



Toxic responses of Sox2 gene in the regeneration of the earthworm *Eisenia foetida* exposed to Retinoic acid

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ABSTRACT

Exogenous retinoic acid delays and disturbs the regeneration of *Eisenia foetida*. The stem cell pluripotency factor, Sox2, can play a crucial role in cell reprogramming and dedifferentiation. In this study, we compared the regeneration of *Eisenia foetida* in different segments after amputation and the effects of retinoic acid on the regeneration of different segments. The results showed that the regeneration speed of the head and tail was slightly faster than the middle part, and retinoic acid disrupted and delayed the regeneration of the earthworm. The qRT-PCR and Western blot analysis showed that the expression of the Sox2 gene and Sox2 protein was highest on the seventh day in different segments ($p < 0.05$). After treatment with retinoic acid, the expression level of the Sox2 gene and Sox2 protein was significantly reduced ($p < 0.05$). The results indicated that the regeneration of earthworms and the formation of blastema are related to the expression of the Sox2 gene and protein. Retinoic acid delays and interferes with the regeneration of the earthworm by affecting the expression levels of the Sox2 gene and protein.

1. Introduction

Regeneration means that when an organ or other structure of an organism is lost, the remainder of the organism can reform a functional copy of the structure. The regenerative ability of different organisms is different, and vertebrate regeneration is weaker than that of invertebrates (Bely and Nyberg, 2010). For example, salamanders and toads, both vertebrates, can regenerate some parts of their bodies, such as tails, limbs, and toes (Godwin and Rosenthal, 2014). However, during limb regeneration in amphibians, only the distal part of a limb regrows (Agata et al., 2007). Newts have particularly robust regenerative abilities, including the capacity to regenerate several anatomical structures and organs, including entire limbs. This remarkable regenerative capacity is thought to depend on cellular dedifferentiation (McGann et al., 2001). Among invertebrates, the ability to regenerate lost body parts is very well developed in echinoderms (Carnevali, 2006). Another invertebrate, the planarian, can generate new tissue at the site of a wound via cell proliferation (blastema formation), and remodeling existing tissues to restore symmetry and proportion in a process called morphallaxis (Reddien and Sanchez Alvarado, 2004). Another worm, the common earthworm, has lived on the earth for billions of years, perhaps partially due to its regenerative abilities

(Sturzenbaum et al., 2004). *E. foetida* has the greatest survival rate when fewer segments are amputated (Xiao et al., 2011). In earthworms, regeneration takes place through a process of de-differentiation, whereby cells revert to an undifferentiated state before cellular reprogramming to form new tissues (Myohara et al., 1999; Pearson, 2001). Park found a number of genes that might be associated with regeneration, such as the labial gene (Cho et al., 2009). *E. foetida* has five identified pluripotent factor genes (Sox2, Oct4, Nanog, Lin28, and C-myc) which are expressed in a specific pattern during regeneration. The results suggested that the cells undergo reprogramming during earthworm regeneration (Zheng et al., 2016).

Stem cells have the potential to self-replicate and have multi-lineage differentiation, which are the origin of all cells in the body. Stem cells have the ability to regenerate, a property called pluripotency. In one experiment, scientists injected four genes, Oct4, Sox2, C-myc, and Klf4, into mouse embryonic fibroblasts, which reversed the developmental state of the cell and introduced cell reprogramming. For the first time, Takahashi and Yamanaka succeeded in producing induced pluripotent stem cells (iPSCs) in mice (Yamanaka and Takahashi, 2006). The research also revealed that the expression of these genes has spatio-temporal specificity. Pluripotent genes were expressed only in cells during early development and in totipotent cells, but not in mature cells

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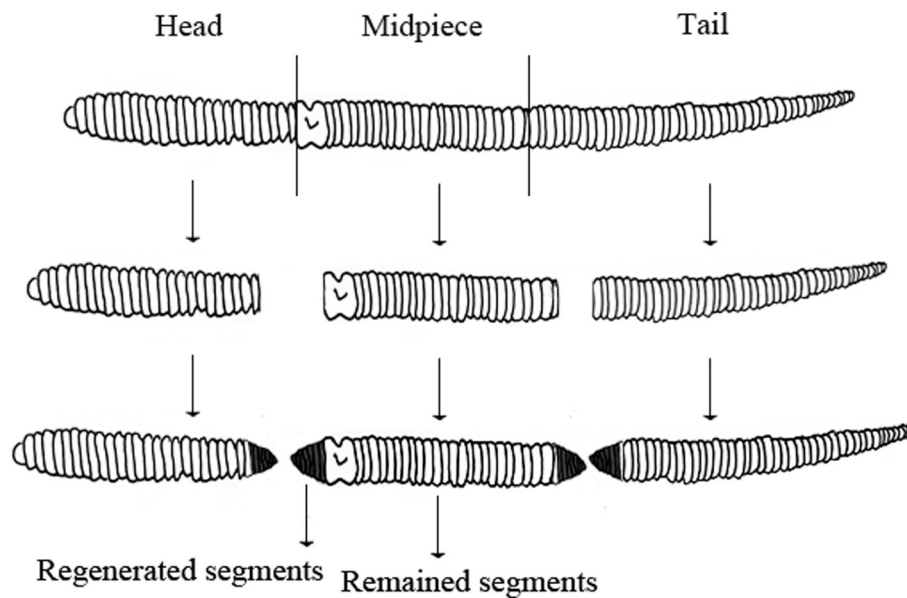


Fig. 1. Schematic diagram of the experiments.

A: Schematic diagram showing the experimental design of tail regeneration; B: Schematic diagram showing the experimental design of different segments.

(Welstead et al., 2008; Zheng et al., 2013). Among them, octamer-binding transcription factor-4 (Oct4) is essential for the self-renewal of embryonic cells and is also expressed in iPSCs (Kobayashi et al., 2016). The transcription factor, Nanog, is specifically expressed in primordial germ cells, inner cell masses, ESCs, and other totipotent cells (Mitsui et al., 2003). The transcription factor, Sox2, is a member of the Y-associated High Mobility Group (HMG) family of proteins in the Sox region. The expression of Sox2 has high sensitivity. The smallest changes in Sox2 will cause ES cells to differentiate into many cell types. Sox2 is not only expressed in ES cells but also has restricted expression in early embryonic neural cells. These genes inhibit the differentiation of neural stem cells (Wakamatsu et al., 2004) and represses melanocyte development (Laga et al., 2010). Sox2 is also used to identify stem cell subsets with other surface markers of stem cells. The abnormal expression of Sox2 is also associated with colorectal cancer and breast cancer (Xiang et al., 2011). When Sox2 is not expressed, cells lose the ability to time development and embryonic defects occur. Therefore, Sox2 acts as a guardian of the developmental clock in stem cells in order to sustain precise control of head development in vertebrates (Mandalos and Remboutsika, 2017). The cells undergo reprogramming during regeneration in earthworms, and this process is closely related to the expression of the Sox2 gene (Zheng et al., 2016).

Retinoic acid (RA) is a metabolite of vitamin A in the body (Blaner et al., 1994). RA signaling plays an important role in the regeneration of vertebrate lens, and Tsonis found that RA signaling is necessary for lens regeneration in newts (Tsonis et al., 2000; Tsonis et al., 2002). Retinoids also influence pattern specification in hydroid polyps (*Hydractinia echinata*) in a way that suggests interference with the generation and transmission of signals responsible for the dimension and spacing of structures (Muller, 1984). Through the study of toads, in amphibians, vitamin A treatment can increase the developmental capacity of the regeneration blastema formed on the amputated limb to the level of the original limb bud, thus the blastema becomes capable of differentiating into a complete limb and the girdle (Niazi and Ratnasamy, 1984). Retinoids alter cell-cell or cell-substrate adhesiveness of fibroblasts, chondrocytes, epithelial cells and various types of carcinoma cells concomitantly with changes in their differentiation or morphology (Crawford and Stocum, 1988). New research suggests that RA could be a major regulator of Schwann cell migration after nerve injury, thus offering a new insight into peripheral nerve repair (Latasa et al., 2016). Retinoic acid can regulate gene expression, and it is combined with receptors that connect to DNA and regulate gene expression to maintain the differentiation of epithelial tissues and other

tissues. Retinoic acid had significant effects on earthworm regeneration. Retinoic acid can delay and interfere with regeneration through the formation of body axis interference (Qi et al., 2004).

It has been reported that the Sox2 gene can affect the regeneration of earthworms, and that RA has an inhibitory effect on the regeneration of the head and tail shaft of earthworms (Xiao et al., 2005; Zheng et al., 2016). However, how RA affects the regeneration of *Eisenia foetida* and how *Eisenia foetida* respond to Sox2 when exposed to RA are unclear. Thus, in the present study, we investigated the expression of Sox2 gene, protein and the influence of RA on earthworm regeneration at the molecular level.

2. Materials and methods

2.1. Experimental animals

Adult earthworms (*E. foetida*) were purchased from Sichuan, Qionglai, and maintained at 21–24 °C under a relative humidity of 60–70%.

2.2. Sample collection

Each earthworm was starved 2 days in beakers with wet filter paper before it was amputated. The filter paper was switched every day. Artificial soil was prepared according to the method described in OECD (1984) and placed into twelve beakers. Six of the beaker had RA at a concentration of 0.1 mmol/kg added. The remaining six beakers served as a control with no added RA. Each beaker was filled with 650 kg of artificial soil.

Earthworms were cut on ice, and the blood of the wound was cleaned with a filter paper. The amputation site is shown in Fig. 1. The head (the first 25 segments of the earthworm), the midpiece (segments 26–51), and the tail (the last 52–95 segments) were also collected. Each of the beakers contained 35 earthworms. The beaker was placed in an artificial climate incubator and maintained at 21–24 °C under a relative humidity of 60–70%.

The researchers collected the regenerating samples at a series of time points: 0 h, 0.5 h, 1 day, 2 days, 3 days, 5 days, and 7 days. Eighteen earthworms were used for sample collection at each time point. Four earthworms were collected (Fig. 1) at each time point.

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