Topic Introduction

Xenopus, a Model to Study Wound Healing and Regeneration: Experimental Approaches

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Xenopus has been widely used as a model organism to study wound healing and regeneration. During early development and at tadpole stages, *Xenopus* is a quick healer and is able to regenerate multiple complex organs—abilities that decrease with the progression of metamorphosis. This unique capacity leads us to question which mechanisms allow and direct regeneration at stages before the beginning of metamorphosis and which ones are responsible for the loss of regenerative capacities during later stages. *Xenopus* is an ideal model to study regeneration and has contributed to the understanding of morphological, cellular, and molecular mechanisms involved in these processes. Nevertheless, there is still much to learn. Here we provide an overview on using *Xenopus* as a model organism to study regeneration and introduce protocols that can be used for studying wound healing and regeneration at multiple levels, thus enhancing our understanding of these phenomena.

XENOPUS AS A MODEL ORGANISM TO STUDY WOUND HEALING AND REGENERATION

Understanding how regeneration processes occur has been an ancient question. Considering *Stedman's Medical Dictionary* definition of regeneration, “reproduction or reconstitution of a lost or injured part,” both the study of organ regeneration per se and wound healing are crucial to understand the cellular, molecular, and physiological mechanisms underlying regeneration. This knowledge can then be used in the pursuit of treatments for different diseases, wounds, or injuries. Herein we recapitulate the advantages of using *Xenopus* as a model organism to study wound healing and regeneration and highlight different experimental approaches and methods to study such processes.

Many animal models have been historically used to study regeneration, from nonmammals to mammals, each of them presenting specific advantages and disadvantages (Table 1). *Xenopus* has been positioned as an ideal model. To start, they can be induced to lay hundreds to thousands of eggs (Cline and Kelly 2012), and their maintenance and breeding is simple and of low cost (Sive et al. 2000; Edwards-Faret et al. 2017), leading to experiments with large sampling and high statistical power. Additionally, performing surgeries and manipulating their embryos is easy because of their size and external development; likewise, they can resist and overcome surgeries of different complexities with simple surgery care (Harland and Grainger 2011). These characteristics, in addition to a well-annotated genome (Hellsten et al. 2010; Session et al. 2016), make *Xenopus* an outstanding animal model to perform high-throughput experiments, such as RNA sequencing and proteomics (Amin et al. 2014;

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TABLE 1. Comparison of some model organisms widely used for regeneration studies

Model organism	Advantages	Disadvantages	References
<i>Xenopus</i>	Regenerative and nonregenerative stages Large offspring External development Genome available Molecular and genetic techniques Transgenic lines available High synteny with mammals	Fewer antibodies available Injuries not closely analogous to those in mammals	Session et al. 2016; Ishibashi et al. 2008; Hellsten et al. 2010; Edwards-Faret et al. 2017; Slack et al. 2004; James-Zorn et al. 2015; Phipps et al. 2020
Axolotl	Excellent regenerative capacities Large offspring External development Genome available Molecular and genetic techniques High synteny with mammals	Only regenerative stages (does not allow comparison with nonregenerative stages) Fewer antibodies available Improved annotation and genomic assembly are needed More extensive transgenic line repository is needed. Injuries not closely analogous to those in mammals	Joven et al. 2019; Joven and Simon 2018; Gerber et al. 2018; Nowoshilow et al. 2018; Smith et al. 2019; Khattak et al. 2014
Zebrafish	Excellent regenerative capacities Large offspring External development Genetics Genome available Molecular and genetic techniques Extensive transgenic lines available	Only regenerative stages (does not allow comparison with nonregenerative stages) Fewer antibodies available Injuries not closely analogous to those in mammals Evolutionarily further from mammals	Azevedo et al. 2011; Itou et al. 2012; Marques et al. 2019; Varshney et al. 2015; Farah et al. 2016
Spiny mouse	Mammal Expectedly high protein homology with <i>Mus musculus</i> (many antibodies available) Genetic information available	Only regenerative stages (does not allow comparison with nonregenerative stages) Lack of an annotated genome Fewer tools and techniques (relatively new model) Small offspring, long gestation More complex maintenance and postsurgery care	Gawriluk et al. 2019; Gawriluk et al. 2016; Haughton et al. 2016; Maden and Varholick 2020; Brant et al. 2019; Seifert et al. 2012

Collart et al. 2014; Lee-Liu et al. 2014, 2018; Sun et al. 2014; Peshkin et al. 2015). Furthermore, transgenic lines can be generated in large numbers and at low cost (Ishibashi et al. 2008). Techniques using transcription activator-like effective endonucleases (TALENs) and clustered regularly interspaced short palindromic repeat (CRISPR)–Cas9 (Ken-ichi et al. 2013; Guo et al. 2014; Sakane et al. 2014; Nakajima and Yaoita 2015) or injection or electroporation of DNA, mRNA, or morpholinos (Eide et al. 2000; Gómez et al. 2003; Bestman et al. 2006; Blum et al. 2015) are effective in elucidating gene functions. Even though *Xenopus laevis* has a relatively long generation time (7–12 mo) and an allotetraploid genome, making genetic experiments harder to perform as a result of more complex genome organization and gene content, the sister species *Xenopus tropicalis* is an alternative for genetics experimentation because of its diploid genome and shorter generation time (3–4 mo). There is extreme similarity between both species, which allows some interchange of experimental results (Karpinka et al. 2015).

Moreover, *Xenopus* tadpole stages are able to regenerate many tissues and organs, including the spinal cord, lens, tail, and limbs; this ability decreases through metamorphosis progression and is almost completely lost after metamorphosis (Filoni et al. 1995, 1997; Slack et al. 2004; Gaete et al. 2012). This transition offers the ability to compare the responses to damage in regenerative versus nonregenerative stages, in order to untangle the mechanisms responsible for regeneration competence (Gaete et al. 2012; Lee-Liu et al. 2014, 2018; Muñoz et al. 2015). Furthermore, functional recovery can be evaluated through simple behavioral tests: Classification of swimming behavior (Gaete et al. 2012) and measurement of free swimming distances (Muñoz et al. 2015; Edwards-Faret et al. 2017) can be used for spinal cord and tail regeneration evaluation, and optomotor response or visual avoidance behavior can be used for visual system regeneration assessment (McKeown et al. 2013). This positions *Xenopus* as a good model organism for performing preclinical trials.

Even though some specific antibodies and reagents are still lacking, the *Xenopus* community has an established database resource, Xenbase (www.xenbase.org), that works as a repository for information about the genome, genes, expression profiles, gene function, and useful reagents and biological data obtained from *Xenopus* research (James-Zorn et al. 2015). Likewise, there are community resources, such as the National *Xenopus* Resource (Marine Biological Laboratory), the European *Xenopus* Resource Centre (University of Portsmouth), the Biological Resource Center *Xenopus* (University of Rennes 1), and the National BioResource Project (Hiroshima University), that rear and distribute transgenic lines.

EXPERIMENTAL SYSTEMS FOR THE STUDY OF WOUND HEALING AND REGENERATION IN *XENOPUS*

Regeneration can be addressed at many levels: (1) epimorphic regeneration, involving the formation of a blastema that guides the regeneration of a complex structure (e.g., limb regeneration); (2) tissue regeneration, considering regeneration without blastema formation (i.e., spinal cord, lens, and embryonic regeneration); (3) cellular regeneration, which encompasses reconstruction of a damaged cell (e.g., axon regeneration); and (4) wound healing, implying scar-free or scar-based repair of a tissue (e.g., epidermis) (Carlson 2007). Here we provide an overview of some of the latest *Xenopus* experimental approaches that allow the study of regeneration at different levels (Fig. 1), many of which are described in detail in the accompanying protocols.

Limb regeneration capacities have been studied during development (Komala 1957; Suzuki et al. 2006; Keenan and Beck 2016); nonetheless, what hinders these studies is variation in the degree of regeneration among tadpoles. For decreasing this variation, special attention should be given to larval maintenance as well as to precision and consistency of limb amputation; see Protocol: **Studies of Limb Regeneration in Larval *Xenopus*** (Fig. 1, Limb amputation; Mescher and Neff 2019). As limb regeneration involves different tissues, it is a great model for studying the effect of different compounds on successful regeneration and patterning of these tissues (King et al. 2012; Mescher et al. 2013).

The inability of mammals to regenerate spinal cord is determined by cellular intrinsic and extrinsic factors (Kaplan et al. 2015). On one hand, mammalian spinal cord axons are able to grow in a regenerative permissive environment (Richardson et al. 1980; David and Aguayo 1981), providing evidence that extrinsic factors, present in the spinal cord environment, restrict or favor axon regeneration. On the other hand, stem cells, grafted into an injured mammalian spinal cord, are able to differentiate into neurons despite the nonpermissive environment (Lu et al. 2012), proving that there are some intrinsic factors, within the neurons, hampering spinal cord regeneration in mammals. Even though specific protocols for generating different types of spinal cord injuries, including their pros and cons, have been published (Polezhaev and Carlson 1972; Lee-Liu et al. 2013; Edwards-Faret et al. 2017; Phipps et al. 2020), they cannot discriminate between intrinsic and extrinsic factors. Transplantation experiments, involving regenerative stages as donors and nonregenerative stages as hosts, allow one to study intrinsic factors of regenerative cells in a nonregenerative environment (Méndez-Olivos et al. 2017). Cell transplantation experiments can be performed in the spinal cord; see Protocol: **Cell Transplantation as a Method to Investigate Spinal Cord Regeneration in Regenerative and Non-regenerative *Xenopus* Stages** (Fig. 1, Transplant; Méndez-Olivos and Larraín 2018).

Stages of lens regeneration are well-characterized (Freeman 1963; Henry 2003; Henry and Tsonis 2010). In *Xenopus*, different stages of lens regeneration can be studied in whole animals after lentectomy; see Protocol: **Methods for Examining Lens Regeneration in *Xenopus*** (Fig. 1, Lentectomy; Henry et al. 2019b). In addition, one can prepare ex vivo eye tissue cultures to examine specific eye tissue interactions; see Protocol: **Ex Vivo Eye Tissue Culture Methods for *Xenopus*** (Fig. 1, Ex vivo eye culture; Henry et al. 2019a).

Finally, to understand the involvement of specific genes, proteins, or signaling pathways in wound healing, oocytes and embryos can be studied. If a quick assay is needed, mechanical wounding can be performed. If greater consistency of wounding is needed or if the interest is on studying the early steps

FIGURE 1. Experimental approaches and methods for studying wound healing and regeneration in *Xenopus*. Shown are some of the *Xenopus* life cycle stages and methods (introduced in the text) that are used to study different phenomena: oocytes for wound healing, tadpoles for limb and lens regeneration, and tadpoles in combination with froglets for transplantation studies.

or the dynamics of wound healing, laser wounding is preferred. The involvement of different signaling pathways in wound healing can be achieved by using glutathione-S-transferase (GST) pull-down assays of signaling molecules. See Protocol: Investigating the Cellular and Molecular Mechanisms of Wound Healing in *Xenopus* Oocytes and Embryos (Fig. 1, Wound healing; Li and Amaya 2019).

CELLULAR AND MOLECULAR METHODS FOR THE STUDY OF REGENERATION MECHANISMS

Many groups have shed light on the mechanisms involved in regeneration (for reviews, see Lee-Liu et al. 2017; Phipps et al. 2020). These include cellular migration (Yoshii et al. 2007; Aztekin et al. 2019), proliferation and differentiation (Yoshino and Tochinai 2004; Gaete et al. 2012; McKeown et al. 2013; Muñoz et al. 2015), and inflammation and the immune response (Mescher et al. 2017), among others.

Considering the cases of tail, spinal cord, limb, and retina injury, the regeneration of nerve connections is crucial (Gaze 1959; Filoni and Paglialunga 1990; Zhao and Szaro 1994; Taniguchi et al. 2008). Thus, relevant questions arise: Which neuronal connections are recovered? Which neuronal nuclei are involved in the regenerative process? Are the axons regenerating and regrowing (e.g., sprouting), or are new neurons being generated? These questions can be addressed by using double axonal tracing in an injured spinal cord; see Protocol: **Tracing Central Nervous System Axon Regeneration in *Xenopus*** (Fig. 1, Axonal tracer; Gibbs and Szaro 2018). This sequential retrograde double-labeling approach uses dextran amines that are incorporated only in terminals or cut axons, allowing the labeling of regenerated axons.

Additionally, understanding the contribution of specific cell types on cell death, proliferation, and regeneration is another recurring question in the regeneration field. This can be addressed through cellular ablation, and the specificity of the observation relies on precise spatial and temporal control of the ablation of a specific cell type. The **nitroreductase/metronidazole (NTR/Mtz) system** addresses these requirements; see Protocol: **Rod-Specific Ablation Using the Nitroreductase/Metronidazole System to Investigate Regeneration in *Xenopus*** (Fig. 1, Ntr/Mtz ablation; Martinez-De Luna and Zuber 2018). The NTR/Mtz system expresses NTR under the control of a cell-type-specific promoter, and, following treatment with Mtz, a cytotoxic product is generated in the NTR-expressing cells, resulting in cell death.

Finally, if the interest focuses on single cells or a cluster of cells for determining (1) the cell fate of a specific cell, (2) the role of a particular gene in a specific cell, or (3) the role of a particular cell during the spinal cord regeneration process, the infrared laser-evoked gene operator (IR-LEGO) system can be used; see Protocol: **Infrared Laser-Mediated Gene Induction at the Single-Cell Level in the Regenerating Tail of *Xenopus laevis* Tadpoles** (Fig. 1, IR-LEGO; Hasugata et al. 2018). IR-LEGO allows gene induction at the single-cell level by generating local heat shock by laser irradiation or ablation of a specific cell by using higher-laser-power irradiation. The IR-LEGO system is highly robust and generates focused cellular damage (Kamei et al. 2009).

In summary, *Xenopus* is clearly an outstanding model organism to study wound healing and regeneration, and the *Xenopus* community has constantly been developing approaches and methods to improve research in this area. Continued advances in understanding regenerative mechanisms in *Xenopus* may provide novel insights to improve regeneration in humans.

COMPETING INTEREST STATEMENT

The authors declare no conflicts of interest.

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