© 2020. Published by The Company of Biologists Ltd | Development (2020) 147, dev180844. doi:10.1242/dev.180844

PRIMER

Model systems for regeneration: *Xenopus*

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ABSTRACT

Understanding how to promote organ and appendage regeneration is a key goal of regenerative medicine. The frog, *Xenopus*, can achieve both scar-free healing and tissue regeneration during its larval stages, although it predominantly loses these abilities during metamorphosis and adulthood. This transient regenerative capacity, alongside their close evolutionary relationship with humans, makes *Xenopus* an attractive model to uncover the mechanisms underlying functional regeneration. Here, we present an overview of *Xenopus* as a key model organism for regeneration research and highlight how studies of *Xenopus* have led to new insights into the mechanisms governing regeneration.

KEY WORDS: Heart, Regeneration, Spinal cord, Tail, *Xenopus*, Appendage

Introduction

The goal of regenerative medicine is to identify novel therapies aimed at stimulating a full regenerative response in humans following injury, disease or during ageing. As such, uncovering the mechanisms employed by organisms with high regenerative capacity may lead to new therapies for humans, which generally have poor regenerative capabilities. As originally discovered by Lazzaro Spallanzani in the mid-18th century, the anuran ('tailless') amphibians, which include *Xenopus laevis* (*X. laevis*) and *Xenopus tropicalis* (*X. tropicalis*), are one such group of animals with high regenerative capacity (Dinsmore, 1991). Originally introduced as a model organism for endocrinology and physiology, but later adopted for cell and developmental biology (Gurdon and Hopwood, 2000), the African clawed frog (*X. laevis*) has been increasingly appreciated over the past 50 years for its regenerative capacity (Deuchar, 1975). Although this species has many advantages, its allotetraploid genome and long generation time make it less attractive for genetic studies. For this reason, its diploid relative, the Western clawed frog (*X. tropicalis*) was introduced as an experimental organism in the late 1990s to mitigate these disadvantages (Amaya et al., 1998).

In this Primer, we give an overview of the life cycle, evolutionary position and regenerative capacity of *X. laevis* and *X. tropicalis*. We then summarise the tools and techniques that are available to study regeneration in these species and review how studies of *Xenopus* have informed our current understanding of the cellular and molecular mechanisms of regeneration. Finally, we discuss the major benefits and limitations of using *Xenopus* as a model system to understand regeneration.

Life cycle, evolutionary position and regenerative potential of *Xenopus*

Although *X. laevis* and *X. tropicalis* diverged between 30 million and 90 million years ago (Evans et al., 2004; Session et al., 2016), there are many similarities between the two species (Fig. 1). Both follow the same morphological changes during development and can be staged using the same developmental staging system (Nieuwkoop and Faber, 1994; Zahn et al., 2017). Embryos can be generated in large numbers following a simple hormone injection and the rate of *Xenopus* development can be manipulated by modifying temperature, which greatly facilitates experimental manipulation during early development. It was this ease of generation of embryos that led to their adoption as models for developmental biology (Amaya et al., 1998; Gurdon and Hopwood, 2000). Although *X. tropicalis* embryos proceed slightly faster through gastrulation and neurulation than *X. laevis* embryos, and optimal housing temperatures differ between the two species (Ishibashi et al., 2017; Khokha et al., 2002), the timing and levels of orthologous gene expression are remarkably similar at comparable developmental stages, particularly after gastrulation (Yanai et al., 2011). Both species reach tadpole stages by 3-4 days post-fertilisation and metamorphosis within 2-3 months, although *X. tropicalis* commonly reaches sexual maturity quicker (Ishibashi et al., 2017) and is therefore the favoured species for genetic studies (Amaya et al., 1998; Tandon et al., 2017). Importantly, both species are supported by advanced genomic resources, including annotated genomes (Amaya, 2005; Hellsten et al., 2010; Session et al., 2016), and genetic approaches, such as transgenesis and gene-editing tools (Blitz et al., 2013; Hartley et al., 2002; Ishibashi et al., 2012; Kroll and Amaya, 1996; Love et al., 2011b; Ogino et al., 2006; Tandon et al., 2017).

Xenopus species occupy a phylogenetic position between urodele amphibians and amniotes and in concordance with this position their regenerative capacity is intermediate between the two (Fig. 2). Whereas urodeles display life-long regenerative capacity of many

Model systems for regeneration

This article is part of a series entitled 'Model systems for regeneration'. This series of articles aims to highlight key model systems and species that are currently being used to study tissue and organ regeneration. Each article provides background information about the phylogenetic position of the species, its life-cycle and habitat, the different organs and tissues that regenerate, and the experimental tools and techniques that are available for studying these organisms in a regenerative context. Importantly, these articles also give examples of how the study of these models has increased our understanding of regenerative mechanisms more broadly, and how some of the open questions in the field of regeneration may be answered using these organisms. To see the full collection as it grows, please visit: https://dev.biologists.org/collection/regeneration_models.

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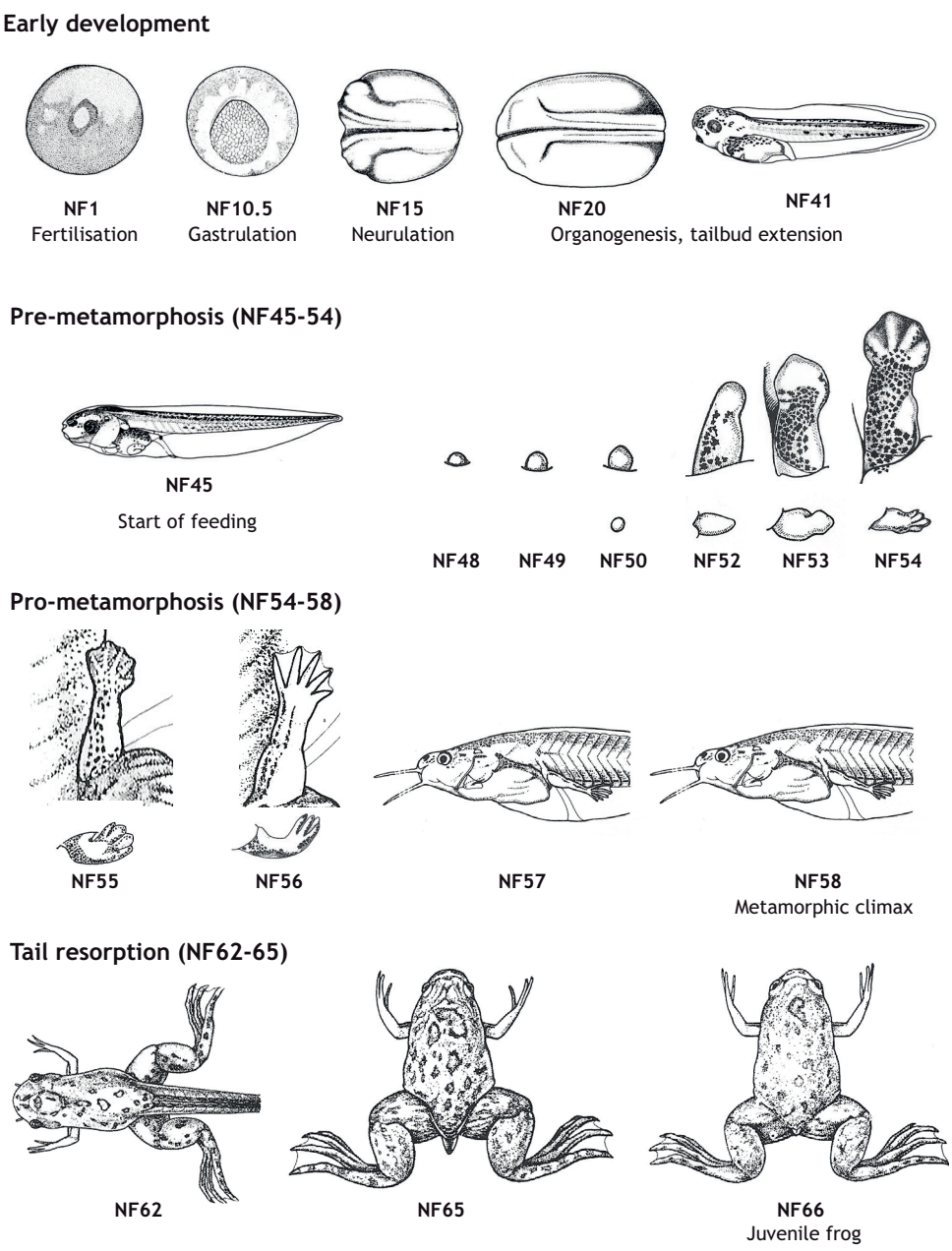

Fig. 1. The key life stages of *Xenopus*. Stages of *Xenopus* development as illustrated by Nieuwkoop and Faber (1994). Eggs are fertilised externally and embryos begin to develop, undergoing gastrulation, neurulation, organogenesis and tailbud extension. They then hatch as larvae within 3-4 days (NF22-27), dependent on temperature. After 5-7 days of development, the larvae/tadpoles begin feeding (from NF45 onwards). From NF48 onwards, during a 'pre-metamorphic' phase, animals develop limb buds, starting with the hindlimbs, which form joints and digits during metamorphosis. Metamorphosis ends with resorption of the tail at NF62-66. Following tail resorption, froglets become juveniles in around 4-6 months and grow until they reach sexual maturity by 6-18 months, depending on sex and species.

organs and appendages (Joven et al., 2019), amniotes and mammals generally display only embryonic or neonatally restricted regeneration, particularly of the central nervous system (CNS) (Bernstein and Stelzner, 1983; Boulland et al., 2013; Chen et al., 1997; Gaillard et al., 2007; Karl and Reh, 2010) and heart (Price et al., 2019). Notably, amniotes fail to regenerate their limbs or tailbuds even during embryogenesis (Schoenwolf, 1977, 1978; Summerbell, 1974). In contrast, *Xenopus* has high regenerative capacity in the pre-metamorphic larval stages, but this capacity is largely lost following metamorphosis, with the exception of a few tissues, such as the retina (see Table 1). Thus, *X. laevis* and *X. tropicalis* are in a useful position to understand the evolutionary transition between organisms with life-long regenerative capacity and those with limited regenerative capacity (a question of phylogeny) and to investigate the mechanisms that underlie the changes from regenerative capacity to non-regenerative capacity during the lifetime of an organism (a question of ontogeny). For brevity, hereafter we focus on regeneration in the context of tissues for which recent findings have led to new insights, namely the appendages (tail/limb), CNS and heart. Where similar regenerative processes have been reported in both *X. laevis* and *X. tropicalis*, we will refer to these species collectively as '*Xenopus*'.

Insights gained from studying regeneration in *Xenopus*

Appendage regeneration

Tail regeneration

Xenopus tadpoles can regenerate their tails, including all of the associated tissues, such as the spinal cord, major blood vessels, muscles and fin (Fig. 3A), from the early tailbud stages (Deuchar, 1975) through to pre-metamorphosis [Nieuwkoop and Faber stage (NF) 53] (Fukazawa et al., 2009; Gaete et al., 2012; Love et al., 2011a; Nieuwkoop and Faber, 1994). This ability is transiently lost at the start of feeding in *X. laevis* (the 'refractory period'; NF45-47) (Beck et al., 2003). Regeneration encompasses three broad phases

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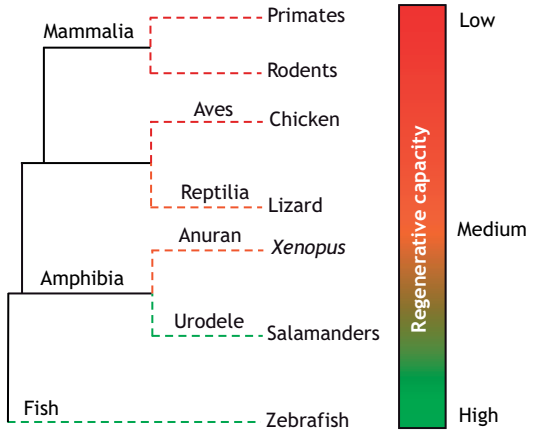

Fig. 2. Phylogenetic tree of taxons and common species used as models of regeneration. Coloured dashed lines denote the general regenerative capacity of each taxon, from high (green) to low (dark red). However, it should be noted that this capacity might vary amongst species within the same taxon (e.g. salamanders, teleost fish) and with age within the same species (e.g. fetal versus adult in mammals, or tadpoles versus adults in *Xenopus*). Lines are not to scale with regards to time.

(Fig. 3B): (1) an initial wound closure and inflammatory response phase at 0-1 days post-amputation (dpa); (2) the formation of a blastema-like regenerative bud at 1-2 dpa; and (3) an outgrowth and overt regeneration phase, starting from 2-3 dpa (Love et al., 2011a). Although the newly formed tail does not regain the entirety of its original tissue patterning, and exhibits differences in muscle, vasculature and axonal organisation, the regenerated tail is fully functional by 7 dpa (Deuchar, 1975; Love et al., 2011a).

A fundamental question in regenerative biology is what drives the process of regeneration? When addressing this question, attention has primarily focused on the 'blastema' – a group of mesenchymal and proliferative cells located at the tip of regenerating tissues. At the cellular level, the *Xenopus* tail blastema was initially postulated to contain de- or trans-differentiating cells, based on early experiments in urodeles (Brockes and Kumar, 2002; Echeverri and Tanaka, 2002; Okada, 1980). However, grafting experiments in the *X. laevis* tail showed that the regenerating muscle, spinal cord and notochord are derived from their respective tissue lineages without de- or trans-differentiation (Gargioli and Slack, 2004; Ryffel et al., 2003). Thus, for a time, the regenerative structure was termed a 'regeneration bud' to distinguish it from groups of de-differentiated blastema cells containing pluripotent stem or progenitor cells (Gargioli and Slack, 2004; Beck et al., 2006; Love et al., 2011a). Subsequently, the presence of lineage-restricted progenitors was also found during limb regeneration in axolotls (Kragl et al., 2009) and digit regeneration in mice (Lehoczky et al., 2011; Rinkevich et al., 2011), suggesting that the existence of lineage-restricted progenitors may be a general property of regenerative tissues. In the *X. laevis* tail, the regeneration bud is now known to contain a number of lineage-restricted progenitors, such as Sox2/3^{+} neural progenitor cells (NPCs) (Gaete et al., 2012) and Pax7^{+} satellite cells (Chen et al., 2006).

Where studies of *Xenopus* appendage regeneration have been particularly instrumental is in promoting our understanding of the molecular mechanisms required to initiate a regenerative programme. Early assessment of drivers of regeneration focused on known developmental signalling pathways. These studies, alongside more recent genome-wide approaches, confirmed that developmental pathways, such as the Notch, bone morphogenetic protein (BMP), Wnt, fibroblast growth factor (FGF), transforming growth factor β (TGFβ) and sonic hedgehog (Shh) pathways, become reactivated during tail regeneration (Aztekin et al., 2019; Beck et al., 2003, 2006; Ho and Whitman, 2008; Lin and Slack, 2008; Lin et al., 2012; Taniguchi et al., 2014). Further studies have also highlighted the importance of additional cellular mechanisms and factors in tail regeneration, such as apoptosis (Tseng et al., 2007) and the extracellular matrix (ECM) (Contreras et al., 2009). Additionally, a subpopulation of epidermal cells, named 'regeneration-organising cells' (ROCs), have been reported to migrate to the amputation edge and release signals that promote the proliferation of the axial tissues (Aztekin et al., 2019). Using genome-wide approaches to assess changes in the transcriptome during the different phases of tail regeneration in *X. tropicalis*, two previously unappreciated processes unfolded (Love et al., 2011a). One was that the expression of many genes associated with metabolism change during regeneration, suggesting a potential role for metabolic reprogramming during regeneration (Love et al., 2014). In addition, genes implicated in the production of reactive oxygen species (ROS) were upregulated during regeneration. A follow-up study revealed that tail regeneration is associated with a sustained increase in ROS levels and that this is required for *Xenopus* tail regeneration to proceed (Love et al., 2013). It has since become apparent that sustained ROS production is a common feature of tissue and appendage regeneration in both invertebrates and vertebrates (Gauron et al., 2013; Khan et al., 2017; Pirotte et al., 2015; Zhang et al., 2016), suggesting that a role for ROS in regeneration is ancient and conserved. The current challenges are to ascertain how ROS levels are regulated during regeneration and to identify the downstream targets of ROS production that promote cell proliferation, growth factor signalling and metabolic reprogramming.

In addition to elevated ROS production, a requirement for bioelectrical changes has been observed following tail amputation in *X. laevis*. This includes the repolarisation of cellular membrane potential (Vm) (Adams et al., 2007; Tseng et al., 2010), the transient activation of a voltage-gated sodium channel, $Na_V1.2$ (Tseng et al., 2010), and the reversal of electric current densities (J_I) driven by changes in trans-epithelial potential (TEP) (Ferreira et al., 2016). In addition, depletion of ROS in regeneration-competent animals using the NADPH oxidase (Nox) inhibitor DPI impairs TEP and J_I dynamics, suggesting that bioelectrical signals are downstream of ROS (Ferreira et al., 2018). An indirect link between extracellular oxygen (O_2) influx and ROS production has also been proposed (Ferreira et al., 2018). Elevated O_2 influx correlates with closure of the wound epithelium and formation of the blastema; a similar increase is also observed following skin wounding in mice, suggesting this process is evolutionarily conserved (Ferreira et al., 2018). This process also appears to be downstream of ROS, as DPI-treated tadpoles show significant decreases in O_2 influx. It should be noted, however, that stabilization of a key mediator of hypoxia, hypoxia-inducible factor α (Hif1α), is not sufficient to rescue regeneration in DPI-treated tadpoles, even though Hif1α is itself necessary and sufficient for tail regeneration, via modulation of J_I current reversal. This suggests that both ROS and Hif1α are independently required for regeneration (Ferreira et al., 2018).

An interesting conundrum is how and why regenerative processes change with age. The ability to study regenerative and non-regenerative phases within the same species makes *Xenopus* a useful model to tackle this question. Although the presence of a 'refractory period' has not yet been documented in *X. tropicalis*, assessment of the refractory period in NF45-47 *X. laevis* tadpoles has confirmed the absence of some pro-regenerative mechanisms during the refractory

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Table 1. Regenerative capacity of tissue and appendages in *Xenopus laevis* and *Xenopus tropicalis*

	Xenopus laevis			*Xenopus tropicalis*		
Tissue/appendage	NF stage	Regeneration?	Reference(s)	NF stage	Regeneration?	Reference(s)
Tail	26-44; 48-50; 52-53	✓	(Beck et al., 2003; Deuchar, 1975; Fukazawa et al., 2009; Gargioli and Slack, 2004)	41-47; 49-51	✓	(Love et al., 2011a, 2013)
	45-47	✗	(Beck et al., 2003)	<49; 51+	?	–
Hindlimb	48-50	?	–	52-53	✓	(Hayashi et al., 2015)
	51-55	✓ (>60% of animals regenerate 3-5 digits)	(Dent, 1962; Hayashi et al., 2015; Muneoka et al., 1986)	48-50; 54+	?	–
	57; 59-60	↓	(Dent, 1962; Muneoka et al., 1986)			
Forelimb	66 (5 months old)	↓	(Géraudie et al., 1990)	All stages	?	–
Spinal cord (after tail amputation)	40-44; 49-50	✓	(Gaete et al., 2012; Taniguchi et al., 2008)	49-50	✓	(Love et al., 2011a)
	45-47	✗	(Beck et al., 2003)	<49; 51+	?	–
Spinal cord (after spinal cord transection)	50-54	✓	(Muñoz et al., 2015)	All stages	?	v
	56-66	✗	(Muñoz et al., 2015)			
Telencephalon	47-53	✓	(Endo et al., 2007)	All stages	?	–
	66	✗	(Endo et al., 2007; Yoshino and Tochinai, 2004)			
Heart	57-58	✓	(Marshall et al., 2019)	<66	?	–
	61-62 to froglet	✓✗				
	Frog (6 months old; 5 years old)	✗	(Marshall et al., 2017; Marshall et al., 2019)	Frog (1 year old)	✓	(Liao et al., 2017)
Eye (full removal)	27-47	✓	(Kha and Tseng, 2018)	All stages	?	–
Eye (partial removal)	32-48	✓	(Ide et al., 1984; Wunsh and Ide, 1990)	All stages	?	–
Retina	47-48; 51-54; 66 (3-9 months old)	✓	(Lee et al., 2013; Yoshii et al., 2007)	66 (3-19 months old; 7 years old)	✓	(Miyake and Araki, 2014)
				<66	?	–
Lens	46-56	✓ (although capacity declines with age)	(Filoni et al., 1997; Freeman, 1963)	50-54	✓	(Henry and Elkins, 2001)
	58; 66	✗	(Filoni et al., 1997; Freeman, 1963)	>54	?	–
Optic nerve	54-58; 66 (<6 months, 1 year and 10 years old)	✓	(Gaze, 1959; Reier and de Webster, 1974; Taylor et al., 1989)	All stages	?	–
Jaw	All stages	?	–	66	✗	(Kurosaka et al., 2008)
Bone (skull injury)	All stages	?	–	66	✓	(Muñoz et al., 2018)
Skin (after full-thickness wound)	66	✓	(Otsuka-Yamaguchi et al., 2017; Yokoyama et al., 2011)	All stages	?	–

Summary of the regenerative ability of *X. laevis* and *X. tropicalis* tissues at different stages of development, as classified by Nieuwkoop and Faber (NF; Nieuwkoop and Faber, 1994). Symbols are used to represent the presence, to the authors' knowledge, of peer-reviewed observations of regeneration as successful (✓), unsuccessful (✗), a mixture of complete, incomplete and unsuccessful (✓ ✗) or lacking of peer-reviewed experimental data (?). ↓ denotes the regeneration of a hypomorphic spike (i.e. impaired regeneration). Where appropriate, specific experimental information is given. Note the exclusion of the intestine from this summary as, although the intestine is used as a stem cell niche model, intestinal remodelling during metamorphosis is fundamentally a developmental process rather than an injury-induced regenerative process.

period. For example, it has been shown that ROS levels, $Na_v1.2$ expression and TEP/J_I dynamics are impaired during the refractory stages in *X. laevis* tadpoles (Ferreira et al., 2018; Tseng et al., 2010), confirming the importance of ROS production and changes in bioelectrical dynamics for establishment of a full regenerative programme. At the cellular level, ROCs fail to migrate to the amputation site during the refractory stages (Aztekin et al., 2019), although the reason for this is currently unknown. Studies of the refractory period have also implicated the importance of inflammatory processes during tail regeneration. In regeneration-competent *X. tropicalis* tadpoles, inflammatory cells are recruited to the wound site over the first 6 h post-amputation (hpa) (Love et al., 2011a), in line with genome-wide studies in *Xenopus*, which demonstrated an enrichment of inflammation-associated genes during the early phase of regeneration (Aztekin et al., 2019; Chang et al., 2017; Love et al., 2011a). In the refractory period, however, inappropriate activation of the immune system may impair tail regeneration, as immunosuppression improves regeneration in these animals (Fukazawa et al., 2009). Contradicting this finding, the presence of bacteria and the activation of complexes downstream of the NF-kB

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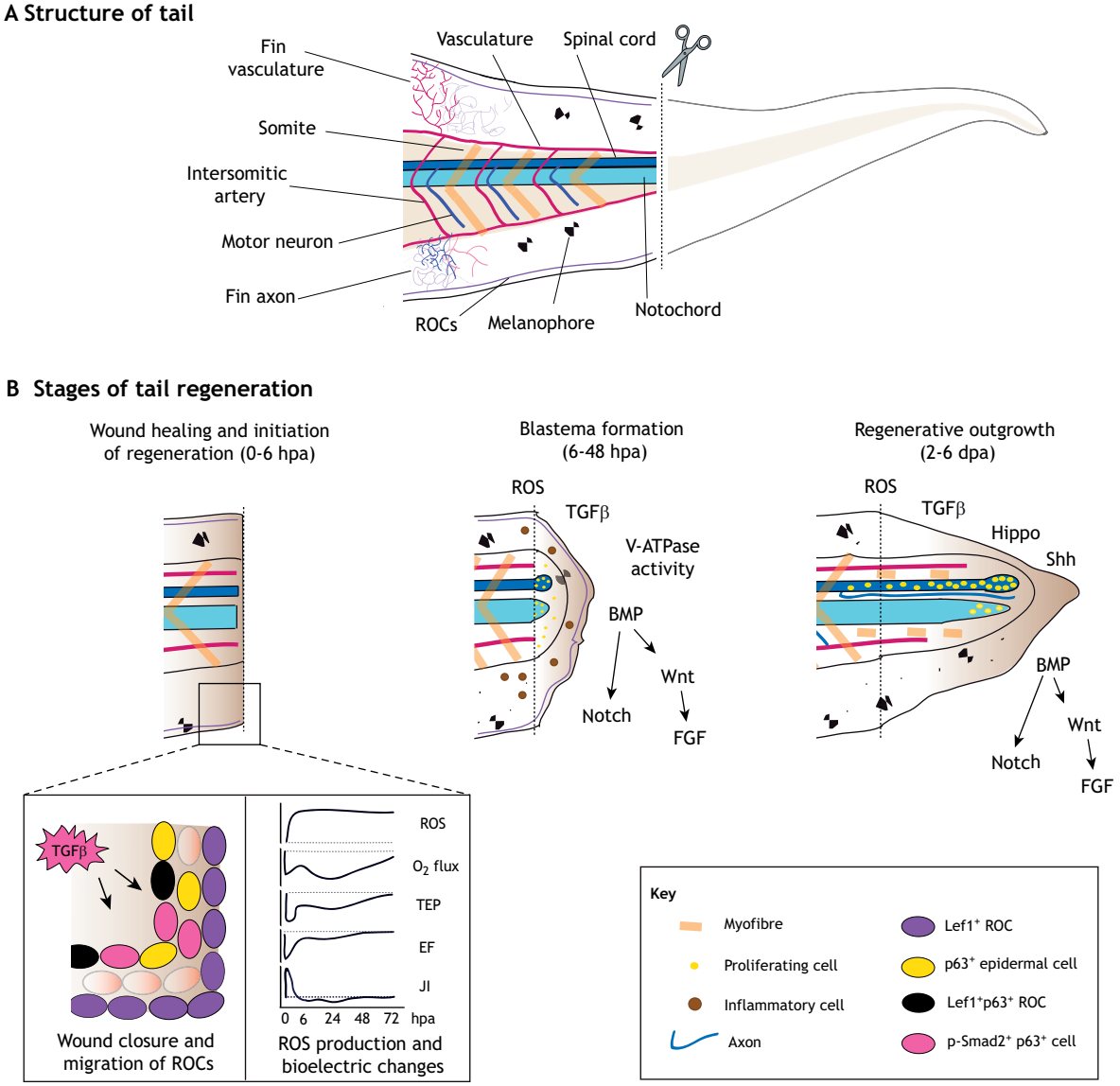

Fig. 3. Tail regeneration. (A) The intact tadpole tail contains somitic muscle (orange), a notochord (light blue), vasculature (red) and epidermal cells, including the recently identified re-organisation cells (ROCs, purple) and melanophores (black). The tail also contains a spinal cord (dark blue), from which axons exit through spinal ganglia to innervate the fin. Motor neurons extend axons along the intersomitic boundaries. Regeneration of the tail can be studied following amputation, which is commonly made at a position halfway to two-thirds of the way along the total tail length (shown here by a dotted line). (B) Key stages of regeneration of the *Xenopus* tail are shown in bold. Injury induces wound healing and the initiation of regeneration (0-6 h post-amputation, hpa), which requires bioelectric changes and the upregulation of signalling molecules (shown in inset), including reactive oxygen species (ROS) and transforming growth factor β (TGFβ). TGFβ signalling is required for wound healing by p63$^+$ epidermal cells. Lef1$^+$ ROCs, which can also express p63, contribute to the wound epithelium and to the initiation of regeneration. From 6-48 hpa, the blastema forms, containing proliferating cells and the regenerative structures of the spinal cord and notochord. From 2 dpa, regenerative outgrowth begins, coinciding with innervation of the regenerate by neuronal axons from the rostral spinal cord and outgrowth of the spinal cord, notochord and vasculature. Myofibres degenerate and are replaced with new myofibres, which originate from Pax7$^+$ satellite cells (not shown). Melanophores arise within the regenerate from melanophore precursors. Some of the key signalling pathways implicated in regeneration are shown alongside the regenerating tail. EF, electric field; JI, electric current density; TEP, transepithelial potential.

pathway have recently been suggested to promote regeneration in the refractory period (Bishop and Beck, 2019 preprint). Further studies are required to fully elucidate the involvement of the immune system, but it is likely that tight regulation of the inflammatory response is required for efficient tail regeneration.

Limb regeneration

Xenopus tadpoles can regenerate their hindlimbs at the pre-metamorphic stages, especially during the early limb bud stages (NF50-52), but regenerative capacity diminishes steadily as limb development progresses (NF53 and onwards) (Keenan and Beck, 2016). In *X. laevis*, the number of digits formed at the completion of regeneration decreases as development proceeds until only a cartilaginous, hypomorphic spike is formed in pro- and post-metamorphic animals (Dent, 1962; Goss and Holt, 1992). Owing to its enclosed development, regeneration of the forelimb has only been studied in post-metamorphic frogs, when amputation also results in the regeneration of a hypomorphic spike (Endo et al., 2000). *Xenopus* therefore offers several advantages as a model system for investigating limb regeneration as one can study: (1) the

successful regeneration during early hindlimb development; (2) the mechanism(s) responsible for progressive loss of regenerative capacity of the limb; and (3) the mechanisms that promote successful limb regeneration and patterning in the limb when regeneration is normally limiting.

Differential expression of genes involved in tissue patterning, inflammation, ECM composition and metabolism (Grow et al., 2006; King et al., 2003; Mescher et al., 2013), and the expression of developmental genes (e.g. *fgf8*, *fgf10*, *msx1*, *sall4* and *hoxa13*) (Christen and Slack, 1997; Endo et al., 2000; Neff et al., 2011; Yokoyama et al., 2001) have all been reported during limb regeneration. Interestingly, it has been found that the developmental stage of the limb mesenchyme determines regenerative capacity far more than the developmental stage of the overlying epidermis (Yokoyama et al., 2000). Recent re-assessment of *X. laevis* limb development has led to the postulation that, like the chick, *Xenopus* has specific regions within the mesenchyme (i.e. the zone of polarizing activity; ZPA) and epidermis (i.e. the apical epithelial ridge; AER) that direct anterior-posterior patterning during development (Christen and Slack, 1997; Christen et al., 1998; Endo et al., 1997; Yokoyama, 2007; for a review, see Keenan and Beck, 2016). As development proceeds, the expression of ZPA and AER markers (*shh* and *fgf8*, respectively) decreases, correlating with the loss of regenerative capacity at ∼NF55-57 (Endo et al., 1997; Wang and Beck, 2014). Chromatin immunoprecipitation has also shown that histone modifications within the limb blastema are unchanged compared with those in the developing NF52-53 limb bud (Hayashi et al., 2015), suggesting that regenerative cells at this stage of development seem to retain memory of what they were prior to amputation. It is plausible that, as the mesenchyme differentiates into subpopulations as the limb develops (Tschumi, 1957), these cells are less able to acquire different identities following amputation, leading to inappropriate tissue patterning. However, other work has suggested distinct regeneration-specific mechanisms, including some regeneration-specific genes (*grem1* and *hsp70*) (Pearl et al., 2008) and the differential involvement of micro-RNAs in regeneration-competent limb buds and regeneration-incompetent limbs (Zhang et al., 2018).

In the adult limb, studies have predominantly focused on assessing treatments that may promote successful regeneration or tissue patterning. This includes the modulation of bioelectricity (Herrera-Rincon et al., 2018; Tseng and Levin, 2013), the stimulation of regeneration with exogenous growth factors and forced gene expression, or cell transplantation (Lin et al., 2013; Wang et al., 2015). Although exogenous application of Fgf10 is sufficient to stimulate *X. laevis* hindlimb regeneration (Yokoyama et al., 2001), forced expression of Shh and Fgf10 is insufficient to enhance forelimb regeneration (Lin et al., 2013). However, regeneration of multiple digits can be stimulated by transplanting larval limb cells with activated Wnt/β-catenin signalling, when combined with a cocktail of Shh, Fgf10 and thymosin-β4 (Lin et al., 2013). Interestingly, the survival of the transplanted grafts relies on host thymectomy prior to metamorphosis, suggesting that immune suppression is required for donor cell survival. However, given that contralateral limbs – which were amputated, but not treated with the cell transplant or growth factor cocktail – fail to regenerate, it is clear that immune suppression alone is not sufficient to promote regeneration in the adult forelimb. Although complex treatment was required in this instance to stimulate regeneration, short-term modulation of bioelectricity has shown similar levels of success. For instance, chemical induction of sodium influx for 1 h is sufficient to induce regeneration of toes and toenails at 45 dpa (Tseng and Levin, 2013), and 24-h treatment with a wearable progesterone-containing bioreactor restores locomotor function to near-control levels by 7.5 months post-amputation, despite the animals failing to regenerate an appropriately patterned limb (Herrera-Rincon et al., 2018). Treated animals also display increased major blood vessel vascularization, increased innervation and re-organisation of bone patterning. This suggests that short-term treatment aimed at modulating bioelectricity is able to stimulate a long-term regenerative programme, which can promote functional regeneration, in spite of differences in morphology compared with intact appendages. Intriguingly, bioelectrical changes following injury can act over a long range, via a process termed bioelectric injury mirroring (Busse et al., 2018). It would be interesting to assess whether longer-term bioreactor treatment would induce improved patterning of the regenerate.

Conserved mechanisms of appendage regeneration

Overall, *Xenopus* appendages have provided useful mechanistic insights into the general properties of a core regenerative programme and suggest that the blastema of both the tail and limb are strikingly similar with regards to the production and pro-regenerative nature of ROS and bioelectric gradients. A recent study has also outlined a previously unknown role for melanocortin signalling in the production of ROS in the regenerating limb bud, showing that the application of melanocortin-stimulating hormone rescues regeneration in denervated limbs (Zhang et al., 2018); reduced ROS levels in denervated limbs further suggests a possible role of nerve signalling in the production of ROS in the blastema. Considering that both tail and limb regeneration are nerve dependent (Cannata et al., 2001; Filoni and Paglialunga, 1990; Suzuki et al., 2005; Taniguchi et al., 2008), it would be interesting to assess whether stimulation of the CNS is a general source of ROS production in regenerating tissues. In the future, it will be important to understand the differential regulation of signalling pathways during development and regeneration, the mechanism underlying the relationship between ROS, bioelectricity and nerve dependency, and the contribution of cellular memory of cells in the blastema to regeneration competency.

Regeneration of CNS tissues

The regenerative potential of the mammalian CNS is very limited. As such, injuries to the brain or spinal cord commonly lead to devastating consequences. This can include motor, sensory and autonomic dysfunction, as well as the development of a chronic inflammatory state (Allison and Ditor, 2015; Sun et al., 2016). Research in mammalian models of CNS injury have focused on improving the ability of axons to grow through the injury site, or transplantation of exogenous stem cells, but these interventions have only shown limited success (Anderson et al., 2018; Dias et al., 2018; Jessberger, 2016). In *X. laevis* tadpoles, however, a crucial feature of the regenerative process in the CNS appears to be the ability to activate endogenous NPCs to proliferate and generate new neurons (Fig. 4A) (Bernardini et al., 2010; Gaete et al., 2012; Muñoz et al., 2015; Yoshino and Tochinai, 2004). Note that, although we term these cells 'NPCs', they are sometimes described in the literature as radial glia (RG) or ependymal cells (Chernoff et al., 2018; Edwards-Faret et al., 2018; McKeown et al., 2013).

Brain regeneration

The *X. laevis* telencephalon and mesencephalon can regenerate up to pre-metamorphic stages (NF47-54). Injury can be achieved by

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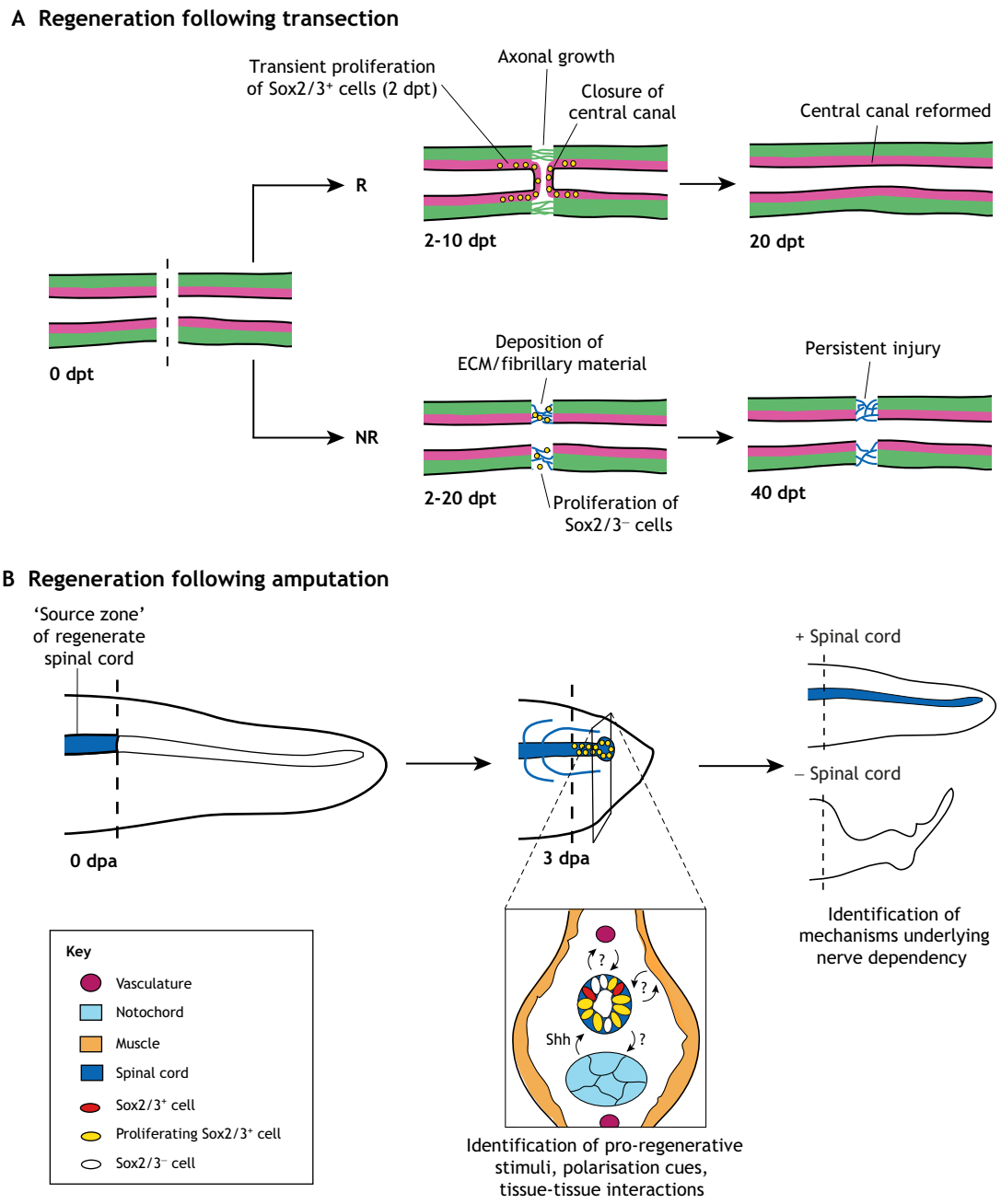

Fig. 4. Spinal cord regeneration. (A) Spinal cord regeneration following spinal cord transection. The rostral spinal cord contains a ventricular zone (pink) containing Sox2/3$^+$ progenitor cells and neuronal cell bodies, and white matter (green) containing axonal tracts. In pre-metamorphosis regenerative stages (R), the cut stumps close, Sox2/3$^+$ cells transiently proliferate and axonal tracts bridge the cut tissue by 10 days post-transection (dpt). Cells migrate to reform the central canal by 20 dpt. In non-regenerative (NR) stages, proliferation is predominantly observed in Sox2/3$^-$ cells outside of the spinal cord stumps. Fibrillary material (blue) is observed within the transection gap by 20 dpt, and injury persists up to 40 dpt. (B) Spinal cord regeneration following tail amputation. The regenerate spinal cord originates from cells rostral to the amputation site (blue). The injured spinal cord first forms a bulbous neural ampulla. This structure (shown in inset) contains Sox2/3$^+$ cells (red) that begin to proliferate (yellow) at 2-3 dpa, correlating with the onset of spinal cord outgrowth. Axons do not exit the regenerate spinal cord; instead, axons from the rostral tail grow into the regenerate to innervate the new tail. This model can be used to identify the molecular mechanisms required for epimorphic regeneration, and to assess interactions with other regenerating tissues. As an example, the notochord (light blue, shown in inset) has previously been shown to express sonic hedgehog (Shh), which promotes proliferation of cells in the spinal cord. This model can also be used to study nerve dependency, as without the spinal cord the tail does not regenerate. Dashed lines indicate amputation site.

partial removal of the olfactory bulb and cerebrum (Yoshino and Tochinai, 2004) or via vacuum aspiration of neurons in the optic tectum (McKeown et al., 2013). Both injuries induce the proliferation of NPCs and the generation of newborn neurons (McKeown et al., 2013; Yoshino and Tochinai, 2004). In the mesencephalon, recovery, as measured by visual avoidance behaviour, is delayed by treatment with cell cycle inhibitors hydroxyurea and aphidicolin (McKeown et al., 2013), showing that proliferation is a key process required for functional regeneration. Newborn neurons integrate into the regenerating tectal circuit; integration and behavioural recovery is significantly enhanced at 24 h post-injury by visual stimulation (Gambrill et al., 2019),

suggesting that experience-dependent plasticity can increase the rate of regeneration. Complete regeneration of the olfactory bulb following injury to the telencephalon requires reconnection to the olfactory nerve, consistent with an activity-dependent regenerative mechanism also in the telencephalon. However, the cerebrum can regenerate independently of this connection, suggesting that this may be a unique feature of sensory brain regions (Yoshino and Tochinai, 2006).

Spinal cord regeneration

Like the brain, the *Xenopus* spinal cord can regenerate up to pre-metamorphosis following tail amputation (Gaete et al., 2012; Love et al., 2011a). An *X. laevis* transection model has also been published (Gaete et al., 2012; Muñoz et al., 2015). In *X. laevis*, both injuries induce the upregulation of Sox2/3 expression and the proliferation of Sox2/3-positive cells (Gaete et al., 2012; Muñoz et al., 2015). Spinal cord-specific manipulation of Sox2 expression, either by morpholino oligonucleotide knockdown or the expression of a dominant-negative form of Sox2, prevents spinal cord regeneration (Gaete et al., 2012; Muñoz et al., 2015). In non-regenerative (NR) post-metamorphic froglets, Sox2/3 expression is very low in the intact spinal cord and, although Sox2/3 expression increases upon transection, these cells do not proliferate and the spinal cord does not recover its integrity, leading to persistent paralysis (Fig. 4A) (Muñoz et al., 2015). These studies suggest that, like in the brain, the proliferation of NPCs is a key feature of successful spinal cord regeneration.

The ability to use two different injury models to study spinal cord regeneration is a key advantage of *Xenopus*. Whereas transection requires the bridging of two stumps, tail amputation requires the growth of multiple tissues in an organised fashion from a single wound (Fig. 4B). Studying how each of these processes is controlled enables researchers to address different questions. For example, transection allows the study of a purely spinal injury and, as this can be assessed in both regenerative (R) and NR stages, key factors that promote successful regeneration in NR stages can be elucidated. For instance, regeneration in NR stages can be improved by transplanting dissociated spinal cord cells from R, but not NR, animals (Méndez-Olivos et al., 2017). In this study, the grafted cells were first treated with a cocktail of epidermal growth factor (EGF), FGF2 and brain-derived neurotrophic factor (BDNF) to aid survival, before retention in a fibrinogen/thrombin matrix. Grafted Sox2/3^{+} cells from R animals self-organised into rosette-like structures and proliferated, and by 60 days post-transection had differentiated into neurons that projected axons into the host spinal cord. In turn, host axons were also able to grow into the graft, suggesting that the transplanted cells support a permissive environment for axonal growth. By contrast, transplanted cells from an NR host in the same paradigm are unable to survive, form rosette structures or differentiate into neurons. This suggests an intrinsic cellular difference between cells from R and NR stages, rather than a non-permissive environment in the injured NR spinal cord. This study also highlights the possibility of using transection in NR animals to assess therapeutics that can promote the regeneration of host axons.

On the other hand, tail amputation allows one to study mechanisms of nerve dependency, reparative organogenesis and tissue-to-tissue interactions. For instance, it has been shown that surgical removal of the spinal cord before amputation prevents the tail from regenerating, in part due to impaired cell proliferation in the notochord (Taniguchi et al., 2008). Conversely, it has been reported that notochord-derived Shh promotes proliferation in the spinal cord during tail regeneration (Taniguchi et al., 2014). These examples highlight the importance of communication between different tissues and cell types during epimorphic regeneration. Furthermore, the requirement for neuronal innervation is a common but poorly understood phenomenon of successful regeneration (Boilly et al., 2017).

Optic nerve and retinal regeneration

Unlike the brain and spinal cord, the *Xenopus* optic nerve and retina retain regenerative capacity throughout life. Interestingly, retinal regeneration is achieved by a different cellular mechanism in *X. laevis* compared with *X. tropicalis*. In adult *X. laevis*, precursors of the ciliary marginal zone (CMZ) repopulate the injured retina following partial removal; however, after full removal of the retina, cells of the retinal pigmented epithelium (RPE) transdifferentiate to form the new neural retina (Yoshii et al., 2007). By contrast, in adult *X. tropicalis*, cells of the CMZ rather than the RPE appear to regenerate the retina following total removal (Miyake and Araki, 2014), similar to retina regeneration in zebrafish (Raymond et al., 2006). Following on from research in non-mammalian vertebrates, a population of retinal progenitors has recently been identified in the developing mouse CMZ (Bélanger et al., 2017), although whether these cells are retained in adulthood, and how they may respond to injury, is unknown.

Unlike other tissues in the CNS, optic nerve regeneration following crush is not predominantly due to the proliferation of existing cells or new neurogenesis (Beaver et al., 2001). Instead, *X. laevis* retinal ganglion cells (RGCs) are protected from death following axonal injury, and these cells regrow their axons to reconnect the retina to the optic tectum by 15 days post-crush (dpc) (Gaze, 1959; Zhao and Szaro, 1994). Regeneration follows two key phases: (1) inflammation and degeneration of injured axons up to 5 dpc; and (2) regeneration of RGC axons across the injury site from 5 dpc (Wilson et al., 1992; Zhao and Szaro, 1994). Translational ribosomal affinity purification has been used to assess transcriptomic changes specifically in regenerating RGCs at 1, 3, 7 and 11 dpc (Whitworth et al., 2017). Interestingly, although axonal growth-associated genes such as *klf6* and *gap43* are upregulated, as well as responses to endoplasmic reticulum stress, oxidative stress, hypoxia and inflammation, the majority of differentially expressed genes are down regulated, including genes involved in axonogenesis, axonal guidance and synaptic communication. This suggests that regenerating RGCs switch from a mature ‘neuronal’ gene expression profile to a programme that prioritises cell survival (Whitworth et al., 2017).

In summary, *Xenopus* tadpoles use a range of different mechanisms to regenerate their nervous system, from the activation of tissue resident cells in the spinal cord to transdifferentiation in the retina. Unlike mammals, the *X. laevis* optic nerve and retina can regenerate throughout life. The ability to regenerate the brain and spinal cord changes during the life cycle of *Xenopus*, from being near perfect before metamorphosis (as in axolotl or zebrafish), to being very limited after metamorphosis (as in mammals). This offers an opportunity to assess experimentally the mechanisms underlying this transition within the same species. Indeed, datasets using transcriptomic and proteomic approaches are starting to provide clues relating to the differences in response to injury in R and NR spinal cords (Gibbs et al., 2011; Lee-Liu et al., 2014, 2018), such as upregulation of metabolism, cell cycle control genes and the immune response in R, but not NR, stages. The challenge now is to identify the important players that will allow us to promote regeneration in non-regenerative animals such as mammals.

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Cardiac regeneration

Heart disease is the leading cause of death worldwide. It is therefore understandable why so many organisms have been investigated for their cardiac regenerative capacity, in the hope of applying lessons learned from those organisms to advance regenerative approaches in humans. Although cardiac regeneration work has largely been dominated by studies in zebrafish and mice, heart regeneration has also recently been evaluated in *Xenopus* (Marshall et al., 2017). Heart regeneration in adult *X. laevis* frogs (>5 years old) is absent 1 year after endoscopy-based resection of the heart apex, which is calculated to remove approximately 4% of cardiac tissue (Marshall et al., 2017). In their study, the authors found evidence of persistent fibrosis, cardiac hypertrophy and deterioration of cardiomyocyte sarcomere structure, with no change in proliferation as evidenced by mRNA expression of cell cycle-related genes (Fig. 5A). They therefore concluded that the adult *Xenopus* heart responds similarly to the mammalian adult heart following injury. By contrast, a separate study (Liao et al., 2017) found that 12-month-old *X. tropicalis* frogs are capable of near scar-free regeneration by 30-60 days following approximately 10% apical resection of the heart (Liao et al., 2017) (Fig. 5B). There are notable differences in methodologies between these two studies, including the injury technique, the techniques used to assess proliferation, and the age of the animals, all of which might influence the observed regenerative outcomes (Liao et al., 2018; Marshall et al., 2018).

Given that *Xenopus* is well known to exhibit a diminishing regenerative capacity from larval to adult stages, a more detailed follow-up study assessed heart regeneration in *X. laevis* across different life stages, including metamorphic onset (NF57-58), climax (NF61-62) and end (NF66), as well as a few weeks after completion of metamorphosis (froglet) and in 6-month-old juvenile

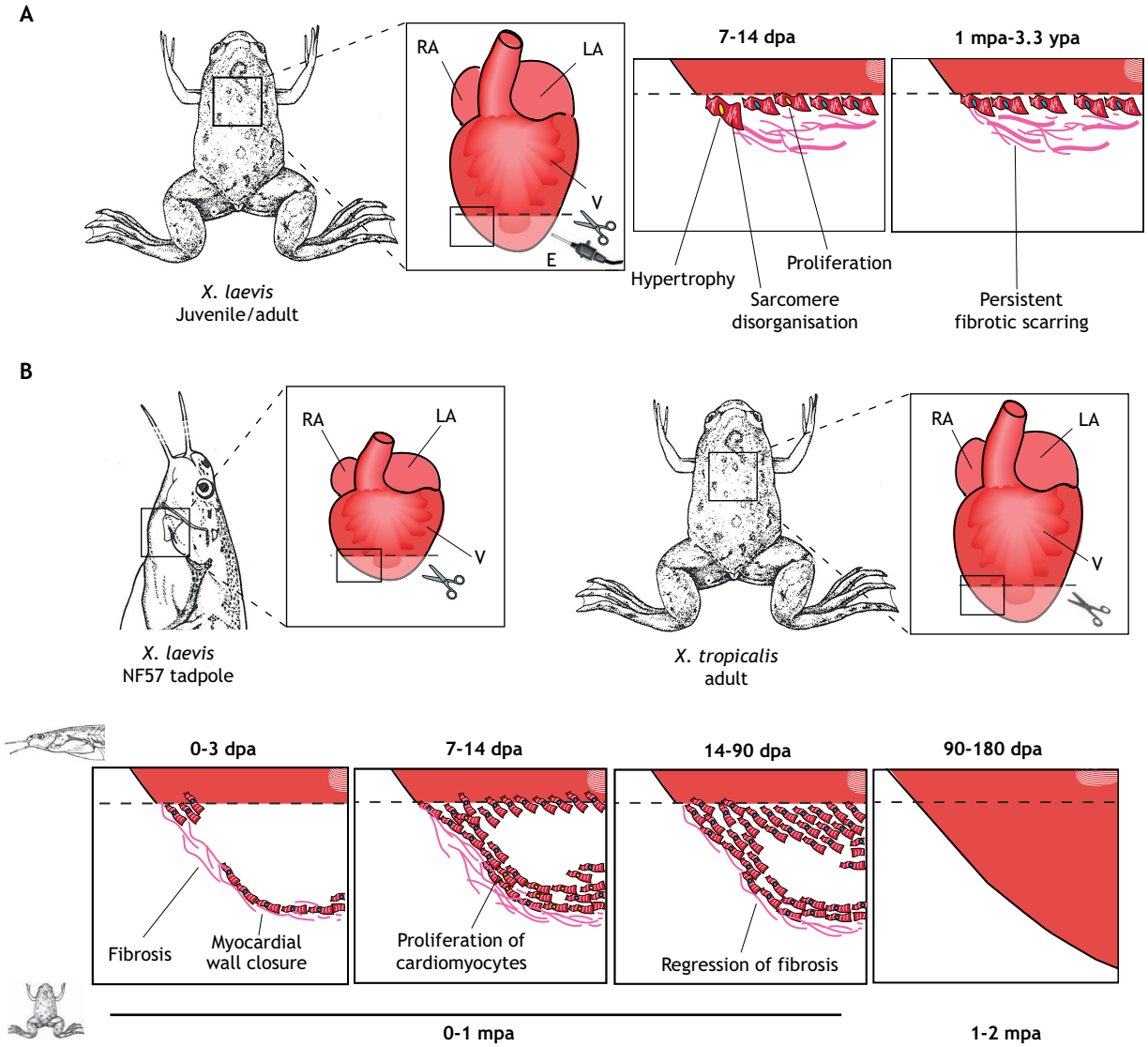

Fig. 5. Cardiac regeneration. (A) In 6-month-old juvenile and >5-year-old adult *X. laevis* frogs, heart regeneration is unsuccessful following apical resection using either surgical scissors (in the case of juveniles) or endoscopy-based resection (E, in the case of adults). Amputation results in hypertrophy of cardiomyocytes, with limited wound closure and fibrotic scarring from 1 month post-amputation (mpa) to 3.3 years post-amputation (ypa). (B) By contrast, heart regeneration is successful in *X. laevis* tadpoles (NF57) and *X. tropicalis* adults (1 year old) following apical resection using surgical scissors. Amputation of the ventricular apex (dashed line) leads to wound healing, cardiomyocyte proliferation (yellow nuclei) and deposition of connective tissue (fibrosis, pink), which gradually reduces as regeneration continues until little to no scar is present. The time required for heart regeneration in the *X. laevis* tadpole (top time line; dpa, days post-amputation) is longer than that required in the *X. tropicalis* adult (bottom timeline; mpa, months post-amputation).

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frogs (Marshall et al., 2019). Although cardiomyocyte proliferation was investigated, as is often the focus in heart regeneration studies, changes relating to the ECM were also studied. Specifically, it was shown that whereas R animals (injured before metamorphosis) are able to clear deposited fibronectin and collagen and successfully complete cardiac regeneration, NR animals (injured after metamorphosis) display persistent fibrotic scars and fail to complete regeneration (Marshall et al., 2019). It therefore appears that cardiac regeneration may be stage and species dependent. However, it is unknown whether pro-metamorphic *X. tropicalis* tadpoles display increased regenerative capacity relative to their *X. laevis* cousins, or what mechanisms allow heart regeneration to proceed better in adult *X. tropicalis*. Similar differences have been observed between the teleost fish zebrafish and medaka (Lai et al., 2017) and in surface-dwelling versus cave-living Mexican cavefish (Stockdale et al., 2018).

But what might underlie the change in regenerative capacity as metamorphosis proceeds? It is known that neonatal mice lose cardiac regenerative capacity at around 1 week after birth (Porrello et al., 2011), a time that is also associated with a peak in serum thyroid hormone (TH) levels (Hadj-Sahraoui et al., 2000). It is also notable that the regeneration of other *Xenopus* tissues is diminished during and after TH-regulated metamorphosis (Beck et al., 2009). This led Marshall and colleagues to ask whether TH levels or availability might be responsible for the switch between regenerative and non-regenerative states in *X. laevis*. They showed that both TH excess and deprivation in tadpoles impair fibrotic clearance and reduce regeneration efficiency. This was associated with increased fibrotic scar extent, a reduction in complete myocardial wall closure, altered ECM gene expression, and changes in the kinetics of tenacin-C deposition and elimination. These observations suggest that ontogenic loss of regenerative capacity is not simply due to increased TH levels, as both too little and too much TH have negative impacts on regeneration. Two plausible hypotheses for this exist: (1) that causing an animal to develop too quickly (i.e. with excess TH) or stunting its development (via TH deprivation) will have a knock-on effect on its ability to regenerate, or (2) that the exact level and/or timing of TH availability and function is key to maintaining a full regenerative response.

In summary, the regenerative capacity of its heart makes *Xenopus* an attractive model for heart regeneration studies. In the future, we expect *Xenopus* to be used more widely and in comparative studies with the mouse model. This should reveal similarities and differences with regard to permitting or inhibiting heart regeneration, findings that will no doubt provide essential information that could aid regenerative medicine approaches following heart injury in the clinic.

Conclusions and future perspectives

A major advantage of using *Xenopus* as a model species for regeneration comes from the fact that it harbours life stages during which it is regeneration competent and others when it is regeneration incompetent. As these stages are experimentally accessible, one can compare the key mechanisms that permit the switch between regeneration competence and incompetence in the same organism. *Xenopus* also provides an accessible testing ground for exploring pre-clinical interventions that may enhance regeneration in NR stages, prior to exploring related approaches in other models that are poor at regenerating, such as mammals.

Of course, as with any model organism, *Xenopus* has its limitations. In particular, the generation of transgenic and mutant lines is time consuming, primarily because of the time required for *Xenopus* to reach sexual maturity, which can range from 6 months to over a year in *X. tropicalis* and *X. laevis*, respectively. In addition, although *Xenopus* is becoming more widely used as a model organism for regeneration studies, it is relatively new compared with other models, and therefore regeneration research in *Xenopus*, especially *X. tropicalis*, is largely in its infancy. Nonetheless, many general principles of regeneration were first found in *Xenopus*, including the lineage-restricted nature of blastemal stem/progenitor cells, the reactivation of developmental signalling pathways during regeneration, and the key role of ROS and bioelectricity for successful regeneration outcomes.

An additional key advantage of *Xenopus* is that it is not only an excellent model for regeneration, but also for developmental studies. Hundreds of animals per fertilisation can be grown cheaply and whole-animal or targeted manipulations can be easily performed. This includes the ability to genetically modify only the left or right side of an embryo, leaving a contralateral control side, which is a unique advantage of *Xenopus*. We and others have also shown remarkable parallels between the mechanisms underpinning tissue regeneration and development. For example, sustained ROS production is required both for appendage regeneration and for early development (Han et al., 2018; Love et al., 2013). *Xenopus* provides an excellent model to study the shared mechanisms, and any interplay between them, that are responsible for both tissue formation and regeneration.

Finally, despite the time required for the generation of transgenic and mutant *Xenopus* lines, there is an ever-growing body of methodologies and resources available for *Xenopus*, including centralised information resources, and reagent and animal suppliers, such as Xenbase, the National *Xenopus* Resource and the European *Xenopus* Resource Centre, that facilitate access to transgenic and mutant lines, but also where researchers can learn techniques and tools especially relevant to *Xenopus* (Gilchrist et al., 2004; Grant et al., 2015; James-Zorn et al., 2013; Pearl et al., 2012). For these reasons, we anticipate that continued studies in *X. laevis* and *X. tropicalis* will continue to provide important new insights relevant to regenerative medicine for decades to come.

Acknowledgements

We apologise to authors whose work could not be included in this Primer owing to space constraints.

Competing interests

The authors declare no competing or financial interests.

Funding

L.S.P. is supported by a Biotechnology and Biological Sciences Research Council Research Training Support Grant (BB/M011208/1). L.M. and E.A. are supported by a grant from the British Heart Foundation Oxbridge Regenerative Medicine Centre (RM/17/2/33380).

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