Mini-Review

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Potworm (*Enchytraeus japonensis*) as a potential platform for drug screening in regenerative medicine

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SUMMARY: Regenerative medicine holds substantial promise for restoring the function of damaged or lost organs and tissues. However, the development of effective small-molecule drugs in this field has been limited, partially owing to the lack of suitable regenerative animal models for drug discovery. *Enchytraeus japonensis*, a small annelid with exceptional regenerative capacity, is emerging as a valuable model for regeneration research. *E. japonensis* regenerates in a shorter period, in only 4 days, than other major regenerative animal models. Moreover, novel experimental systems have recently been developed to enhance the utility of *E. japonensis*, including a soaking RNA interference system for easy, non-invasive gene knockdown, an imaging system for quantifying cell distribution in the blastema, and soaking-based pharmacological inhibition using small-molecule compounds. This review highlights *E. japonensis* as a potential platform for chemical screening in regenerative medicine.

Keywords: regeneration, blastema, annelid, chemical screening, RNA interference

1. Introduction

"Regenerative medicine replaces or regenerates human cells, tissues, or organs to restore or establish normal function" (*1*). This approach is often based on the principles of stem cell technology and tissue engineering (*2*). For instance, recombinant human growth factors have been used as biopharmaceuticals to promote fibroblast proliferation during wound healing (*3*). However, only a limited number of effective small-molecule compounds have been developed, and one reason for this is the lack of suitable regenerative animal models for drug discovery. Therefore, model systems that are easy to use and effective for chemical screening, such as nematodes (*4*) and silkworms (*5*), are required in the field of regenerative medicine.

Annelids, including earthworms, ragworms, and leeches, exhibit remarkable regenerative capacity (*6*). The potworm (*Enchytraeus*) is a small annelid species measuring approximately 1 cm in size that is typically found in garden plant pots. The genus *Enchytraeus* derives from the Greek words *en* ("in") and *chytra* ("pot") (*7*). When some potworms are cut transversely, undifferentiated proliferative cell masses named "blastemas" form at the cut ends, which serve as the material for regenerating lost organs, mirroring the regenerative phenomena observed in many living organisms (*6*). This review discusses the utility of potworms in regeneration research and explores their potential applications as chemical screening platforms for regenerative medicine.

2. *Enchytraeus japonensis* as a regenerative animal model

Annelid species such as *Pristina leidyi* (freshwater annelid), *Capitella teleta* (marine annelid), and *Platynereis dumerilii* (marine annelid) have been extensively used as model annelids for studying regeneration (*6*). In some annelids, pharmacological inhibition using small-molecule inhibitors has been used to investigate various biological processes, such as key signaling pathways. For example, Wnt/β-catenin signaling has been studied during regeneration in *Capitella teleta* (*8*), *Aeolosoma viride* (*9-11*), and *Lumbriculus variegatus* (*12*) and during development in *Platynereis dumerilii* (*13-16*). Because these pharmacological studies employed the soaking method, annelids offer the advantage of a technically simple model that can be used to study regeneration in living organisms.

Enchytraeus japonensis (a Japanese potworm) was first reported in Japan in 1993 (*17*) and has since been developed as an experimental model for regeneration research (*18-31*). In laboratory settings, *E. japonensis* can be easily maintained on agar plates at 24°C with

powdered oatmeal used as a food source. Its body length is approximately 1 cm (Figure 1A), facilitating easy handling. Once matured, it asexually reproduces by fragmenting its body; however, sexual reproduction can be induced under specific culture conditions (*18*). Its body surface is whitish and semi-transparent, enabling the direct visualization of internal structures, including the brain, pharynx, testes, ovaries, intestine, and ventral nerve cord. Upon transverse amputation, *E. japonensis* completes blastema formation within 24 h and achieves full regeneration within four days (*18*). Compared to other regeneration model organisms, *E. japonensis* exhibits faster blastema formation (~1 day versus ~2 weeks in axolotl (*32*) and ~2 days in zebrafish (*33*)) and has a smaller body size (~1 cm in length versus 30 cm for axolotl (*34*) and ~4 cm for zebrafish (*33*)). Collectively, these features make *E. japonensis* a powerful model system for investigating the cellular and molecular basis underlying blastema formation.

Invertebrate blastema models, including the potworm blastema, may appear relatively simple compared with the cellular behaviors underlying organ regeneration in vertebrates such as zebrafish. However, recent advances in single-cell analysis now make it possible to directly compare cellular homology and similarity even between evolutionarily distant organisms (*35*). Consequently,

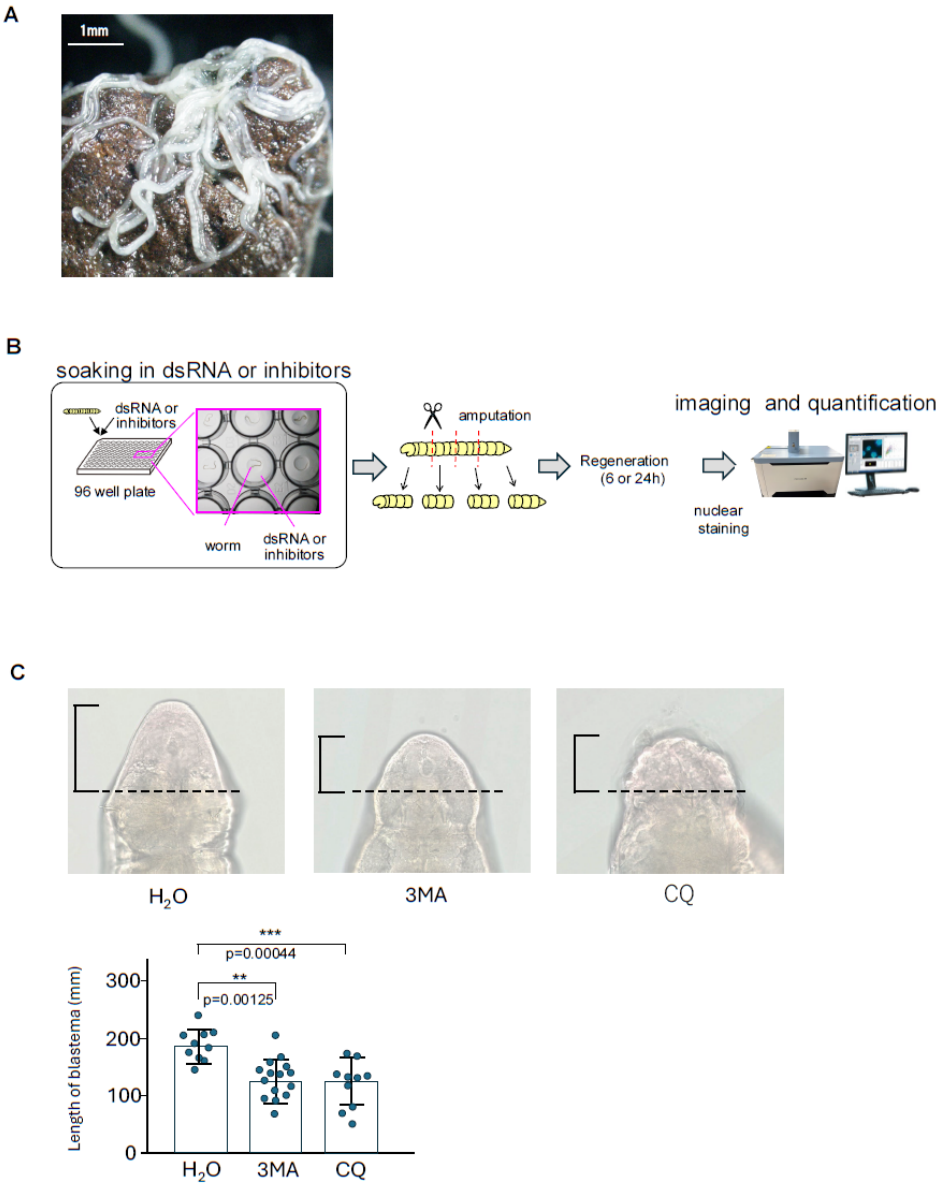

Figure 1. (**A**) Photograph showing multiple *E. japonensis* potworms. (**B**) Schematic illustration of recent experimental systems developed for regeneration research using *E. japonensis*: (1) A soaking RNAi system, (2) an imaging system for quantitative analysis of cell distribution, and (3) a soaking chemical inhibition system. These systems have enhanced the utility of *E. japonensis* as an animal model for regenerative research. (**C**) Example of a soaking chemical inhibition system. The autophagy inhibitors 3-methyladenine (3MA; 100 μM) and chloroquine diphosphate (CQ; 100 μM) were used. Both treatments reduced blastema length, indicating that pharmacological inhibition with both 3MA and CQ using this system was effective in *E. japonensis*. Brackets indicate blastema length. Dotted lines indicate the amputation sites. The bars and the error bars in the graph indicate the mean and standard deviation (SD), respectively. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Dunnett's test). dsRNA: double-stranded RNA.

future studies could clarify which vertebrate regenerative or developmental processes the potworm blastema model most closely resembles, and which cell types can serve as useful models. In this context, the simplicity of the potworm system and its amenability to quantitative analysis are expected to offer substantial advantages, thereby advancing our understanding of these processes. A comparison of key features among regeneration model organisms is summarized in Table 1.

3. Recent advances in experimental systems for regeneration research using *E. japonensis*

Recently, *SoxC*-expressing cells were demonstrated to play an important role as the cellular basis, and *mmpReg* is a key molecular basis for blastema formation in *E. japonensis.* Three experimental systems were developed in this study: (1) a soaking RNA interference (RNAi) system, (2) an imaging system for the quantitative analysis of cell distribution, and (3) a soaking chemical inhibition system. These advances have enhanced the utility of *E. japonensis* as a regenerative animal model.

3.1. Soaking RNAi system

The first RNAi study in *E. japonensis* employed microinjection of double-stranded RNA (dsRNA) (*27*). Although this system was effective, it was also complex and invasive. A recently developed soaking RNAi system overcomes these issues (*31*). In this system, worms were immersed in dsRNA solution in a 96-well plate for 24 h, followed by amputation and quantitative polymerase chain reaction (qPCR) analysis. For instance, treating worms with *soxC* dsRNA reduced *soxC* expression levels by approximately 40% compared to that in worms treated with *GFP* dsRNA as a negative control. Similarly, qPCR analysis revealed that treatment with *mmpReg* dsRNA reduced *mmpReg* expression levels by 50%. Notably, this soaking RNAi system is particularly advantageous because it provides a straightforward, easily reproducible, and minimally invasive method for inducing gene knockdown in *E. japonensis*.

3.2. Imaging system for quantitative analysis of cell distribution within the blastema

A simple cell quantification system is useful for measuring cell proliferation and accumulation. Fujita *et al*. (2024) developed such a system for *E. japonensis* (*31*). In this system, intact worms were first treated with dsRNA using a soaking method, followed by amputation to initiate regeneration. Regenerated blastemas were sampled over time, and their nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) and subsequently analyzed using an image cytometer CQ-1 (Yokogawa). The number of nuclei in the blastemas was quantified using the CellPathwayFinder software (Yokogawa). This system has enabled the evaluation of the effects of RNAi on blastema formation. For instance, under *soxC* RNAi conditions, analysis of blastema size and cell number at 6 and 24 h post-amputation revealed a reduction in blastema size, accompanied by a marked decrease in cell number (~30% at 6 h; ~45% at 24 h) (*31*) (Figure 1B). Similarly, soaking RNAi was used to knock down *mmpReg* during blastema formation, resulting in reduced blastema size and decreased cell numbers at 6 and 24 h post-amputation (~ 40% at 6 h; ~ 40% at 24 h) (*31*) (Figure 1B). Therefore, these results indicate that *soxC* and *mmpReg* are essential for blastema formation (*31*). Overall, this imaging system enables robust and reproducible quantification of blastema cell distribution.

3.3. Pharmaceutical inhibition in *E. japonensis*

Similar to other annelids, pharmacological inhibition using the soaking method is also effective in *E. japonensis*. For example, treatment with a metalloproteinase inhibitor (MMP2/MMP9 inhibitor

Table 1. Comparison of features among regeneration model organisms

	Potworm	Nematode	Zebrafish	Salamander
Regenerative organs	whole-body	axons	heart, fins, spinal cord, retina, *etc.*	tail, limbs, spinal cord, heart, *etc.*
Compatibility with 96-well plate	Yes	Yes	No	No
Manual/automated operation	possible	possible	possible	possible
Cost (money and space)	small	small	moderate	moderate
Genetic homogeneity	high (clone)	high (clone)	high (inbred strain available)	not high (inbred strain not available)
Functional genetics	RNAi only	Many approaches established	Many approaches established	Many approaches established
phylogenetic proximity to humans	distant	distant	intermediate	intermediate

RNAi: RNA interference.

I; *N*-([1,1′-biphenyl]-4-ylsulfonyl)-D-phenylalanine) *via* the soaking method reduced blastema size (*31*). The resulting phenotype was identical to that obtained by RNAi-mediated gene silencing, highlighting the effectiveness of this approach (*31*). Another example is the use of the small-molecule autophagy inhibitors 3-methyladenine (3MA) and chloroquine diphosphate (CQ). Worms were immersed in water containing 3MA or CQ, and blastema length was measured two days post-amputation (Figure 1C). In both treatments, blastema length was reduced, indicating that pharmacological inhibition using both 3MA and CQ was effective in *E. japonensi*s. Thus, this demonstrates that the effects of chemical inhibitors can be easily assessed in *E. japonensis*.

4. Future perspectives

This review highlights the utility and recent advances in experimental systems using *E. japonensis* in regeneration research. First, a soaking RNAi system was developed that provides a simple, reproducible, and minimally invasive method for inducing gene knockdown in *E. japonensis*. Second, an imaging system was established to quantitatively analyze cell distribution within the blastema following dsRNA exposure, enabling efficient measurement of changes in cell number in the blastema (*31*). Third, pharmacological inhibition using the soaking method was effective in *E. japonensis*, offering a technically simple approach. Collectively, these systems enhanced the utility of *E. japonensis* as a regenerative animal model. Specifically, combining the imaging system with soaking-based pharmacology can be used to screen for chemical compounds that modulate cell numbers during blastema formation (Figure 1B). Importantly, by combining the soaking RNAi system with pharmacological inhibition may enable preliminary genetic-level investigations into the relevant signaling pathways and the mechanisms of action of the identified compounds. Similarly, although genomic annotation remains incomplete, transcriptomic approaches integrated with pharmacological inhibition may facilitate informative preliminary studies of compound mechanisms of action. Overall, *E. japonensis* could serve as a regenerative animal model for screening chemical compounds in regenerative medicine.

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