1. Introduction
1.1. The earthworm, *Eudrilus eugeniae*:

An earthworm is a member of oligocheata, belongs to phylum Annelida. It is a beneficial organism and commonly called as “the farmers friend” (Ghabbour, 1966; Rossner, 1981; Hamilton *et al*., 1998; Stephens *et al*., 1995, Stephens *et al*., 1996) which has not only having the beneficial roles such as soil fertility and increase water holding capacity (Edwards and Lofty, 1968; McCredie and Parker, 1992) but also have an important role in the scientific field of regeneration and stem cell research as a model organism (Johnson *et al*., 2012). The earthworm, *Eudrilus eugeniae* (Fig.3) has been proposed as a novel model organism to explore regeneration and stem cell research, because of the following reasons; the worm has the powerful regeneration ability and it was noted that complex organs were also regenerated; the worm *E.eugeniae* has higher growth rate, as little as 5 weeks it reaches the maturity (Rodriguez and Lapeire, 1992) and attains 12 mg body weight per day; Economical and easy to maintain in the laboratory (soil, leaf litter and cow-dung are enough); It is extremely fertile, (Dominguez and Edwards, 2001) and it can tolerate wide temperature difference, ranges from 15-30°C (Dominguez and Edwards, 2001; Viljoen and Reinecke, 1992).

In the systemic position, the earthworm, *E.eugeniae* has been noticed in the following position.

1.2. Taxonomy (systematic position):

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Animalia</th>
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<tbody>
<tr>
<td>Phylum</td>
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<td>Class</td>
<td>Clitellata</td>
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<tr>
<td>Subclass</td>
<td>Oligochaeta</td>
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<tr>
<td>Order</td>
<td>Haplotaxidae</td>
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<tr>
<td>Family</td>
<td>Eudrilidae</td>
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<td>Genus</td>
<td><em>Eudrilus</em></td>
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</table>
Species : *eugeniae*

1.3. Anatomy of the earthworm, *E.eugeniae*:

The earthworm, *E.eugeniae* is a segmented worm, which has about 80 to 200 segments (Oboh et al., 2007). Each segment was separated into a compartment like structure called the septum. The body of the earthworm simply defined as a two tubes, arranged like a “tube within a tube”. The outermost tube consists of three layers namely: ECL (Epithelial Cell Layer) (50 ± 10 µm in thickness) is the first outermost layer followed by CML (Circular Muscle Layer) (90 ± 8 µm in thickness) as a middle layer and the innermost of the outer tube is the LCL (Longitudinal Cell Layer) (112 ± 8 µm in thickness). The inner tube contains the digestive system. The space between the tubes is filled with coelomic fluid, which has several types of cells commonly called as ‘coelomocytes’. They are derived from the chloragogen layer, which is the outermost layer of inner tube. Coelomocytes are divided into two types namely, 1. Amoebocytes; and 2. Eleocytes.

The segments of the earthworm, *E.eugeniae* was divided into three parts; namely, 1. Pre-clitellar (from segments 1<sup>st</sup> to 12<sup>th</sup>); 2. Clitellar (from segments 13<sup>th</sup> to 18<sup>th</sup>) and 3. Post- clitellar region (from segments 19<sup>th</sup> to till last). The Pre-clitellar segments start from the mouth of the 1<sup>st</sup> segment and ends at 13<sup>th</sup> segment. The important organs such as; Brain, Heart, Seminal vesicle and Testis were positioned at 3<sup>rd</sup>, 6<sup>th</sup> to 9<sup>th</sup>, 10<sup>th</sup> to 12<sup>th</sup> and 10<sup>th</sup> to 11<sup>th</sup> segments respectively, which were present at the pre-clitellar segments (Fig.4). The position of different organs in the body of an earthworm, *E.eugeniae* is segment specific.

Mouth is arranged in the first segment, which was also called as peristomium. Based upon the structure of prostomium, the worm was classified into epilobous. The Clitellar segments start from 13<sup>th</sup> segment and end at 18<sup>th</sup> segment. The Clitellum in the mature worm looks prominent like thick cylindrical collar, starts with ovary and its accessory glands were positioned at the 13<sup>th</sup> segment and end with the start point of a pair of the prostate gland in
the 18th segment. The Prostate gland projection till stands to 24th segment. The structure of
the prostate gland is tubular in the earthworm, *E. eugeniae*. The post-clitellar region starts
with the projections of prostate gland & intestine and ends with a ‘periproct’, which is the last
segment of the earthworm.

The earthworm, *E. eugeniae* has the two types of setae namely; 1. Body setae and 2.
Penail setae. The body setae is used for movement by the earthworm, *E. eugeniae*, while the
penail setae is important for copulation. In earthworm, *E. eugeniae* totally four pairs of body
setae are noted which are arranged by ventrolateral form and anchored till LCL of the outer
layer. In contrast, the arrangement of setae is like bristle, namely four pairs arranged ventrally
and both lateral body segments of the worm. The arrangements of setae in the body segments
were termed as Lumbricine. Except the first and the last segment, all the body segments have
the body setae.

The vital organs in the earthworm, *E. eugeniae* has been listed below and the function
of each organ has been described elaborately.

1.4. Nervous system:

The earthworm central nervous system composed of bilobed cerebral ganglion located
on the 3rd segment of the pre-clitellar region. A pair of large nerve trunks, namely
circumpharyngeal connectives (CPC), which passes from the lateral borders of the ganglion
and covers the pharynx. Then, a pair of large nerve unites at the ventral side of the pharynx
and forms the subpharyngeal ganglion at the 4th segment (Walter Hess, 1925). Nerves from
the lateral region of the cerebral ganglion passes to the prostomium, while, nerves from the
near mid and the ventral region of the circum-pharyngeal connective (CPC) passes to the 1st
and the 2nd segment of the worm respectively. All the succeeding segments, except the first
and the last were supplied with three pairs of nerve trunk, arrives from the nerve cord of the
concerned segment.
Commonly in oligochaete, the Central Nervous System is composed of a ventral nerve cord (VNC). The dorsal region of the VNC of oligochaete worms contains three giant nerve fibers, namely one medial and a pair of lateral giant fibers. Each Medial Giant Fiber axon has ventrally projecting collaterals and one cell body. Collaterals also project ventrally from each LGF axon. One of these collaterals, after crossing the VNC midline becomes the neurite of the LGF cell body. In each segment (except segments 1\textsuperscript{st} and 2\textsuperscript{nd}) the VNC gives rise to pairs of segmental nerves. Some fibers within these nerves are sensory, which function as sensory neuron and other fibers serve as motor neurons and innervate the musculature within the body wall.

The earthworm, \textit{E.eugeniae} has four pairs of pseudoheart, arranged from the 6\textsuperscript{th} segment to 9\textsuperscript{th} segment. The function of the pseudoheart is not known.

1.5. Reproductive organs in the earthworm, \textit{E.eugeniae}:

The earthworm, \textit{E.eugeniae} is a hermaphrodite – which has both male and female reproductive organs in a single worm. The reproductive organs such as Testis, seminal vesicle, ovary and its accessory glands were positioned at 10\textsuperscript{th} to 11\textsuperscript{th}, 10\textsuperscript{th} to 12\textsuperscript{th} and 13\textsuperscript{th} segment respectively.

1.5.1. Female reproductive organ and its accessory glands:

The female reproductive organ consists of two pairs of ovaries, namely; 1. One pair of fully developed & functional ovary and, 2. One pair of rudimentary ovary. A pair of rudimentary ovary is present at the 13\textsuperscript{th} segment & one pair of fully developed and functional ovary was present at the 14\textsuperscript{th} segment, which was rounded, berry shaped solid structure, with 800 µm in diameter. Notably, the structure of earthworm, \textit{E.eugeniae} ovary is closely resembles with the mammalian ovary.

In addition to the ovaries, two pairs of oviduct and a pair of spermathecae were also present at the similar manner respectively. Spermatheca is a thin walled band shaped sac,
normally 3 mm length in young worm, which is attached at septum of 13th and 14th segment, whose function is to store sperm. A pair of atria begins from the 13th segment and penetrates through the septum of 13th – 14th segment and opens to the exterior at the 14th segment. Each atrium is a thick-walled muscular tube with uniform diameter. In addition a pair of small rounded accessory glands were also present at the 13th segment, located at the junction of spermatheca and atrium. As described by various authors, the accessory gland consists of several compartments, separated by thin muscle fibers. The function of the rounded accessory gland has been identified that it secretes nourishing fluid for sperm, which are stored in the large spermatheca. The reproductive organs are located freely in the ceolomic fluid of the ceolomic cavity.

1.5.2. Male reproductive organ and its accessory glands:

The male reproductive organ of the earthworm, *E.eugeniae* consists of two pairs of testis sac and two pairs of the seminal vesicle. One pair of the testis sac is located in the 10th segment and the other pair is located on the 11th segment. Alternatively, each pair of seminal vesicle is located on the 11th and 12th pre-clitellar segments. The bulbous funnel like testis sac are thin walled tube choked, full of mature sperm, which connects the prostate gland through two pairs of Vasa deferentia from the 10th - 11th segment to 19th segment. The structure of male and female reproductive organs and its corresponding histological sections were shown in fig.5E-G.

The earthworm, *E.eugeniae* has a pair of prostate gland located on the 19th segment. Its projection is long till 24th segment of post-clitellar segments. The prostate gland is sausage shaped in structure, enclosed of thick muscular envelope composed of longitudinal and circular muscle fibers.
1.6. Nephridia:

Nephridia is one of the excretory systems in the earthworm, which is a sort of diffuse network which opened to the exterior with the help of excretory pores present on the skin and communicated with the body cavity. Two types of nephridial system have been recognized in oligochaeta; 1. The ordinary or meganephric kind, which consists of large and separate nephridia; 2. The plectouephric or diffuse variety – in this case nephridia are numerous, small in size, each connected with one another by a network of ducts, whose openings are present on the surface of the skin.

Nephridia play a major role in eliminating or segregating the unwanted substances from the earthworm body and retain or reabsorb the constituents which are necessary to the organism (Wigglesworth, 1939). There are two circulating fluids present in the earthworm, namely; 1. Blood and, 2. Coelomic fluid, both of which separated from each other but both of them together forms the greater part of the internal environment of the body. The coelomic fluid receives the food materials from the gut and passes it to various cells of the body. The metabolic wastes thus obtained were then passed into nephridia, through which they were eliminated.

The number of nephridia present in each septum varies within limits. On an average, there are 20 to 25 anteriorly and the same number posteriorly on each half of the septum. Each septum will thus have 40 to 50 nephridia on its anterior face and the same number on its posterior face, so that in a single coelomic compartment has about 80 to 100 septal nephridia.

1.6.1. Structure and topography of nephridium:

Each nephridium consists of; (i) a funnel followed by a short narrow tube which is bent on itself before joining the body of the nephridium; (ii) the main body of the nephridium consisting of a short straight lobe and a long spirally twisted loop. The straight lobe is rounded at its free end, and is continued at its other end into one of the-limbs of the long
twisted loop, which is more than twice the length of the straight lobe, and (iii) a terminal nephridial duct, which is a continuation of the other limb of the twisted loop and leads from the nephridium to the septal excretory canal.

1.7. Identification of earthworm, *E.eugeniae* species:

In order to characterize the earthworm species, following strategies has been used; 1. Number of setae in the segment (Lumbricine - 8 setae per each segment; anisochaetine – arrangement of setae is uneven in each segment; perichaetine – Less than 8 Setae per segment). In addition, to the above classification, based upon the arrangements of setae it was further classified into, closely paired setae, widely paired setae and separate setae. 2. Based upon the Prostomium, the worm may be classified into prolobous, epilobous and tanylobous. 3. Based upon the position and shape of the Prostate glands; it may be tubular, tubuloracemose and racemose. 4. Position of clitellum in the earthworm. 5. Presence of Nephridia per segment (either holoic – one pair per each segment; or meroic – more than one pair per each segment). 6. Molecular characterization has also been carried out nowadays, using; i) mitochondrial cytochrome-c oxidase I, COI (Pop *et al*., 2003; Chang *et al*., 2007; Huang *et al*., 2007; Pop *et al*., 2007; Iglesias Briones *et al*., 2009; Otomo *et al*., 2009; Pérez Losada *et al*., 2009; Richard *et al*., 2010), ii) 18S ribosomal DNA (Pop *et al*., 2003; Pop *et al*., 2007), iii) 28S ribosomal DNA (Pérez Losada *et al*., 2009) and iv) 16S ribosomal DNA (Pop *et al*., 2003; Pop *et al*., 2007; Iglesias Briones *et al*., 2009; Pérez Losada *et al*., 2009).

1.8. Earthworm, *E.eugeniae* Life cycle:

The earthworm, *E.eugeniae* is a fast growing organism as reported by many groups. The total duration for one complete life cycle is only about 60 days. Though, earthworm is a hermaphrodite (both male and female reproductive organs are present in the same animal), the mode of reproduction has been classified as biparentally (mutual exchange of genetic
material with another mature worm of the same species) or uniparentally (no sexual fertilization by another worm).

The earthworm, *E. eugeniae* comes under the category of biparental mode of reproduction, where mature worm of same species exchange the genetic material mutually. After mutual exchange of sperm and egg, cocoon has been developed and released. During the formation of cocoon, the colour of the cocoon is grayish-white, it further hardens, and the colour changes from grayish-white to orange-brown. The cocoon became strong and leathery. Later the orange-brown colour cocoon was changed to brown, which further changed to dark-brown colour before hatching. It was also identified that the maximum rate of cocoon production was noted as 3.6 cocoons per week at 25 ºC (Jorge Dominguez *et al.*, 2001). The released cocoon has been incubated for 16 to 18 days, and hatches the new worm.

The shape of the cocoon is irregular oval; one side is flatter when compared with the other side. Each cocoon has sharply pointed tips on both sides. Average length and diameter of the cocoon has been found out that, 6.02 ± 0.05 mm (with minimum range from 4.3 to maximum 7.8) and 3.11 ± 0.03 mm (with minimum range from 2.1 to maximum 4.0) respectively (Reinecke and Viijoen, 1988).

Hatching success has been noted on cattle manure at the rate of 84%. Nearly a mean of 2.12 ± 0.09 hatchings were emerged per cocoon (Reinecke and Viijoen, 1988). After cocoons were hatched, the time to attain the sexual maturity of the worm has been noted as 35 to 45 days (Viljoen and Reinecke, 1989b; Viljoen and Reinecke, 1994). In case of cocoons, which was cultured in the cow dung medium, attains maturity in 42 ± 2 days. The mature worm has been identified by means of prominent clitellum, which is buffy in nature. The mature worm further copulated biparently and produce cocoons accordingly. Thus, the total time duration for one complete life cycle of the earthworm, *E. eugeniae* was 60 days.
1.9. Regeneration studies in earthworms:

The earthworms has wonderful regeneration ability, and the regeneration studies were reported in the following earthworms such as *Eisenia andreï*, *Eisenia fetida*, *Lumbricus rubellus*, (Blakemore, 1999; Gates, 1972; Blakemore, 1998; Slims and Gerard, 1985; Stephenson, 1930), *Ptychodera flava* (Rychel and Swalla, 2008) and *Enchytraeus japonensis* (Takeo et al., 2008). In addition to the earthworms, flatworm taxa also has the capable of regeneration, example; *Macrostomum lignano* (Egger et al., 2006).

It was reported that in most of the oligocheate worms, neoblast has a common morphological characteristics of undifferentiated cell types, such as high nucleo-cytoplasmic ratio, a large nucleus with a large nucleolus and a basophilic cytoplasm (Randolph, 1892; Kreeker, 1923; Turner, 1934; Turner, 1935; Bilello and Potswald, 1974). It was also noted that within the species of earthworm, the regeneration ability varies, as a classical example, *Lumbricus terrestris*, do not have the ability to regenerate segments as regenerated in *Eisenia fetida* (Edwards and Lofty, 1972). Similarly, the regeneration capability of earthworms, *E.eugeniae* and *P.excavatus* is different.

Even though the earthworm is easy, economic to rear and it is a wonderful model system to study the regeneration. There was no report on the regeneration of earthworm, *E.eugeniae*. To study the mechanism and to identify the genes behind the regeneration process, the earthworm, *E.eugeniae* is the excellent model system.

1.10. Stem cells and regeneration:

Stem cells are specialized cells have a self-renewal capability in addition it has the ability to generate more differentiated progeny. Pluripotent embryonic stem cell (ESC) lines has been derived and established from blastocyst-early-stage embryos (Trounson, 2006; Richards et al., 2004). Human embryonic stem cell (hESC) lines express many markers that are common to Pluripotent and undifferentiated cells, such as CD9, CD24, Octamer-binding
protein (Oct-4), Nanog, Alkaline phosphatase, LIN28, Rex-1, Cripto/TDGF1, DNMT3B, SOX2, EBAF, Thy-1, Stage-specific embryonic Antigen-3 & -4 (SSEA-3 & -4) and tumor-rejection antigen-1-60 & -1-81 (TRA-1-60 & -1-81) (Trounson, 2006; Richards et al., 2004; Chambers and Smith, 2004). Stem cells have a main role in the field of regeneration therapy and they are important for the regeneration of central nervous tissue, myocardium, β-cells & dystrophic muscle and musculoskeletal tissues (David Stocum et al., 2008).

1.11. Vitamins in regeneration process:

Vitamins are the organic compounds needed for the organisms as a essential nutrients and they are required very less amount, which promote the health. Vitamins are classified based upon their biological and chemical function, namely; Vitamin A, Vitamin B1, Vitamin B2, Vitamin B3, Vitamin B5, Vitamin B6, Vitamin B7, Vitamin B9, Vitamin B12, Vitamin C, Vitamin D, Vitamin E and Vitamin K. In addition to the above classification, Vitamins are classified into two types, based upon the solubility, namely; 1. Water-soluble Vitamins (Vitamins – B1, B2, B3, B5, B6, B7, B9, B12, and C) and; 2. Fat-soluble Vitamins (Vitamins - A, D, E, and K). Vitamins have diverse biochemical functions, such as 1. Hormone-like functions (Example: as a regulator of mineral metabolism (such as vitamin D), regulators of cell and tissue growth and differentiation (such as some forms of vitamin A)) 2. Act as antioxidants (Example: vitamin E and vitamin C). 3. Act as a precursor for enzyme cofactors, which help enzymes in their work as catalysts in metabolism. (Example: biotin is part of enzymes involved in making fatty acids). Vitamins function as to carry chemical groups or electrons between molecules. For example, folic acid may carry methyl, formyl, and methylene groups into the cell.

In addition to the aforementioned biological process, Vitamins have a role in the regeneration process. It was reported that vitamin B2 called as ‘riboflavin’ (Fig.1), which was
important for the blood regeneration (Spector et al., 1943). The role of vitamin A during the regeneration was discussed briefly in the review of literature session.

In this thesis, earthworm, *E.eugeniae* was used as a model system for regeneration and stem cell studies. The worm, *E.eugeniae* has the highest growth rate, extremely fertile and easy to maintain in the laboratory. For the above said characteristic nature and greater regeneration ability, the worm *E.eugeniae* was chosen as an experimental animal for the present regeneration studies. Following objectives were designed to study the regeneration capability of earthworm, *E.eugeniae* and to identify and characterize the regeneration specific genes.

1.12. Objectives:


2. Cellular level mechanism in the formation of regeneration blastema.

3. Identification and characterization of stem cell niche.

4. Identification of regeneration specific genes by cDNA library construction and proteomics approach.

5. The function of TCTP (Translationally Controlled Tumor Protein) in the anterior regeneration of the earthworm, *E.eugeniae*. 