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Research Article

# EXPRESSION OF ALPHA-FETOPROTEIN IN THE EARTHWORM *EUDRILUS EUGENIAE*

**Subburathinam Balakrishnan, Saranya Lathakumari, Paulkumar Kanniah, Sudhakar Sivasubramaniam***

*Department of Biotechnology, Manonmaniam Sundaranar University, Tirunelveli, Tamilnadu, India*

**Corresponding author: sudhakar@msuniv.ac.in*

## ABSTRACT

The earthworm *Eudrilus eugeniae*, commonly called as "African night-crawler" is a segmented animal which is divided into three major segments such as anterior, clitellar and posterior segments. The earthworm has enormous ability to regenerate the autotomized or lost body parts. The clitellar region plays a pivotal role in the regeneration of both anterior and posterior region of earthworm. In the present study, the Alpha-fetoprotein (AFP) sequence was retrieved from *Eudrilus eugeniae* transcriptome dataset and we found a contig in the size of 1873bp with 609 amino acids. The expression of the AFP was not detected in the body segment but the expression of the protein was confirmed only in clitellar and regenerative blastema by the immunoblot technique. Both in the clitellum and regenerative blastema, the AFP was located in the nucleus. The data suggest that AFP has a decisive role in the regeneration of earthworms.

**Keywords:** Alpha-fetoprotein (AFP), *Eudrilus eugeniae*, Regeneration, Immunoblot, Immunohistochemistry.

## 1. INTRODUCTION

*Eudrilus eugeniae* resides on the damp ground surface layer and is often seen wherever organic matter is present. The worm is reddish-brown and has overlapping pinkish white ventral and convex dorsal sides. The mature worm has approximately 25-30 cm long, diameter 5-7 mm long body. It has approximately 120-300 separate segments with 5600 mg of individual biomass [1]. The pale colour puffy clitellar segment starts from 13 and ends at 18$^{th}$ segment. The clitellum is important for the reproduction of the worm.

The amazing regeneration ability and easy maintenance conditions make this an ideal animal model for studies on regeneration and stem cells [1-6]. The regeneration in *E. eugeniae* begins with a proliferating mass of undifferentiated progenitor cells and subsequently forms the regenerative blastema [6]. Previously, Subramaniam *et. al.*, found that the expression translationally-controlled tumor protein homolog (*TPT1*) is important for the successful regeneration. Sayan Paul *et. al.*, reported the proteins such as disulfide-isomerase A3-like (*PDIA3*), translationally-controlled tumor protein homolog (*TPT1*), and DnaJ homolog subfamily B member 1 (*DNAJB1*) were highly upregulated and proteins include ribonucleoside diphosphate reductase (*RRM1*), IS630 family transposase (*IS630*) and rRNA intron-encoded homing endonuclease (*PAE1850*) were drastically down regulated in the anterior regeneration of earthworm *E. eugeniae* [7]. Subramanian *et. al.*, found that differentiation of granular epithelial cells in the clitellum is a normal event during the posterior segmental regeneration of the worm and at the time point, the puffiness of the clitellum also has been disappeared. The knockdown of *TPT1* gene expression resulted the failure of both the disappearance of clitellar segmental puffiness and differentiation of clitellar granular epithelial cells [2]. It has been reported that the clitellum is essential for the successful regeneration [5, 8, 9]. But a further detail of clitellar segmental role in the successful regeneration is still unknown.

Alpha-fetoprotein (AFP) family has three different proteins. They are serum albumin protein, group-specific component/vitamin D-binding protein (GC) and afamin/alpha-albumin (AFM) protein. The increase of AFP level was reported in serum of mice and humans in hepatocellular carcinoma, hepatoblastoma, germ cell tumors and gastrointestinal carcinomas [10,11] but AFP is absent in normal adult serum. Besides, AFP is expressed in embryo but not in the adult body tissue. In addition, the protein normally produced by fetal yolk sac, regenerating adult liver and fetal gastrointestinal epithelium [12]. Hence, it is used as a marker to detect cancer during embryogenesis [13].

In the current work, the expression of Alpha-fetoproteins (AFP) was tested in the clitellum, anterior and posterior regenerative blastema of the earthworm, *E. eugeniae*. These studies confirm that AFP was expressed in the clitellar segment, anterior and posterior regenerative blastema but the protein was not detected in the other body segments. The data generated will be helpful to understand the molecular function of AFP in the clitellum and in the events of the complex regeneration.

## 2. MATERIAL AND METHODS

### 2.1. Maintenance of earthworm *E. eugeniae*

The *E. eugeniae's* culture stock was raised in Department of Biotechnology, Manonmaniam Sundaranar University, Tirunelveli, Tamilnadu, India (8.7637 °N, 77.6486 °E). The worm was fed the same ratio of cow dung and leaf litter and maintained at sufficient humidity in a plastic container [3, 4, 6].

### 2.2. Amputation and regeneration of *E. eugeniae*

The mature worms have been selected in the presence of a matured clitellum that was subjected to the regeneration process. The worm *E. eugeniae* was amputated in the $10^{th}$ segment of the anterior and $30^{th}$ segment of the posterior with a sterile surgical blade. After blastema formation on $6^{th}$ day, the amputated worms were taken and used for further regeneration studies.

### 2.3. Multiple sequence alignment and phylogenic tree construction of Alpha-fetoprotein (AFP)

The *E. eugeniae* Alpha-fetoprotein (AFP) cDNA sequence was translated by the ExPASy translation tool, and the AFP sequences of various animals from NCBI source were retrieved. The multiple sequence alignment was achieved with the Clustal W tool. The translated protein sequence of worm *E. eugeniae* AFP was aligned to its homologue sequence in *Homo sapiens* (Human), *Vulpes vulpes* (Red fox), *Ranitomeya imitator* (Mimic poison frog), *Mus musculus* (Mouse), *Asarcornis scutulata* (White-winged duck), respectively. The phylogenic tree was constructed by using Clustal Omega tool (https://www.ebi.ac.uk/Tools/msa/clustalo/).

### 2.4. Elution of protein from the SDS-PAGE

The AFP is the predominant protein in the cocoon of the earthworm. Freshly laid cocoon was collected and cleaned with double distilled water. A portion of the coccon was taken and protein lysate was prepared for SDS-PAGE. The proteins (1 mg) in the lysate were resolved in 8% preparative SDS-PAGE. The coomassie blue staining revealed distinct 70 kDa protein and the band was sliced out. The slice was rinsed in PBS saline a couple of time. Then, the sliced was smashed in 200 µl PBS buffer. The sample was incubated in 4 degree for overnight. Then, the sample was spin at 10K for 10 min at 4 degree. The supernatant was collected and used for neutralization of anti-AFP antibody.

### 2.5. Immunoblot (IB)

Using the protocol of previously published articles, SDS PAGE and Immunoblot analyses have been done [2,3]. *E. eugeniae* tissues were collected and gently washed with 1X PBS icecold. 2X sample buffers used to grind the tissue. 30 µg of tissue lysates were loaded in the each well of the 12% SDS-PAGE. Proteins have been transferred to the PVDF membrane (Cat. 1620177, Biorad United States) and the western blot has been carried out in dilution 1:1000 and 1:3000, with the following key antibodies: anti-Alpha-fetoprotein (AFP) (Cat.A8452; Sigma Aldrich) and anti-β-actin (Cat. A2066;Sigma Aldrich, India). The primary antibody incubation was allowed for 12 hrsat 4°C. Following Washing, the membrane was incubated with the goat's anti- rabbit IgG- conjugated with alkaline phosphatase secondary antibodies (Cat. A 9919;Sigma Aldrich) for 1 hr at room temperature. The BCIP/NBT (Cat.E116 Ameresco, USA) colorimetric substrate has been used as a development solution.

### 2.6. Immunohistochemistry( IHC)

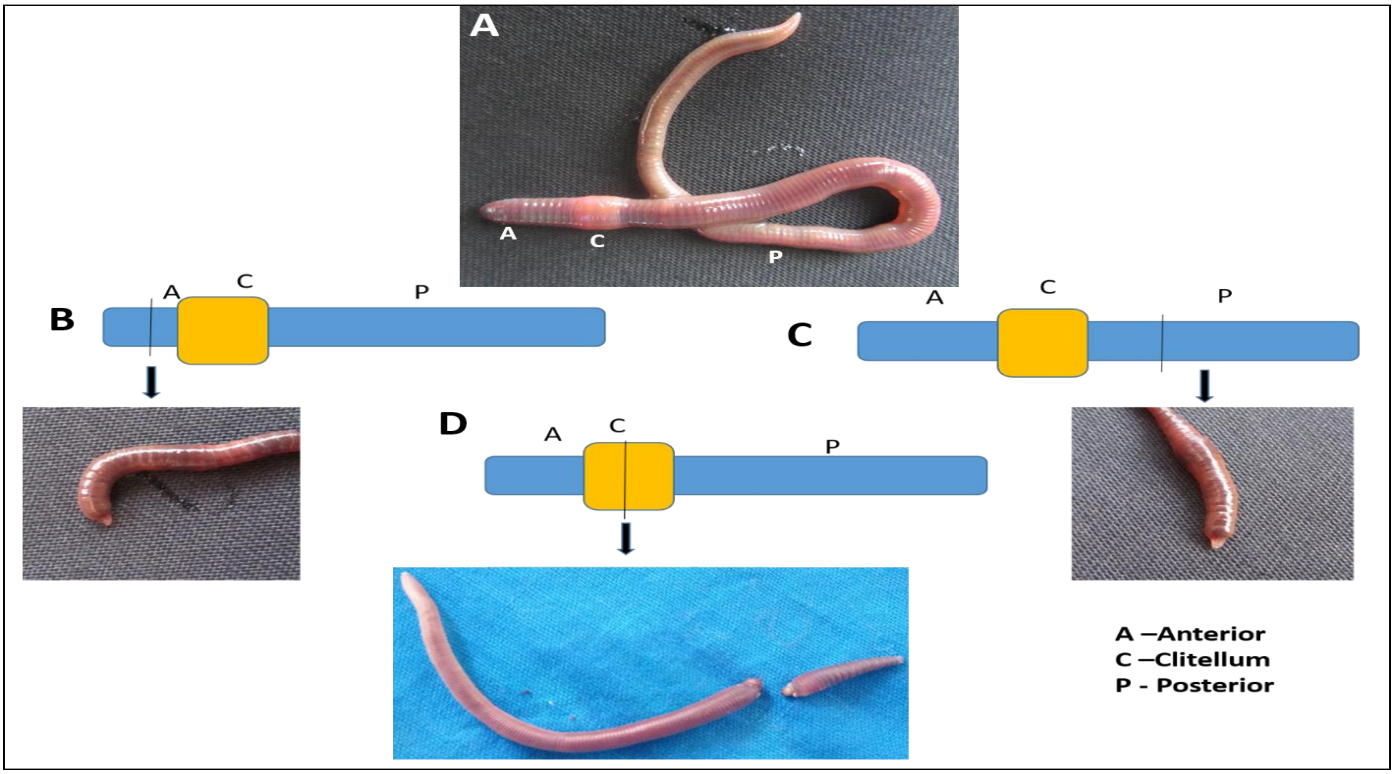
Several tissues of *E. eugeniae* were subjected to IHC the following by the standard protocol of the previously published article [3, 2, 6]. The 6 µm section was used to immunohistochemistry (IHC) for Alpha-fetoprotein (AFP) expression analysis. Following removal of paraffin from the sections, and treatment with 10% $H_2O_2$ (Cat.1072090500; MERKCA, India) and 10% methanol (Cat. AS059; Himedia, India) and endogenous peroxidize stopped using in 1X PBS and trypsin for (0.1% trypsin in 0.1% $CaCl_2$ for 10 minutes). The non-specific blocking was done by keeping tissue slides at room temperature in 2% BSA for 1 hr. The anti-Alpha-fetoprotein (AFP) (Cat.A8452; Sigma Aldrich) antibody was used in 1:100 dilution in 2% BSA solution. Besides, the membrane was incubated in the secondary antibody, Goat anti-mouse IgG conjugated with horseradish peroxidize (Cat.031050, Sigma-Aldrich, India) in 1:2000 dilution in 1X PBST at room temperature for 1 hr. The

developing substrate in Diaminobenzidine (Cat.E733; Amresco, USA) was used. The counter-stained with Mayer's hematoxylin (Cat. MHS1, Sigma-Aldrich). Finally, DPX mounted slide was observed and the images were documented under an Olympus BX53 microscope.

## 3. RESULTS AND DISCUSSION

### 3.1. Role of clitellum in the regeneration of earthworm *E. eugeniae*

The anterior region of the earthworm *E. eugeniae* starts from the 1-12$^{th}$ segments. It consists of the mouth, prostomium, brain, heart, testis, and oviduct. The clitellar region starts from the 13$^{th}$ to 18$^{th}$ segment. Finally, the posterior regions contain prostate glands, intestine and anus from the 19$^{th}$ to remaining segments (Fig1.A). Previous studies revealed that clitellar segments acted a pivotal role in earthworm *E. eugeniae* [2,5,8]. The Fig.1 B to D clearly explain the importance of clitellum in the regeneration. Amputation at both the anterior and posterior ends, the worm with the clitellar segments could successfully regenerate but the segments which were detached from the clitellum failed to regenerate (Fig. 1. B & C). Fascinatingly amputation at the clitellar segments started the abnormal regeneration with multiple blastemas and eventually resulted failure in the regeneration process and subsequently both parts of the worm died (Fig. 1. D).




*(A) The earthworm, E. eugeniae, A -anterior region, C - clitellum, P - posterior region (B) Amputation of anterior segments and their regenerative blastema on 4$^{th}$ day.(C) Amputation of Posterior segments and their regenerative blastema on 4$^{th}$ day. (D) Amputation in the middle of the clitellum and their formation of abnormal regenerative blastema on 4$^{th}$ day*

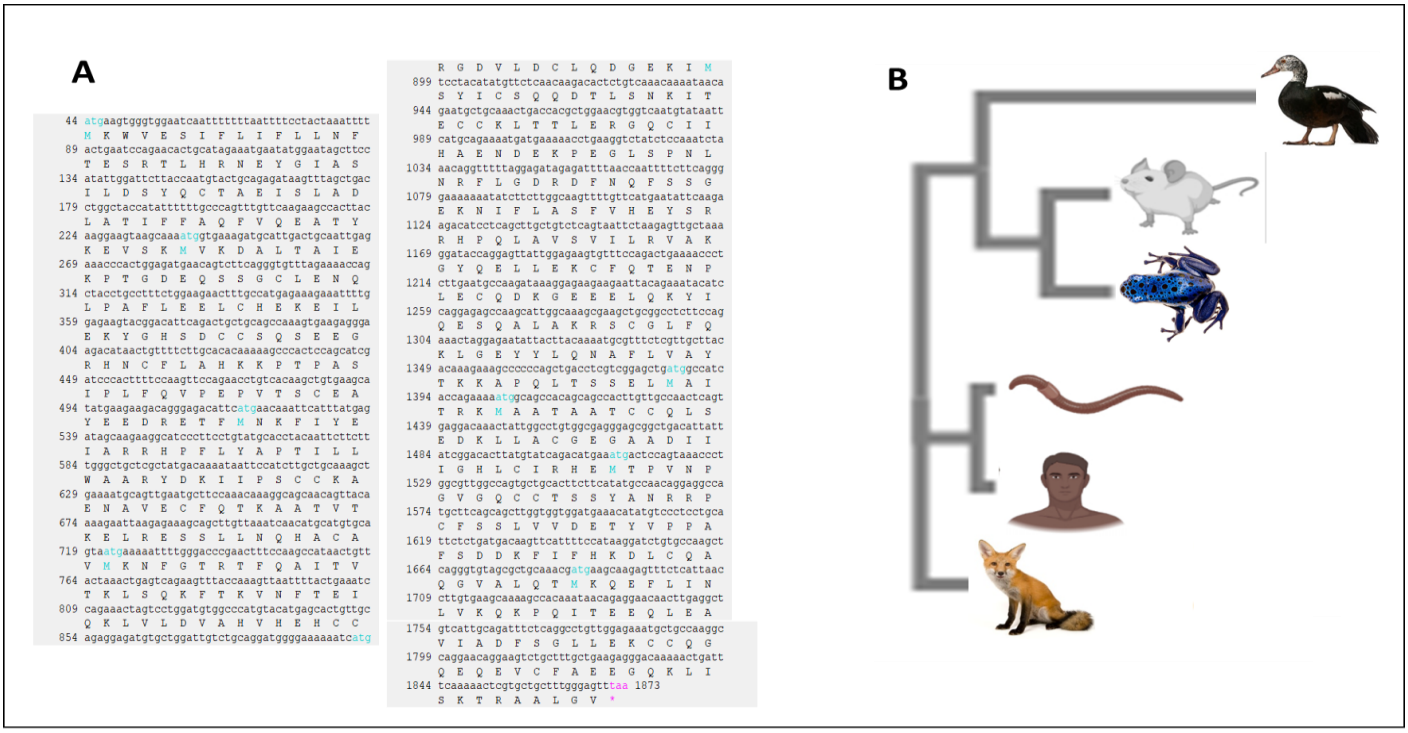
**Fig. 1: Morphology of *E. eugeniae* and role of clitellum**

### 3.2. Alpha-fetoprotein (AFP) protein identification for earthworm *E. eugeniae*

The AFP sequence was retrieved from the transcriptome dataset of *E. eugeniae* and size of the contigis 1873 bp and it has an ORF which encodes 609 amino acids sequence (Fig. 2. A). Besides, the AFP sequences of different *Homo sapiens* (Human), *Vulpes vulpes* (Red fox), *Ranitomeya imitator* (Mimic poison frog), *Mus musculus* (Mouse), *Asarcornis scutulata* (White-winged duck), were retrieved from the NCBI. AFP sequence was not present in the protein databases of insect (taxid: 50557) and *Caenorhabditis elegans* (taxid: 6239) and the retrieved sequences were aligned by Clustal W tool. The data showed that the AFP sequence has 100% homology with that of human AFP isoform 1. Using the multiple sequence alignment, the

phylogenetic tree was generated by the Clustal Omega software has been shown in the fig. 2. B. The worm belongs to the phylum Annelida but the AFP of the worm has 100% protein sequence homology with that of human.

Previously Subramanian *et al.*, mentioned the phylogenetic tree was constructed using the worm *E. eugeniae* TCTP protein and their homologues and found that the sequence of worm TCTP has highest homology with that of *Drosophila melanogaster* [2].




*(A)The open reading frame (ORF) of Alpha-fetoprotein in the transcript of AFP of E. eugeniae, (B) Molecular phylogeny of earthworm E. eugeniae based on the AFP.*

**Fig. 2: Alpha-fetoprotein (AFP) sequence and their phylogenetic tree**

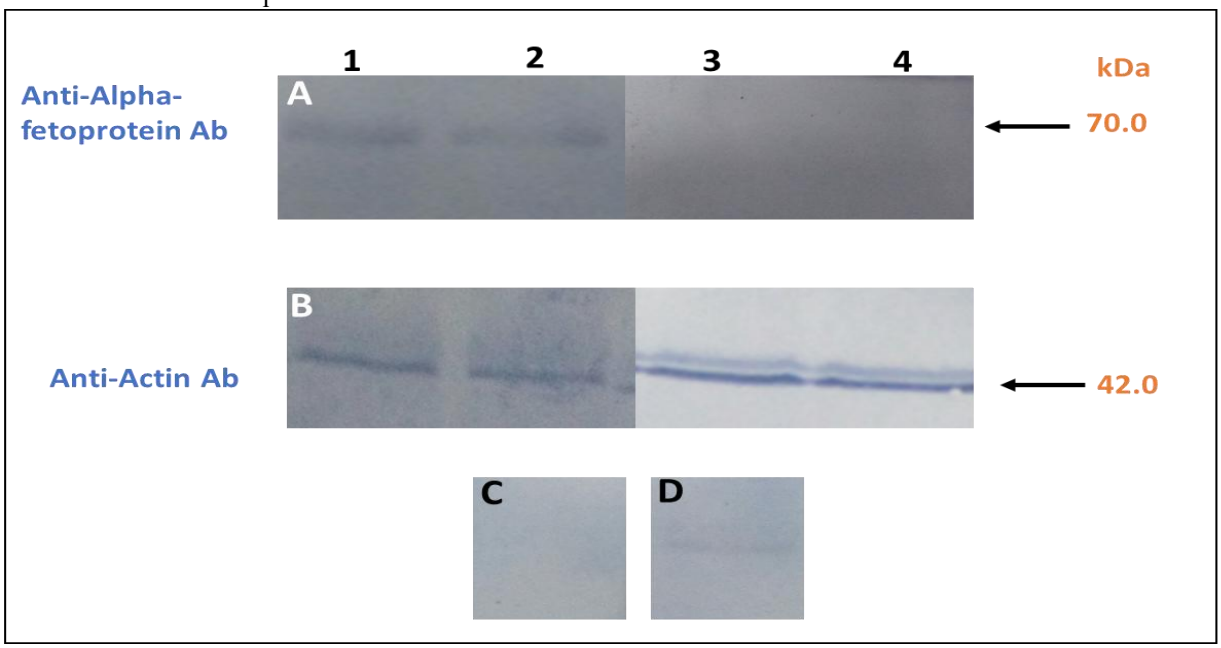
### 3.3. Expression of Alpha-fetoprotein (AFP) in *E. eugeniae*

We performed the SDS PAGE with earthworm tissue samples. Then immunoblot experiment was performed to confirm the AFP expression in the earthworm *E. eugeniae*. The data are shown in fig. 3. The expression of β-actin was used as a control (Fig. 3B). The immunoblotting of fresh cocoon and clitellar segmental lysates showed a single band of AFP at 70 kDa size (Fig.3A lane 1 and 2) and the protein was not detected in samples of anterior and posterior segments (Fig. 3A lane 3 and 4). To confirm the quality of the antibody specificity, the gel eluted AFP protein was incubated with the anti-AFP antibody and then it was used to detect the AFP protein with protein lysate of cocoon. In the case, the antibody didn't detect any band shows (Fig. 3C). For the positive control, the same protein same was immunoblotted with the anti-AFP antibody. Here the expected size protein band at ~70.0 kDa was detected (Fig. 3.D).

### 3.4. AFP expression of the control clitellum of *E. eugeniae*

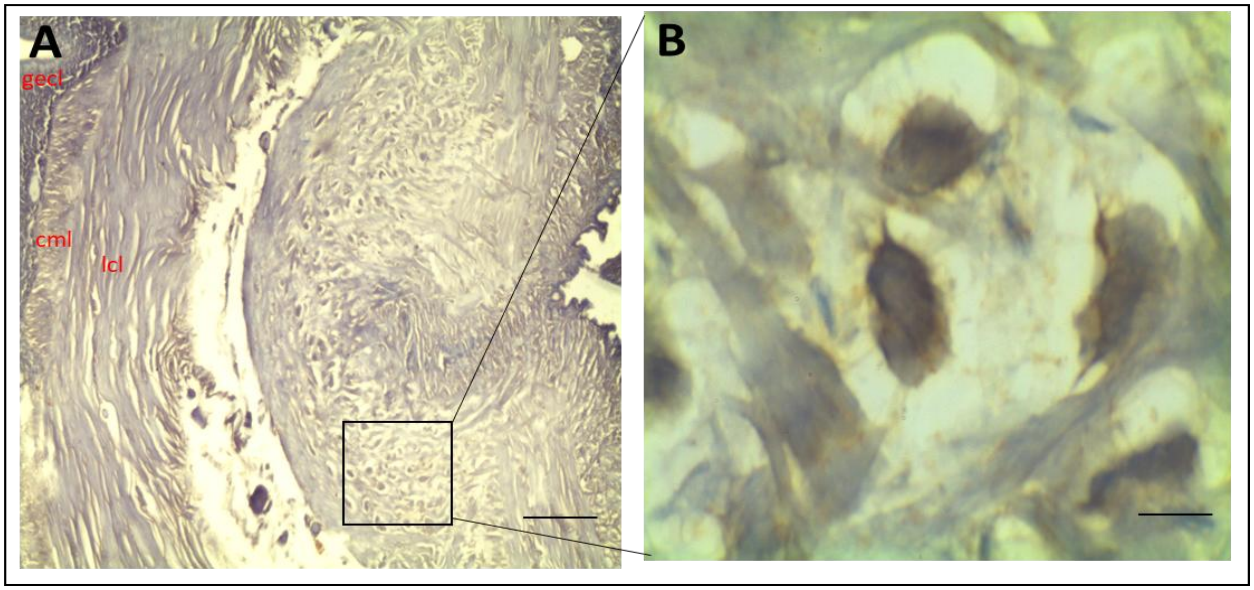
We prepared paraffin embedded control clitellar tissue of a mature earthworm for immunohistochemistry (IHC). Then, 5 µm thick section was made. The tissue was subjected to IHC using the anti-AFP antibody. Then, the tissue was observed under microscope and the abundant expression of AFP was found in the cells of the different layers clitellum segment (Fig 4. B, C). The granular epithelial cell layer is the first in the skin of clitellum. The second layer is called circular muscle layer and finally the third layer is called as longitudinal cell layer. The protein expression was maximum in the granular epithelial cell layer and its expression also has found in the circular muscle layer. A few cells of longitudinal cell layer also produced the protein. The

interior muscle of the clitellum also synthesized the protein. Also, it was found that localization of the AFP in the nucleus. It has be reported that the AFP is a secretory protein [14]. This is the first report which show the AFP in the nucleus.




*(A) The protein lysate was prepared and subjected for the Immunoblot with anti-AFP antibody (lane 1). A single band was detected at ~70.0 kDa size. The second lane was loaded with the clitellar tissue protein lysate and the same band was noted. The lane 3 and 4 were loaded with the lysate of anterior (lane 3) and posterior (lane 4) segments, respectively. (B) The PVDF membranes of the 42 kDa range were used for detect the level of actin protein with anti-actin antibody. It recognized β-actin protein at 42.0 kDa size. (C)To confirm the quality of the antibody specificity, the gel eluted AFP protein was incubated with the anti-AFP antibody and then it was used for immunoblot with protein lysate of cocoon. In the case, the antibody didn't detect any band. (D)For the positive control, the same protein was immunoblotted with the anti-AFP antibody. Here the expected size protein band at ~70.0 kDa was detected*

**Fig. 3: Expression of AFP in the earthworm *E. eugeniae***




*The immunohistochemistry experiments were carried out with the clitellar tissues of E. eugeniae to find the expression of AFP. (A) Immunohistochemistry experiment was carried out with anti- AFP antibody using the clitellar tissues. It shows the expression of AFP scale bar: 50 μm. The different layers of skin the clitellar segment: gecl- granular epithilial cell layer, cml-circular muscle layer and lcl-longitudinal cell layer. (B) 60X enlarged image of panel B the prominent AFP expression in the nucleus scale bar 18μm.*

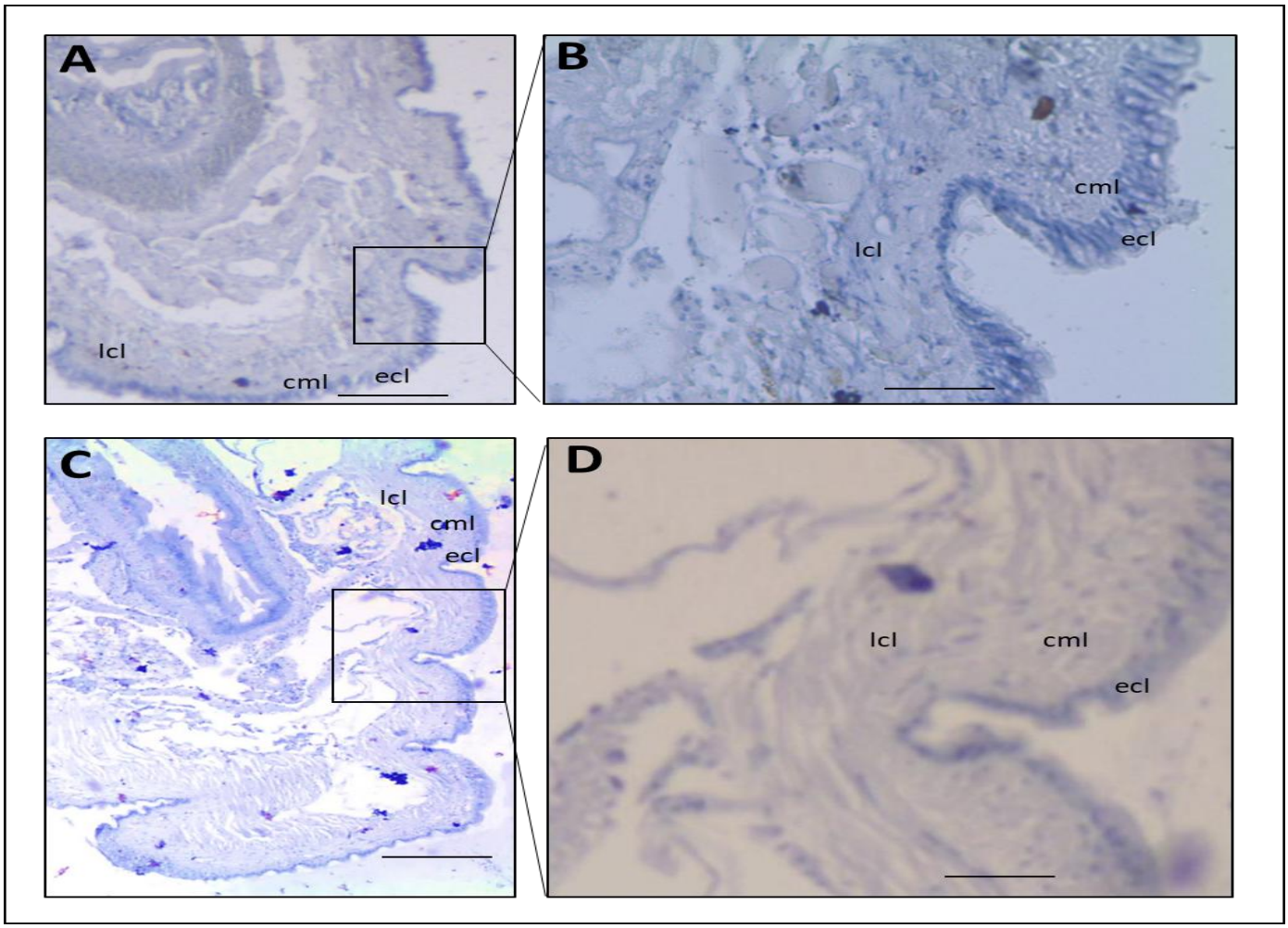
**Fig. 4: Expression of AFP in clitellum segments of *E. eugeniae***

### 3.5. AFP expression in the anterior and posterior blastema

The expression of AFP was performed on the regeneration blastema of anterior and posterior segments of earthworm *E. eugeniae*. The worm has an abundant regeneration ability of both anterior and posterior parts [2, 6]. We examined the expression of AFP in the body segments using IHC technique. The data has been shown in the figs. 5 & 6. The data clearly show that the protein was not expressed both in the anterior (Fig. 5A & B) and posterior segments (Fig.5A & B) of the control worm. The data clearly matches with that of immunoblotting results shown in the fig. 3C.

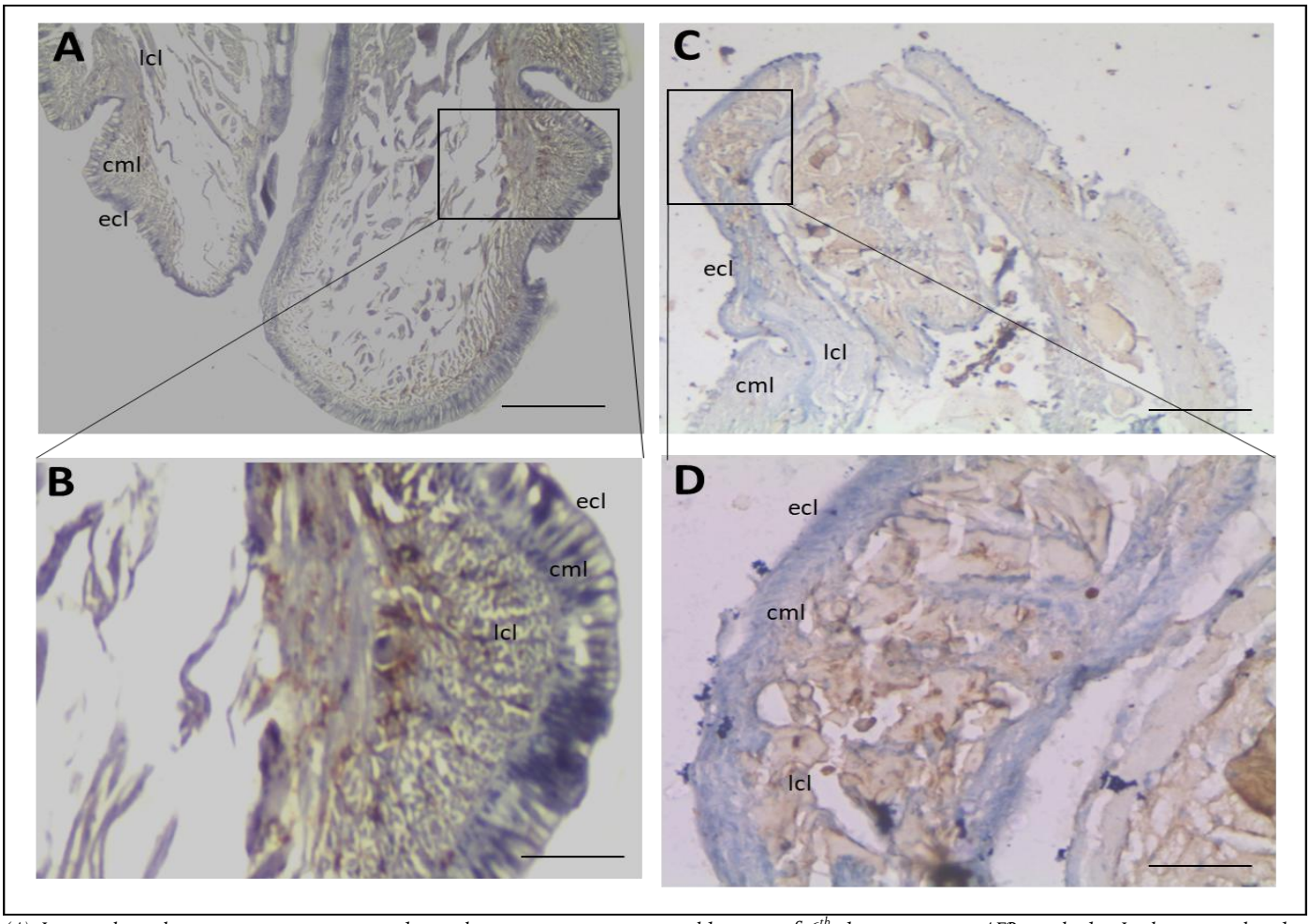
The sixth day blastema of the worm amputated at 10$^{th}$ segment was subjected to IHC with anti-AFP antibody. The data has been shown in the fig. 6A & B. The data clearly show the expression of AFP in the longitudinal layer of the blastema but the protein expression is absent in the epithelial cells. Johson *et.al.*, showed that the cells in the longitudinal layer initiate the blastema formation and migratory cells from the adjacent segment also found in the longitudinal layer. Those cells had autofluorecent property which was due to rich amount of riboflavin [6]. Smilarly, the sixth day blastema of the worm amputated at 30$^{th}$ segment was subjected to IHC with anti-AFP antibody.

The data shown in the fig.6 C & D show the expression of AFP in the cells of longitudinal layer and also the localization of the protein has been noted in the nuclus. The AFP expression was found in the yolk sac and liver, with a much lower concentration in the intestines [15].




*(A) Immunohistochemistry experiment was carried out with anterior normal tissue sample using anti-AFP antibody. The expression of AFP was not found. (B) 20X enlargement of panel A (C) Immunohistochemistry experiment with the posterior tail sample also didn't show positive signal for the anti-AFP antibody. (D) 20X enlargement of panel C Scale bar 50 µm for A &C, 18 µm for B &D. The different layers of skin the body segment: eml-epithilial cell layer, cml-circular muscle layer and lcl-longitudinal cell layer*

**Fig. 5: Control anterior and posterior segments of the worm *E. eugeniea***




*(A) Immunohistochemistry experiment carried out the regenerative anterior blastema of 6th day using anti-AFP antibody. It shows an abundant expression of AFP (B) enlarged image of panel A (C) Immunohistochemistry with anti-AFP antibody using the 6th day posterior regenerative blastema. It shows an abundant expression of AFP at the nucleus of the worm (D) enlarged image of penal C. scale bar 50 µm for A and C, 20 µm for B and D.*

**Fig. 6: Alpha-fetoprotein (AFP) expression in the 6th day anterior and posterior regenerative blastema of the worm *E. Eugeniae***

The expression of alpha-fetoprotein in adults is often linked to hepato-carcinoma and teratoma and has a predictive value for advanced gastric cell cancer [13, 16]. However, in people without an obvious pathology, hereditary persistence of alpha-fetoprotein may also be found [17]. The protein is thought to be the fetal counterpart of serums albumin and, in the same transcriptional orientation of chromosome 4, the alpha-fetoprotein and albumin genes are present together. It is found in monomeric and dimeric and trimeric forms, binding copper, nickel, bilirubin and fatty acids. Alpha-fetoprotein A level was elevated in renal functional loss in spinal bifida and anencephaly in amniotic fluids [18].
It was reported that AFP antagonize apoptosis in the liver cancer cell [19]. The findings suggest that the APF promotes the rapid cell division. Also the finding of AFP expression in embryonic development supports the prediction. The protein expressing cell has been extracted from murine liver [20]. The cells proliferation is under control of the Jagged1/Notch2 signal. Carbon tetrachloride is a liver damaging chemical. The injection of carbon tetrachloride injections elevated the proliferation of the cells. The cells also produce albumin and interestingly the expression stem cell marker protein SOX 9 also found in the cells. The finding confirms that the AFP synthesizing cells are stem cells [20]. Based onthe previous reports and our data, it is sure that AFP has a major role in the regeneration.

## 4. CONCLUSION

The AFP transcript has been found in *E. eugeniae* and the protein sequence shares 100% homology with human counterpart. The earthworm *E. eugeniae* molecular phylogeny has been documented. An AFP expression was identified in the control clitellum, also in the blastema of anterior and posterior regeneration. Several experiments clearly show the localization of the protein in the nucleus. From the experimental data, we conclude that AFP plays a major role in the regeneration of the worm.

## 5. ACKNOWLEDGMENTS AND FUNDING

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### *Conflict of Interests*

The authors declare no potential conflicts of interest.

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