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Cellular and molecular mechanisms of regeneration in *Xenopus*

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We have employed transgenic methods combined with embryonic grafting to analyse the mechanisms of regeneration in *Xenopus* tadpoles. The *Xenopus* tadpole tail contains a spinal cord, notochord and segmented muscles, and all tissues are replaced when the tail regenerates after amputation. We show that there is a refractory period of very low regenerative ability in the early tadpole stage. Tracing of cell lineage with the use of single tissue transgenic grafts labelled with green fluorescent protein (GFP) shows that there is no de-differentiation and no metaplasia during regeneration. The spinal cord, notochord and muscle all regenerate from the corresponding tissue in the stump; in the case of the muscle the satellite cells provide the material for regeneration. By using constitutive or dominant negative gene products, induced under the control of a heat shock promoter, we show that the bone morphogenetic protein (BMP) and Notch signalling pathways are both essential for regeneration. BMP is upstream of Notch and has an independent effect on regeneration of muscle. The *Xenopus* limb bud will regenerate completely at the early stages but regenerative ability falls during digit differentiation. We have developed a procedure for making tadpoles in which one hindlimb is transgenic and the remainder wild-type. This has been used to introduce various gene products expected to prolong the period of regenerative capacity, but none has so far been successful.

Keywords: *Xenopus*; regeneration; limb; spinal cord; bone morphogenetic protein; Notch

1. INTRODUCTION

One of the problems of regeneration research is that many of the most interesting phenomena are found in organisms that are not traditional 'laboratory models' for experimental work. This has been a handicap to investigation of regeneration at the molecular level although it is now being overcome by the development of new techniques (see Slack 2003). A model organism that does show significant regenerative capacity is the tadpole of the frog *Xenopus*. Much of the molecular biology of early *Xenopus* development was determined using a single technique for gene overexpression, the injection of synthetic mRNA into fertilized eggs. As the mRNA will decay after 1–2 days this method cannot be used to investigate late events, including those of tadpole regeneration and metamorphosis. The ability to investigate late events has depended on the transgenic technique introduced by Kroll & Amaya (1996), which enables the introduction of genes into every cell in the tadpole. It is possible to generate lines of transgenic frogs, although because of the long generation time of *Xenopus laevis*, most experiments are performed on founder tadpoles. We have used three methods in combination with the basic transgenesis protocol. First, we always ensure that the transgenics are identifiable with a marker such as GFP. To achieve this, we often use double cassettes in which the active transgene is controlled by one promoter and the marker transgene by another, often the *γ-crystallin* promoter, which directs expression to the lens of the eye (Offield *et al.* 2000). Second, we have made extensive use of a heat shock promoter to induce transgene expression during the experiment (Wheeler *et al.* 2000). This enables the tadpoles to undergo normal embryonic development, which might otherwise be compromised by transgene expression at earlier stages. Third, as well as tissue-specific promoters to control the spatial extent of transgene expression, we have also used grafts from transgenic embryos to non-transgenic hosts. This combination of techniques has provided a flexible repertoire of techniques for research, and recent results represent a significant advance in our understanding of the mechanisms of regeneration.

We have focused on the regeneration of the tail and the limb, and this paper will briefly review our main conclusions. In general, we find that *Xenopus* regeneration differs significantly from that of the urodele amphibians, and tends more to resemble the tissue renewal mechanisms found in mammals.

2. TAIL

(a) *Tail development*

Tail development appears to be similar in all vertebrates (Catala *et al.* 1995; Kanki & Ho 1997; Goldman *et al.* 2000; Cambray & Wilson 2002) and, at present, we know more about the mechanisms in *Xenopus* than in any other vertebrate. The *Xenopus* tadpole tail arises from the posterior part of the neurula. The anterior half of the tail is actually trunk tissue laid out during gastrulation, which

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becomes displaced into a post-anal position by a later anterior migration of the endoderm (Tucker & Slack 1995*b*). The posterior part of the tail forms from the tail bud of the eponymous embryonic stage. We have shown that this tail bud will not arise 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 various types of tissue explant (Tucker & Slack 1995*a*). We believe that this tissue interaction eventually results in the activation of the BMP and the Notch signalling pathways (Beck & Slack 1999, 2002; Beck *et al.* 2001), 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 becomes extended into the dorsal and ventral fins (Tucker & Slack 2004). After amputation, the tail will regenerate fully within 10–20 days, depending on stage. All the main tissue types are restored, although the spinal ganglia may be defective. The limited amount of experimental work in the past has focused on the process of spinal cord regeneration (e.g. Filoni & Bosco 1981) but the tail does illustrate genuine regeneration of a complex multi-tissue pattern. Despite the different origin of the anterior and posterior halves of the tail (trunk versus tail bud) we are not aware of any difference in regenerative capacity between them. It should be noted that the anuran tadpole tail does not contain vertebrae, as these are found only in the definitive trunk region that will be retained as the post-metamorphic frog (Smit 1952).

It was formerly considered that the tail could regenerate at all stages, but we have recently found that this is not the case. From stage 49 (*ca*. 7 days) virtually 100% of tadpoles will regenerate, but at earlier stages the proportion is lower and in particular there is a refractory period in stage range 46/47 during which almost all tadpoles heal over the wound and fail to regenerate. If such tadpoles are kept to a later stage and then re-amputated, the tail does regenerate, but if they are simply kept they will persist with half a tail until metamorphosis. The causes of the refractory period are not fully understood but it is experimentally very useful as it provides us with an assay for gene products that will promote regeneration, and well as those that will inhibit regeneration at the later stages.

(b) *Cell lineage*

Studies on urodele regeneration have revealed considerable evidence for de-differentiation of mature cell types including neural ependymal cells and multinucleated muscle fibres (Lo *et al.* 1993; Kumar *et al.* 2000; Echeverri *et al.* 2001; Echeverri & Tanaka 2002). Not only is there de-differentiation, but there is also some metaplasia of the resulting blastemal cells to become cells of different histological types in the regenerate. We were interested to find out if either or both of these processes (de-differentiation and metaplasia) occurred in the anuran tadpole tail.

An initial morphological study showed that the regenerating bud does not have the appearance of a uniform undifferentiated blastema (figure 1). Rather, the termini of the spinal cord and notochord remain structured. The spinal cord forms a terminal bulb or 'ampulla', formerly described by Stefanelli (1951), and the notochord forms a bullet-shaped mass of cells continuous with the sheath

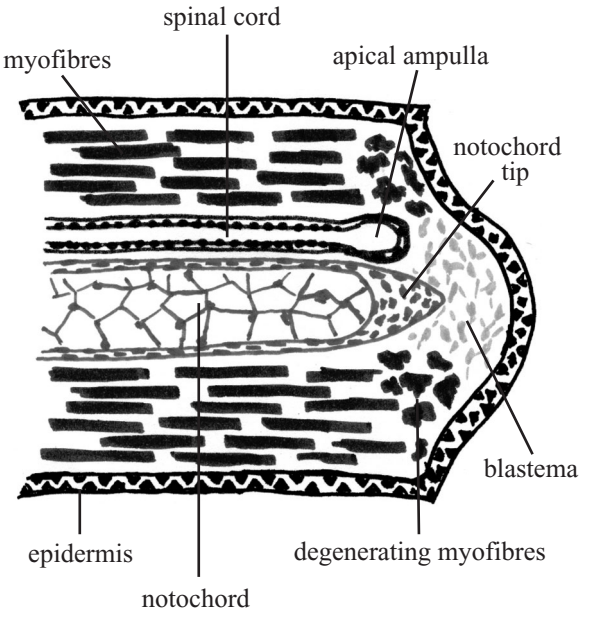

Figure 1. Schematic diagram of *Xenopus* tail regeneration bud at *ca*. 3 days after amputation.

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'. This does contain some mononuclear cells positive for the muscle-specific antigen 12/101, although as will be described below we consider these to be differentiating myocytes rather than products of de-differentiation of multinucleate fibres.

Morphological study can be informative about cell lineage but it cannot really prove any particular mechanism. For this reason we have used the transgenic technology to label specific tissues within the tail to discover their fate during regeneration (Gargioli & Slack 2004). Most experiments have involved grafting tissues from transgenic neurulae that are constitutively expressing GFP. It is possible at this stage to make very clean tissue separations and thus to graft explants of either neural plate, notochord or presomite mesoderm into the corresponding position of an unlabelled embryo. Provided the grafts are made to a sufficiently posterior position the host embryo will develop into a tadpole with one tissue type in the tail labelled. We have used the CMV promoter, which remains active in all the tissue types of the tail. Thus, a cell should still continue to express GFP if it de-differentiates, and if it re-differentiates to the same or to a different tissue type.

For the spinal cord and notochord the results are very simple (figure 2*a*,*b*). The regenerated spinal cord and notochord grow from the corresponding tissues in the stump, and there is no gain of cells from other tissues, or export of cells to other tissues, judging from the conservation of the labelling. These data are consistent with the morphological picture, suggesting that the spinal cord and notochord each regenerate as self-contained compartments.

The situation for the muscle is more complex. Tadpoles that are transgenic for GFP driven by a muscle-specific

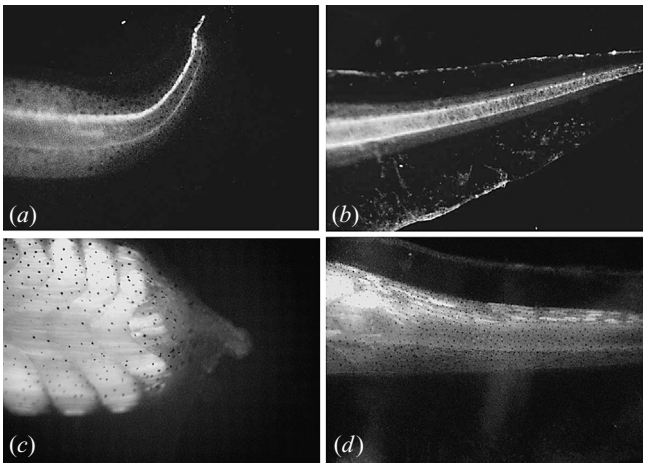

Figure 2. Evidence for the absence of de-differentiation and metaplasia in *Xenopus* regeneration. (*a*) Ten-day regenerate including *CMV–GFP* labelled spinal cord. (*b*) Twenty-day regenerate including *CMV–GFP* labelled notochord. (*c*) Three-day regenerate of transgenic for *cardiac actin–GFP*. Despite abundant GFP expression in the myofibres of the stump there is no GFP in mononuclear cells of the blastema. (*d*) Thirty-day regenerate including *CMV–GFP* labelled presomite mesoderm from late neurula.

promoter (the *cardiac actin* promoter in *Xenopus* drives expression in cardiac and striated muscle; Mohun *et al.* (1989)) show no mononuclear cells labelled with GFP in the blastema (figure 2*c*). Although the *cardiac actin* promoter would be expected to be turned off in the event of de-differentiation, we should expect the GFP protein product to persist for a couple of days, so the complete absence of green mononuclear cells argues against de-differentiation of myofibres. 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 in the tail does not mean that myofibres will necessarily be labelled in the regenerate from that tail. In fact, myofibres in the regenerate are only labelled in large numbers if the grafts are taken from late stage neurulae (figure 2*d*), or in small numbers if they are taken from the lateral region of early neurulae. This suggests that the precursor cells for the regenerated muscle are not the myofibres themselves, but are some other class of cell that arise from the lateral part of the presomite plate, and move to a medial position in the late neurula because of the overall dorsal convergence of the mesoderm (Pourquié 2001).

We believe that the precursor cell type is the muscle satellite cell. 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 (Seale & Rudnicki 2000). 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 (Seale *et al.* 2000). We have examined how many satellite cells are labelled with GFP in the various types of graft of presomite mesoderm, and we find that there is a good correlation between the number of satellite cells labelled and the number of myofibres labelled when the tails are amputated and allowed to regenerate.

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, 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 the three major tissue types. Although there has been persistent speculation about the existence of pluripotential cells that give rise to more than one of the tissues in development, the actual evidence is quite weak for the presence of individual cells in the tail bud that can populate more than one tissue type (Griffith *et al.* 1992; Davis & Kirschner 2000; Cambray & Wilson 2002). Therefore, the compartmentation of tissue type seen in regeneration follows on from a similar compartmentation during development.

(c) *Molecular pathways in regeneration*

We had previously established that the BMP and Notch signalling pathways are critical for tail bud outgrowth during embryonic development (Beck & Slack 1998, 1999, 2002; Beck *et al.* 2001). 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, that BMP signalling was upstream of Notch, and that BMP signalling also, independently, could provoke the formation of tail muscle.

We have now investigated the role of these pathways in regeneration and find that it is remarkably similar to the situation in development (Beck *et al.* 2003). We were assisted by being able to use the early refractory phase of the *Xenopus* tadpole as an assay for gene products promoting regeneration, as well as the normal later tail as an assay for gene products inhibiting regeneration. Because both the BMP and Notch pathways have numerous functions during development, it was essential to use the heat shock promoter for this work so that transgene activity could be induced just over the period of the experiment. This involves exposure to 34 °C for 30 min each day during the experiment.

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 (Hsu *et al.* 1998). To inhibit BMP signalling, we used either tBR, a truncated BMP receptor lacking its cytoplasmic domain, which behaves as a dominant negative (Suzuki *et al.* 1994), or noggin, an extracellular inhibitor of BMP (Smith & Harland 1992). 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. To stimulate this pathway we have used the isolated NICD (Coffman *et al.* 1993).

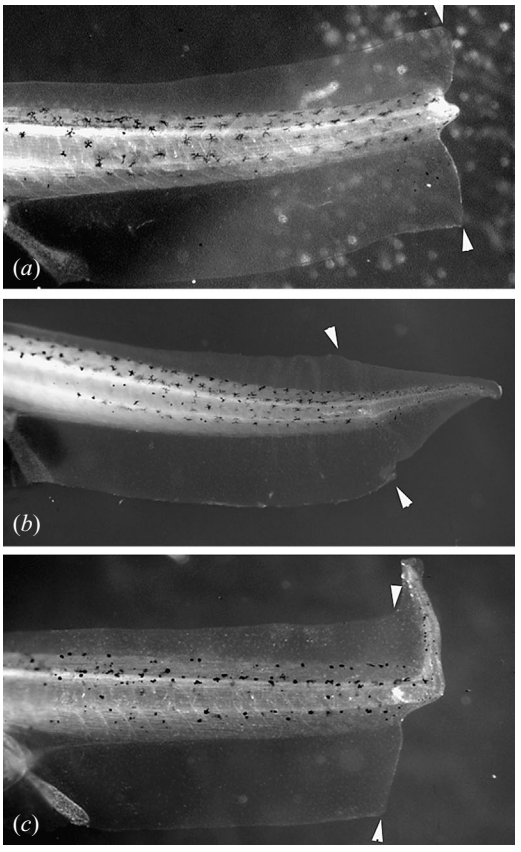

Figure 3. Stimulation of tail regeneration during the refractory period. White arrowheads show amputation levels. (*a*) Normal tadpole fails to regenerate. (*b*) *Alk3* (constitutive BMP receptor) stimulates complete regeneration. (*c*) NICD (constitutive form of Notch) stimulates regeneration of spinal cord and notochord.

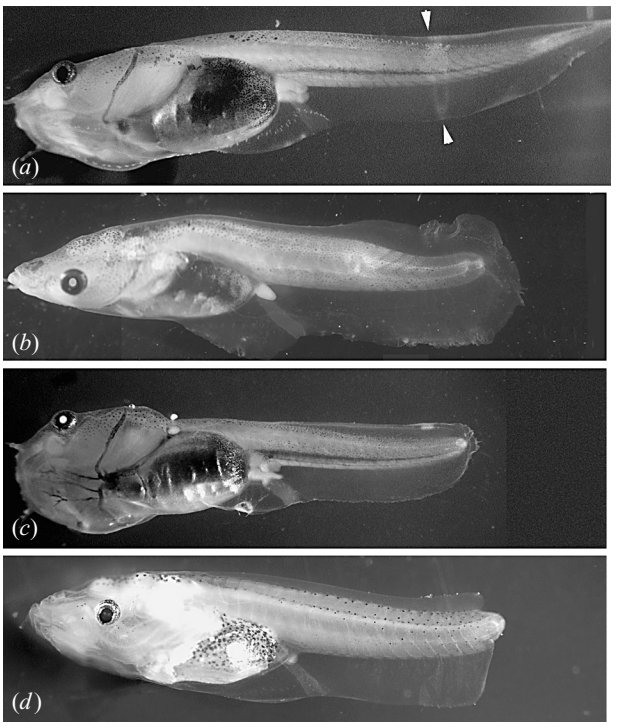

Figure 4. Inhibition of tail regeneration. (*a*) Normal tadpole regenerates completely (white arrowheads show amputation level). (*b*) *tBR* (dominant negative BMP receptor) inhibits regeneration. (*c*) *noggin* (extracellular BMP inhibitor) inhibits regeneration. (*d*) MG132 (inhibits proteolytic cleavage of Notch) inhibits regeneration.

When either of the activators, Alk3 or NICD, is induced shortly before amputation of a tail during the refractory phase, tail regeneration is stimulated (figure 3). 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 (figure 4). We have not succeeded in producing effective inhibition of Notch signalling with any gene product, but have previously shown that inhibitors of the calpain-like protease that performs the intramembranous cleavage of Notch will inhibit tail outgrowth (Beck & Slack 2002). In this work we have used the inhibitor MG132, which inhibits tail regeneration effectively at 10 μM.

Because both pathways seem to activate regeneration during the refractory period, and both seem to be necessary for regeneration later on, 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. As we now know that muscle regeneration depends on the activation of satellite cells we are in a position to investigate this further. We shall also be examining the effects of the two pathways on the spinal cord and the notochord separately, and trying to identify the initial genetic targets involved in regenerative behaviour.

3. LIMB

In contrast to the situation with the tail, where *Xenopus* is the leading model organism, relatively little experimental work has been done on limb development (Tschumi 1957). This means that we must be extrapolate from chick and mouse to infer the developmental mechanisms. Although gene expression patterns in *Xenopus* limb buds are generally similar (e.g. expression of *FGF8* in the apical epidermis, *Shh* in the posterior mesoderm, and *Lmx1* in the dorsal mesenchyme; Christen & Slack (1997); Endo *et al.* (1997); Matsuda *et al.* (2001)), there are also some differences that may be significant (Christen & Slack 1998).

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 mosaic behaviour of the chick limb bud was long held to represent evidence for the progress zone model (Summerbell *et al.* 1973). However, like other anurans, and unlike urodele amphibians, *Xenopus* can produce only the occasional hypomorphic unbranched spike if amputated after metamorphosis (Goss & Holt 1992).

Table 1. Transgenic experiments, stage 56 tadpoles.

	stump	spike	1 toe	2 toes	3 toes	regeneration (%)	regeneration quality[a]
FGF-8	18	2	2	0	1	22	0.26
FGF-10	12	4	10	2	2	60	0.73
wt	10	4	4	1	0	47	0.42

[a] Regeneration quality: stump scores 0; spike, 0.5; 1 toe, 1; 2 toes, 2; 3 toes, 3. Quality = Σ score/number of experimental animals.

Table 2. FGF-10 bead experiments, stage 56 tadpoles.

	stump	spike	1 toe	2 toes	3 toes	regeneration (%)	regeneration quality[a]
PBS	15	3	1	1	2	32	0.48
1 mg ml^{-1} FGF-10	12	3	5	2	0	45	0.48
2 mg ml^{-1} FGF-10	3	2	1	0	0	50	0.33

[a] Regeneration quality: stump scores 0; spike, 0.5; 1 toe, 1; 2 toes, 2; 3 toes, 3. Quality = Σ score/number of experimental animals.

One hundred per cent of complete regenerates can only be formed up to stage 52, when the developing limb is a flattened paddle shape, and the ability to regenerate a complete limb declines progressively until stage 57 when only the occasional toe is formed (Dent 1962).

Although this ability is of some interest, it is not clear, *prima facie*, that it is really the same sort of phenomenon as regeneration of the urodele limb, which involves de-differentiation of mature cells to form a blastema (Nye *et al.* 2003). The histology of the regenerating stages shows that they contain a large proportion of undifferentiated cells and there is no obvious sign of de-differentiation of muscle or cartilage following amputation. Thus, is this perhaps really more akin to embryonic regulation than to regeneration of a mature body part? If so, could regenerative ability be prolonged by prolonging the period of apical signalling, or activating the target genes of such a signal?

We demonstrated some years ago that *fgf8* was re-expressed in the epidermis that grew across the cut surface (Christen & Slack 1997), and subsequent work by the laboratory of Ide has shown that the regenerative capacity is a property of the mesoderm and is correlated with expression of *fgf10* (Yokoyama *et al.* 2000). Moreover, in both *Xenopus* and urodeles there is some evidence for a neurotrophic signal stimulating regeneration and this has many properties of of the an FGF-like factor (Filoni & Paglialunga 1990; Cannata *et al.* 2001). Both of these lines of work suggested that FGFs or the mitogen-activated protein (MAP) kinase pathway might stimulate regeneration in this system. We were further encouraged by reports that regenerative ability could be prolonged in *Xenopus* by implantation of heparin beads soaked in FGF10 protein (Yokoyama *et al.* 2001).

(a) *Making genetically modified limbs*

We have used the transgenic method, combined with grafting, to prepare tadpoles in which one hindlimb is transgenic but the remainder of the organism is wild-type. This is a useful procedure for cases where the overexpression, or even the slight leakiness of an uninduced inducible gene, would disrupt normal development. This work depended on finding the prospective region for the hindlimb on the flank of the lozenge-stage embryo, which was done using orthotopic grafts from *CMV–GFP* labelled donors. We found that the hindlimb is formed from the mid-region of the body of the lozenge-stage embryo, with the epidermis being derived from a somewhat more anterior position than the mesoderm. Examples of such grafts are shown in figure 5*a–c*. The use of an inducible promoter is shown in figure 5*d*,*e*, in which a myc-tagged protein is detected in the transgenic limb after heat shock but not before.

The objective of this procedure was to overexpress specific genes that might activate signal transduction pathways or growth-controlling mechanisms that might prolong the period of regeneration. Various genes including *fgf8*, *fgf10*, *draf1* (constitutive form of *Raf*), *Shh*, *cyclinE3*, *msx1*, have been tested but none has produced a clear result (table 1; figure 5*f*). Most of the effort was directed towards experiments with the FGFs, because of the report that FGF10 beads would prolong the phase of regeneration. However, we could not reproduce this effect of FGF10 beads and we find only a marginal effect of overexpressed FGF10, which does not attain statistical significance (table 2). We consider that the reason for the discrepancy between our results and those of the Ide laboratory is that there is a certain variation in regenerative behaviour between batches of tadpoles. They used tadpoles at stage 56, but we find that some batches will lose most regenerative ability as early as stage 55 whereas others will give significant regeneration at stage 57 (which may be *ca.* 10 days later in age, depending on temperature). This means that small effects of experimental intervention are hard to detect reliably, whereas at the same time a limited comparison may yield a spurious difference.

4. CONCLUSIONS

We have shown that *Xenopus* is a very useful organism for regeneration research. The ability to combine transgenesis and grafting between embryos creates a

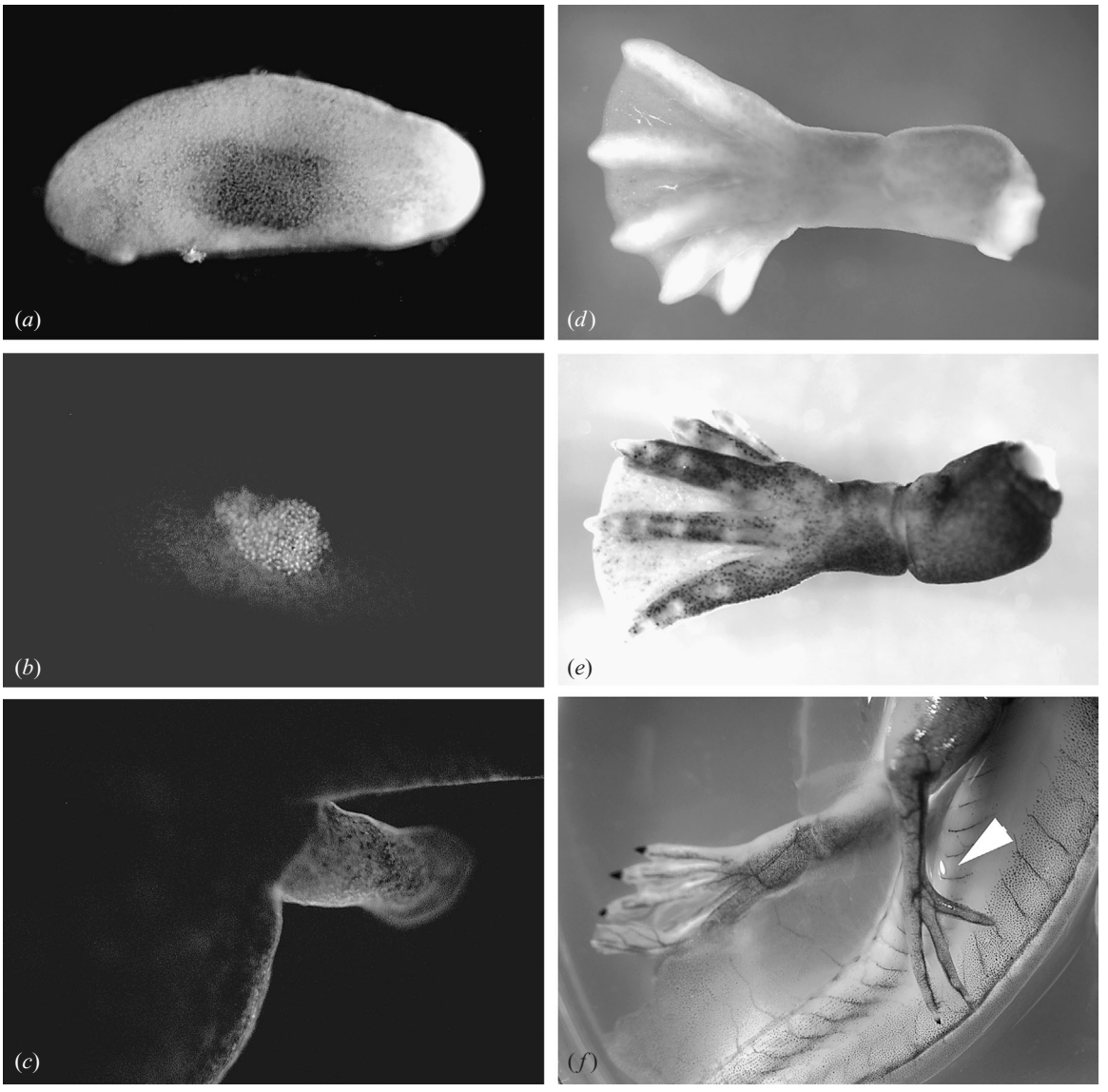

Figure 5. Experiments on *Xenopus* limb regeneration. (*a*) Late lozenge-stage embryo bearing a lateral graft of epidermis and mesoderm taken from a *CMV–GFP* transgenic embryo. (*b*) The same specimen viewed in fluorescence. This graft includes the prospective region of the hindlimb. (*c*) GFP-labelled limb bud from a similar specimen at tadpole stage 52. (*d*,*e*) Induction of expression of a myc-tagged protein by heat shock. The tadpole carries *Shh–myc* under the control of the *Xenopus hsp70* heat shock promoter, and the protein product is detected by whole-mount immunostaining for the myc sequence. (*d*) Unheated. (*e*) Heated. (*f*) A three-toed regenerate (white arrowhead) from a tadpole with a limb transgenic for *hsp70–FGF10*.

powerful technology with which to advance this work. Our results show that the situation in both the limb and the tail is very 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 by using the normal mechanisms of cell renewal. In this regard, *Xenopus* is much closer to the situation in mammals, and it may be that in future it will not only represent an opportunity to solve some interesting biological problems, but also be a useful model for more practical development of the new technology of regenerative medicine.

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GLOSSARY

BMP: bone morphogenetic protein
CMV: cytomegalovirus
GFP: green fluorescent protein
NICD: Notch intracellular domain