Cell. Mol. Life Sci. 65 (2008) 54 – 63
1420-682X/08/010054-10
DOI 10.1007/s00018-007-7431-1

Cellular and Molecular Life Sciences

The *Xenopus* tadpole: a new model for regeneration research

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Online First 20 November 2007

Abstract. The *Xenopus* tadpole is a favourable organism for regeneration research because it is suitable for a wide range of micromanipulative procedures and for a wide range of transgenic methods. Combination of these techniques enables genes to be activated or inhibited at specific times and in specific tissue types to a much higher degree than in any other organism capable of regeneration. Regenerating systems include the tail, the limb buds and the lens. The study of tail regeneration has shown that each tissue type supplies the cells for its own replacement: there is no detectable de-differentiation or metaplasia. Signalling systems needed for regeneration include the BMP and Notch signalling pathways, and perhaps also the Wnt and FGF pathways. The limb buds will regenerate completely at early stages, but not once they are fully differentiated. This provides a good opportunity to study the loss of regenerative ability using transgenic methods. (Part of a Multi-author Review)

Keywords. *Xenopus*, regeneration, spinal cord, notochord, muscle, satellite cells, limb, lens.

Introduction

Xenopus is not normally thought of as a regeneration model, because the regenerative capacity of urodele amphibians (newts and salamanders) exceeds that of the anurans (frogs and toads). However, *Xenopus* does show an interesting range of regeneration behaviours which deserve more study than they have received in the past. First, the tadpole tail regenerates very nicely [1], and this leads us to ask why the tail can regenerate when the main body axis cannot, even though they are composed of the same tissue types (spinal cord, notochord and myotomes). Second, the limb buds of the *Xenopus* tadpole will regenerate perfectly at early stages; then the ability falls with the degree of differentiation and is eventually lost [2]. By contrast, avian limb buds do not regenerate at all, and in most urodeles limb regeneration occurs through larval life and sometimes through adult life as well [3]. This poses two interesting questions: Why can *Xenopus* buds regenerate, and why is this ability lost at later stages? Third, the lens of the eye will regenerate if removed. Unlike in the newt, the new lens does not come from the iris by the well-known process of Wolffian regeneration, but instead from the cornea [4]. Finally, in a less well known process, the epithelium of the gut regenerates during metamorphosis. Most of the intestinal epithelium degenerates, and it is replaced by growth from a number of imaginal cell nests. This regeneration process can occur *in vitro* in response to thyroid hormone [5]. The behaviour of these nests represents an accessible model system for studying the establishment of the structural-proliferative units of the gut, such as the glands of the stomach or the crypts of the intestine.

So there are plenty of regenerative phenomena to study in *Xenopus*, and unlike the urodeles, *Xenopus* has the huge advantage for experimental work of being a standard laboratory model organism [6]. This means that there is a good probe inventory available, a whole range of microsurgical techniques for labelling tissues or altering their relative positions during embryonic development, and a set of transgenic techniques for introducing genes or antagonising specific gene function.

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Transgenic techniques

Especially important is the transgenic method that was introduced in 1996 [7]. This depends on the introduction of DNA into isolated sperm heads and then the injection of these modified sperm into unfertilised eggs. With good-quality eggs the method generally works well, and it is possible to make a large number of transgenic individuals in one day. If a fluorescent label is expressed along with the transgene, the transgenic individuals can be identified at an early stage and the expression of the transgene subsequently monitored in living specimens [8]. We have shown that mammalian promoters often give good control of spatial and temporal expression patterns in *Xenopus* embryos, so numerous tissue-specific promoters are already available 'off the shelf' and do not need to be cloned again from *Xenopus* [9]. Most transgenic experiments are performed with founder tadpoles, but it needs to be remembered that each individual founder represents a different transgene insertion site and copy number compared to the others. If a transgenic line is bred from a founder with a single insertion site, this will yield a more homogeneous population of tadpoles [10]. Breeding does take time with *Xenopus laevis*, but the related species *Xenopus tropicalis* has a shorter generation time and is now the organism of choice if breeding experiments are envisaged. *X. tropicalis* has a generation time of 4–5 months [11,12]. It has a completely known genome sequence and is a true diploid, whereas *X. laevis* is pseudotetraploid (i.e. has two copies of everything diverged in sequence by 5–10%). All the normal microsurgical procedures work with *X. tropicalis*, and it is also possible to use probes cloned from *X. laevis*, as the evolutionary divergence between the two species is small enough to enable cross hybridisation.

Although there is no homologous recombination technology that can be used for making knockouts, a wide variety of types of dominant negative reagents have been designed that enable individual genes to be inhibited [13]. These include truncated receptors or transcription factors that sequester the wild-type molecule in inactive complexes, and 'domain swap' transcription factors where an activation domain is replaced by an inhibitory domain, or vice versa. This offers a facility for gene inactivation comparable to mouse knockouts, but with the advantage that a whole gene family is often affected, so a loss-of-function phenotype is more likely to be forthcoming than when just a single family member is removed. Also, RNA interference by transgenesis has been described in *X. laevis*, providing a further method to silence gene function [14].

There are also now in existence several effective methods for conditional regulation of gene activity. The first operates at the protein level and depends on making a fusion of the target gene with a nuclear hormone receptor such as the glucocorticoid receptor [15]. Normally the fusion protein is sequestered in the cytoplasm by endogenous heat shock proteins and thereby kept in an inactive configuration. When the glucocorticoid agonist dexamethasone is added to the embryos, it binds to the receptor, liberates the fusion protein from the complex and enables it to perform its function. For example, if the fusion protein is a transcription factor, it becomes free to enter the nucleus and bind to its target genes. The second method operates at the DNA level and uses a heat shock promoter to control gene activity. This normally controls the expression of the *hsp70* gene, and its activity can be induced by exposure of the tadpoles to a temperature of 34° for 30 min [16]. Third, the Gal4 system has been adapted from its original use in *Drosophila* [17] to *Xenopus* [18]. Here, two constructs are introduced, a specific promoter driving production of the yeast transcription factor Gal4, and the gene of interest driven by the specific upstream activating sequence (*UAS*), which is recognised and bound by Gal4. This enables any promoter of interest to be used to regulate any gene. This system can be enhanced by the use of hormone-inducible forms of Gal4, which incorporate binding sites for oestrogen or glucocorticoids. Fourth, the Cre-lox system in its gene-activating mode has been adapted from mice [19,20]. Again, two constructs need to be introduced. One carries the gene for the Cre recombinase driven by a promoter of choice. The other carries the gene of interest which will be activated by Cre activity. It has a strong ubiquitous promoter such as *CMV*, then a short sequence containing transcriptional stop signals, then the main coding region of the gene of interest. The stop sequence is flanked by *loxP* sites, which are recognised by the Cre recombinase. If Cre is active in a cell, then the region between the *loxP* sites will be excised and an active product will be produced.

Combination of these methods means that a transgene, or a specific inhibitor, can be introduced into the egg, its expression controlled by a promoter of choice and its activity induced at a time chosen by the experimenter. Although transgenic axolotls have recently been made and used to study cell lineage [21], the level of transgenic technology available for *Xenopus* is currently superior to that available for other regeneration models and offers an unparalleled opportunity to investigate a whole variety of problems.

The tail

Tail development

Tail development is similar in all vertebrates [22–25], but we currently know more about the mechanisms in *Xenopus* than in any other vertebrate. The tail is defined as the region posterior to the proctodeum (anal opening). Contrary to popular perception, the *Xenopus* tadpole tail does not derive just from the tailbud. About the anterior 60 % of the tail is actually trunk tissue from the posterior part of the neurula that becomes displaced into a post-anal position by a late anterior migration of the proctodeum [26]. Only the posterior 40 % or so of the tail arises from the tail bud. We have shown that this tail bud will not form in the first place unless the neural plate, posterior mesoderm and posterior notochord all meet at one point, a condition that occurs in the normal embryo but not in the exogastrula or in various types of tissue explant [27]. We believe that this tissue interaction eventually results in the activation of the BMP and Notch signalling pathways [28–31], which are both necessary for tail bud outgrowth. When formed, the *Xenopus* tadpole tail consists of a spinal cord, a notochord and segmented myotomes, surrounded by some connective tissue, and the epidermis, which is drawn into dorsal and ventral fins containing neural crest-derived mesenchyme. Each of these tissue types is formed by a specific process in early development: mesoderm induction forms the notochord and muscle [32], neural induction forms the spinal cord [33], neural crest induction forms the fins [34]. Anatomical study of the tail bud showed that it is divided into gene expression zones corresponding to these tissue types [28, 35, 36]. It should be noted that the anuran tadpole tail does not contain vertebrae, as these are found only in the anterior trunk region that will be retained as the body of the post-metamorphic frog [37]. It is possible that there is a small region of pluripotential cells in the tail bud, associated with *oct4* homologue expression [38], but experimental evidence for the presence of multipotential cells in the tail bud remains weak [39].

Following amputation, the tadpole tail will regenerate over about 20 days (Fig. 1). Over this period the whole tadpole is growing, and the new tail does not quite catch up with the length that it would have attained without amputation. However, tail regeneration represents an excess of growth over the normal rate, and leads to the formation of a structured regenerate similar to the original.

Cell lineage in the tail regenerate

Studies on urodele tail and limb regeneration have revealed evidence for de-differentiation of mature cell types, including neural ependymal cells and multinucleated muscle fibres [40–43]. Not only is there de-differentiation, but there is also some metaplasia of the resulting blastemal cells such that they may become cells of different histological types in the regenerate. We were interested to find whether either or both of these processes (de-differentiation and metaplasia) occurred in the anuran tadpole tail.

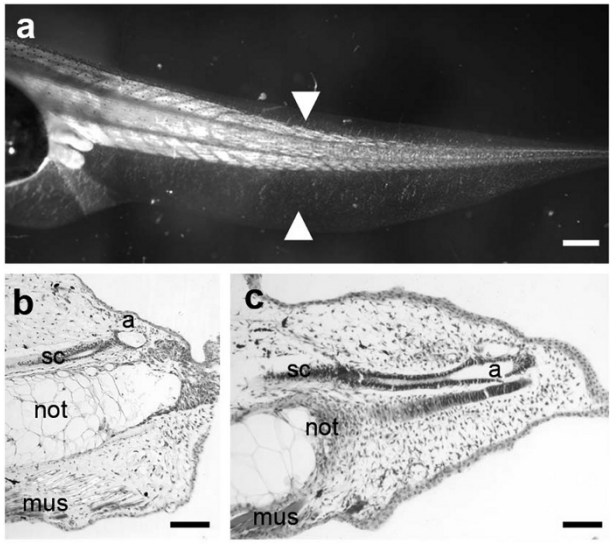

Figure 1. (*a*) A 20-day regenerating tail. White arrowheads indicate the level of amputation. (*b*) A sagittal section of a 3-day regenerating tail. (*c*) A sagittal section of a 5-day regenerating tail. not, notochord; sc, spinal cord; a, neural ampulla; mus, muscle. Scale bar in (*a*), 500 μm. Scale bar in (*b*, *c*) 100 μm.

An initial morphological study showed that the regenerating bud, although somewhat different from the embryonic tail bud, also does not have the appearance of a uniform undifferentiated blastema (Fig. 1b, c). Instead, the termini of the spinal cord and notochord remain distinct and coherent. The spinal cord forms a 'neural ampulla', originally described by Stefanelli [44], and the notochord forms a bullet-shaped mass of cells continuous with the sheath of the more proximal region. For the first few days after amputation the multinucleate myofibres in the region of the cut surface show massive degeneration with large amounts of extracellular proteinaceous debris. In addition to these structures, there is some undifferentiated tissue around them, which might properly be called a 'blastema'.

Morphological study can be suggestive about cell lineage, but it cannot prove any particular mechanism. We have used transgenic *CMV-GFP* grafts to label specific tissues within the tail to find what happens to them during regeneration. Although we have found that less than 100 % of cells express the transgene, the *CMV* (cytomegalovirus) promoter does remain active in most cells of all the tissue types of the tail. So if a cell de-differentiates, it should continue to express GFP

(green fluorescent protein), and if it re-differentiates to a different tissue type, it should also still express GFP. It is possible at the neurula stage to make very clean tissue separations and so to graft explants of either neural plate, or notochord, or presomite mesoderm into the corresponding position of an unlabelled embryo. Then the host embryo will grow to become a tadpole with just one tissue type in the tail labelled. The tail can be amputated through the graft, and the fate of the labelled tissue in the regenerate can be observed. It is also possible to make single tissue grafts at tadpole stages, although it is then harder to avoid contamination with other tissues.

For the spinal cord we found that about 0.5 mm of labelled spinal cord will populate the entire regenerated spinal cord with labelled cells, indicating that this extent of spinal tissue in the stump serves as the precursor for the whole regenerate ([45] and Fig. 2a, b). There is no visible export of GFP-labelled cells to other tissues or import of unlabelled cells from other tissues, although the latter might be difficult to detect. The regenerated spinal cord contains neurons of both motor and sensory type, in their normal positions ([1] and Fig. 2c–f). It also contains fibre tracts and innervates the regenerate. However, there is some defect in the regenerated nervous system compared to the original. The most obvious is the almost complete lack of spinal ganglia. These are composed of sensory neurons and glial cells and are normally located in a ventrolateral position relative to the spinal cord, one per somite. In the regenerate there are no well-formed ganglia, just a few extramedullary neurons in the same positions. Also, examination of the innervation of the regenerated part of the tail shows that the segmental pattern of spinal nerves is not regenerated. There is extensive motor and sensory innervation in the regenerate, but it comes from cell bodies located in the stump of the spinal cord (Fig. 3). This situation is found as the normal, non-regenerated condition in some species of anuran tadpole other than *Xenopus* [46].

The notochord probably has an important mechanical role in stiffening and elongating the tail. The process of its regeneration appears to be quite simple (Fig. 1b, c). Most cell division is found in the sheath region of non-vacuolated cells, and the bullet-shaped mass of cells at the growing tip is composed of such cells. Tadpoles including a heat shock-inducible *noggin* gene can be prevented from regenerating by inducing noggin expression shortly after amputation [10]. The main effect seems to be on the rate of cell division in the notochord and spinal cord. The surrounding tissues show cell division at the normal rate, but this seems inadequate to prevent healing of the epidermis over the cut surface and the cessation of

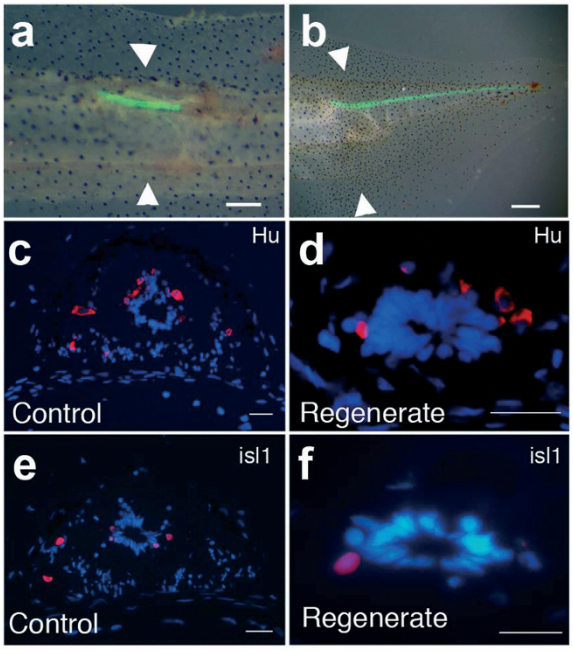

Figure 2. (*a*) A stage-48 tadpole with green fluorescent protein (GFP)-labeled spinal cord graft. (*b*) The same tadpole shown in (*a*) following amputation through the graft and 7 days' regeneration. White arrowheads indicate amputation levels. (*c–f*) Immunostaining of all neurons with Hu (*c*, *d*) and motorneurons with islet 1 (*e*, *f*) on transverse sections. (*c*, *e*) Unoperated control; (*d*, *f*) 2-week-old tail regenerates. Scale bars: 500 μm in (*a*, *b*); 20 μm in the rest.

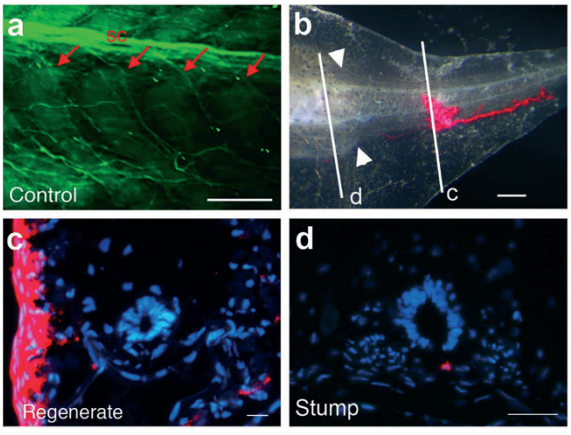

Figure 3. (*a*) β III tubulin detection (green) in a control tadpole tail, showing the arrangement of nerve tracts. Red arrows indicate spinal nerves. (*b*) Retrograde DiI labelling of a 7-day regenerating tail. Two white lines show the position of cross-sections in (*c*) and (*d*). (*c*, *d*) Cross-section through the DiI-injected tail regenerate and the stump. DiI is shown in red, and nuclei are conterstained with DAPI. DiI fails to label the regenerating spinal cord (*c*) but does label cell bodies in the stump (*d*). Arrowheads in (*b*) mark the amputation levels. Scale bars: 250 μm in (*a*, *b*); 20 μm in (*c*, *d*).

regeneration. Likewise, some batches of frogs produce tadpoles showing a refractory period at stage 46–48, during which an amputated tail simply heals without regenerating. This is also associated with a low rate of cell division in the notochord. So it is

probable that continued growth and elongation of the notochord is necessary to prevent healing and to establish regeneration.

The situation for the muscle is somewhat different. It is possible to label myofibres in the tail with grafts of presomite mesoderm from *CMV-GFP* donors. Many labelled fibres arise from grafts from any position within the presomite mesoderm. However, the presence of labelled myofibres at the amputation level of the tail does not mean that myofibres will necessarily be labelled in the regenerate [19,20]. In fact, myofibres in the regenerate are only labelled in significant numbers if the grafts are taken from the lateral region of early neurulae, or from late-stage neurulae in which this lateral tissue has moved more dorsally (Fig. 4a). This suggests that the precursor cells for the regenerated muscle are not myofibres, but are some other class of cell that arises from the lateral part of the presomite plate.

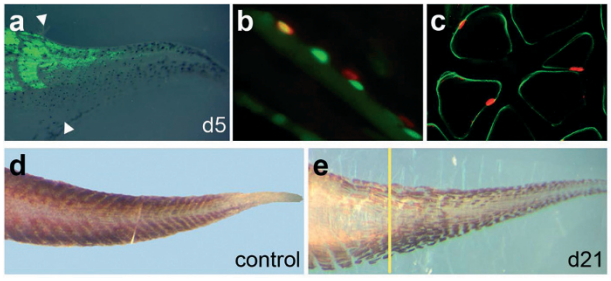

Figure 4. (*a*) Muscle regeneration in tadpoles in which GFP-labelled grafts are taken from the lateral region of early neurulae. Labelled myofibres appear in the regenerate. (*b*) Labelled satellite cell following such a graft (pax7 red, GFP green). (*c*) Location of satellite cells below the basement membrane of myofibres, shown by immunostaining for pax7 (red) and laminin (green). (*d*) Muscle fibres showing segmentation in normal developing tadpole tail (12/101 monoclonal antibody stain). (*e*) Presence of non-segmented muscle in a 21-day regenerating tail (12/101 stain).

We have shown that this precursor cell type is the muscle satellite cell ([20, 47]; Fig. 4b, c). Satellite cells are small mononuclear cells lying within the basement membrane of the myofibres, which can re-enter mitosis and contribute to growth and regeneration of the muscles [48]. Satellite cells can be identified by the flat morphology of their nuclei and the fact that they express the transcription factor Pax7 but not the major muscle proteins [49]. We have shown that the Pax7-positive cells arise predominantly from the lateral regions of the presomite plate [Y. Chen, unpublished]. At the electron microscope level the Pax7-positive cells correspond to satellite cells in morphology, and they also show significant labelling with BrdU, unlike the myofibre nuclei, which are quiescent. If satellite cells are depleted by heat shock-induced expression of a dominant negative reagent, Pax7-EnR, then the formation of muscle in the regenerate is seriously reduced [47]. The fact that satellite cells originate from lateral mesoderm may seem in contradiction with labelling experiments in mouse and chick [50, 51] that indicate an origin from the somites, but the *Xenopus* grafts are done at an earlier, presomite stage, and it is likely that the lateral precursor cells are incorporated into the somite on segmentation. It has recently been shown that satellite cells also contribute to the regeneration of muscle in the newt *Notophalamus viridescens*, although the relative contribution of satellite cells relative to de-differentiation in this organism remains uncertain [52].

The overall conclusion from this study of cell lineage in *Xenopus* tadpole tail regeneration is that there is no de-differentiation and no metaplasia. What we see is complete regeneration of the distal tail by processes that closely resemble those of normal tissue renewal: cell proliferation in the ependymal layer of the spinal cord and in the sheath region of the notochord, and renewal of myofibres from the satellite cells associated with, but distinct from, the differentiated muscle fibres. Interestingly, the tail buds of all vertebrates contain distinct regions giving rise to each of these three major tissue types. Although at the cellular level the process seems very similar to normal growth, it is worth emphasising that regeneration must involve more than this. In some experimental situations in which tail regeneration is blocked (described below), growth of the tadpole and its tail stump continues at the normal rate but without formation of a regenerate. Since growth of all the component tail tissues can occur without regeneration, this implies that regeneration must involve some additional elements as well.

Molecular pathways in tail regeneration

So far global studies of gene expression in tail regeneration are limited to one macro-array study of changes at 1.5 and 3 days following amputation [53]. Although this contains useful data it is probably very incomplete.

We previously established that the BMP and Notch signalling pathways are critical for tail bud outgrowth during embryonic development [29–31]. The main assay in that work was the ability to provoke formation of an ectopic tail from a graft of animal cap tissue in the posterior neural plate. The results showed that Notch signalling is essential for outgrowth of the neural tube and notochord, and that BMP signalling was upstream of Notch and could also, independently, provoke the formation of tail muscle. We subsequently found that the same pathways are needed for tail regeneration [54].

BMPs activate cell surface receptors that phosphorylate Smad proteins in the cytoplasm, which then

migrate to the nucleus and turn on their target genes. To stimulate the BMP pathway, we have made use of Alk3, a mutated, constitutively active form of the type 1 BMP receptor, which phosphorylates its targets in the absence of BMP [55]. To inhibit BMP signalling, we have used either tBR, a truncated BMP receptor lacking its cytoplasmic domain, which behaves as a dominant negative [56], or noggin, an extracellular inhibitor of BMP [57]. Normal activation of the Notch pathway involves a ligand-induced intramembranous cleavage of Notch to yield a free intracellular domain, which forms a complex with bHLH proteins of the Su(H) family and enters the nucleus to turn on target genes. In order to stimulate this pathway, we have used the isolated Notch intracellular domain (NICD) [58].

When either of the activators, Alk3 or NICD, is induced shortly before amputation of a tail during the refractory phase, tail regeneration is stimulated (Fig. 5a). The Alk3-stimulated regenerates are normal, and contain spinal cord, notochord and myotomes. The NICD-stimulated regenerates contain spinal cord and notochord, but little or no muscle. When either of the inhibitors of BMP signalling, noggin or tBR, are induced just before amputation of a later tail, the normal regeneration is inhibited (Fig. 5b). We have previously shown that protease inhibitors that inhibit the intramembranous cleavage of Notch will inhibit tail outgrowth [31]. In this work we have used the inhibitor MG132. This inhibits tail regeneration effectively at 10 μM, and although it is not specific for Notch, also inhibiting proteasome-mediated proteolysis, it has remarkably few other effects on developing embryos or tadpoles.

Because both pathways seem to activate tail regeneration during the refractory period, and both seem to be necessary for regeneration at later stages of tadpole development, it is possible that they are components of a linear pathway. To test this we have examined young, refractory stage tadpoles to find out what happens if one pathway is stimulated and the other inhibited. The results suggest that the BMP pathway is upstream of Notch. This is because MG132 will inhibit regeneration induced during the refractory period by Alk3. Furthermore, NICD will provoke regeneration at these stages even when tBR is also present. However, the effect of NICD/tBR is similar to NICD alone in that no muscle is formed in the regenerate. This suggests that the BMP pathway has a separate effect on muscle regeneration, independent of Notch.

There are a few other studies on tail regeneration. The group of Levin has shown that regeneration is inhibited by two quite dissimilar reagents. Inhibitors of caspase 3 are inhibitory, suggesting that dying cells secrete some pro-regenerative signal [59]. Also, inhibitors of the proton pump V-ATPase suppress regeneration [60]. If V-ATPase is inhibited, then the membrane potential of the distal cells is much reduced. Overexpression of a yeast proton pump can partially rescue both the membrane potential and the ability to regenerate. It has long been considered that electric fields may have a causal role in regeneration, and the V-ATPase may have a role in their establishment.

In zebrafish it has been shown that FGF signalling, in this case due to FGF20, is necessary for caudal fin regeneration [61]. Wnt signalling is also required upstream of the FGF signalling [62]. We believe that a similar mechanism exists in *Xenopus* [G. Lin, unpublished results].

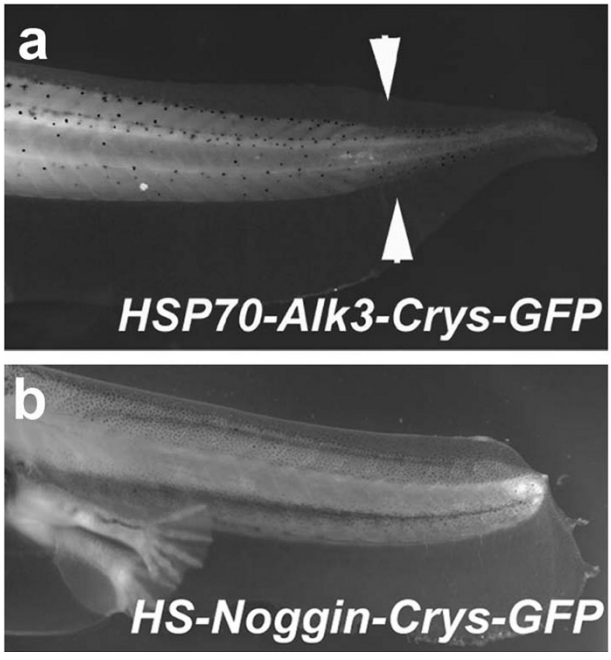

Figure 5. (*a*) Tail regeneration is stimulated when Alk3 is induced shortly before amputation of a tail during the refractory phase. (*b*) Tail regeneration is inhibited when noggin, an inhibitor of BMP signalling, is induced just before amputation of a later tail. Both are shown 7 days after amputation.

Differences between original and regenerated tail

As mentioned above, the regenerated nervous system is not quite the same as the original: there are no spinal ganglia and, at least initially, the peripheral innervation comes from neurons in the stump rather than newly regenerated ones. The regenerated muscle is also not identical to the original. Although the tadpoles can swim well using their regenerated tails, the muscle is not segmented into myotomes and instead forms a disorganised mass of fibres (Fig. 4d, e). This is probably because there is no 'somite oscillator' in the regenerating tail comparable to that existing in the

posterior region of the neurula stage embryo. Although elements of the regeneration mechanism are the same as those of the embryonic tail bud it is noticeable that some aspects are missing, and these are the ones where an embryological process has finished and cannot easily be repeated for anatomical reasons. The neural crest in the embryo is formed by an inductive interaction between epidermis and neuroepithelium at gastrula-neurula stages. But in the regenerating tail there is no junction between the spinal cord and the epidermis. Instead, the spinal cord closes to form the neural ampulla, and the epidermis heals over the cut surface. So the circumstances for forming a neural crest by the process used in embryogenesis do not exist. Likewise for myotomal segmentation. This derives from somite segmentation, which is set up by the somite oscillator. In the regenerating tail the new muscle comes from satellite cells which are released into the blastemal region of the regeneration bud. In this situation there is no coherent mass of tissue within which a somite oscillator could operate. A regenerate which develops somewhat differently from the embryo, or in which the final arrangement of structures is not quite the same as the original, is not unique to *Xenopus*. The regenerated lizard tail is also an imperfect copy of the original in which the spinal cord regenerates ependyma and fibre tracts but no new neurons [63]. Even the urodele tail also is not quite identical to the original. The muscle is initially unsegmentated and seems to acquire some segmentation over a prolonged period by rearrangement of the terminal attachments of the myofibres to bring them into register. Although the urodele tail contains vertebrae, and these do regenerate, they are formed initially as a cartilage rod which later segments in anteroposterior sequence [64]. Both these processes are somewhat different from those seen in embryogenesis.

The lesson from these studies is that if close attention is paid to the details of regeneration, it may be found that the processes are not the same as those of development and that the end product organ is also not quite the same.

The limb

In contrast to the situation with the tail, where *Xenopus* is currently the leading model organism, rather little experimental work has been done on limb development in this species [65]. This means that we must be extrapolate from chick and mouse to infer most of the developmental mechanisms. Although gene expression patterns in *Xenopus* limb buds are generally similar to higher vertebrates (e.g. expression of *fgf8* in the apical epidermis, *shh* in the posterior mesoderm and *lmx1* in the dorsal mesenchyme [66–68]), there are also some differences that may be significant [69]. A subtractive hybridisation study has identified a number of genes associated with regeneration [70], and a microarray study showed extensive activation of genes involved with embryonic patterning, stress responses and inflammation [71].

The main biological difference between the *Xenopus* limb and that of higher vertebrates is that the developing *Xenopus* limb shows significantly more regenerative capacity. Indeed, the failure of the chick limb bud to compensate following surgical rearrangement (mosaic behaviour) was advanced as evidence for the progress zone model of proximo-distal patterning [72]. But like other anurans, and unlike urodele amphibians, *Xenopus* produces little or no regenerate if amputated after metamorphosis [73] (Fig. 6a, b). In the hindlimb, complete regenerates are formed up to stage 52, when the limb bud is a flattened paddle shape; then the ability to regenerate falls off progressively until about stage 57, by which time the limb is well differentiated and only the occasional toe is formed following amputation [2]. The forelimb tends to regenerate better and post-metamorphosis will still form a cartilaginous spike, wheras the hind limb normally forms nothing [74].

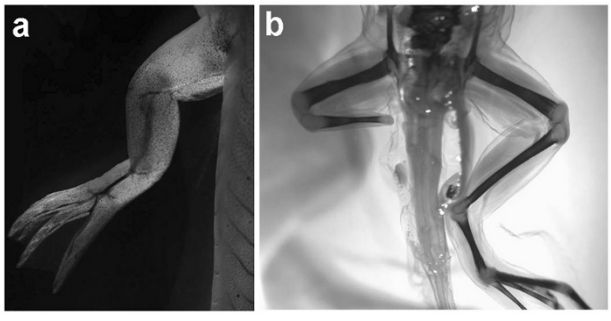

Figure 6. (*a*) A complete limb formed following amputation of the limb bud at stage 52. Figure kindly supplied by Dr Caroline Beck [10]. (*b*) A short spike formed when a post-metamorphic limb is amputated. Alizarin-stained to show the bone. Figure kindly supplied by Dr Bea Christen.

It is often argued that *Xenopus* limb regeneration is not epimorphic regeneration, such as is found in urodeles [75], but rather embryonic regulation of a committed but undifferentiated organ rudiment. The histology of the regenerating stages of the hindlimb shows that they contain a high proportion of undifferentiated cells and there is no obvious sign of dedifferentiation of muscle or cartilage following amputation. Moreover, the dorsoventral pattern is still labile and under the control of the epidermis during

the regeneration-competent stages [67]. However, the forelimb does seem to form a genuine blastema post-metamorphosis, and the fact that regenerative ability gradually declines in concert with differentiation provides a ready-made experimental system for trying to understand what the permissive conditions are for regeneration to occur. Experiments on this topic need to be conducted carefully to avoid artifacts resulting from variation in the regenerative performance of limbs. Even for limbs at a particular stage, it has been shown that regeneration is better if the amputation plane goes through a prospective joint region containing mostly fibroblastic cells than through a major cartilage condensation containing mainly differentiating chondrocytes [76, 77].

Following amputaton, *fgf8* becomes re-expressed in the epidermis of young, regeneration-competent limb buds [68], and work by the lab of Ide has shown that the regenerative capacity is a property of the mesoderm and is correlated with expression of *fgf10.* A regeneration-stage mesoderm can cause *fgf8* re-expression in post-regeneration-stage epidermis [78]. This group subsequently reported that beads carrying FGF10 protein could cause re-expression of *shh*, *msx1* and endogenous *fgf10* in post-regeneration stage mesenchyme, and also prolong the period of regeneration competence to later stages [79]. However, we were not able to reproduce these results in our laboratory. We have also attempted transgenic overexpression of *fgf8* and *fgf10* in late limb buds, but without effects on their regeneration capacity [80]. There is also some evidence for a role for Wnt signalling. Introduction into *Xenopus* limb buds of Wnt agonists and antagonists using adenovirus has indicated some ability, respectively, to increase or diminish the regenerative potential during the period in which it normally declines [81].

It is well known that urodele limb regeneration requires a trophic signal from the nerves which is needed for proliferation of the blastema and without which regeneration will not occur [82, 83]. Studies on *Xenopus* limb regeneration have shown that the early buds will regenerate without nerves, but the later buds do need them; otherwise the loss of regenerative ability occurs about two developmental stages earlier than usual [84]. The synthesis of FGF2 is found in early but not later mesenchyme, suggesting that FGF-like factors may underlie the neurotrophic effect [85]. In the post-metamorphic forelimb, denervation causes cessation of growth of the blastema in the period of 4–7 days. The genes *tbx5* and *prx1* are re-expressed as normal, but their expression is not sustained without nerves. *Fgf8* in the epidermis and *fgf10* and *msx1* in the mesenchyme are normally expressed after 4 days but do not become activated at all in denervated blastemas [86]. So there is good evidence for some sort of neurotrophic effect here, but as in urodeles, the nature of the factors or factors responsible is still uncertain.

The lens

In some but not all urodeles, the well-known process of Wolffian lens regeneration can occur [87] whereby after the lens of the eye is removed it will regenerate from the dorsal iris. This does not occur in *Xenopus* or other anurans. However, the lens of *Xenopus* tadpoles does regenerate from the cornea [4]. The tadpole cornea consists of two layers: an outer layer of squamous epithelium two cells thick, continuous with the epidermis, later becoming the corneal epithelium, and an inner layer of mesenchymal cells, continuous with the sclera, later becoming the substantia propria. During normal development and during regeneration the lens arises from the inner layer of the outer cornea. Tadpoles of all stages will regenerate a lens, but post-metamorphic frogs will not. Following lens removal, the inner layer of the outer cornea becomes cuboidal, forms a cell cluster and develops into a new lens. This eventually detaches from the surrounding cornea, and the cells become differentiated into lens fibres. The whole process takes between 1 and 2 weeks depending on the age of the tadpole.

The related species *X. tropicalis* shows a very low rate of lens regeneration. This is not due to an intrinsic block but rather to rapid healing of the inner layer, which forms a physical barrier between the outer cornea and the inducing stimuli in the vitreous humour. This may be shown by grafting outer corneal fragments into the vitreous humour, upon which they will develop into lens tissue [88].

Conclusions

The works reviewed above are sufficient to show that *Xenopus* is a very useful organism for regeneration research. The ability to combine transgenesis and grafting between embryos creates a powerful technology to progress this work. The various results show that the situation in tail, limb and eye is somewhat different from the situation for urodele regeneration. Whereas urodeles have specific mechanisms for achieving de-differentiation of mature differentiated cells, *Xenopus* appears to achieve its objectives mostly by using the normal mechanisms of cell renewal. In this regard, *Xenopus* is much closer to the situation in mammals, and because of this it may be that it will not only be a useful model for solving some interesting biological

problems, but also for more practical development of the technology of regenerative medicine.

Acknowledgements. Funding for work in the author's laboratory was supplied by the Wellcome Trust.

1 Filoni, S. and Bosco, L. (1981) Comparative analysis of the regenerative capacity of caudal spinal cord in larvae of serveral Anuran amphibian species. Acta Embryol. Morphol. Exp. 2, 199–226.
2 Dent, J. N. (1962) Limb regeneration in larvae and metamorphosing individuals of the South African clawed toad. J. Morphol. 110, 61–78.
3 Scadding, S. R. (1977) Phylogenic distribution of limb regeneration capacity in adult Amphibia. J. Exp. Zool. 202, 57–67.
4 Casci, T., Vinos, J. and Freeman, M. (1999) Sprouty, an intracellular inhibitor of Ras signaling. Cell 96, 655–665.
5 Shi, Y. B. and Ishizuya-Oka, A. (1996) Biphasic intestinal development in amphibians: embryogenesis and remodeling during metamorphosis. Curr. Top. Dev. Biol. 32, 205–235.
6 Sive, H. L., Grainger, R. M. and Harland, R. M. (2000) Early Development of *Xenopus laevis:* A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
7 Kroll, K. L. and Amaya, E. (1996) Transgenic Xenopus embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. Development 122, 3173–3183.
8 Pownall, M. E., Isaacs, H. V. and Slack, J. M. W. (1998) Two phases of Hox gene regulation during early Xenopus development. Curr. Biol. 8, 673–676.
9 Beck, C. and Slack, J. M. W. (1999) Gut specific expression using mammalian promoters in transgenic *Xenopus laevis.* Mech. Dev. 88, 221–227.
10 Beck, C. W., Christen, B., Barker, D. and Slack, J. M. W. (2006) Temporal requirement for bone morphogenetic proteins in regeneration of the tail and limb of Xenopus tadpoles. Mech. Dev. 123, 674–688.
11 Amaya, E., Offield, M. F. and Grainger, R. M. (1998) Frog genetics: *Xenopus tropicalis* jumps into the future. Trends Genet. 14, 253–254.
12 Carruthers, S. and Stemple, D. L. (2006) Genetic and genomic prospects for *Xenopus tropicalis* research. Sem. Cell Dev. Biol. 17, 146–153.
13 Lagna, G. and Hemmati-Brivanlou, A. (1998) Use of dominant negative constructs to modulate gene expression. Curr. Top. Dev. Biol. 36, 75–98.
14 Li, M. and Rohrer, B. (2006) Gene silencing in *Xenopus laevis* by DNA vector-based RNA interference and transgenesis. Cell Res. 16, 99–105.
15 Kolm, P. J. and Sive, H. L. (1995) Efficient hormone-inducible protein function in *Xenopus laevis.* Dev. Biol. 171, 267–272.
16 Wheeler, G. N., Hamilton, F. S. and Hoppler, S. (2000) Inducible gene expression in transgenic Xenopus embryos. Curr. Biol. 10, 849–852.
17 Brand, A. H. and Perrimon, N. (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118, 401–415.
18 Hartley, K. O., Nutt, S. L. and Amaya, E. (2002) Targeted gene expression in transgenic Xenopus using the binary Gal4-UAS system. Proc. Natl. Acad. Sci. USA 99, 1377–1382.
19 Ryffel, G. U., Werdien, D., Turan, G., Gerhards, A., Goosses, S. and Senkel, S. (2003) Tagging muscle cell lineages in development and tail regeneration using Cre recombinase in transgenic Xenopus. Nucl. Acids Res. 31, 1044–1051.
20 Gargioli, C. and Slack, J. M. W. (2004) Cell lineage tracing during Xenopus tail regeneration. Development 131, 2669–2679.
21 Sobkow, L., Epperlein, H. H., Herklotz, S., Straube, W. L. and Tanaka, E. M. (2006) A germline GFP transgenic axolotl and its use to track cell fate. Dual origin of the fin mesenchyme during development and the fate of blood cells during regeneration. Dev. Biol. 290, 386–397.
22 Catala, M., Teillet, M. A. and Le Douarin, N. M. (1995) Organization and development of the tail bud analyzed with the quail-chick chimaera system. Mech. Dev. 51, 51–65.
23 Goldman, D. C., Martin, G. R. and Tam, P. P. L. (2000) Fate and function of the ventral ectodermal ridge during mouse tail development. Development 127, 2113–2123.
24 Kanki, J. P. and Ho, R. K. (1997) The development of the posterior body in zebrafish. Development 124, 881–893.
25 Cambray, N. and Wilson, V. (2002) Axial progenitors with extensive potency are localised to the mouse chordoneural hinge. Development 129, 4855–4866.
26 Tucker, A. S. and Slack, J. M. W. (1995) The *Xenopus laevis* tail-forming region Development 121, 249–262.
27 Tucker, A. S. and Slack, J. M. W. (1995) Tail bud determination in the vertebrate embryo. Curr. Biol. 5, 807–813.
28 Beck, C. W. and Slack, J. M. W. (1998) Analysis of the developing Xenopus tail bud reveals separate phases of gene expression during determination and outgrowth. Mech. Dev. 72, 41–52.
29 Beck, C. and Slack, J. M. W. (1999) A developmental pathway controlling outgrowth of the Xenopus tail bud. Development 126, 1611–1620.
30 Beck, C. W., Whitman, M. and Slack, J. M. W. (2001) The role of BMP signaling in outgrowth and patterning of the Xenopus tail bud. Dev. Biol. 238, 303–314.
31 Beck, C. W. and Slack, J. M. W. (2002) Notch is required for outgrowth of the Xenopus tail bud. Int. J. Dev. Biol. 46, 255–258.
32 Woodland, H. R. and Jones, E. A. (1988) Mesoderm induction in the future tail region of Xenopus. Rouxs' Arch. Dev. Biol. 197, 441–446.
33 Kelly, O. G. and Melton, D. A. (1995) Induction and patterning of the vertebrate nervous system. Trends Genet. 11, 273–278.
34 Tucker, A. S. and Slack, J. M. W. (2004) Independent induction and formation of the dorsal and ventral fins in *Xenopus laevis.* Dev. Dyn. 230, 461–7.
35 Gont, L. K., Steinbeisser, H., Blumberg, B. and de Robertis, E. M. (1993) Tail formation as a continuation of gastrulation: the multiple cell populations of the Xenopus tailbud derive from the late blastopore lip. Development 119, 991–1004.
36 Gawantka, V., Pollet, N., Delius, H., Vingron, M., Pfister, R., Nitsch, R., Blumenstock, C. and Niehrs, C. (1998) Gene expression screening in Xenopus identifies molecular pathways, predicts gene function and provides a global view of embryonic patterning. Mech. Dev. 77, 95–141.
37 Smit, A. L. (1952) The ontogenesis of the vertebral column of Xenopus laevis (Daudin) with special reference to the segmentation of the metotic region of the skull. Annals University Stellenbosch 29, 79–136.
38 Morrison, G. M. and Brickman, J. M. (2006) Conserved roles for Oct4 homologues in maintaining multipotency during early vertebrate development. Development 133, 2011–2022.
39 Davis, R. L. and Kirschner, M. W. (2000) The fate of cells in the tailbud of *Xenopus laevis.* Development 127, 255–267.
40 Lo, D. C., Allen, F. and Brockes, J. P. (1993) Reversal of muscle differentiation during urodele limb regeneration. Proc. Natl. Acad. Sci. USA 90, 7230–7234.
41 Kumar, A., Velloso, C. P., Imokawa, Y. and Brockes, J. P. (2000) Plasticity of retrovirus-labelled myotubes in the newt limb regeneration blastema. Dev. Biol. 218, 125–136.
42 Echeverri, K., Clarke, J. D. W. and Tanaka, E. M. (2001) In vivo imaging indicates muscle fiber dedifferentiation is a major contributor to the regenerating tail blastema. Dev. Biol. 236, 151–164.
43 Echeverri, K. and Tanaka, E. M. (2002) Ectoderm to mesoderm lineage switching during axolotl tail regeneration. Science 298, 1993–1996.

44 Stefanelli, A. (1951) I fenomeni rigenerativi e degenerativi del midolo spinale caudale degli anfibi a dei rettili. Boll. Zool. 18, 279–290.
45 Lin, G., Chen, Y. and Slack, J. M. W. (2007) Regeneration of melanophores and other neural crest derivatives in the Xenopus tadpole tail. BMC Dev. Biol. 7, 56.
46 Nishikawa, K. and Wassersug, R. (1989) Evolution of spinal nerve number in anuran larvae. Brain Behav. Evol. 33, 15–24.
47 Chen, Y., Lin, G. F. and Slack, J. M. W. (2006) Control of muscle regeneration in the Xenopus tadpole tail by Pax7. Development 133, 2303–2313.
48 Seale, P. and Rudnicki, M. A. (2000) A new look at the origin, function, and 'stem-cell' status of muscle satellite cells. Dev. Biol. 218, 115–124.
49 Seale, P., Sabourin, L. A., Girgis-Gabardo, A., Mansouri, A., Gruss, P. and Rudnicki, M. A. (2000) Pax7 is required for the specification of myogenic satellite cells. Cell 102, 777–786.
50 Gros, J., Manceau, M., Thome, V. and Marcelle, C. (2005) A common somitic origin for embryonic muscle progenitors and satellite cells. Nature 435, 954–958.
51 Relaix, F., Rocancourt, D., Mansouri, A. and Buckingham, M. (2005) A Pax3/Pax7-dependent population of skeletal muscle progenitor cells. Nature 435, 948–953.
52 Morrison, J. I., Loof, S., He, P. and Simon, A. (2006). Salamander limb regeneration involves the activation of a multipotent skeletal muscle satellite cell population. J. Cell. Biol. 172, 433–440.
53 Tazaki, A., Kitayama, A., Terasaka, C., Watanabe, K., Ueno, N. and Mochii, M. (2005) Macroarray-based analysis of tail regeneration in *Xenopus laevis* larvae. Dev. Dyn. 233, 1394–1404.
54 Beck, C. W., Christen, B. and Slack, J. M. W. (2003) Molecular pathways needed for regeneration of spinal cord and muscle in a vertebrate. Dev. Cell 5, 429–439.
55 Hsu, D. R., Economides, A. N., Wang, X. R., Eimon, P. M. and Harland, R. M. (1998) Mol. Cell 1, 673–683.
56 Suzuki, A., Thies, R. S., Yamaji, N., Song, J. J., Wozney, J. M., Murakami, K. and Ueno, N. (1994) Proc. Natl. Acad. Sci. USA 91, 10255–10259.
57 Smith, W. C. and Harland, R. M. (1992) Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in Xenopus embryos. Cell 70, 829–840.
58 Coffman, C. R., Skoglund, P., Harris, W. A. and Kintner, C. R. (1993) Expression of an extracellular deletion of Xotch diverts cell fate in Xenopus embryos. Cell 73, 659–671.
59 Tseng, A. S., Adams, D. S., Qiu, D. Y., Koustublian, P. and Levin, M. (2007) Apoptosis is required during early stages of tail regeneration in *Xenopus laevis*. Dev. Biol. 301, 62–69.
60 Adams, D. S., Masi, A. and Levin, M. (2007) H+ pump-dependent changes in membrane voltage are an early mechanism necessary and sufficient to induce Xenopus tail regeneration. Development 134, 1323–1335.
61 Whitehead, G. G., Makino, S., Lien, C. L. and Keating, M. T. (2005) Fgf20 is essential for initiating zebrafish fin regeneration. Science 310, 1957–1960.
62 Stoick-Cooper, C. L., Weidinger, G., Riehle, K. J., Hubbert, C., Major, M. B., Fausto, N. and Moon, R. T. (2007) Distinct Wnt signaling pathways have opposing roles in appendage regeneration Development 134, 479–489.
63 Simpson, S. B. (1970) Studies on regeneration of the lizard's tail. Am. Zool. 10, 157–165.
64 Iten, L. E. and Bryant, S. V. (1976) Stages of tail regeneration in the adult newt, *Notophthalmus viridescens*. J. Exp. Zool. 196, 283–292.
65 Tschumi, P. (1957) The growth of the hindlimb bud of Xenopus laevis and its dependence upon the epidermis. J. Anat. 91, 149–173.
66 Endo, T., Yokoyama, H., Tamura, K. and Ide, H. (1997) Shh expression in developing and regenerating limb buds of Xenopus laevi. Dev. Dyn. 209, 227–232.
67 Matsuda, H., Yokoyama, H., Endo, T., Tamura, K. and Ide, H. (2001) An epidermal signal regulates Lmx-1 expression and dorsal-ventral pattern during Xenopus limb regeneration. Dev. Biol. 229, 351–362.
68 Christen, B. and Slack, J. M. W. (1997) FGF-8 is associated with anteroposterior patterning and limb regeneration in Xenopus. Dev. Biol. 192, 455–466.
69 Christen, B. and Slack, J. M. W. (1998) All limbs are not the same. Nature 395, 230–231.
70 King, M. W., Nguyen T., Calley J., Harty M. W. , Muzinich M. C., Mescher A. L., Chalfant C., N'Cho M., McLeaster K., McEntire J. et al. (2003) Identification of genes expressed during *Xenopus laevis* limb regeneration by using subtractive hybridization. Dev. Dyn. 226, 398–409.
71 Grow, M., Neff, A. W., Mescher, A. L. and King, M. W. (2006) Global analysis of gene expression in Xenopus hindlimbs during stage-dependent complete and incomplete regeneration. Dev. Dyn. 235, 2667–2685.
72 Summerbell, D., Lewis, J. H. and Wolpert, L. (1973) Positional information in chick limb morphogenesis. Nature 244, 492–496.
73 Goss, R. J. and Holt, R. (1992) Epimorphic vs. tissue regeneration in Xenopus forelimbs. J. Exp. Zool. 261, 451–457.
74 Endo, T., Tamura, K. and Ide, H. (2000) Analysis of gene expressions during Xenopus forelimb regeneration. Dev. Biol. 220, 296–306.
75 Nye, H. L. D., Cameron, J. A., Chernoff, E. A. G. and Stocum, D. L. (2003) Regeneration of the urodele limb: a review. Dev. Dyn. 226, 280–294.
76 Wolfe, A. D., Nye, H. L. D. and Cameron, J. A. (2000) Extent of ossification at the amputation plane is correlated with the decline of blastema formation and regeneration in *Xenopus laevis* hindlimb. Dev. Dyn. 218, 681–697.
77 Nye, H. L. D. and Cameron, J. A. (2005) Strategies to reduce variation in Xenopus regeneration studies. Dev. Dyn. 234, 151–158.
78 Yokoyama, H., Tamura, S. Y., Endo, J. C., Izpisua-Belmonte, J. C., Tamura, K. and Ide, H. (2000) Mesenchyme with fgf-10 expression is responsible for regenerative capacity in Xenopus limb buds. Dev. Biol. 219, 18–29.
79 Yokoyama, H., Ide, H. and Tamura, K. (2001) FGF-10 stimulates limb regeneration ability in *Xenopus laevis*. Dev. Biol. 233, 72–79.
80 Slack, J. M. W., Beck, C. W., Gargioli, C. and Christen, B. (2004) Cellular and molecular mechanisms of regeneration in Xenopus. Philos. Trans. R. Soc. Lond. B Biol. Sci. 359, 745–751.
81 Kawakami, Y., Rodriguez Esteban, C. R., Raya, M., Kawakami, H., Marti, M., Dubova, I. and Belmonte, J. C. I. (2006) Wnt/beta-catenin signaling regulates vertebrate limb regeneration. Genes Dev. 20, 3232–3237.
82 Wallace, H. (1972) The components of regrowing nerves which support the regeneration of irradiated salamander limbs. J. Embryol. Exp. Morph. 28, 419–435.
83 Brockes, J. P. and Kintner, C. R. (1986) Glial growth factor and nerve-dependent proliferation in the regeneration blastema of Urodele amphibians. Cell 45, 301–306.
84 Filoni, S. and Paglialunga, L. (1990) Effect of denervation on hindlimb regeneration in *Xenopus laevis* larvae. Differentiation 43, 10–19.
85 Cannata, S. M., Bagni, C., Bernardini, S., Christen, B. and Filoni, S. (2001) Nerve-independence of limb regeneration in larval Xenopus laevis is correlated to the level of fgf-2 mRNA expression in limb tissues. Dev. Biol. 231, 436–446.
86 Suzuki, M., Satoh, A., Ide, H. and Tamura, K. (2005) Nerve-dependent and -independent events in blastema formation during Xenopus froglet limb regeneration. Dev. Biol. 286, 361–375.
87 Tsonis, P. A., Madhavan, M., Tancous, E. E. and Del Rio-Tsonis, K. (2004) A newt's eye view of lens regeneration. Int. J. Dev. Biol. 48, 975–980.
88 Henry, J. J. and Elkins, M. B. (2001) Cornea-lens transdifferentiation in the anuran, *Xenopus tropicalis*. Dev. Genes Evol. 211, 377–387.