5. Discussion
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Regeneration is the remarkable ability of animals and widely diverse among the species. In nature, the ability of regeneration varies among the animals. Higher the evolution of animals holds lower the ability of regeneration (Fig. 43).

Fig. 43 Regeneration ability among the animal kingdom.

The mechanism of regeneration process will help to find the therapeutic agents for the regenerative medicine. Worldwide investigators are using hydra and planaria as a model system for regeneration studies but they are facing following problems 1. No well developed organs like circulatory system, reproductive organs and nervous system, 2. The handleings are highly difficult because of the microscopical size animal’s 3.
Comparatively evolutionarily lower system than Earthworm. When compared to planaria and hydra, we propose earthworm as a wonderful model system because 1) the animal has closed vascular system. 2) It has the ability to regenerate the lost segments and organs; such as brain, heart, prostate, 3) it is easy to maintain in the laboratory; 4) Higher growth rate (Rodriguez and Lapeire, 1992) and attains 12 mg body weight per day; 5) It is extremely fertile, will reach sexual maturity within 35 days (Dominguez et al., 2001) and 6). It can tolerate extreme temperature difference (ranges from 15 - 30°C) (Viljoen and Reinecke, 1992; Dominguez et al., 2001).

5.1. Regeneration and pattern formation of mouth and anus in *Eudrilus eugeniae*

The regeneration and pattern formation are varying among animals. The skin epidermis plays the main role in commencement of regeneration process. The process of regeneration starts from wound healing. The wound healing is a complex event (Gurtner et al., 2008) and it is the first step in regeneration and organogenesis. In human, the new tissue development and epithelialization takes 2 - 10 days of post injury (Gurtner et al., 2008). In contrast, the amphibian axolotl can complete the wound healing within 8 hours (Kawasumi et al., 2013). It has been known that the wound healing process is varying among the earthworm species. In the earthworm, Eisenia fetida the wound epithelialization takes 1 to 3 days; in contrast the present investigation shows epithelialization was completed within 2 days (Fig. 10 A and B) in E. eugeniae. We suggest that the prognosis of E. eugeniae wound healing will lend a hand to study about the regenerative medicine and the accelerated wound healing in mammals. Notably, there is higher vascularization occurred in the regeneration blastema (Fig. 11 C and D). The same was documented in several animals. There was drastic amount of microvascularation was formed and helps for the entire liver regeneration from partial
hepatectomic liver in mice (Drixler et al., 2002). The angiogenic process is very much important for zebrafish fin regeneration (Bayliss et al., 2006). It has been studied in adult newts, the neovascularation plays prevail role during blastema development (Rageh et al., 2002). The same was also observed in E. eugeniae (Fig. 11 C and D). It is well known that the blastemal cells are mononucleated in nature and the same was noted in E. eugeniae regeneration (Fig. 12 C and D). In our study, we have observed and documented the higher population of BrdU positive cells in 3rd day blastema (Fig. 17 A). The observation corroborates the previous reports and confirms the blastema replenish with stem cells (Johnson Retnaraj Samuel et al., 2012). Differentiation and pattern formation are the major processes in early development and organogenesis. The stem cell differentiation and positional cues are regulated by the particular spatial and temporal gene expression. In current investigation, it is noted that on 6th day, abundant of differentiated cells were noted in layers ECL, LCL, CML and IECL of regenerated anterior and posterior parts. The observations are highly similar to the structure of intact mouth and anus (Fig. 13 A-J). However the functional recovery of mouth and anus remain uncovered, the impairment of mouth and anus function should cause food deprivation. Organogenesis is a mechanistic process; it needs more energy because of huge cell proliferation and turn over. The impact of food deprivation in embryonic organogenesis was studied in few animals, for instance, in development process of C. elegans, it has been studied that the food scarcity has been effectively tackled by undertaking growth and gate check point adaptations (Schindler et al., 2014). Likewise, E. eugeniae has adapted with unique starvation behaviour on regeneration (Fig. 14 and 15). In addition, the present study elucidates either the loss of mouth or anus in E. eugeniae cannot impair its regeneration instead of that there was mere body weight loss documented in serial days. The accumulative evidence affirmed that some of the factors
of *E. eugeniae* can regulate the organogenesis at the time of regeneration and homeostasis.

### 5.2. Role of clitellum during regeneration

In the present study it has been found that, the regeneration ability was restricted in clitellum lost parts (Fig. 20). When compared the amputated worm having clitellum with the anterior and posterior part of worm without clitellum region, have no regeneration ability and the pieces has been dead in following days (Fig. 20 G and I). Besides, the posterior part without clitellum has the ability to form the regenerative blastema (Fig. 20 F). However the histological, stereozoom microcopical results authenticate that the posterior non clitellar region regenerates the anus instead of mouth (Fig. 21 G and H). The data suggested that clitellum is important for proper regeneration in *E. eugeniae*.

Stem cells are the important unit for development, the loss of stem cells lead to lethality and reduce the growth (Nichols et al., 1998). It is assumed that the decrease in regeneration rate and survival of worm may be due to the insufficient stem cell population or it lacks particular stimuli for stem cell activation. The BrdU experimental results show that clitellum enriched with higher stem cells act as a stem cell reservoir (Fig. 22 A). The loss of clitellum caused loss of stem cells. The secreted product from the clitellum triggers the stem cell migration from clitellum to the regenerating blastema and also triggers the differentiation of the blastema cell into septum and organ development. The paracrine effect of stem cell can also determine the regeneration rate.

### 5.3. Finding stem cell niche of *E. eugeniae*

The micro environment of stem cells is known as stem cell niche. The stem cell niche of *E. eugeniae* has been studied by several methods. The BrdU labelling retention
assay shows the blastema replenish with higher amount of BrdU positives cells and the BrdU positive signals were noted at the ECL layer of adjacent segments. The experimental result was highly correlated to our previous report (Johnson Retnaraj Samuel et al., 2012). It has been found that the pool of BrdU positive cells were observed at the blastemal region and also the positive cells reside at the ECL layers in anterior regenerating blastema and adjacent segments. In addition, similar to the report of Johnson Retnaraj Samuel et al., 2012, the autofluorescence of BrdU positive cells were documented in posterior region also.

The transcription factor Oct4 preferentially expressed only in undifferentiated pluripotent stem cells of embryo (Nichols et al., 1998; Takahashi and Yamanaka, 2006). Accumulative reports show that protein Oct4 is highly conserved from invertebrates to humanoids (Millane et al., 2011; Kalidas et al., 2015a). In the present study, the expression of E. eugeniae Oct4 was observed at the same molecular size 48 kDa of human Oct4. The IHC results of Oct4 expression more correlated the result of BrdU experiment, the blastema and ECL layer of adjacent segments show higher expression of Oct4 protein (Fig. 23 C and H). In addition, the abundant of Oct4 also noted at the ECL of clitellum region (Fig. 23 I). The experimental results confirm the evolutionarily conserved nature of Oct4 and its localization in stem cell niche.

Lamin A is an intermediate filament protein and the C-terminal region of the Lamin A is conserved from human to the earthworm. Constantinescu et al., 2006 found the expression of Lamin A in nuclear periphery of the differentiated cells and not in the stem cells. The same result has been observed in present study that the blastema and ECL of E. eugeniae failed to express the lamin A protein (Fig. 24 A and B). The comparison of Oct4 and Lamin A IHC showed the Lamin A positive cells were not detected on the Oct4 expressing region. Interestingly, the previous report of
Constantinescu et al., 2006 and Kalidas et al., 2015a shows no Lamin A expression in stem cells and they proposed Lamin A as negative marker to identify the stem cells.

The expression of alkaline phosphatase was documented in the embryonic stem cells (Pera et al. 2000), adult stem cells and the induced pluripotent stem cells (Takahashi et al., 2007). Similar to this, the alkaline phosphatase activity experimental results show the expression of ALP on the blastema and ECL layer of adjacent segments (Fig. 22 A and B).

The expression analysis of stem cell marker proteins in regeneration is important to understand the biology of stem cell in epimorphic regeneration of animals. Several investigators Pfister et al., 2008 have studied the protein vasa as the stem cell marker in neoblast (totipotent stem cell) of flatworm M. lignano. The piwi, one of the important gene for stem cell maintenance in Drosophila and it was proposed as stem cell marker (Lin and Spradling, 1997). The expression of stem cell markers, Myc, Nanos, Vasa, and Piwi has been reported in hydra (Millane et al., 2011). The foot regeneration specific protein CnNK-2 of homeobox gene has been identified in hydra. It has been known that CnNK-2 was expressed, when animal start to regenerate and form foot in fresh water hydra (Grens et al., 1996). Then, the gene CnNK-2 has been reported as marker for foot regeneration (Grens et al., 1996). Here the stem cell niche of E. eugeniae studied by several experiments such as BrdU, and stem cell marker protein Oct4, Lamin A and ALP. The accumulative results of the experiments clearly confirm that the stem cell niche of E. eugeniae resides at the ECL layer and the blastemal cells.
5.4. Transcriptome analysis in *E. eugeniae* posterior regeneration process

The Transcriptome analysis and Next-generation sequencing is the high throughput method to study the expression of total RNA in cells (Mutz et al., 2013). It is very much helpful to study the gene expression of animals (Vera et al., 2008). The underlying factors of regeneration process of several mammalian and other living systems have been studied by transcriptome analysis method (Vera et al., 2008; Schulz et al., 2012; Sousounis et al., 2013). In earthworm *E. eugeniae*, transcriptome analysis shows the stem cell factors Sox2, c-Myc, KLF and growth regulators Ras, TCTP were expressed at the posterior regeneration. The BLAST analysis more authenticated the previous results that the stem cell factors of *E. eugeniae* SOx2, c-Myc, Klf have more than 80% similarity with the human stem cell factors (Fig. 26 C, 27 C, 28 C).

The protein Ras has more than 87% similarity with the human Ras (Fig. 29 C) and the higher expression of Ras was documented in *E. eugeniae* regeneration (Fig. 29 F). Similarly, the elevation of Ras was documented in regeneration of other animals including mammals (Goyette et al., 1983; Komatsu and Ruoslahti, 2005). It shows evolutionarily conserved function of Ras from invertebrate to vertebrate region.

5.5. The Functional analysis of Translationally Controlled Tumour Protein (TCTP) in posterior regeneration

The growth regulator protein TCTP is evolutionarily highly conserved among the organisms which has a prevail role in development of both plants and animals. In present investigation, the TCTP gene was identified and sequenced (Fig. 30 A). The sequence of *E. eugeniae* TCTP shows higher similarity with higher vertebrates such as mouse and human Fig. 30 C. In addition, when compared the *E. eugeniae* TCTP
sequence with other earth worm TCTP sequences, there was a mere variation occurred (Fig. 30 C). The phylogenetic tree analysis shows E. eugeniae TCTP was more close to the Drosophila TCTP sequence.

The result of western blot shows that rich expression of TCTP was noted at the early regeneration of E. eugeniae and in later stage, the expression level was reduced Fig. 31 C. In addition, the IHC results authenticate the result of western blot and it shows higher expression of TCTP in early regeneration blastema (3rd day). However in later stage (6th day), the expression was noticed at the terminal growing part (Fig. 33 A). The result is more supportive to the result of Chen et al., 2007b, they observed the higher expression of TCTP mRNA at the early cleavage of amphioxus embryo and in later stage the expression was reduced (Chen et al., 2007b). The previous report shows that TCTP was expressed in both nucleus, cytoplasm of the cells and also the protein secreted out from the cells (Yoon et al., 2000).

By the keen observation of the expression status in the tissue layers of earthworm, it was found that the protein is present in extra cellular matrix region, Similarly, TCTP is secretary protein and none of the cells in the both normal and regenerated tissues showed the nuclear localization of the TCTP protein.

Nutlin-3 is a chemical inhibitor of TCTP, it can stabilize the expression of endogenous p53 level which influence the p53, mdm2 binding (Amson et al., 2012). In present investigation, it has been found that nutlin-3 treatment reduces the expression of TCTP and perturbs the regeneration process of posterior amputated E. eugeniae (Fig. 35 D). It causes the phenotypical variation and regeneration arrest in all the injected worms (Fig. 35 D). Similarly, the pharmacological treatment of nutlin-3 shows reduction
of TCTP level in human cancer cells. It is well known that suppression of TCTP causes
the tumour reversion in cancer cells (Amson et al., 2012).

The target inhibition of TCTP with siRNA shows the regeneration arrest and it
also caused the lethality of ± 1 TCTP RNAi in animals. The previous report of Chen et
al. 2007a proposed that TCTP is an important molecule for the early development and
the homozygous mutant of TCTP readily caused the lethality in embryo of mice. On the
other hand, null mutant of TCTP in Drosophila causes the lethality in larval stage and
reduction of TCTP level readily reduces the body and organ size of Drosophila (Hsu et
al., 2007). Moreover, the cellular and morphological changes of clitellum were not
occurred in the TCTP knocked down worm (Fig. 39). In our unpublished data we have
found out clitellum plays an active role in regeneration. Outer most GECL of clitellum
dilated during regeneration, however, the process was failed to occur in TCTP RNAi
(Fig. 40 E and F). The collective evidences suggested that TCTP play a profound role in
early development and also in regeneration of animals. The analysis of tunnel apoptotic
assay shows significant tunnel positive cells were documented in nutlin-3 injected not in
the TCTP RNAi. The experimental results suggested that enrichment of p53 might be the
reason for the programmed cell death in nutlin-3 injected worm. The same was
documented in several in vitro and in vivo systems (Secchiero et al., 2006; Gu et al.,
2008; Amson et al., 2012).

Cell cycle is one of the mechanistic processes of actively growing part. The
proliferating region of animals is studied by the experiment mitotic index, which helps to
assess the mitotic stage cells. Interestingly, there were very low mitotic index observed in
the nutlin-3 and TCTP RNAi tissues (Fig. 41 A and B). The experimental evidences are
correlated with the previous reports perfectly (Brioudes et al., 2010). Interestingly, the
protein TCTP is implicated in the regeneration process, embryonic development and also in cancer. It was reported that the stem cell factors Oct4 and Nanog’s were regulated by the TCTP and reduction of TCTP level decreased the rate of stem cells in mammary tumour cells (Koziol et al., 2007; Amson et al., 2012). The important signalling molecules of the developmental pathways such as Ras, Wnt, Akt are also activated by the higher expression of TCTP (Kim et al., 2009; Gu et al., 2013). In addition, TCTP stabilizes the protein hypoxia-inducible factor 1α (HIF1α), which promotes the expression of key angiogenic molecule Vascular endothelial growth factor (VEGF) (Chen et al., 2013). In current study, the pharmacological and specific knockdown of TCTP causes the regeneration failure. However, the mechanism behind the role of TCTP in regeneration is needed to study.
6. Summary
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The African worm *E. eugeniae* is a segmented annelid made with several well developed body systems such as neuronal system, digestive system (mouth, pharynx and digestive system), vascular system (heart, blood vessels), reproductive system (testis, ovary, seminal vesicle, prostate glands, pineal glands) excretory system (nephridia, anus) and similar to other animals, earthworm was also developed with locomotive organ known as setae. The broad spectrum autofluorescence occurred in setae of *E. eugeniae*. The wound healing process was completed on 2nd day of anterior and posterior amputated *E. eugeniae* by the epithelial cell layers. The undifferentiated blastema of *E. eugeniae* arose on the 3rd day of post amputation which is enriched with higher vascular network. The cells of blastema were unpigmented and mononucleated in nature. The differentiation and pattern formation was noted on the 6th day of post amputation. The regenerated mouth and anus was developed on the 6th day of post amputation. The worm, *E. eugeniae*, would usher in starvation during the regeneration of anterior and posterior segments. Stem cells of *E. eugeniae* reside on the blastema and epithelial cell layer of adjacent segments. The stem cells have the autofluorescence nature. Clitellum act as stem cell reservoir and it plays a unique role in proper regeneration. The loss of clitellum resulted in monster regeneration and regeneration failure. The stem cell markers (Oct4, Lamin A, Alkaline phosphatase activity) authenticate the localisation of stem cells in blastema and epithelial cell layer of adjacent segment. The transcriptome analysis shows the expression of mammalian orthologous genes present in *E. eugeniae* regeneration. The *E. eugeniae* stem cell factors Sox2, Klf, c-Myc have higher homology with other vertebrate and invertebrate stem cell factors. The developmental regulatory protein Ras and TCTP were involved in *E. eugeniae* regeneration. The pharmacological suppression of TCTP by siRNA and...
inhibitor caused severe cell death and also arrested the regeneration process in posterior amputated worms. The TCTP RNAi also stops clitellar change in posterior amputated E. eugeniae. It is concluded that the TCTP has early functions in the complex regeneration process. The current work found the following five solid findings. 1) The mouth and anus are ventrally and dorsally opened respectively. 2) The stem cells in the earthworm is fluorescence in nature. 3) The amputation of earthworm clitellum region causes monster worm with anus in both end; 4) The worm regenerates not only mouth but also the anus under starvation condition; 5) TCTP is a protein which has important role in early events of cell differentiation signalling in the regeneration process.