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Medial regeneration in earthworm (Eisenia foetida): preference in segmental regenerative growth

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Abstract

Earthworm, *Eisenia foetida* has a good regenerative potential and has been used in various studies to understand the regenerative process. Regeneration in earthworm is a sequential process starting with blastema formation by dedifferentiation and redifferentiation to restore the lost parts. Regenerative potential is dependent on the position of amputation and site specific.*Eisenia foetida* were cultured and two types of amputation were performed i.e. 20 segments amputated from anterior and posterior ends and 40 segments amputated from anterior and posterior end resulting in medial segments for regenerative studies. The growth and regenerative potential were compared and correlated. The anterior ends of the medial segment showed a better regenerative growth as compared to posterior end and quantified by the calculating coefficient of regeneration. It is also observed that medial fragment with more segments have a higher potential of regeneration and a good correlation was seen between the site of amputation and time for regeneration. Various theories are proposed relating to regeneration potential and site of amputation. A preference in anterior regeneration to posterior regeneration is hypothesized due to the presence of vital organs for survival. Regeneration should be studied at a genetic level to understand the mechanism.

Keywords: Medial regeneration, amputation, *Eisenia foetida*, regenerative growth, blastemal

1. INTRODUCTION

Members of the phylum Annelida has a wide distribution, cosmopolitan in nature and occupy a variety of niche from soil to marine environments. Some species sieve into the soil for soil fertility, some burrow deep into marine sediments while others are parasitic in nature[1, 2]. Annelids can be divided into two broad classes i.e. Clitellata and Polychaeta. Clitellata is further divided into Oligocheates (earthworms)and Hirudinea (leeches)[3]. Regeneration is a phenomenon by which an organism can restore its lost parts caused due to injury and this phenomenon has appeared and disappeared during the course of evolution. Various species of earthworms show the ability to regenerate its lost parts and often preferred as models to study regeneration. Earthworms are mostly chosen as regeneration models due to its availability, rapid regeneration, easy to culture and handle in laboratory[4, 5].*Eisenia foetida* is one of the lumbricid species which are predominantly used for regenerative research. Lumbricid species including *E.foetida* are triploblastic in nature with a true coelom, metameric, a closed vascular system, centralized brain ganglia and a nervous system with longitudinal cords and sense organs. Survival after decapacitation of earthworms are crucial for regeneration as the earthworms are cut into various segments by predators and soil cultivators.

Regeneration is a gradual process and involves the formation of blastema at first. The formation of blastema is the characteristic of epimorphotic regeneration. The process of epimorphic regeneration involves dedifferentiation and cell proliferation and provides a useful model to investigate the mechanism of normal development as well as differentiation[6]. Different tissue such as myofibers, satellite cells, cartilage, dermis and nerve cells contribute to blastema formation after amputation[7, 8].Dedifferentiation, a switch in cell type from fully differentiated to pluripotent status, is initiated by the rearrangement of extracellular matrix and integrins[9]. Earthworm regeneration is proposed to follow the epimorphotic type of regeneration where dedifferentiation leads to the formation of blastema cells and subsequent redifferentiation without any contribution from totipotent stem cells. Formation of blastema is crucial for the regeneration and subsequently restoration of the lost parts by differentiation as reported by various studies of early wound healing[4, 10]. The course of blastema development and redifferentiation is well documented in a study by Park et al [11]. This study shows that intestine extrusion occurs first, followed by wound healing. The wound covers the intestine by forming an intact epithelial covering and occurs in the initial 3-5 days of regeneration. After wound healing, a small blastema (group of undifferentiated cells) is formed on day 7.Blastemas are derived by dedifferentiation from the tissues adjacent to the injured surface and longitudinal muscles of the coelomic side contribute maximally to blastema formation. The blastema cell population is suggested to increase the cell recruitment and division producing extracellular matrix and leads to tissue swelling. Blastema formed beneath wound dermis is considered to begin segmentation within 1 week of amputation, although active redifferentiation is not initiated after 1 week.The blastema continues to grow undifferentiated and regenerating tissue outgrowths were seen between day 14-17. The dark red color of the normal segments was in marked contrast to the much lighter color of new regenerating tissue during the 3 weeks of regeneration. Rapid growth and pigmentation occurred after 26 days. The intestine reopened and the worm was able to feed and excrete digested material after 30 days post amputation[11].

Regenerative capacity in earthworms is dependent on the position of the lost body segments as documented in various studies. These studies are focused on anterior or posterior regeneration i.e. amputation of variable number of segments along the anterior or posterior end. A decrease in the regenerative capacity was observed as the number of segments amputated increases in earthworm *E.foetida*[12]. It was also tested that the regenerative growth rate was faster when the amputation site was closer to the anterior end[13]. Liebmanm's study showed that the regeneration rate of head decreased slightly after amputation of nine segments. Moreover, the decrease became more prominent behind the seventh segment, with each additional metamere removed decreasing the degree of regeneration[14]. The length of regenerated portion is variable and a significant co-relation could not be derived between the number of regenerated segments and number of amputated segments as well as number of total body segments. However, the study on *E.andrei* for distal regeneration suggest to restore an average of 16 segments when 20 segments were amputated from the distal end[11]. Moment reported that when the level of amputation is moved 10 segments more posteriorly, the average number of segments regenerated declined by 10. This result supports the idea that amputation level influences the average number of regenerated segments[10, 15]. These documented studies based on either anterior or posterior regeneration where the amputation of the segments is fixed from either of the ends i.e. proximal and distal. Anterior and posterior regeneration in earthworms are worked upon to a greater extent as compared to medial anterior and medial posterior regeneration. Significant differences in anterior and posterior regeneration of medial segments were seen after 30 days.The regeneration length and capacity for posterior regenerated segments are significantly more than anterior regenerated segments[16]. Considering the importance and minimal number of studies related to medial regeneration, our work was focused on segmental regeneration of anterior and posterior medial regeneration.

Environmental factors such as temperature and nutrition influence the regenerative capacity in earthworms. Earthworms cultured at 25°C regenerated faster as compared to those that were cultured at 30°C and 20°C. Furthermore, sexual activity also influences the regeneration rate. Farther the position of site of amputation from the sexual organs, more decrease in the rate of regeneration[14]. The process of regeneration occurs by dedifferentiation and redifferentiation and this is regulated by a family of genes known as *Hox* gene. *Hox* gene family includes three labile genes *Pex-lab01, Pexlab02, and Pex-lab03*which play a crucial role in blastema formation and wound healing in early head regeneration[17].Pluripotency factors *oct4, nanog, sox2, c-myc* and *lin28 genes* are upregulated during cellular reprogramming and dedifferentiation [18].Pituitary adenylate cyclase-activating polypeptide (PACAP)like compounds have similar trophic functions during caudal regeneration of the earthworm, *E. foetida*, which is known for PACAP in vertebrate species[19].The ways in which the regeneration capacities of earthworms depend on the numbers or position of amputation, has not been demonstrated in detail to date.

In this study, we estimate the segmental regeneration in the trunk region of *E.foetida* by amputation of different number of segments from the proximal and distal ends and the number of days required for the regeneration. The number of segments regenerated in anterior and posterior ends of the medial segment are compared and correlated. In our study, we also estimated the time required for the blastema development in both the ends of the medial segment and prostomium development in the anterior end of the medial segment.

2. MATERIALS AND METHODS

2.1. Earthworm culture

Earthworms, *E. foetida* (Annelida: Oligochaeta) were reared in vermicompost at 25 ± 1 °C, in a laboratory climate chamber for several generations. All earthworms that hatched from cocoons after20 days were regarded as immature until they developed a clitellum and were left in the culture chamber to develop into adult earthworms. Reproductive adult earthworms, which developed a clitellum about 60 days after hatching, were selected for the experiments. The adult earthworms had a well-developed clitellum consisting of seven segments with 26–32 segments before the clitellum and 76 segments behind it. Since, earthworms are hermaphrodites, the clitellum contains the male and female reproductive organs and cocoons are formed when the clitellum passes over the head. Each cocoon contains one or more eggs. Earthworm, *E.foetida* has typically 108 segments in adults.The head is composed of seven segments that are heteronomous, equipped with specific organs such as the prostomium, cerebral ganglion, pharynx, or septal glands. The cerebral ganglion is a bi-lobed structure, which occurs dorsally in segment 3 and segment 4, and contains the sub-oesophageal ganglion. The posterior end of the body is an anal segment with an anteriorly adjacent growth zone.

2.2.Earthworm amputation

Adult earthworms with a distinct clitellum were chosen for amputations. Two types of amputations were performed including : (i) medial parts of earthworms whose 20 segments from anterior and posterior ends were removed (M21/88), medial anterior and posterior ends were labelled MA20/21 and MP88/89 respectively (Figure 1(A)) and (ii) medial parts of earthworms whose 40 segments from anterior and posterior ends were removed (M41/68), medial anterior and posterior ends were labelled MA40/41 and MP68/69 respectively (Figure 1(B)). These manipulations were designed to observe the effects of position of amputation on lengths of regeneration in medial segments. The amputation sites with respect to the number of segments are depicted in figure1. Earthworms without any amputation were treated as controls.All amputation treatments were performed with 5 earthworms per treatment, each kept in a 200 ml plastic container with tissue culture.

Before amputation, each earthworm was rinsed with de-ionized water, placed on a fresh piece of blotting paper, and viewed under a stereomicroscope. The earthworms were kept in a relaxed position to facilitate the precise amputation at the particular sites. Earthworms were amputated quickly with a sharp scalpel, and after amputation were cultured in polyethylene plastic containers (200 ml) with fresh tissue culture, and kept moist by sprinkling with water weekly in a climate chamber at 25 ± 1°C. The opening of the plastic containers was covered with muslin cloth to facilitate gas exchange and prevent the organism from escaping. The progress in regeneration were checked regularly for 30 days. The number of regenerated segments in anterior and posterior medial fragments were observed under a stereomicroscope regularly and the regenerative capacities of both the ends were analyzed. Pearson Correlation was used to analyze correlation coefficient between number of regenerated segments and time. The data were analyzed using Microsoft excel version 8. The coefficient of regeneration with respect to number of segments is calculated as:

$$Coefficent\ of\ regeneration\ (\varepsilon) = \frac{number\ of\ segments\ regenerated\ (observed)}{number\ of\ segments\ amputated (expected)}$$

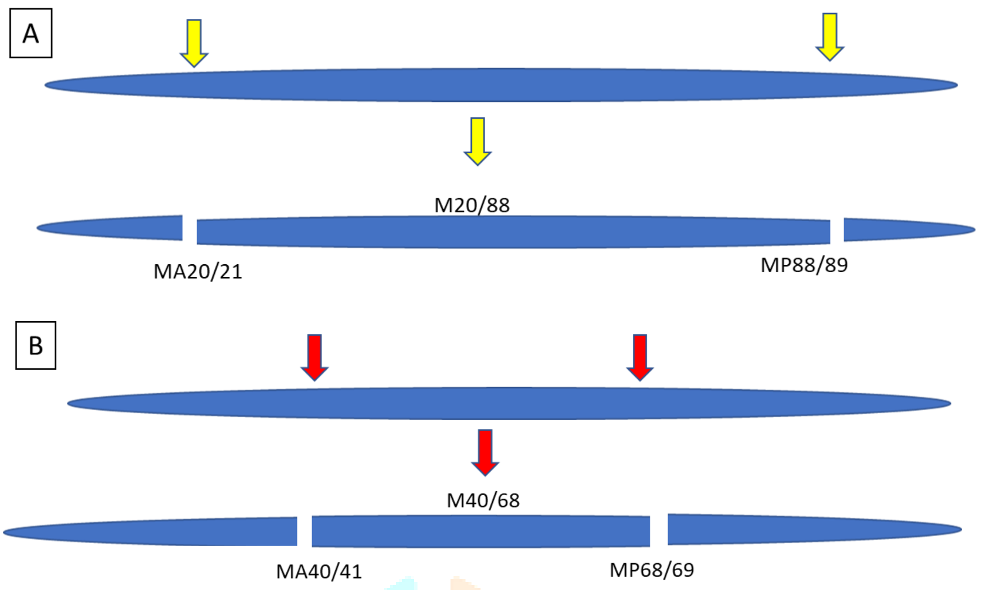

Figure 1) Schematic representation of amputation in *E.foetida*for(A) amputation of 20 segments from anterior and posterior end with medial fragment M20/88 with anterior end (MA20/21) and posterior end (MP88/89).(B) amputation of 40 segments from anterior and posterior end with medial segment M40/68 with anterior end (MA40/41) and posterior end (MP68/69).

3. RESULTS

3.1. 20 segment medial regeneration

Earthworms were amputated 20 segments from the anterior and posterior ends resulting in medial fragment of 68 segments (M21/88). The anterior end (MA20/21) showed no growth for 3 days. Intestine extrusion and wound healing occur in the initial 2 days post amputation. This was observed by the closure of the anterior end on the 2nd day. Blastema is a group of undifferentiated mass of cells is formed by the dedifferentiation of the cells near the amputated region which is seen on the 3th day post amputation (Figure 2(A)). The blastema could be identified by a cap like transparent structure without segmentation. From the 4th day, segmentation is seen in the growing regenerative region. The number of segmentations increases from the 4th day and attains a saturation point on the 12th day where the segmentation increases till the 12th day post amputation (Figure 2(B)). The number of segments were constant after the 14th day and the regenerative growth was documented till 30th day post amputation. However, the number of segments did not increase after the 14th day, but pigmentation was seen initially which approximately continued till the 18th day. The average number of regenerated segment in MA20/21 were recorded to be 16.6. The slope of the number of regenerated segment vs time graph was highest for MA20/21 which had the largest number of regenerated segments.

The posterior end (MP88/89) of the medial segment showed a similar trend of regenerative growth but showed a delayed growth. There was no growth seen till the 6th day post amputation and the blastema growth occurs on the 7th day post amputation (Figure 2(C)). The morphology of blastema was similar to that seen at the anterior end

MA20/21. The segmentation was not seen as the subsequent step after blastema formation but the blastema continued to grow to a certain extend. The segmentation occurs on the 10-11th day post amputation with 2 segments. The segmentation increases till the 15th day post amputation and did not increase afterwards till the 30th day. The average number of regenerated segments were recorded to be 4.75. the posterior growth of the medial segment failed to show a significant growth with respect to the number of segments amputated.

From the above results, it can be concluded that the anterior end of the medial segment showed a faster and a better regenerative growth as compared to the posterior ends despite the number of segments amputated being constant. This result of differential regenerative growth is hypothesized due to the presence of vital organs in the anterior end such as pharynx, prostomium and cerebral ganglion. The graphical representation is depicted in figure 2(D).

3.2. 40 segment medial regeneration

Earthworms were amputated 40 segments from the anterior and posterior ends resulting in medial fragment of 28 segments (M41/68).The posterior end (MP68/69 showed no growth for 3 days. Intestine extrusion and wound healing occur in the initial 3 days post amputation. The blastema formation is the next step of wound healing which occurs between day 4-5 post amputation. The segmentation in blastema is not seen and represents a transparent mass of undifferentiated cells which is responsible for the formation of growth trajectory. Segmentation in the growing part was seen at day 5 post amputation and increased till day 14 post amputation with the maximum number of segments recorded to be 12.8. The growth of the regenerated section was not seen after day 14 and gradually the organisms with the regenerated parts could not survive. None of the organism survived after day 27 post amputation.

The posterior end (MP68/69) of the medial segment showed a similar trend of regenerative growth but showed a delayed growth. There was no growth seen till the 4th day post amputation and the blastema growth occurred between the 5-6thday post amputation. The segmentation started from the 8th day and continued till 12th day with only 3 segments. And interesting observation that seen on 8th day was that the organism could excrete the feaces from its posterior end. However, this is not a proof for the development of anal segment since the excreta could easily be removed via muscular contraction and relaxation. The regenerative growth stopped till 3 segments and no further growth was recoded till 25th day. The organism did not survive post 27 days.

From the above results, it can be concluded that the anterior end of the medial segment showed a faster and a better regenerative growth as compared to the posterior ends despite the number of segments amputated being constant. This result of differential regenerative growth is hypothesized due to the presence of vital organs in the anterior end such as pharynx, prostomium, gizzard, sexual organs and cerebral ganglion.The slope of the number of regenerated segment vs time graph was more for MA40/41 as compared to MP68/69. The graphical representation is depicted in figure 2(D).

3.3. Comparison between segments regenerated and site of amputation

Comparison between the anterior regenerative growth after amputation at M20 and M40 reveals the importance of amputation site and regenerative potential. The coefficient of regeneration for anterior and posterior segments were calculated using the formula. The coefficient of regeneration for M20/21 is calculated to be 0.83 whereas for M40/41 it is 0.237. the coefficient of regeneration is a measure of the regenerative potential and is directly proportional. Therefore, the potential for regeneration is higher for MA20/21 than MA40/41. Similarly, the coefficient of regeneration for posterior ends were calculated to be 0.32 and 0.175 for MP88/89 and MP68/69 respectively. The potential for MP88/89 is therefore higher than MP68/69. Therefore, the site of amputation has a correlation with the regenerative capacity as it is seen thatthe segments could grow faster and restore maximum segments when a smaller number of segments were amputated.

3.4 Correlation between the regenerative capacity and time for regenerative growth

A partial regeneration correlation showed significant correlation between number of segments regenerated and time for MA20/21 (*R^2=0.475, df=3, P=0.41*) and MP88/89 (*R^2=0.287, df=3, P=0.63*).A correlation between number of segments regenerated and time was also seen for MA40/41 (*R^2=0.92, df=1, P=0.25*) but for MP68/69 the correlation was not significant (*R^2=1, df=1*).

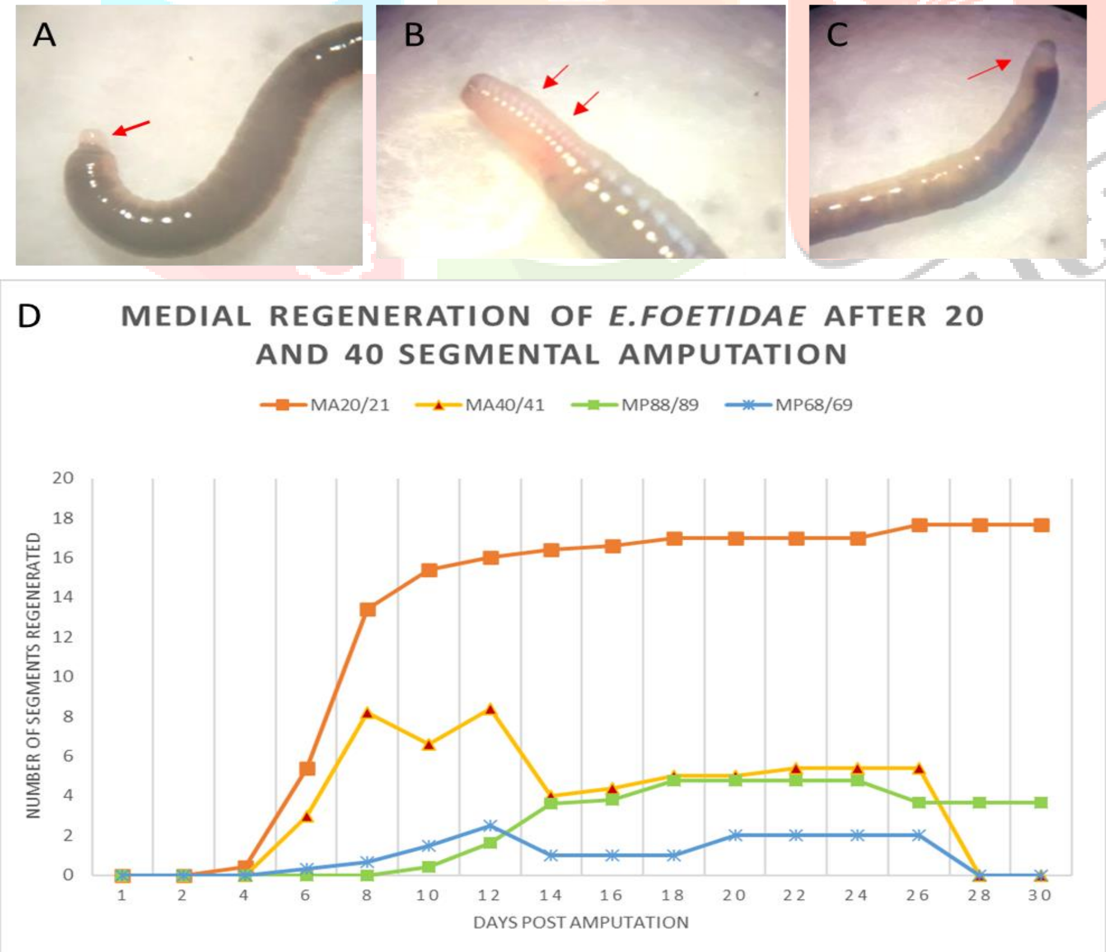

Figure 2) (A) Blastema formation at the anterior end of medial segment MA20/21 at day 3 post amputation (B) Segmentation and growth of the regenerative part in the anterior end medial segment M20/21 at day 7 post amputation (C) Blastema formation at the posterior end of medial segment MP88/89 at day 7 post amputation (D) Graphical representation of regenerative growth (number of segments regenerated vs time in days).

4. DISCUSSION

Earthworms are classified under the phylum Annelida which are placed at a higher phylogeny than lower organisms such as hydra and planaria. Earthworms are also preferred as regenerative models due to the presence of metamere and segmentation. The regenerative pathways are also different depending on the organism such as in hydra regeneration where the preexisting tissue give rise to the regenerative parts[20]. Earthworms however adopt the path of dedifferentiation and redifferentiation at the amputation site and hence they are more suitable to explore the regenerative capacities in vertebrates[4]. The regeneration process in *E.foetida* initially begins with wound closure and formation of blastema at the amputation site. Blastema formation is followed by head or tail bud formation. The number of days required for the formation of blastema is different for anterior and posterior amputated ends. After the development of blastema, segmentation of the regenerative parts occurs and is terminated after a certain number of days. The occurrence of segmentation and termination is also observed to be different in different cases depending upon the site of amputation and number of segments amputated[21]. The segmental growth for anterior ends is observed to be faster than in posterior ends.

In our study, *E.foetida* showed a good regenerative growth for anterior ends when compared to posterior end. The regenerative growth is more when fewer segments are amputated in earthworm, *E.foetida*.A positive correlation was seen between the number of segments regenerated and time for anterior endof medial regeneration. Two type of amputations were performed in our study which includes amputation of 20 segments and 40 segments from anterior and posterior ends. The anterior end after 20 segment amputation showed a better regenerative growth than posterior end and could restore maximum segments in a specified amount of time.This differential regenerative growth is hypothesized due to the presence of vital organs in the anterior end such as pharynx, prostomium and cerebral ganglion. These organs are vital for the basal metabolism and survival. The posterior segment could not restore all the segments. The anterior end after 40 segment amputation showed a better regenerative growth than posterior end but could not restore all the segments. The medial segment contains very few segments and could not survive beyond 27 days. The coefficient of regeneration(ε) is the measure of regenerative potential as described earlier. Coefficient of regeneration is the highest for anterior end of 20 segment amputation (MA20/21) followed by MP88/89,MA40/41 and MP68/69.According to "Morgan's law of regeneration" it is stated that closer the amputation was to the anterior part of the body, the longer the length of regeneration[22]. This law was also proved by Moment in his work on *E.foetida*[13]. Our study also followed the Morgan's law of regeneration as the amputation sites were different for both the experiment and regenerative length was calculated to be more whose amputation site was closer to the ends. Our study also showed that anterior length of regeneration was significantly high than the posterior length of regeneration. However, Xiao reported that the posterior length of regeneration was significantly high than the anterior length of regeneration in immature earthworms and did not match with our observation. Therefore, the regenerative potential of posterior segments were significantly high as compared to anterior segments in immature earthworms. The posterior regeneration was seen to be highest in M7-32 as compared to M33-60,M33-90 and M26-90. Adult clitellar earthworms however showed a reduced regenerative potential. The overall trend in lengths of posterior regeneration was to compensate as far as possible for the lost segments. When the earthworms retained 20-30 segments after amputation, they became long. However, when they had 50-60 segments, their lengths of regeneration did not increase until they reached a certain critical length, i.e. when they had 80–90 segments remaining, [16]. Park demonstrated that the earthworm, *E.andrei* could regenerate 74% of the amputated segments in the tail regeneration. By histological studies, it was also shown that longitudinal muscle layer from the coelomic side contributes maximally to the blastema development[11].

Myohara's work on *Enchytraeus japonensis* showed a combination of epimorphosis and morphallaxis in its regeneration. The epimorphosis type of regeneration was seen in anterior and posterior ends where the anterior blastema grew to form the necessary segments of the head and the posterior blastema formed the anal segment. However, posterior blastema did not grow as much as the anterior blastema. The intermediate regions showed a different response towards regeneration as the old segments transformed morphallactically to establish proper proportions in the digestive tract[4]. Apart from cell signaling for repatterning, neurosecretory pathways are also important in initiating regeneration process in *E.foetidae*[23]. Clitellum Factor (CF) produced by neurosecretory cells in the supraoesophageal ganglia level plays a role in sexual reproduction and rates of regeneration which cannot function at the same time[24]. There are also several theories to predict and explain the regenerative process. One such theory was proposed by Liebmanm that the chloragogenous system regulates various aspects of regeneration[25].Another such theory is a critical inhibitory voltage theory which suggests that the formation of new segments continue at the tip of the earthworm until a critical inhibitory voltage is built up[26].Various morphological studies reveal that regeneration requires the presence of nervous system[27]. Intervention into the genetic and molecular mechanism is necessary to understand these theories. *Hox* gene family represent those group of gene which were expressed at different time during regeneration process. *Pex-lab01* and *Pex-lab02* were expressed as soon as 3 and 12 hours postamputation. The expression of *Pex-lab03* appeared to be up-regulated within the first hours, and reached distinct peak 1h after amputation, followed by a gradual decrease to the intact level[17].Pluripotency factors *oct4, nanog, sox2, c-myc* and *lin28* genes are upregulated during cellular reprogramming and dedifferentiation. mRNA expression of *Sox2* gene is seen to be higher in the head and tail than in the midpiece. At protein level, the *Sox2* protein expression level is similar in the head and tail, while expression in the midpiece is lower. Therefore, expression of *Sox2* gene promotes the blastogenesis and the regeneration[20]. Experimentation on earthworms can prove to be an excellent study for the process of regeneration and used as an excellent model of study. Amputation of segments can also have practical application in population buildup.

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References

1. Kristensen, E. and J. Kostka, *Macrofaunal Burrows and Irrigation in Marine Sediment: Microbiological and Biogeochemical.* Interactions between Macro–and Microorganisms in Marine Sediments. Washington, DC: American Geophysical Union, 2005: p. 125-157.
2. Uebelacker, J.M., *A new parasitic polychaetous annelid (Arabellidae) from the Bahamas.* The Journal of Parasitology, 1978: p. 151-154.
3. Bhambri, A., et al., *Large scale changes in the transcriptome of Eisenia fetida during regeneration.* PloS one, 2018. **13**(9).
4. Myohara, M., et al., *Fragmenting oligochaete Enchytraeus japonensis: a new material for regeneration study.* Development, growth & differentiation, 1999. **41**(5): p. 549-555.
5. Drewes, C.D., E.P. Vining, and M.J. Zoran, *Regeneration of rapid escape reflex pathways in earthworms.* American zoologist, 1988. **28**(4): p. 1077-1089.
6. Mescher, A.L., *The cellular basis of limb regeneration in urodeles.* International Journal of Developmental Biology, 2004. **40**(4): p. 785-795.
7. Kintner, C.R. and J.P. Brockes, *Monoclonal antibodies identify blastemal cells derived from dedifferentiating muscle in newt limb regeneration.* Nature, 1984. **308**(5954): p. 67-69.
8. Muneoka, K., W. Fox, and S. Bryant, *Cellular contribution from dermis and cartilage to the regenerating limb blastema in axolotls.* Developmental biology, 1986. **116**(1): p. 256-260.
9. Wei, Y., et al., *Monoclonal antibody MT2 identifies the urodele α1 chain of type XII collagen, a developmentally regulated extracellular matrix protein in regenerating newt limbs.* Developmental biology, 1995. **168**(2): p. 503-513.
10. Moment, G., *The possible roles of coelomic cells and their yellow pigment in annelid regeneration and aging.* 1974.
11. Park, S.K., S.-J. Cho, and S.C. Park, *Histological observations of blastema formation during earthworm tail regeneration.* Invertebrate reproduction & development, 2013. **57**(2): p. 165-169.
12. Gates, G., *Regeneration in an earthworm, Eisenia foetida (Savigny) 1826. II. Posterior regeneration.* The Biological Bulletin, 1950. **98**(1): p. 36-45.
13. Moment, G.B., *The relation of body level, temperature, and nutrition to regenerative growth.* Physiological Zoology, 1953. **26**(2): p. 108-117.
14. Liebmann, E., *The coelomocytes of Lumbricidae.* Journal of Morphology, 1942. **71**(2): p. 221-249.
15. Moment, G.B., *Variation and its causation in earthworm regeneration.* Journal of Experimental Zoology, 1974. **190**(3): p. 297-303.
16. Xiao, N., F. Ge, and C.A. Edwards, *The regeneration capacity of an earthworm, Eisenia fetida, in relation to the site of amputation along the body.* Acta Ecologica Sinica, 2011. **31**(4): p. 197-204.
17. Cho, S.-J., et al., *Differential expression of three labial genes during earthworm head regeneration.* Bioscience, biotechnology, and biochemistry, 2009. **73**(12): p. 2609-2614.
18. Zheng, P., et al., *Expression of stem cell pluripotency factors during regeneration in the earthworm Eisenia foetida.* Gene, 2016. **575**(1): p. 58-65.
19. Varhalmi, E., et al., *Expression of PACAP-like compounds during the caudal regeneration of the earthworm Eisenia fetida.* Journal of molecular neuroscience, 2008. **36**(1-3): p. 166-174.
20. Tao, J., et al., *Toxic responses of Sox2 gene in the regeneration of the earthworm Eisenia foetida exposed to Retnoic acid.* Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 2018. **204**: p. 106-112.
21. Jamieson, B.G.M., *The ultrastructure of the Oligochaeta*. 1981: Academic Press Inc.(London) Ltd.
22. Morgan, T.H., *The physiology of regeneration.* J. exp. Zool, 1906. **3**(3).
23. Moment, G.B., *A theory of growth limitation.* The American Naturalist, 1953. **87**(834): p. 139-153.
24. Bilej, M., *Cellular defense mechanisms.* Immunology of annelids, 1994: p. 167-200.
25. Liebmann, E., *New light on regeneration of Eisenia foetida (Sav.).* Journal of Morphology, 1943. **73**(3): p. 583-610.
26. Moment, G.B., *On the relation between growth in length, the formation of new segments, and electric potential in an earthworm.* Journal of Experimental Zoology, 1949. **112**(1): p. 1-12.
27. Yoshida-Noro, C., et al., *Nervous system dynamics during fragmentation and regeneration in Enchytraeus japonensis (Oligochaeta, Annelida).* Development Genes and Evolution, 2000. **210**(6): p. 311-319.