currently has 218 regenerative research farms widely spread across 57 different nations. For this research, an earthworm is chosen as an animal model because it has an amazing

capability of regenerating their lost body parts (Subramanian, Daisy, et al., 2017)(Paul et al., 2021)(Gopi Daisy et al., 2016). Although earthworm shares similar morphological species, it shows diverse regeneration ability in tissues and organs. The clitellum is the most important feature of an earthworm's body because this region serves as a stem cell niche involving various biological aspects of cell survival involved in organogenesis, innate immune response, and autotomy (self-amputation).

1.2.1: Regeneration in earthworm, *Eudrilus eugeniae*

During regeneration, the clitellum experiences significant cellular alterations. In the course of regeneration, clitellar segments move. Furthermore, it is observed that neighbouring segments of stem cells can reach blastema. The migrating clitellar cells should be stem cells and they should arrive at the site of the amputation and the stem cells should travel to the site of the amputation and finally enter the posterior regenerative blastema. Then, in order to replace the missing organs, they specialize into distinct cell types. Interestingly, the worm is killed when one of the clitellar segments is amputated. Upon amputation at any other body parts, the segment with an intact clitellum can survive and effectively regenerate, but the worm portion that is severed from the clitellum perishes. In addition to growing a new tail, an amputated section of the earthworm's head, heart, and clitellum can also grow new. Some of the most important and emphasized regeneration experiments performed on earthworms which are then used in stem cell biology studies are highlighted here,

1.3: ROLE OF STEM CELLS IN REGENERATION

Because they can differentiate into a variety of different cell types and replicate themselves millions of times, stem cells serve a crucial role in regeneration that more specialized cells like nerve cells cannot. Stem cells primarily function to repair and maintain the region where they are present. It is possible to direct stem cells to differentiate into cells that can be employed in individuals to regenerate and restore tissues that have been harmed or affected by illness. Stem cells can be developed into new tissue for use in transplant and regenerative medicine. Researchers continue to expand their understanding of stem cells and their uses in transplant and regenerative medicine.

1.4: ROLE OF HOX GENES IN REGENERATION

HOX genes are a set of transcription factor genes that works by translating the proteins into their respective region and function. These proteins thus confer segmental or positional identity but do not form the actual segments themselves. Hox code is an attribute of a subpopulation of mesenchymal stromal cells. But, the mechanism by which the *HOX* genes encode proteins to give out patterns of recognition in earthworms is not widely studied and so it stays unknown till today. Juvenile earthworm has more *HOX* genes than matured worms because, during the growth phase in juveniles, *HOXD3* proteins encode and specify the characteristics of 'position', ensuring that the correct structures form in the correct places of the body. As the worm matures and reaches its ultimate structure the amount of *HOX* genes reduces. For this research problem, the earthworm serves as a link that interconnects both hox gene and its role in regeneration and patterning out of tissues and organs. After cell or tissue injury regeneration happens, for this, wound healing is the primary step and earthworms serve as an animal model that exhibits fast wound healing abilities, the epithelial cells cover the wound, and the regenerative segments heal with the presence of stem cells present in the clitellum and the pattern for regeneration is given by the *HOX* genes. *HOX* gene expression as regulators of correct tissue reconstruction after injury. The protein encoded by this gene may play a role in the regulation of cell adhesion processes. There is a chance of making even a non-regenerative part/ segment in the higher animals heal and regenerate if we find out the pattern by which the hox genes help in forming correct structures at the right places. Apoptosis, receptor signaling, motility, and angiogenesis are just a few of the activities that are controlled by hox genes, a highly conserved subsection of the homeobox superfamily (Shah & Sukumar, 2010). The Hox genes have also been the subject of research on regeneration. Hox genes have been associated with blastema patterning and wound healing during the early stages of regeneration (Christen et al., 2003).

1.5: ROLE OF RIBOFLAVIN IN REGENERATION

The worm needs riboflavin to regenerate, and riboflavin supplementation enhances its regeneration. Riboflavin has also been discovered to help speed up planarian regeneration. The liver in vertebrates contains a higher concentration of riboflavin than any other organ. It has been established that earthworms and the liver of vertebrates both have increased levels of riboflavin. It is interesting that the liver and earthworms both possess strong regeneration abilities. Riboflavin is a crucial component of the intricate regeneration process, according to studies.

1.6: EARTHWORM AS AN ANIMAL MODEL FOR REGENERATIVE BIOLOGY

Earthworms are often used as a model organism in regeneration biology research because they have a remarkable ability to regenerate lost body parts. When an earthworm is injured, it can regenerate its lost tissue within a matter of weeks, due to the presence of clitellum initiating blastemal formation. During regeneration, the clitellum produces a thick, protective cocoon that covers the regenerating tissue, helping to provide a safe and supportive environment for the process followed by blastema formation. Blastemal cells are able to differentiate into the various tissue types needed for regeneration, including muscle, nerve, and connective tissue. Thus, Earthworms possess enormous regeneration ability which can regenerate - Symmetrically - successful regeneration with proper positioning of tissue and organs and also under *In-vitro* conditions leading to abnormal regeneration.

In addition to their regenerative abilities, earthworms are also relatively simple organisms with a well-understood anatomy and physiology. They are easy to culture in the laboratory, making them a convenient model organism for research purposes.

2: LITERATURE SURVEY

2.1: ROLE OF CLITELLUM IN REGENERATION - A SOURCE OF STEM CELLS IN CLITELLUM-DEPENDENT WORMS

Regeneration is the ability of an organism to restore or regain its lost body parts (Brookes & Kumar, 2008). To initiate regeneration, wound healing is the first step. Annelids, nemertean worms, planarians and chordates are some of the well-known examples with diverse regeneration ability (Tanaka & Reddien, 2011). In earthworms, two distinct regeneration phenomena are observed:

2.1.1 Epimorphosis

Epimorphosis is a process of re-establishment of tissues by the multiplication of undifferentiated stem cells to form blastema with the help of stem cells present in the clitellar region (Özpolat & Bely, 2016). Earthworm regeneration is typically thought to be an epimorphosis, which is characterised by the dedifferentiation and redifferentiation process of somatic stem cells to form a highly proliferating initiative cell mass known as a blastema. (Ribeiro et al., 2018)(Agata et al., 2007)(Alvarado & Tsonis, 2006). Neoblast stem cells, a type of planarian somatic stem cells exhibits high levels of plasticity and pluripotency enabling the development of blastema thus re-specifying the blastema into the proper organs during the wound-healing process (Martinez-Acosta & Zoran, 2015; Martinez et al., 2008; Tanaka & Reddien, 2011).

2.1.2 Morphallaxis

Contrary to epimorphosis, morphallaxis is a complementary form of regeneration in which no blastema develops because tissue remodelling occurs without any obvious stem cell differentiation while the rest of the body reorganises itself with new positional identities. Transdifferentiation occurs in the multipotent atrial epithelium when morphallactic bud develops and plays a crucial role even in clitellum-dependent worms.(Zoran, 2010),(Özpolat & Bely, 2016). However, compared to epimorphosis, research on the characteristics of morphallaxis in relation to regeneration is sparse. Upon regeneration the bulged clitellum segments disappear (*Notes on Regulation in Stylaria Lacustris* Author (s): E . H . Harper, 2014)(*The Relation of the Glandular Elements of the Clitellum of the Brandling Worm (Eisenia Foetida, Sav.) to the Secretion of the Cocoon.*, 1926) which represents the migration of cells from clitellum which may involve in trans-differentiation or morphallaxis. In the future, it will be

important to unveil the clitellum and its regulation system in relation to morphallaxis.

Table 2.1 : Amputation and Regeneration Studies Using Foldscope (Selvan Christyraj et al., 2020)

AMPUTATED SEGMENTS	OBSERVATIONS
1 to 10 (Amputated at 10 th body segment) -1 to 10: pre-clitellar body segments - presence of minimal stem cells	-The anterior portion of earthworms amputated at the 10 th segment failed to heal its wound. -Amputated sites 1-10 were unable to survive, and death occurred within 12 hours of amputation.
10 to anus (Amputated at 10 th body segment) - 13 to 18: clitellum region is present - presence of stem cells	-Worms were able to heal the wounds 12 hours after amputations (presence of clitellum) -Blastema formation 24 hours after amputation -Differentiation after 72 hours of amputation with the initiation of segment formation -Functional prostomium formation after 96 hours of amputation
1 to 15 (Amputated at 15 th body segment) - 1 to 12: pre- clitellar body segments - Presence of minimal stem cells	-Lacked wound healing capacity and regeneration ability.
16 to anus (Amputated at 16 th body segment)	-Covered with surrounding tissues followed by sealing with an epithelial cell

- Presence of minimal stem cells	layer but was able to regenerate and survive
1 to 30 (Amputated at 30 th body segment) 1 to 12: pre-clitellar body segments 13 to 18: clitellar region 19 to 30: post- clitellar body segments - Presence of intact stem cells	-Wound healing ability and also capacity to regenerate.
31 to anus (Amputated at 31 st body segment) - Absence of clitellum	-Had only wound healing ability but lacked the regeneration capacity and was unable to survive

The above experiment conducted by Kamarajan Rajagopalan (Rajagopalan et al., 2022) suggest that it is better to focus on the posterior body segments of the earthworm to comprehend the regeneration mechanism because anterior segments of the worm have varieties of complicated organs in the initial 13 segments such as mouth, brain and hearts but the posterior region from the 30th body segment to anus have only nerve cord, blood vessels, gut covered by skin with repetitive segments making up the entire body.

2.2 EXPRESSIONS OF STEM CELL PLURIPOTENCY IN DIFFERENT REGIONS OF EARTHWORM

In a study conducted by Pengfei Zheng and Qiang Shao on the earthworm *Eisenia foetida* (Zheng et al., 2016), it has been found out that higher concentration of transcription is happening in the clitellum compared to those in the tail and head region. SOX2 factor has been observed to be present highest in the cocoon region. OCT-4 expression was detected in several different embryonic carcinoma cell lines, unfertilized oocytes, primordial germ cells, and early embryos, but not detected in adult tissues(Jerabek et al., 2014).

The OCT-4 expression was much higher in the cocoon than that present in the clitellum, and there was very little expression of oct4 in the head and tail segments. The difference between the cocoon and clitellum with levels of Oct4 expression may be caused by the source from which it arises. They serve as the hotspot for the transcription regulatory hierarchy that governs pluripotency. It is because these factors are very essential to be present in the clitellum and cocoon as it contains the reproductive organs of the earthworm. (Zheng et al., 2016)

2.3 HOX-D3- SOURCE FOR PATTERNING OF ORGANS DURING REGENERATION

The HoxD3 have garnered interest in regeneration studies as a result of being linked to genes involving dedifferentiation and wound healing during the early phases of regeneration, as well as blastema patterning during the later stages (Christen et al., 2003)(Chuong, 2003)

it was discovered that *HOX* genes, a subset of homeobox genes that encode transcription factors and are typically repressed in undifferentiated pluripotent stem cells, are master regulators of cell identity and fate during embryogenesis, in maintaining this positional identity throughout life, and in defining various regional properties of respective tissues. Intricate molecular circuits that are balanced between stem cell maintenance, proliferation, and differentiation are controlled by many signaling pathways common to stem cells. However, it is still unclear how stem cell-related signaling pathways create and control the *Hox* code (Jennifer et al., 2022)

The juvenile earthworms has more *Hox* genes than the matured worms because, during the growth phase in juveniles, proteins encode and specify the characteristics of 'position', ensuring that the correct structures form in the correct places of the body so there is the presence of more hox genes (Dayem et al., 2019). As the worm matures and reaches its ultimate structure the amount of *Hox* genes reduces. The protein encoded by *Hox* genes play an important role in adhesion processes of cells (Abate-Shen, 2002)

2.4 STEM CELL BIOMARKERS AS A COMPARITIVE ANALYSIS TOOL

For development and regeneration, stem cell factors are crucial (Gurley et al., 2008)

The application of Wnt3A protein is to support hESC-derived cells' in vitro myogenic commitment and subsequent in vivo function (Goessling et al., 2009)

Researchers demonstrated that hESC-derived progenitors that express a myogenic gene are much more committed to myogenesis when Wnt3A is present in the culture medium expressing a platelet-derived growth factor receptor, mesodermal marker (Hwang et al., 2014) (Anon, 1909)

2.5 EARTHWORM AND ORGANOGENESIS

Organogenesis is the process of regeneration of the vital organs of an animal, it is a series of organized processes that transforms the mass of cells and tissues into a complete organ.

Regeneration of the anterior, and posterior body segments and the organs of earthworms was investigated in four different species, namely *Perionyx excavatus* (Johnson Retnaraj Samuel et al., 2012)(Kalidas et al., n.d.) and *Eisenia Andrei* (Park et al., 2013). From one of the studies, it has been confirmed that any damage to the anterior segments of the earthworm, *Eudrilus eugeniae*, *Perionyx excavatus* leads to organ loss, here organ loss of the mouth. Now, During the process of regeneration, the earthworm would suffer to starve until the regeneration of the lost mouth happens (Subramanian, Sudalaimani, et al., 2017). The process of regeneration starts when the stem cells from the clitellum track themselves to the injured body segments initiating organogenesis by the formation of blastema at the wounded region these blastemal cells are generally found in myofibers (Subramanian, Daisy, et al., 2017) and satellite cells (Subramanian, Daisy, et al., 2017) that are found beneath the damaged tissue which is then re-differentiated into organs followed by brain formation nerve regeneration, ganglion appearance (Bae et al., 2020).

In a research conducted by Park, Cho (Park et al., 2013) on earthworm *Eisenia andrei* for tail regeneration, amputated at the 48th body segment, it is observed that the regeneration occurs through a temporal process. After 5 days, small blastema appears and elongates and the blastemal cells proliferate initiating reconstruction the nerve cord, regenerating tissue outgrowth was observed after 14 to 15 days. These instances help us comprehend that earthworm has an amazing capacity to regenerate

the organs using stem cells present in the clitellum region and interestingly, the organs formation is triggered at their correct and exact positions with the help of *Hox* genes. More evidences are still needed to understand the mechanisms by which the *Hox* gene helps in recognizing the patterns of regeneration.

CHAPTER 3: AIM & SCOPE

CHAPTER 3.1: AIM

Finding out the molecular mechanism by which the *HOX* gene regulates the stem cells to give out positioning and patterning to the organs in regenerative segments of earthworm, *Eudrilus eugeniae*

CHAPTER 3.2: SCOPE OF THE PRESENT INVESTIGATION

- To understand the regulatory role of HoxD3 in different regeneration patterns of earthworm, *Eudrilus eugeniae* by maintaining the different positioned amputated segments of the worm in *In-Vivo* and in *In-Vitro conditions*.
- To assess the different regeneration patterns based on morphological, developmental, and functional examination.
- Molecular level characterization of different regeneration patterns using position-determining protein, HOX-D3
- To understand the link between HOX-D3 and stem cells by comparing HOX-D3 expression in relative to WNT3 expression (Stem cell marker) in different regeneration patterns which possess variable tissue reconstructor ability.

4. MATERIALS AND METHODS

4.1: EARTHWORM CULTURE AND MAINTENANCE

Earthworms are collected in the Regeneration and Stem Cell Biology Lab, International Research Center of Sathyabama Institute of Science and Technology. Worms were kept in a plastic container with soil, cow manure, and leaf litter. A diet containing an equal mixture of cow dung and jaggery is offered three times each week. Periodically, the vermicast and earthworm bed were removed and replaced with leaf litter and cow dung.

4.2 PROCESSING OF EARTHWORM SEGMENT FOR IN-VITRO CULTURING

Sexually matured *E.eugeniae* worms were selected for amputation and regeneration. A couple of worms were dissected at the 30th segment with the help of sterile scalpel blade. After all reformed Blastema was again amputated and washed with sterile distilled water frequently all pieces will be soaked in Ice cold filtered 1X PBS Buffer. Within a few minutes, those segments were transferred to the fresh 24mm small petri-discs under sterile condition; sterile blunt tips were used for the transformation of segments from the one disc to another disc. Dissection of the regeneration of anterior segments is only processed in outside of the chamber, once those are eliminated from the worms in step 2 after pre-treatment it will transfer to the fresh petri dish which contains filtered ice-cold 1X PBS buffer, rest of all processing steps will be undertaken by the sterile condition. After 10 minutes incubation, 1X PBS buffer removed and antibiotic solution (Streptomycin (1µg/1ml), Amphotericin B (1µg/1ml), Penicillin (1µg/1ml) was added in the disc which contains dissected segments. In this process, antibiotic processing is crucial, and the strategy employed here is antibiotics at high concentrations with varying time intervals and stress condition maintenance. For example, the first antibiotic wash is a 15-minute processing step, but it won't completely get rid of all the microbes because the bud contains coelomic fluid, which contains microbes (Otuk, A., and Dales, R.P., 1984). The following five-minute segments were gently washed without damaging the tissue. To completely avoid the microorganisms, repeat the same steps twice. The segments are under stress right now, so they are soaked in filtered serum-free L15 medium for five minutes, after which amputated segments are moved to the 10% serum-containing L15 medium the collected regenerative worm coelomic fluid was filtered with 0.22 micron filter and

added as a main supplement on fresh 24mm petri disc. Processed segments (30-37) was stored in incubator at 28 °C.

4.3: PROTEIN ISOLATION

Protein disulphide links are broken with beta-Mercaptoethanol (β -ME, reducing agent). 5% of Beta-Mercaptoethanol was added to 2X sample buffer (stock 1). Samples were transferred into a new 1.5 ml vial at 4 °C with an equal amount of sample buffer. After manually homogenising the samples, they were then placed into a dry bath at 100 °C for 5 minutes, centrifuged for 5 minutes at 10,000 rpm, and the supernatant was transferred to a new 1.5 ml vial and maintained at -20 °C.

4.4 SDS PAGE

4.4.1 Principle

SDS PAGE is an analytical method that separates proteins according to their molecular weight. The study of how charged molecules travel in an electric field is known as electrophoresis. The intensity of the field, the net charge, the size, and form of the molecules, as well as the ionic strength, viscosity, and temperature of the medium in which the molecules are travelling, all affect how quickly they move. For separating bigger molecules like proteins, polyacrylamide gels are frequently utilised. Proteins are divided based on the gel matrix's resistance, with smaller proteins often having less resistance and bigger proteins typically having more resistance. The denaturing effect of SDS is very strong, and it disrupts the secondary, tertiary, and quaternary structure of the protein to produce a linear polypeptide chain coated with negatively charged SDS molecules. This linear polypeptide chain is then coated with negatively charged SDS molecules, which is how the molecular weight of the protein was determined. Through the reduction of all disulphide linkages, β -mercaptoethanol aids in the denaturation of proteins (both inter-disulphide and intra-disulphide bonds). Protein separation is aided by the mesh-like matrix that polyacrylamide produces. Proteins were appropriately stacked or organised during PAGE electrophoresis, and once they reached the separating gel, they began to separate depending on their size.

4.4.2 Materials

Eppendorfs; Micro-pipettes; Micro-tips; Centrifuge; Water bath; Vortex and Distilled water.

4.4.3 Chemical Preparation for SDS PAGE

30% Acrylamide and 0.3% Bis acrylamide:

Acrylamide	- 30 g
N, N' 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

Makeup into 100 ml with distilled water.

4X Stacking Buffer:

Tris HCl	- 6.05 g
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 Bromophenol blue was added.

10% Ammonium Per Sulphate (APS):

APS - 100 mg

Make up 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

Make up into 100 ml with distilled water.

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

Destaining Solution:

Methanol - 30 ml

Glacial Acetic acid - 7 ml

Make up into 100 ml with distilled water.

Table 4.1: Concentrations of separating gel

Sl. No.	Stock Solution	5%	6%	7%	8%	9%	10%	12%	13%	15%
1	30% Acrylamide + 0.3% Bis-acrylamide (ml)	5.0	6.0	7.0	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
3	Distilled water (ml)	17.5	16.5	15.5	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
5	TEMED (μl)	20	20	20	20	20	20	20	20	20

Table 4.2: Stacking gel concentration

Sl. No.	Stock Solution	5 ml	10 ml	15 ml	20 ml
1	30% Acrylamide + 0.3% Bis-acrylamide (ml)	1.25	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

4.4.4 Protein Sample Preparation

Protein disulphide links are broken with beta-Mercaptoethanol (β -ME, reducing agent). 5% of Beta-Mercaptoethanol was added to 2X sample buffer (stock 1). Samples were transferred into a new 1.5 ml vial at 4 °C with an equal amount of sample buffer. After manually homogenising the samples, they were then placed into a dry bath at 100 °C for 5 minutes, centrifuged for 5 minutes at 10,000 rpm, and the supernatant was transferred to a new 1.5 ml vial and maintained at -20 °C.

4.4.5 Lowry's Method

Prepare BSA stock solution, reagent A and reagent B before starting off the experiment

Table 4.3 : Reagent preparation for Lowry's method

Reagents	Chemicals required	Quantity
BSA stock solution	Bovine Serum Albumin	5 mg
	Distilled water	1 ml
Reagent A	Sodium hydroxide	25 mg
	Sodium bicarbonate	50 mg
	Distilled water	5 ml
Reagent B	Copper sulphate	25 mg
	Sodium potassium tartrate	50 mg

	(C ₄ H ₄ O ₆ KNa.4H ₂ O)	
	Distilled water	5 ml
Reagent C	Reagent A	4 ml
	Reagent B	1 ml

Take a 96-well plate when reagent preparation is complete. Depending on the number of samples, we will need one well for the blank, seven for the standards, and maybe more. Put 100 µl of distilled water into each well. The 7 standard wells should each have 5, 10, 15, 20, 25, 30, and 35 µl of distilled water removed from them respectively. 2 µl of distilled water should be taken out of the sample wells. Except for the blank and sample wells, add BSA solution to every well. Replace the distilled water withdrawn with BSA solution for the standard wells. Add 2 µl of the sample to the sample wells. Then add 100 µl Reagent C in all the wells and incubate for 10 minutes. Post incubation, add 10 µl of Folin-Ciocalteu reagent to all the wells. Make sure that dark conditions are maintained while adding Folin's reagent. Now keep this well plate on the shaker, still in dark conditions for 30 minutes. A graph is plotted with the sample values in correlation with the standard values. These sample values can then further be utilized for SDS-PAGE and Western Blotting.

4.4.6 Preparation of Gel

The glass plates were carefully put together after being cleaned with ethanol. To be utilised as a main baseline on gel and aid in the process of agarose sealing, glass plates were attached in the correct equalise way with each corner covered by spacers. The corners of the spacer's surface and bottom were then sealed with prepared 1% agarose that had been cooked until liquid. Separating gel was carefully produced by hand and put onto the gel. Isopropyl alcohol was applied on top of the gel to seal the air out. Following the removal of the IPA and washing with distilled water, draining, and preparation of the stacking gel and its addition, the comb was immediately fixed onto the stacking gel. After giving the gel some time to set, the comb was gently removed, and the wells were cleaned with water to get rid of the bubbles. Carefully removing the spacer's bottom, the setup was installed in the lower chamber, which housed the 1X running buffer as well as the top chamber.

4.4.7 Sample Loading and Electrophoresis

A 10 g protein sample and the necessary molecular markers were added to the wells. 60V was applied while the device was properly polarised and linked to the power pack. The glass plate assembly was removed from the buffer chamber following electrophoresis. The upper glass plate of the spacer was gently bent to separate it from the gel.

4.4.8 Staining and Destaining of Gel

The stacking gel was gently cut off with a cleaver and removed from the glass plate. By carefully gripping the gel's two corners, the separating gel was extracted and put into the Coomassie Brilliant Blue R 250 container (stainer solution). Gently rocking on a rocker should be used to completely immerse the gel in the staining solution for 30 minutes. Once the protein bands could be seen, it was rinsed with distilled water and moved to the destaining solution. Typically, the first destainer wash takes approximately 30 minutes, and the second wash takes roughly overnight.

4.5: WESTERN BLOTTING

For 15 minutes, gels were immersed in 1X transfer buffer. The transfer buffer was used to moisten the blotting equipment. On either side of the apparatus, sponges, tissue paper, and gel are placed in that order, with the gel being placed lightly on the tissue paper after the sponges. After the carbinol on the nylon membrane has been put into effect, the blotting paper is applied to the gel. The twin tissue sheets and sponges were then carefully applied and punctured. Subsequently, the entire blotting setup is moved inside the chamber, which is filled with 1X transfer buffer. The temperature is kept between 4°C and 8°C via the use of ice packs.

4.5.1 Buffer Preparation

10X Transfer Buffer (Stock)

Glycine	- 72.1 g
Tris-base	- 15.2 g
Distilled 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 litre,

10X Transfer Buffer	- 100 ml
Methanol	- 200 ml
Distilled water	- 700 ml

4.5.2 Blocking

All proteins that would bind to both targetable and non-targetable proteins were blocked using a blocking buffer. In 20 ml of 1X TBST buffer, 5% of Milk Powder or Fetal Bovine Serum (FBS) was prepared for blocking.

4.5.3 Primary Antibody

In the presence of either 5% milk solution or FBS, the suitable primary antibody was added in the ratio 1:5000 (1 part of antibody to 5000 parts of milk solution or FBS). For instance, HOX-D3, which is a Rabbit anti-Mouse Polyclonal Antibody, is selected as a primary antibody. This is poured onto the blot placed in a box which is kept on the rocker and incubated at 4°C overnight. Onto the blot, only HOX-D3 will be bound.

4.5.4 Secondary Antibody

The following day, the setup is kept outside until it reaches room temperature. Post 3 washes with TBST buffer, the secondary antibody is added.

In the 5% milk solution or FBS, the secondary antibody is added in the dilution ratio of 1:10000 (1 part of antibody to 10000 parts of milk solution or FBS). For instance, the secondary antibody HOX-D3, which is a Rabbit anti-Mouse Polyclonal Antibody. This is added onto the blot and rocked continuously for 30 - 40 mins after which another 3 washes with TBST buffer are performed. The secondary antibody binds exclusively in the regions where the primary has already bound on the blot.

4.5.5 Visualization of the blot using ChemiDock

After the addition of the secondary antibody and 3 washes with TBST buffer, the blot is transferred to another box with a small amount of TBST buffer to avoid drying and patchy spots on the blot. Before visualizing, the ECL substrate solution is prepared by the addition of solution A and solution B in equal parts (1:1 ratio). The blot is visualized under Chemidock after one minute of incubation in the ECL solution.

The resulting blot images are then stored in .jpg or .epg format. Dark conditions are maintained so as for the development of the blot image.

4.6: HISTOLOGICAL STUDIES FOR REGENERATION OF BUD

The *E. eugeniae in-vivo* regenerated bud was dissected and rinsed with distilled water. The removed portion was then overnight preserved in 50% formaldehyde at 50°C. The bud segment was then treated with 5 concentrations of isopropyl alcohol (IPA) – 60%, 70%, 80%, 90% and 100% for 1 hour. This is to treat the dehydration of the bud section after being incubated in 50% formaldehyde. This was done to remove the formaldehyde from the tissues. The bud section was once more immersed in 100% IPA at 50°C overnight. The IPA was then removed from the tissues by transferring them to xylene. Finally, tissues were immersed in wax overnight. The intended temperature range is between 58-60°C. Tissues were carefully moved to the second wax after incubation, and after three hours, tissues were placed on the last wax. For sectioning, tissues were moved into the boxes. 7µm slices were obtained from the roto-microtome and put on albumin-coated clean glass slides containing warm water. Slides were placed in a xylene jar for five minutes to remove wax after parts of the wax had been formed into a liquid condition. The 100% IPA Jar was then filled with slides, where they remained for 3 minutes. Slides were mounted with DPX mountain, and visible results were achieved with hematoxylin and eosin stains.

4.7: STATISTICAL ANALYSIS OF *IN-VIVO* BUD REGENERATION OF *E.eugeniae*

Image J software was used to analyze the statistical data. The length of *in-vivo* regenerated buds was measured and compared. More than 50 angles and their lengths were measured, equalized, and manually computed. Measuring angles were set using several fixing points in various locations. A variety of features are offered by ImageJ for image processing and analysis, including: Analyzing data quantitatively: ImageJ offers capabilities for quantifying image attributes like length, area, and intensity. It may also be used to carry more advanced image analysis tasks like segmentation and colocalization. The open-source nature of ImageJ ensures that a sizable developer community continuously updates and improves this robust and adaptable tool for image processing and analysis.

5. RESULTS AND DISCUSSION

5.1 *IN-VIVO* REGENERATION OF BUD WITH OUTGROWTH OF BLASTEMA

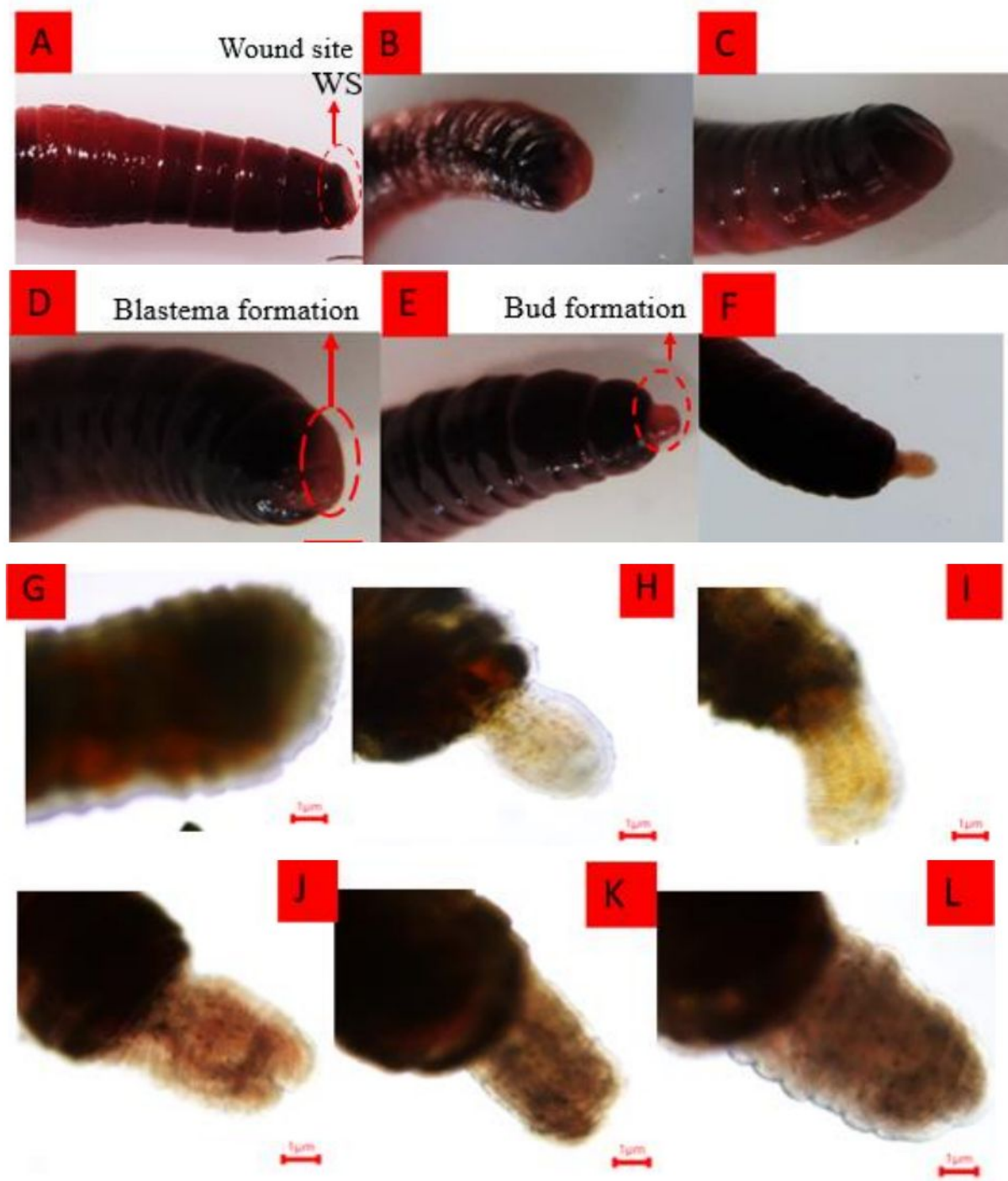


Fig: 5.1: Asymmetrical cut at 10th body segment – successful regeneration

A. 24th hour observation after amputation (1st day) at 10th segment with not much changes observed. **B.** 2nd day observation of blastema formation retaining same phenotypical features of day 1. **C.** 3rd day observation of blastema (wound healing

initiated). **D.** 4th day observation after the initiation of wound healing. Formation of blastema started. **E.** 5th day bud formation **F.** 6th day observation after amputation. **G-H.** – Juvenile earthworm blastemal regeneration observed with results showing faster rates of regeneration compared to juvenile.

From these results we can infer that, earthworms have a remarkable ability to regenerate its lost body parts when they are damaged or lost. This is made possible by the presence of special cells called blastemal cells. The blastemal cells undergo a process of dedifferentiation, which means that they revert back to a less specialized state, similar to stem cells. This allows them to differentiate into the various tissues needed to form a new tail, such as muscle, nerve, and connective tissue. Here, we can find prominent blastema formation in (Fig. E). Following this, the already regenerated posterior blastema was elongated and differentiated into segments indicating successful regeneration when earthworm is asymmetrically cut at the 10th segment. Asymmetric regeneration is more defined with proper segmental restoration

5.2 SYMMETRICAL CUT AT THE CLITELLUM SEGMENT – SUCCESSFUL REGENERATION (7 days observation)

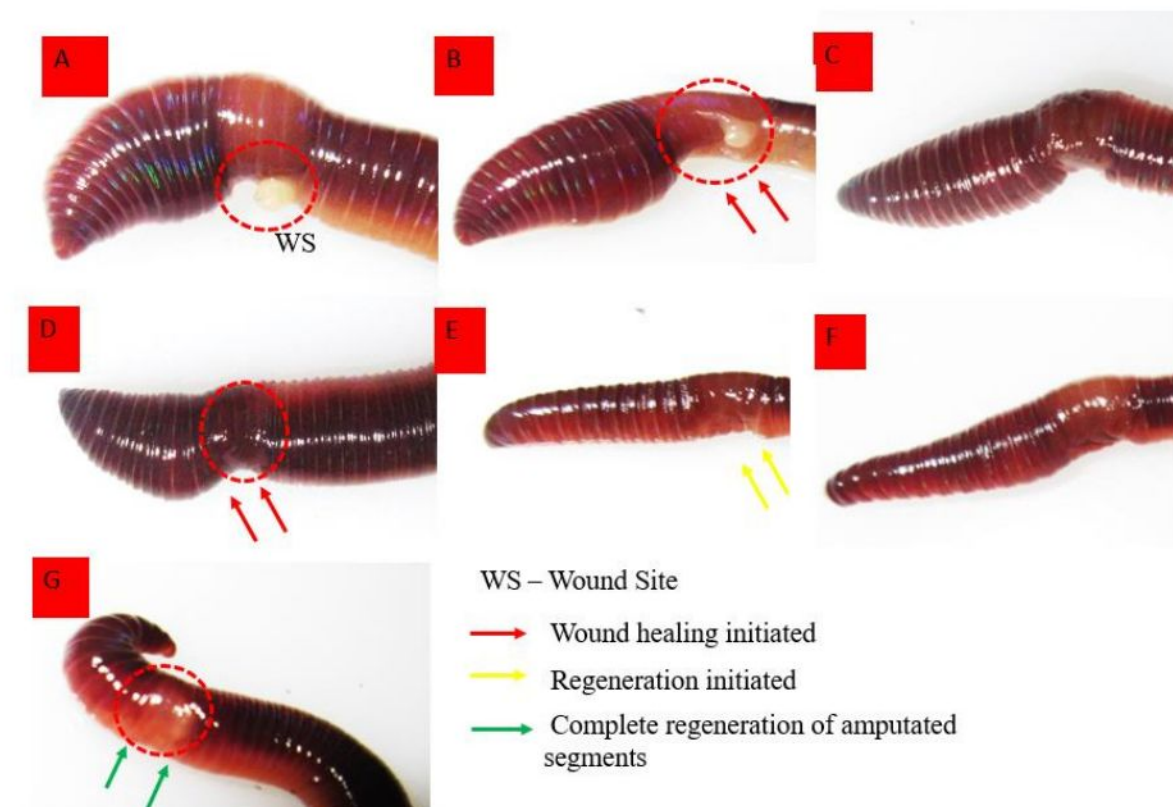


Fig: 5.2: Symmetrical amputation at the clitellum segment (13 to 18th)

A. Adult *E.eugeniae* clitellum segments on 1st day after amputation. **B – D.** Changes have been observed on 2nd, 3rd and 4th day respectively upon amputation on clitellum segments (wound healing initiated). **E – G.** Formation of the amputated segments initiated on 5th day and regeneration of proper clitellum through 6th and 7th days after amputation.

From these changes, it may be inferred that the existence of clitellum factors contributes to the appropriate positioning of cells and tissues during symmetrical and asymmetrical regeneration in earthworms. When a worm sustains a wound or is amputated, it results in the loss of body segments and organs, but the clitellum that is present in earthworms (13th to 18th segment) makes it special because the clitellum factors have the ability to migrate to the injured area or body segment and activate the wound healing steps, (Selvan Christyraj et al., 2020) causing it to successfully regenerate until it once again resembles a normal worm completely. The movement of factors from the clitellum is one of the main reasons for successful regeneration and those factors are assumed to be stem cell factors. Additionally, the clitellum segments may include the stem cell niche, which could provide the microenvironment necessary for the formation and maintenance of stem cells (Sivasubramaniam, 2021). Following the production of a wound in planarians, stem cells gather at the site of the wound and await signals for healing and tissue regeneration (Owlarn et al., 2017). Symmetric regeneration is incomplete and with less defined segments.

5.3: REGENERATION OF JUVENILE CLITELLUM SEGMENTS (13-18) - 7 DAYS
MAINTENANCE UNDER *IN-VITRO* CONDITIONS

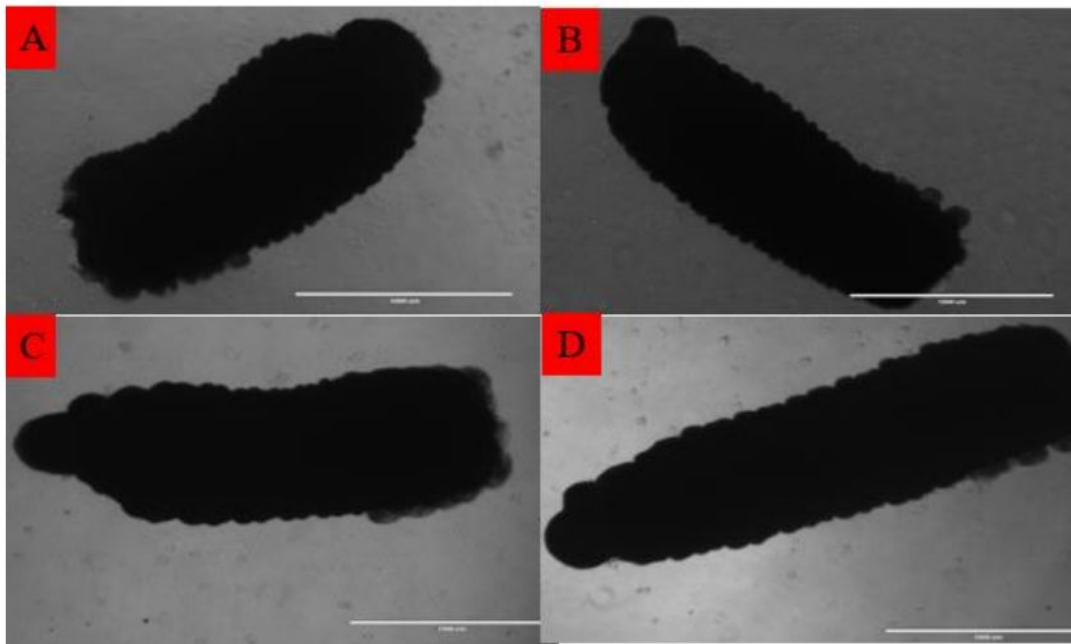
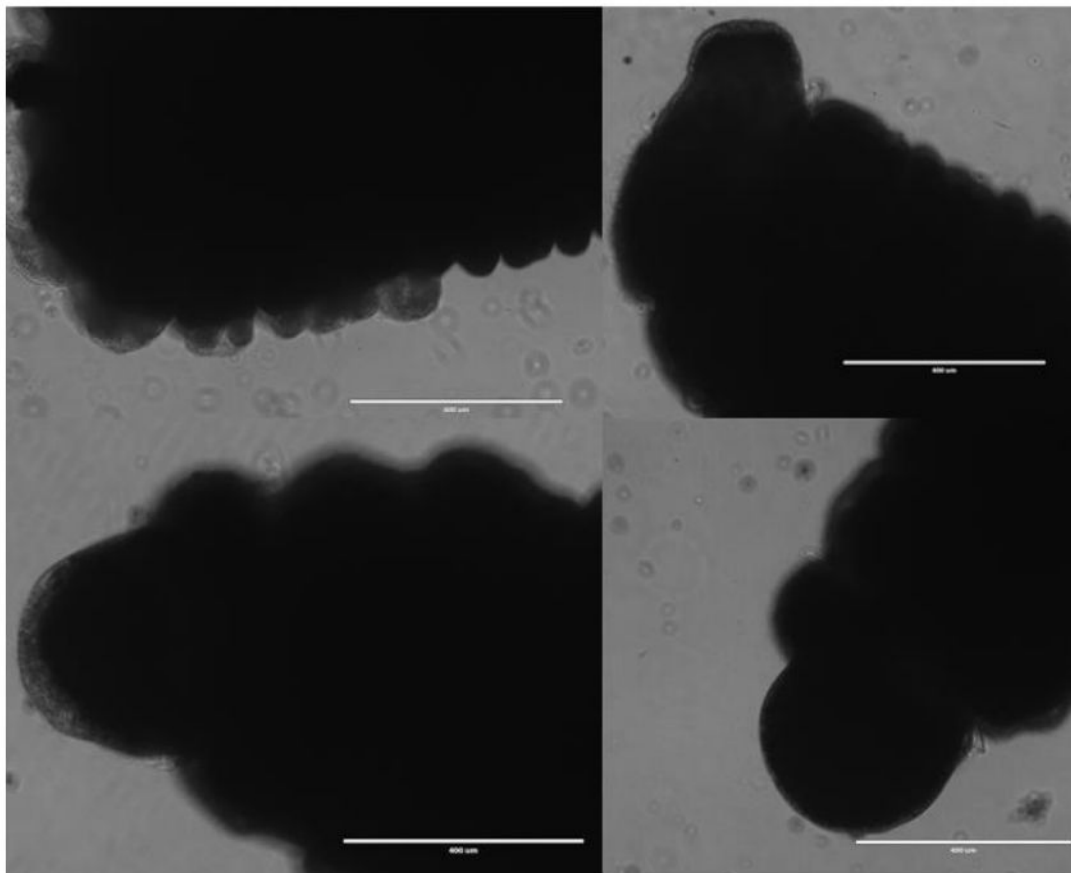


Fig 5.3: *in-vitro* maintenance of juvenile earthworm clitellum segments



5.4 ABNORMAL REGENERATION OF THE BODY SEGMENTS (30-37) - 7 DAYS MAINTENANCE UNDER *IN-VITRO* CONDITIONS

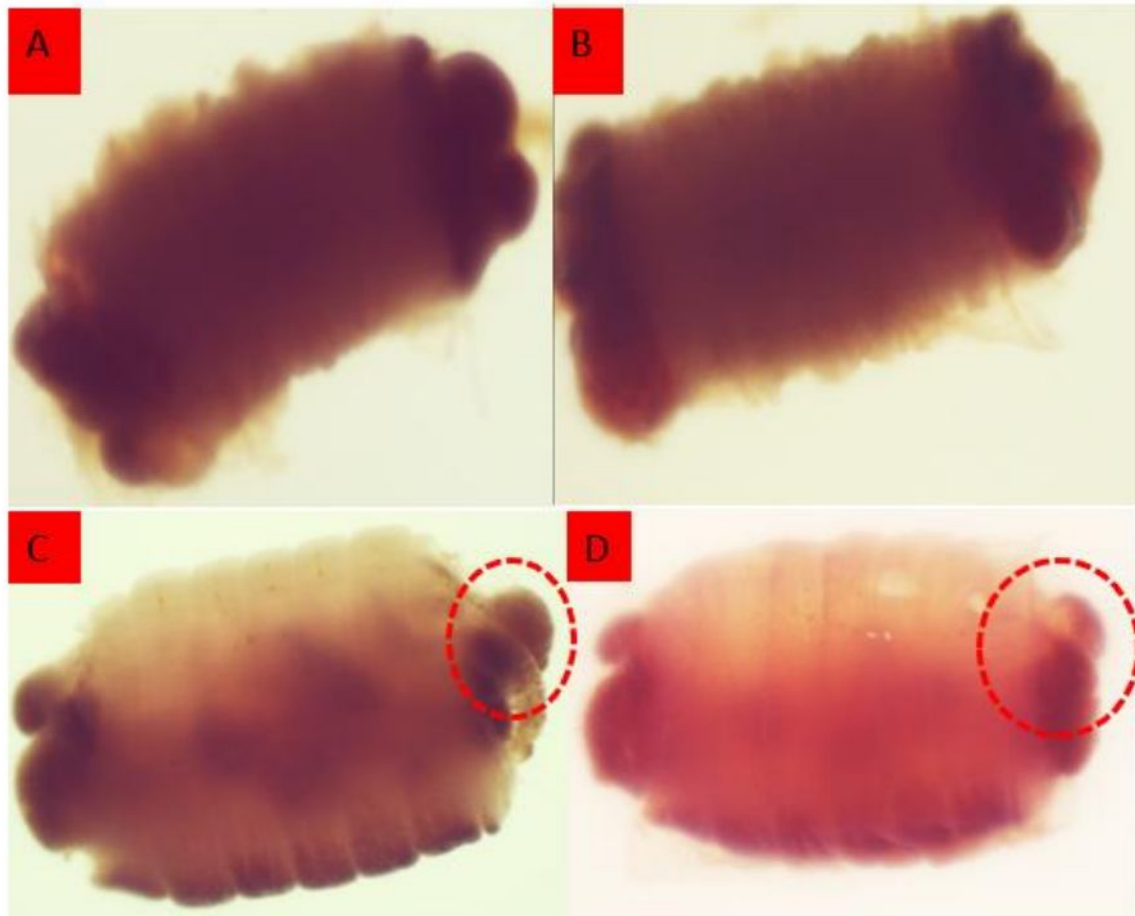


Fig: 5.4: In-vitro maintenance of adult posterior segments (30 to 37th)

(A) Adult *E. eugeniae* posterior segments on 0th day. (B), (C) No segment restorations were observed in day 2 and 4 but there is formation of abnormal buds on either side of the posterior segment. Red circle represents multiple blastema formations in one end which are transparent in nature. (D) Elongation of regenerative blastema in both wound sites; transparent tissue turns to dark and the size of the blastema is elongated.

Although the survival ability under in vivo conditions is influenced by segment length, the existence of clitellum segments, and clitellum factors, such a requirement is not shown in the in vitro maintained earthworm tissues. Regardless of segment length, all *in vitro* maintained tissues were able to survive, but there were differences in the potential for regeneration. The *in vitro* maintained posterior segments (30th-37th segments) of adult worms were able to survive for more than 7 days but lacked the capacity to generate proper blastema. Interestingly, multiple blastema formation was

initiated at one end of amputated sites. Numerous other factors also determine a worm's ability to survive and regenerate, and worms kept in *in vivo* circumstances have some drawbacks due to the fact that, after being amputated, they are kept in an environment devoid of aseptic conditions in an undefined worm bed composition. The amputated worm segments, on the other hand, that are kept under *in vitro* environments are kept in defined medium in a sterile environment. It's significant that the FBS-supplemented L15 medium delivered hormones, growth factors, vitamins, proteins, trace elements, and embryonic growth-promoting substances that aided in the growth and cellular reprogramming that improved survival and blastemal initiation.

5.5 WESTERN BLOTTING RESULTS AND STATISTICAL ANALYSIS

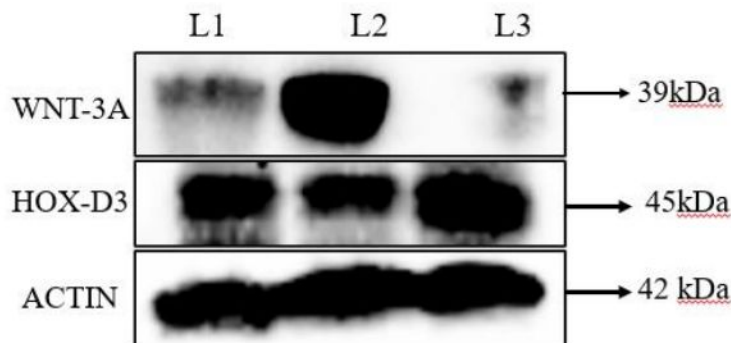


Fig: 5.5: Western blotting results

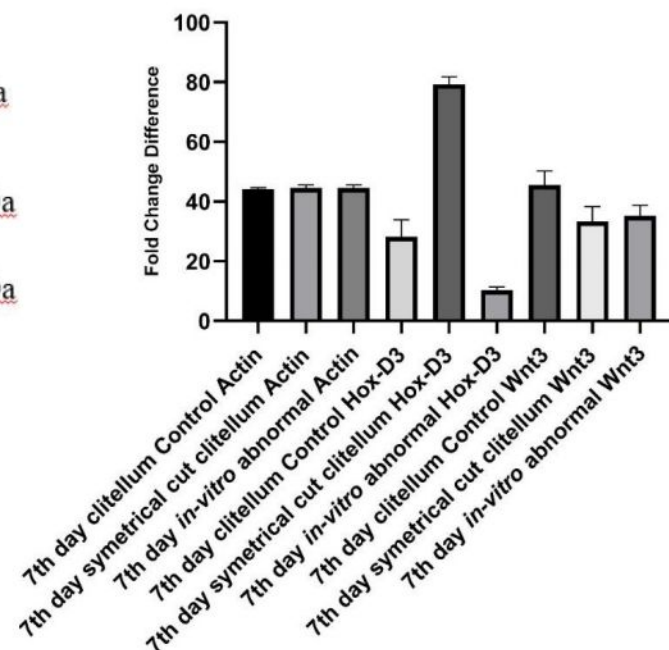


Fig 5.6: Statistical analysis using Image J

L1 – Ladder 1 – Asymmetrical cut of 10th segment – SUCCESSFUL REGENERATION

L2 – Ladder 2 - Symmetrical cut at clitellum segment - SUCCESSFUL REGENERATION

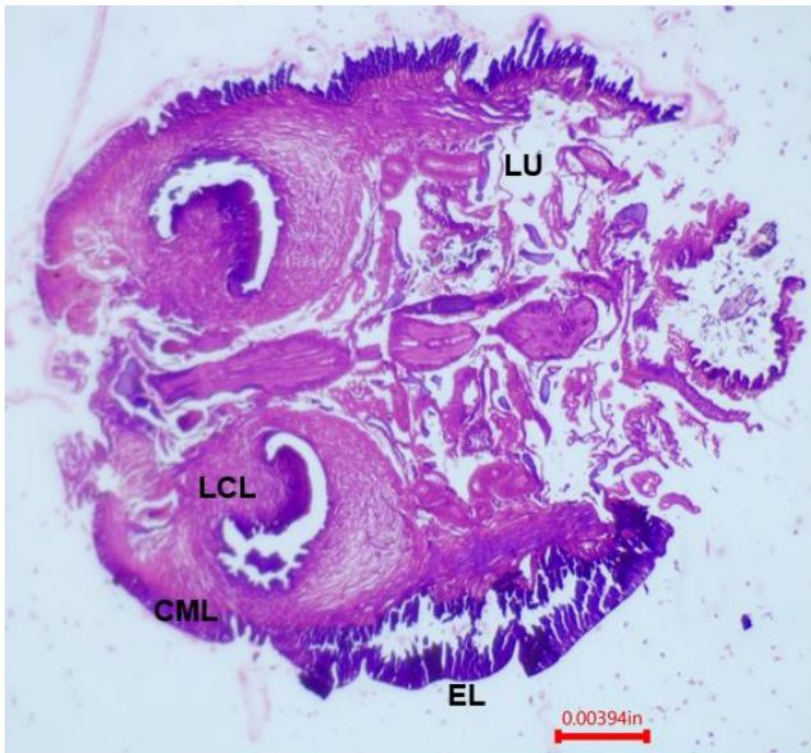
L3 – Ladder 3 – *In-vitro* maintenance of 30-37 body segments – ABNORMAL/ UNSUCCESSFUL REGENERATION

The executioner apoptotic caspases execute apoptosis as well as stem cell activating factors like Wnt3a, this is the common context towards many animal models namely, *Hydra*, *Xenopus*, *Drosophila*, newt and mouse. We have analyzed the key regenerative protein expressions of Wnt3a, HOX-D3, HOX-D3 to unravel the mechanism by which it gives patterning and positioning to organs in earthworm, *E.eugeniae*. Wnt3a protein commonly known for stem cell proliferation has been reported in many animal models. In general, HOX-D3 should be activated along with Wnt3a to achieve regeneration and Caspase-3 acts in execution of apoptosis (Trompouki et al., 2011)

Fig 5.5: According to the western blotting results obtained after seven days of maintenance under in vivo and in vitro conditions, we can infer that under in vivo conditions during symmetrical (amputated at the clitellum segment) and asymmetrical regeneration (amputated at the 10th segment), the patterning identity protein HOX-D3 and stem cell activator protein Wnt3a is activated leading to successful regeneration. But in contrary to this, when under in-vitro conditions where we were able to observe multiple bud formations; in this particular scenario, HOX-D3 is highly expressed whereas Wnt3a protein expression remains suppressed. These results indicate that Wnt3a protein and Homeobox protein HOX-D3 expression must take place at the same time for successful and normal regeneration to occur; otherwise, when only the positional identity protein I(HOX-d3) is expressed, abnormal buds form on either side of the segment under in-vitro conditions, resulting in improper regeneration.

Fig 5.6: Image J software was used to analyze the statistical data allowing you to relatively (no absolute values) quantify protein bands from western blot films. The length of *in-vivo* regenerated buds was measured and compared. More than 50 angles and their lengths were measured, equalized, and manually computed. Measuring angles were set using several fixing points in various locations. The quantification will reflect the relative amounts as a ratio of each protein band relative to the lane's loading control.

5.6 HISTOLOGY RESULTS



- EL** = Epithelial Layer
- CML** = Circular Muscle Layer
- LU** = Lumen
- LCL** = Longitudinal Cell Layer

Fig 5.7: Histology of regenerated clitellum segment

In a healthy earthworm, the Epithelial Layer refers to the outermost layer of cells that cover the body of the worm. This layer is composed of a single layer of flattened or columnar cells, depending on the region of the body. The epithelial layer serves several important functions in the earthworm's body, including protection from physical and chemical damage. The layer also contains specialized cells that secrete mucus, which helps to keep the worm's skin moist and reduce friction as it moves through the soil. The cells in the epithelial layer are tightly packed together, which helps to maintain the worm's shape and prevent fluid loss.

The Circular Muscle Layer of an earthworm refers to a layer of smooth muscle tissue that encircles the body of the worm is responsible for the worm's movements. The circular muscle layer is made up of small, spindle-shaped cells that are arranged in a ring-like pattern around the worm's body. In addition to facilitating movement, the circular muscle layer also plays an important role in the earthworm's digestive system. The muscles contract and relax in a coordinated manner to move food through the digestive tract and mix it with digestive enzymes and gut bacteria. Overall, the circular

muscle layer of an earthworm is a complex and important structure that plays a critical role in the worm's movement and digestive processes.

The Lumen of an earthworm refers to the internal space or cavity within the digestive system of the worm. It is the central space that runs through the length of the digestive tract, from the mouth to the anus. The lumen plays an important role in digestion, as it is where food is broken down and nutrients are absorbed. The walls of the lumen are lined with cells that secrete mucus, which helps to lubricate and protect the delicate tissues of the digestive tract. The lumen also contains various structures, such as the typhlosole, which increases the surface area of the digestive tract and enhances nutrient absorption.

The Longitudinal Cell Layer of an earthworm refers to a layer of smooth muscle tissue that runs along the length of the worm's body, parallel to its nerve cord. This layer is located just above the circular muscle layer and is responsible for the worm's elongation and shortening movements. The longitudinal muscle layer plays an important role in the earthworm's reproductive system. It is responsible for the contractions that help to move eggs or sperm through the worm's reproductive ducts. Overall, the longitudinal muscle layer of an earthworm is a complex and important structure that plays a critical role in the worm's movement and reproductive processes. It works in conjunction with the circular muscle layer to facilitate the earthworm's locomotion and digestion.

Fig 5.7: In this histology of undamaged clitellum, we can clearly distinguish all the layers. Circular muscle layer and longitudinal cell layer are both exquisitely characterized. When the worm is damaged, these two layers are crucial for regeneration because they work together to help in the development of blastemal cells, which are created when the longitudinal muscle layer elongates and/or widens to create the blastema.

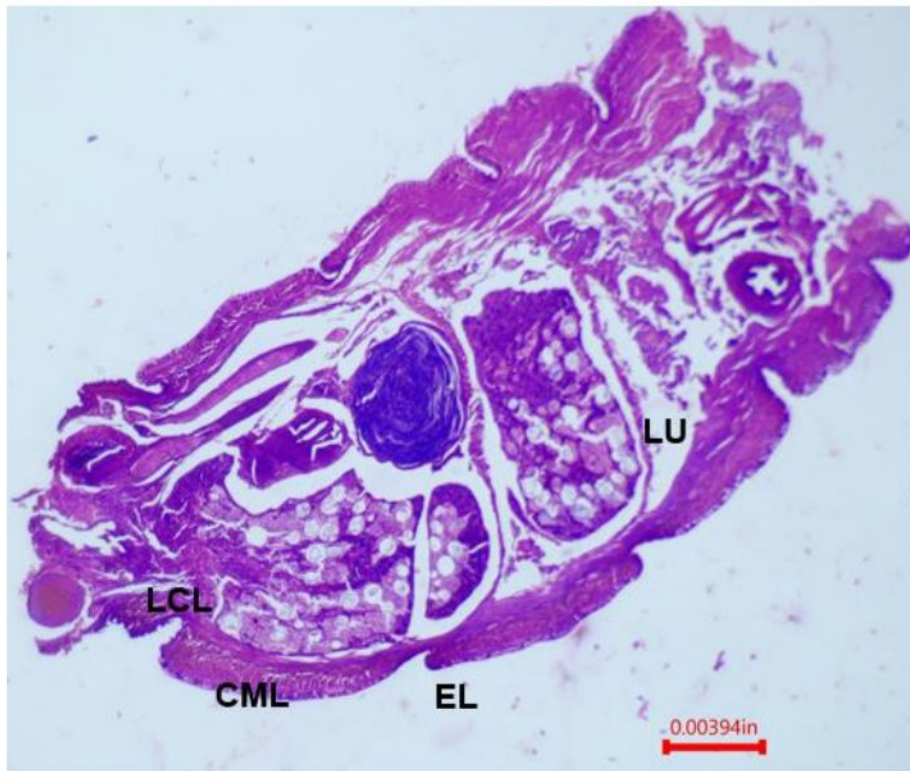


Fig 5.8: Histology of in-vivo maintained asymmetrical cut at 10th segment

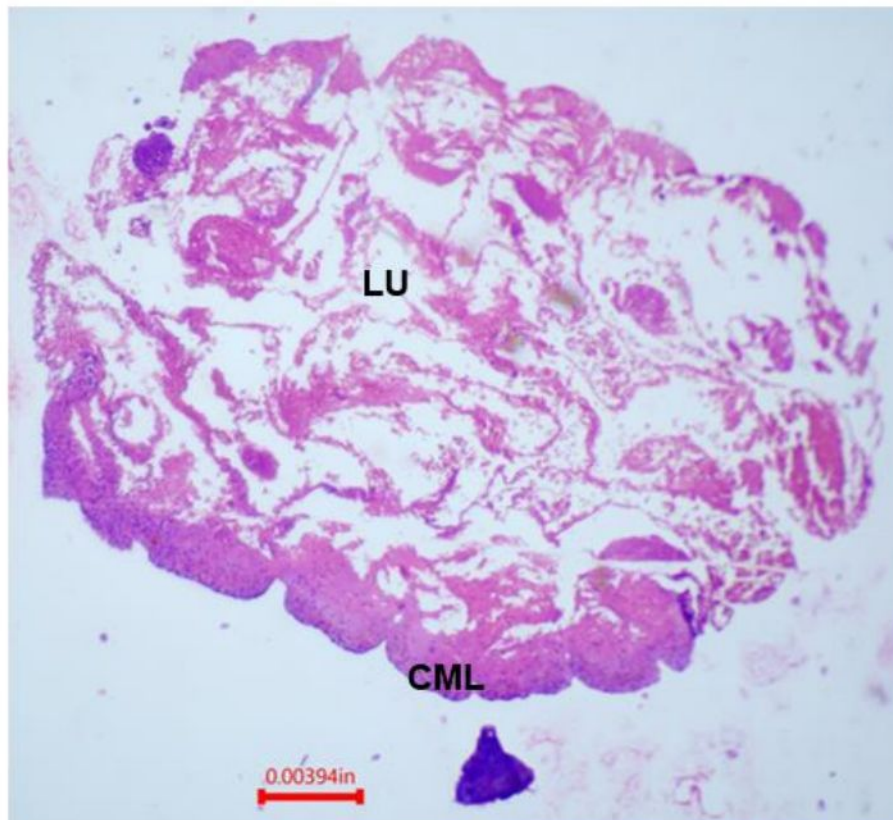


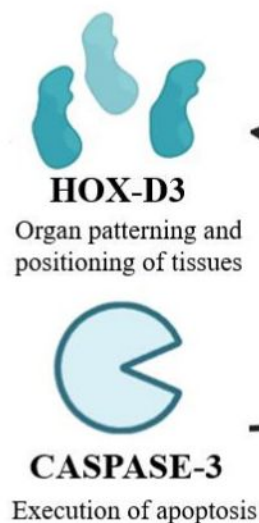
Fig 5.9: Histology of in-vitro maintained body segments (30-37)

Fig 5.8: From the histology results of in-vivo maintained asymmetrical cut we can observe the formation of Defined layers formed with proper organs and tissue patterning after amputation. The segment was restored successfully due to the presence of clitellum factors which was activated along with the positional identity protein HOX-D3. The CML and LCL together forms the blastema regeneration the bud after the worm was amputated at the 10th segment.

In **Fig 5.9:** This result is in contrary to **Fig 5.7** we can observe “Formation of Prematurely defined layers only, Further development of missing tissue context reformation requires Clitellum Factors in *Eudrilus eugeniae*” The LCL layer is not present and is not in conjugation with CML which results in abnormal regeneration when the worm is maintained under *in-vitro* conditions.

5.7 MOLECULAR MECHANISM OF HOMEBOX PROTEIN, HOX-D3 IN RELATION TO PROTEIN WNT-3a

SUCCESSFUL REGENERATION WITH PROPER POSITIONING OF ORGANS



ABNORMAL REGENERATION WITH IMPROPER POSITIONING OF ORGANS

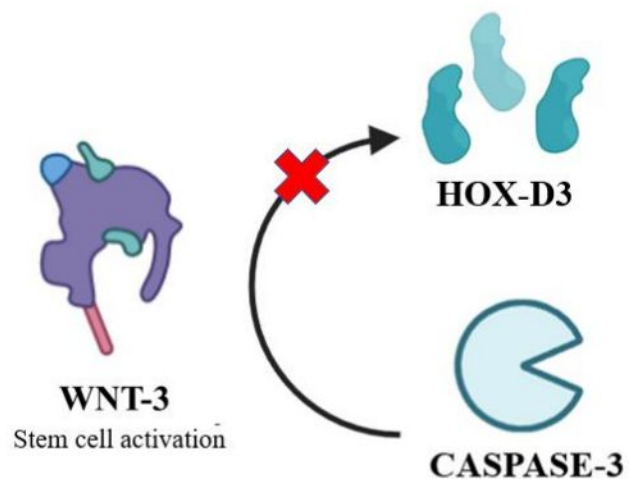


Fig 5.10: Molecular mechanism of HOX-D3

By this mechanism we can elucidate that, Caspase 3 is a type of cysteine protease that plays a crucial role in programmed cell death or apoptosis. It is responsible for cleaving and activating other caspases, including caspase 6, 7, and 9. Caspase 3 is regulated by various factors, including Inhibitor Of Apoptosis Proteins (IAPs), which

bind and inhibit caspase 3 activity. It is also involved in various physiological processes, such as tissue remodeling. Abnormal regulation of caspase 3 has been linked to various diseases such as abnormal tissue growth, cancer, and Alzheimer's disease. Therefore, targeting caspase 3 is a potential therapeutic strategy for the treatment of these diseases. Wnt3a on the other hand is a secreted signaling protein that belongs to the Wnt family. Wnt3a activates the canonical Wnt signaling pathway by binding to its receptors. Studies have shown that Wnt3a is important for embryonic development, including the formation of the neural tube, limbs, and heart. In adults, Wnt3a is involved in the maintenance of stem cells and the regeneration of tissues, such as the liver and intestine. Abnormal regulation of Wnt3a has been linked to various diseases, including cancer. Therefore, understanding the function and regulation of Wnt3a is important for the development of therapies for these diseases.

Fig 5.10: Molecular mechanism of HOX-D3 was elucidated after confirming and standardizing the western blotting and histology results. From this we can understand that, during successful regeneration, Caspase 3 is responsible for execution of apoptosis aiding in the formation of new cells and tissues when Wnt3a (stem cell activation) is activated. But in this, the prominent role is played by HOX-D3 giving as positional identity of organ patterning resulting in normal and successful regeneration.

In contrary, the second scenario where caspase 3 is activated along with Wnt3a but the HOX-D3 protein is not expressed, there abnormal regeneration takes place resulting in formation of premature and undefined layers of cells and tissues with no patterning of organs. This proves that for successful regeneration to take place, apoptosis, stem cell activation (Wnt3a) and positional identity aiding protein HOX-D3 should be expressed together. These proteins should be studied together to understand why and how regeneration of only certain organs take place in our body with the help of stem cells

6: SUMMARY AND CONCLUSIONS

SUMMARY

In summary it can be understood that by using stem cell biomarkers as a comparative analysis tool, we can comprehend the mechanism by which the external factors affect the internal biological functions of the worm. So, understanding the function of the clitellum is crucial to unlock the secret of regeneration because the intact clitellum of earthworms is required for successful regeneration. So, to conclude, HOX-D3 regulates Wnt3a in different regeneration patterns of earthworm, *Eudrilus eugeniae* is proven.

CONCLUSIONS

- The observations show that clitellum creates a pathway for stem cell migration, which needs to be thoroughly investigated before performing molecular biology experiments.
- Subsequent research should focus on uncovering specific functions of every stem cell present in the clitellum region which will provide a broad scope for future advancements in the field of regenerative medicine.
- So, to conclude HOX-D3 regulates Wnt3a in different regeneration patterns of earthworm, *Eudrilus eugeniae*

6.2.2 Applications

It has been demonstrated that HOXD3 is overexpressed in breast cancer tissue as compared to normal breast tissue (Zhang et al., 2018).

Our findings of molecular mechanisms open the possibility of improving the current standard of care for breast cancer patients by designing targeted molecular therapies in relation to WNT-3 stem cell biomarkers helping to overcome the barriers of cancer cell stemness and drug resistance.

They may function as components of enhancer-protein that affect the expression of crucial growth factor receptors and hormone receptors leading to new drug discovery efforts (Shah & Sukumar, 2010)

To date, the evidence only hints at the mechanisms underlying the roles of these genes in oncogenesis. Future work will be aided by molecular therapies as it has tumor suppression properties, for which this particular molecular mechanism will be useful. To understand how invertebrates still retain the ability of regeneration and to find out reasons why it was lost during evolution. More research has to be done to prove it evidently.

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ANNEXURE

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Authors: Chandini Sengupta (Co-author), Srishti Raja (Co-author), Kamarajan Rajagopalan (Co-author), Karthikeyan Subbiah Nadar Chelladurai (Co-author), Sudha S (Co-author), Inbathamizh L (Co-author), Jackson Durairaj Selvan Christyraj (Corresponding Author)

Date submitted: 2023-04-19

Dear Miss Sengupta

The above manuscript has been submitted online by Dr. Selvan Christyraj and you have been listed as a co-author.

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[Author files]	Manuscript ID: CTO-2023-4-11 Type: Review Article Authors: Chandini Sengupta (Co-author), Srishti Raja (Co-author), Kamarajan Rajagopalan (Co-author), Karthikeyan Subbiah Nadar Chelladurai (Co-author), Sudha S (Co-author), Inbathamizh L (Co-author), Jackson Durairaj Selvan Christyraj (Corresponding Author) Submitted: 2023-04-19	

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Jackson Durairaj S
to bio, Srishti, me ▾

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----- Forwarded message -----

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Date: Sat, 25 Mar 2023, 17:42

Subject: Acceptance of Book Chapter and Camera Ready Chapter Submission

To: Jackson Durairaj S <jacksondurairaj@sathyabama.ac.in>

Dear Dr. Jackson,

Greetings of the day!

Your chapter entitled "Future Trends and Directives for Research on Phytochemicals in Neurological diseases" has been accepted to be sent for publication. **(Chapter 12)**



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FOR THE MANUSCRIPT TITLED

Coriandrum Sativum Seeds And Its Fiber Applications

presented at the **MAHSA International Medical
Technology (Mi-MED) Conference 2022** organised
by the Faculty of Medicine, Bioscience, and
Nursing on the 22nd and 23rd of July 2022

**PROF. DR. SRIKUMAR
CHAKRAVARTHI**

Director, Postgraduate Studies,
Research and Innovation,
MAHSA University, Malaysia

**PROF. DR. RUSLI
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Dean, Faculty of Medicine,
Bioscience and Nursing
MAHSA University,
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Functional significance of earthworm clitellum in regulating the various biological aspects of cell survival and regeneration

1. Introduction Earthworms are economically important organisms belonging to the Phylum Annelida and Kingdom Animalia. They have 120 segments and each segment has potential organs that help in various biological aspects of cell survival and regeneration such as organogenesis, wound healing, autotomy, and reproduction events due to the presence of stem cells and other vital proteins; stem cells- the specialized progenitor cells that have self-rejuvenating properties.

100%

MATCHING BLOCK 1/31

W

It has a higher growth rate as little as 5 weeks to reach maturity (

Zirbes et al. 2012). This review highlights all the important features of clitellum and other factors that are responsible for various biological processes associated with the everyday living cycle of earthworms by involving clitellum. As suggested by various scientists' earthworm

96%

MATCHING BLOCK 2/31

W

is a great model for cell and molecular biological experiments. Since it has an amazing ability

to regenerate and heal itself within a week or two without changing its internal functions and keeping their economic importance intact (Subramanian, Daisy, et al. 2017)(Paul et al. 2021)(Gopi Daisy et al. 2016).

76%	MATCHING BLOCK 10/31	W
Autotomy is the ability of certain animals to detach their segments/parts of the body that has been caught or grasped by an external agent		

that is a predator. Autotomy can also mean 'self-severing' or 'self-amputation', which is an important phenomenon involving the loss and often the subsequent regeneration of body parts such as appendages. It is an advantage in a number of invertebrates (e.g., arthropods) and vertebrate (e.g., salamanders) taxa where it usually serves as a self-defence mechanism enabling a potential prey organism to escape or confuse an attacking predator (Maginnis 2006)(Fleming, Muller, and Bateman 2007)

Invertebrates lacking appendages, such as annelid worms, are also known to adaptively autotomize body segments, followed by their regeneration, as an anti-predatory strategy Frequent electrostimulation of worms can lead to the autotomy of posterior segments followed by their regeneration and differentiation of body parts.

78%	MATCHING BLOCK 11/31	W
The animals which can trigger autotomy also have the potential for regeneration. The lizard,		

88%	MATCHING BLOCK 12/31	W
the crab, spider, and octopus, also can trigger autotomy for escaping from predators and then they can regenerate the lost body part (		

Sivasubramaniam 2021) (Walton and John Monteith,1936).
It has been found in autotomy of Peregrine Earthworms