

Cell lineage tracing during *Xenopus* tail regeneration

Cesare Gargioli and Jonathan M. W. Slack *

Centre for Regenerative Medicine, Department of Biology and Biochemistry, University of Bath, Bath BA2 7AY, UK

*Author for correspondence (e-mail: j.m.w.slack@bath.ac.uk)

Accepted 3 March 2004

Development 131, 2669-2679
Published by The Company of Biologists 2004
doi:10.1242/dev.01155

Summary

The tail of the *Xenopus* tadpole will regenerate following amputation, and all three of the main axial structures – the spinal cord, the notochord and the segmented myotomes – are found in the regenerated tail. We have investigated the cellular origin of each of these three tissue types during regeneration.

We produced *Xenopus laevis* embryos transgenic for the CMV (Simian Cytomegalovirus) promoter driving GFP (Green Fluorescent Protein) ubiquitously throughout the embryo. Single tissues were then specifically labelled by making grafts at the neurula stage from transgenic donors to unlabelled hosts. When the hosts have developed to tadpoles, they carry a region of the appropriate tissue labelled with GFP. These tails were amputated through the labelled region and the distribution of labelled cells in the

regenerate was followed. We also labelled myofibres using the Cre-lox method.

The results show that the spinal cord and the notochord regenerate from the same tissue type in the stump, with no labelling of other tissues. In the case of the muscle, we show that the myofibres of the regenerate arise from satellite cells and not from the pre-existing myofibres. This shows that metaplasia between differentiated cell types does not occur, and that the process of *Xenopus* tail regeneration is more akin to tissue renewal in mammals than to urodele tail regeneration.

Key words: *Xenopus*, Tail, Regeneration, Metaplasia, Spinal cord, Notochord, Muscle, Satellite cells, Green fluorescent protein, Cre-Lox recombination

Introduction

The tail is a major region of the body of most vertebrates, constituting that part of the body posterior to the caudal opening of the digestive tract. It consists of a continuation of the axial structures of the body, with segmented muscle flanking the spinal cord and the vertebral column. Even humans exhibit a tail in embryonic life, vestiges of which may be observed on any human skeleton as the lowest three or four caudal (coccygeal) vertebrae. During embryonic stages, a notochord is present instead of the vertebral column. In the larvae of anuran amphibians, the tail is a transitional structure that becomes resorbed during metamorphosis mediated by thyroid hormones. The tail of *Xenopus laevis* tadpoles retains this core organization common to the other vertebrates: with a spinal cord and notochord flanked by paired myotomes, in continuation with the axial trunk.

Some capacity for regeneration of missing structures is found in several classes of vertebrates, especially the urodele amphibians, but complete tail regeneration can also occur in anuran tadpoles. Since the 18th century (Spallanzani, 1768) and more recently (Morgan and Davis, 1902; Locatelli, 1924; Stefanelli, 1947; Baita, 1951; Holtzer, 1956; Roguski, 1957; Niazi, 1966; Hauser, 1972), the tail regeneration of urodele and anuran amphibians has attracted interest, but the mechanism of new tissue formation from the stump is still not yet clearly understood. Elsewhere, we have addressed the molecular signalling processes involved in regeneration (Beck et al., 2003), and in this paper we address the issue of cell lineage. Does the regenerate originate from mature

differentiated cells or from undifferentiated 'reserve' cells? If it is the former, then does each tissue give rise to just its own cell type in the regenerate, or can it switch to different cell types (metaplasia)? These questions are still being answered. In the axolotl, a urodele amphibian, Echeverri and Tanaka (Echeverri and Tanaka, 2002) have demonstrated neural cell plasticity during tail regeneration. During regeneration, the ependymal or radial glial cells of the axolotl spinal cord are able to give rise to neurons, melanocytes, muscle and chondrocytes, cell types of both ectodermal and mesodermal origin. In previous experiments on limb regeneration in newts it has been shown that the multi-nucleate muscle fibres can de-differentiate. The nuclei re-enter S phase, and the multinucleate cells break up into mononuclear cells. These enter the undifferentiated blastema of the limb and some can later differentiate into other tissue types, such as cartilage (Lo et al., 1993; Kumar et al., 2000). These experiments clearly show that de-differentiation of mature differentiated cells, and metaplasia of one cell type to another, can take place during urodele regeneration. Recently the term 'transdifferentiation' has become popular as an alternative to 'metaplasia', particularly in the context of the ability of bone marrow stem cells to populate various other tissue types after grafting. In our own publications we have used the term 'transdifferentiation' in its original sense to refer to direct transformation of one differentiated cell type to another with or without cell division, and the term 'metaplasia' to refer to any conversion of one differentiated tissue type to another regardless of pathway or mechanism (Tosh and Slack, 2002).

Given the uncertainty about the nature of the intermediate cell states in amphibian regeneration, we shall refer to them here as examples of 'metaplasia'.

There is currently much interest in 'regenerative medicine' or stimulation of regeneration of damaged or defective human tissues (e.g. Cavazzana-Calvo et al., 2002). In this context it is important to establish whether metaplasia is a necessary feature of regeneration, or whether tissue-specific regeneration can also take place.

In the present work, we have developed a technique to label specifically the three principal tissues of the tail by orthotopically grafting into a non-transgenic host explants of the appropriate tissue from a transgenic embryo expressing GFP. The resulting tadpoles then have one tissue permanently labelled in a manner that is unaffected by subsequent changes in cell differentiation. After amputation of the tail and its subsequent regeneration, we are able to follow the fate of the labelled tissue cells into the regenerate. Using this method, we demonstrate that metaplasia does not occur during *Xenopus* tadpole tail regeneration. Each of the three main tissue types – the notochord, the spinal cord and the myofibres – behaves independently. The spinal cord regenerates via the formation of a distal bulb ('ampulla') as also described by Stefanelli (Stefanelli, 1951) in urodele spinal cord regeneration. The notochord regenerates directly by growth at the tip region. The muscle does not de-differentiate in the manner seen in urodeles. Instead, the multinucleate fibres in the vicinity of the amputation site degenerate completely and the regenerated muscle is derived from satellite cells that contribute to a blastema-like zone around the tips of the notochord and spinal cord.

Our results indicate that metaplasia is not a necessary component of appendage regeneration. Does this have any significance beyond the anuran tadpole? In fact, the mode of regeneration of each tissue resembles the normal mode of growth or cell turnover seen in mammals. During their evolution the mammals have lost almost all the regenerative capacity seen in urodeles and presumably possessed by urodele-like primitive vertebrates. Although many urodeles regenerate their tails throughout life, the anuran amphibians regenerate the tail only during the larval stages. This places the anurans in an intermediate position, and it may be that the regeneration that might be stimulated in mammals will be closer to the anuran than to the urodele type.

Materials and methods

Embryos and tadpoles

Xenopus laevis embryos and larvae were obtained by standard procedures and staged according to Nieuwkoop and Faber (NF) (Nieuwkoop and Faber, 1967). Embryos were dejellied with 2% cysteine (BDH) pH 7.9, and then cultured in NAM/10 with 5 mM HEPES (NAM, Normal Amphibian Medium: 110 mM NaCl, 2 mM KCl, 1 mM Ca(NO₃)₂·4H₂O, 1 mM MgSO₄·7H₂O, 0.1 mM Na₂EDTA) at 24°C, in Petri dishes coated with 1.5% noble agar. They were fed on nettle powder from 1 week and at about stage 48 transferred to a recirculating aquarium at 25°C. Conditions in this aquarium are such that the ammonia level is kept near zero and the pH is around 8.0. They are fed three times a week on tadpole diet (Blades Biological, Redbridge, UK).

Specimen analysis

Tadpoles were killed with an overdose of MS222 (Sigma) and fixed

overnight in Zamboni's fixative (40 mM Na₂HPO₄, 120 mM NaH₂PO₄, 2% PFA and 0.1% saturated picric acid), washed in 70% ethanol overnight, dehydrated and embedded in paraffin wax. A Leitz microtome was used to cut 6 µm serial sections. Then the sections were dewaxed, re-hydrated and immunostained, then counterstained with Haematoxylin and mounted in Aquatex (Merck).

Plasmid construction

The construction of *pcDNA3/CMV-nucGFP2* was performed by excising *nucGFP2* from *CS2/nucGFP2* (a kind gift from E. Amaya) with *Bam*HI and *Xba*I, and cloning into *Bam*HI/*Xba*I sites in *pcDNA3* (Invitrogen). It was linearized with *Sma*I and diluted to 500 ng/µl final concentration for addition to the sperm nuclei.

For the specific expression of the GFP in the muscles *pCarGFP* was used (a kind gift from E. Amaya). It was linearized with *Not*I and diluted to the final concentration of 500 ng/µl for the transgenesis injection.

For the Cre-Lox system two different constructs were used together: *pCarCre* (i.e. *Cre recombinase* driven by the *cardiac actin*, muscle-specific, promoter) and *pcDNA3/CMV-loxSTOPlox-GFP* (i.e. behaves like *CMV-GFP* following excision of the *STOP* sequence). *pCarCre* was made by excising *Cre+polyA addition sequence* from *pCS2Cre* (a kind gift from D. Werdien) with *Hind*III and *Not*I, and cloning into *pCar*, after removal of *GFP+polyA* with the same enzymes. *pCarCre* was linearized with *Not*I and diluted to a final concentration of 500 ng/µl. The construction of *pcDNA3/CMV-loxSTOPlox-GFP* was performed by substituting *nucGFP2* by *loxP-tpA-loxP-nucGFP* from *pCS2/CMV-lox-tpA-lox-nucGFP* (made by M. Horb). *pcDNA3/CMV-nucGFP2* was cut with *Xba*I and blunted with Klenow (Roche), then the *nucGFP2* was excised with *Hind*III. The *loxP-tpA-loxP-nucGFP* was removed with *Xba*I (blunted with Klenow) and *Hind*III. The *loxP-tpA-loxP* sequence came from *pGK/neo-tpA-lox2* (a kind gift from P. Soriano) and the *tpA* has numerous stop codons to prevent translation. *pcDNA3/CMV-loxSTOPlox-nucGFP* was linearized with *Sma*I and diluted to 500 ng/µl final concentration.

Transgenesis

In order to obtain *X. laevis* transgenic embryos, we followed the procedures previously described (Kroll and Amaya, 1996; Amaya and Kroll, 1999), except for the omission of restriction enzyme from the reaction and the fact that sperm nuclei were frozen in aliquots in sperm suspension buffer before use.

Graft technique

The operations were performed on neurula stage embryos (NF 13-17), in agar-coated dishes, in NAM/2 with 10 µg/ml trypsin (Sigma, type 9) to promote the separation of the three embryonic tissue layers. The embryos were manually demembrated with sharp forceps, then incised using a tungsten needle in the posterior region next to the blastopore, at the level of the presumptive tail-forming region (Tucker and Slack, 1995). The neural plate was lifted using a hair loop to free access to the underlying notochord and somites or, in the case of spinal cord labelling, to be transplanted itself. An explant of neural plate, notochord or pre-somite plate was replaced by a similar sized one from a *CMV-GFP* transgenic donor embryo. To immobilise the graft and promote healing the operated region was covered by a small glass bridge. The bridges are made from cover-slip fragments that are slightly bent over a microburner. The grafted embryos were allowed to heal under the bridges, in agar-coated dishes, for 30 minutes in NAM/2 + 20 µg/ml trypsin inhibitor (Sigma). Then they were moved to NAM/10 + 50 µg/ml gentamicin (Sigma, sulphate salt) for long-term culture.

BrdU injection

The tadpoles at stage 49 NF were anaesthetized in 1/3000 MS222, and injected with 2 µl of the thymidine analog 5-Bromo-2'-

deoxyuridine (BrdU): aqueous solution of (10:1) ratio from the Cell Proliferation Kit (Amersham). The injection was performed 24 hours before fixation.

Tail amputation

The tadpoles were anaesthetized in 1/3000 MS222, and were kept in the anaesthetic solution for the duration of the operation (about 5 minutes). The distal 50% of the tail was removed with a pair of iridectomy scissors (Vannas straight small, John Weiss). The tadpoles were allowed to heal in tap water with aeration (1 hour) and subsequently were returned to the aquarium.

Immunohistochemistry

Sections were prepared as described above. Where necessary, antigen unmasking was performed by boiling the slides in distilled water for 3 minutes at full power in a microwave oven. Anti-GFP polyclonal antibody from Molecular Probes was used at a dilution of 1:100 in 2% Boehringer Blocking Buffer. The secondary antibody (Sigma goat anti-rabbit IgG whole molecule peroxidase conjugate) was used at a dilution of 1:100. Myofibres were stained with 12/101 mAb (Kintner and Brockes, 1984) at 1:100 dilution. Satellite cells were stained with anti-Pax7 mAb (Developmental Studies Hybridoma Bank) at 1:500 dilution. For both of these the second antibody (Sigma rabbit anti-mouse IgG whole molecule peroxidase conjugate) was used at 1:100 in 2% Boehringer Blocking Buffer. All the detection reactions were carried out using an AEC (3-Amino-9-ethylcarbazole) staining kit (Sigma). BrdU incorporation was revealed with anti 5-bromo-2'-deoxyuridine mAb (Amersham 'Cell Proliferation Kit'), antibody provided with nuclease for DNA unmasking. The secondary antibody was Sigma rabbit anti-mouse IgG whole molecule alkaline phosphatase conjugate and the staining reaction was carried out using Fast Red tablets (Sigma).

For simultaneous detection of GFP and Pax7, a fluorescence method was used. The primary antibodies were as specified above. For secondary antibodies were used a H+L IgG fragment goat anti-rabbit FITC conjugated (Vecta Lab) for GFP detection and a H+L IgG fragment rabbit anti mouse Texas Red conjugate, Vecta Lab) for Pax7

detection. The double immunostaining was performed by making first the immunoreaction against Pax7 and then that against GFP. The procedure was followed as described above, the fluorescent secondary antibodies were incubated for 3 hours at room temperature, the slides were mounted in Aquatex (Merck) and analysed with a Leica stereomicroscope equipped with UV lamp and GFP and RFP filters. Images were taken with a colour CCD camera (diagnostic instrument RT spot camera) operating with Advanced Spot RT 3.0 software. Images were processed with Adobe Photoshop 7.0.

Results

Morphology of the regeneration bud

Several regenerates were sectioned in order to establish the overall morphology of the regeneration process. The cases shown in Fig. 1 are from 3-day-old regenerates, and are stained with haematoxylin and with 12/101 antibody to highlight muscle cells. After amputation, the epidermis rapidly forms a thin wound epithelium over the cut surface, which later becomes several cells thick. The spinal cord closes off and forms a terminal ampulla (Fig. 1A,B,E) (Stefanelli, 1951). The notochord terminates with a bullet-shaped mass of cells in continuity with the sheath of the more proximal tissue (Fig. 1A,C,F). Myofibres in the vicinity of the cut surface (1 mm) appear to degenerate, and large amounts of cellular debris and acellular protein masses are present over the first 3 days (Fig. 1G,H). Over the same period, a mass of undifferentiated cells accumulates in a ring around the neural ampulla and notochord tip, especially well visualised in Fig. 1F. We shall refer to this undifferentiated region as a 'blastema' and to the whole regenerating region as the 'regeneration bud'.

The morphological picture does not provide any evidence for the existence of de-differentiation. However, morphology can only be suggestive. It is not possible to arrive at firm

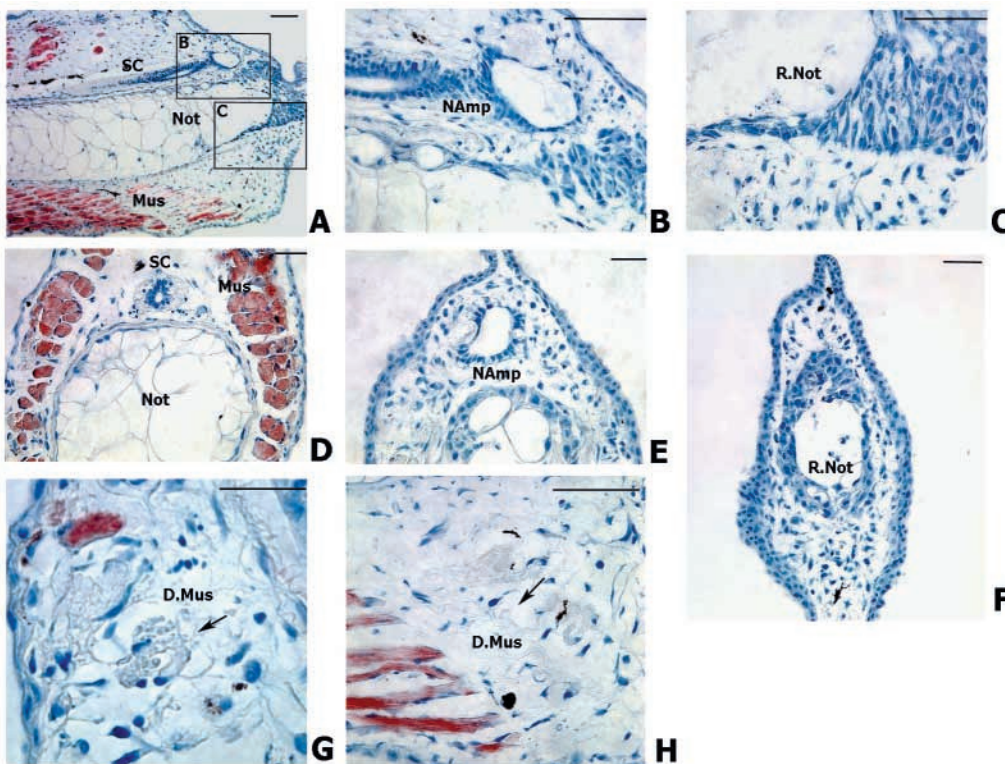


Fig. 1. Three-day regenerating tails in longitudinal (A-C,H) and transverse (D-G) sections immunostained with 12/101 mAb and counterstained with Haematoxylin. (A) Low-power magnification showing neural ampulla (N.Amp), regenerating notochord (R.Not) and blastema (i.e. visibly undifferentiated cells) formation close to the cut surface. (B) Enlarged view of boxed area in A, showing in detail the N.Amp. (C) Enlarged view of boxed area in A showing R.Not. (D) Stump transversal section, proximal to amputation surface, showing notochord (Not), spinal cord (SC) and muscle (Mus). (E) Detail of the enlargement of the ependymal layer in the N.Amp. (F) Transverse section distal to amputation level showing R.Not and the surrounding undifferentiated blastema cells. (G,H) Degenerating muscle (D.Mus) (black arrows) near the amputation level. Scale bars: 100 μm.

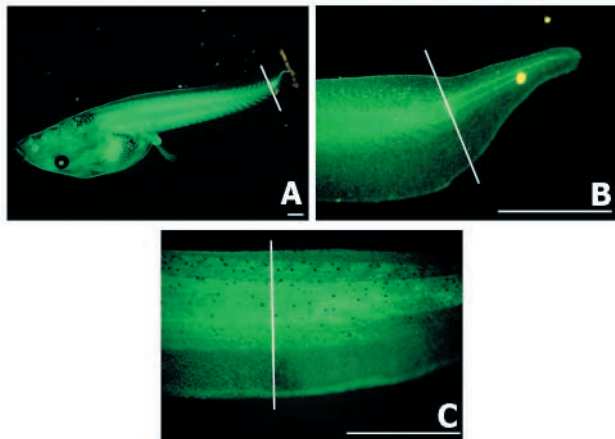


Fig. 2. Transgenic tadpoles expressing GFP under control of the CMV promoter. (A) Five days after tail amputation, the regenerate keeps expressing GFP. (B) Ten-day regenerating tissue. (C) Complete tail regeneration, all the re-formed tissues still express GFP. White line indicates amputation level. Scale bars: 500 μm .

conclusions about cell lineage without prospective labelling of the cell populations in question.

Tissue-specific labelling by neurula grafts

In order to label specific tissue types, we needed a promoter that was active, at high level, in all cell types, and that was independent of any specific controls operating during tail regeneration. This is because the label must persist if cells de-differentiate, and if they re-differentiate with a different phenotype. We made transgenics using three ubiquitous promoters to drive GFP [cytoskeletal actin, CSKA; elongation factor 1 α (EF1 α); and cytomegalovirus early promoter, CMV]. The comparison suggested that the CMV promoter was best, both in terms of the level of activity and in terms of retention of good labelling throughout the regeneration process. This is shown in Fig. 2. The GFP used contains a nuclear localisation signal, and so tends to concentrate in nuclei, but some fluorescence is also visible in the cytoplasm.

Grafting experiments were conducted on neurula stage embryos, at stage 13 or 17. Explants of transgenic notochord, presomite plate or neural plate, expressing GFP, were transplanted into non-transgenic host embryos in the presumptive tail region, next to the blastopore (Table 1; Fig. 3) (Tucker and Slack, 1995). The neurula stage was selected for these operations because it is possible, with the use of trypsin as described in the Materials and methods, to obtain a very

Table 1. Summary of cell population labelling experiments

Graft	Specific label in one tissue Cases	Tissue regeneration labelled		
		Neural plate	Notochord	Somites
Neural plate	12	12	0	0
Notochord	13	0	13	0
Somites				
Medial PSM stage 13	20	0	0	0
Lateral PSM stage 13	10	0	0	7
Medial PSM stage 17	12	0	0	12
Cre-Lox (3d reg)	10	0	0	0

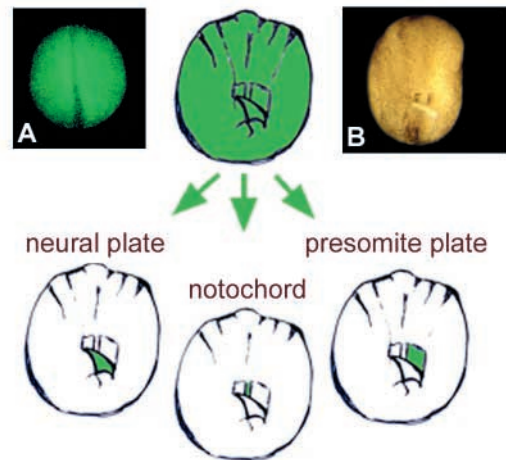


Fig. 3. The grafting technique for the three tail main tissues performed at neurula stage (stage 14/15 NF). (A) *CMV-GFP* donor embryo. (B) Normal host showing retraction of posterior neural plate section.

clean separation of tissue layers and thereby obtain pure grafts with the label confined to a single tissue. Twenty-four hours after the transplantation the GFP fluorescence was visible localised specifically in the position of the grafted tissue (Fig. 4A-C). Sections immunostained for GFP showed that the GFP was confined to the tissue grafted (Fig. 4G-I). At stage 48-49, the stage of the feeding tadpole, the specificity of the labelling was extremely obvious. Because of the transparency of the tadpole tail it was possible to visualise the fluorescent cells at single cell resolution *in vivo* and confirm that the expression of GFP was limited to the tissue originally transplanted, with no fluorescence signal in other cells outside the targeted tissue (Fig. 4D-F). It should be noted that the neural plate grafts took tissue from the central part of the neural plate which end up in the ventral half of the neural tube. The dorsal half of the tube is not labelled and so we should not expect to see, and did not see, any migratory neural crest cells labelled. Individual myofibres either had all nuclei labelled or no nuclei labelled. This is because if some myoblasts contributing to a fibre carry the nucGFP gene then the protein will be synthesized in the fibre and will enter all of the nuclei. It has previously been shown that the first formed myofibres in *Xenopus* tadpoles are mononuclear, but that they become multinucleate over the stage range 40-46 (Muntz, 1975). Again, the results of the *in vivo* examination were confirmed by sectioning and immunostaining for GFP (Fig. 4L-N).

Behaviour of spinal cord and notochord during tail regeneration

The grafted larvae were allowed to reach stage 49-50 and were then amputated at 50% of the tail length. The tail regeneration process was followed *in vivo* by anaesthetising the tadpoles at intervals and taking pictures under a stereomicroscope equipped with UV lamp and GFP filter, as described in the Materials and methods.

Spinal cord

At 5 days after amputation, the regenerating spinal cord was already visible and was in continuity with that of the stump

(Fig. 5A). The blastema surrounding the re-forming spinal cord was completely negative for the GFP fluorescence, as was the notochord. The labelled cells expressing GFP were localised only in the regenerating spinal cord, all other surrounding tissue cells being completely negative. In the following days the regenerating spinal cord kept elongating, always in continuity with that of the stump (Fig. 5B,C). The blastema was not involved at all in the formation of the new spinal cord. Once the stage of complete tail regeneration was reached, the GFP was still exclusively and specifically expressed in the spinal cord as in it had been in the original tail.

Notochord

The regenerating notochord behaved in a similar manner to the spinal cord. After 5 days, the re-forming notochord was already visible in continuity with that of the stump. The blastema around the regenerating notochord was negative for the expression of the GFP (Fig. 5D). The labelled cells, as in the spinal cord regeneration, were not visible outside the notochord itself. With the elongation of the regenerating notochord the fluorescent labelling remained confined to the notochord, and all the other tail tissues were still completely negative (Fig. 6E,F).

The labelling of these two major tissues gives a clear answer to the question about their origin in regeneration. For spinal cord and notochord there is no metaplasia. The spinal cord arises from the spinal cord of the stump and the notochord arises from the notochord of the stump.

Origin of muscle in the regenerate

Car-Cre/Lox labelling compared to *Car-GFP*

Before describing the results from the grafts of presomite mesoderm, it will be convenient to describe one other experiment that set limits to the origin of the regenerated muscle. This is an experiment with the *Cre/Lox* system, where a proportion of myofibres were permanently labelled with GFP after an intrachromosomal recombination to remove a translational stop signal. This experiment was conducted in founder tadpoles, not by crossing *Cre*-expressing and reporter lines together. The *Cre* was driven by the muscle-specific cardiac actin promoter (*Car*) and the reporter plasmid

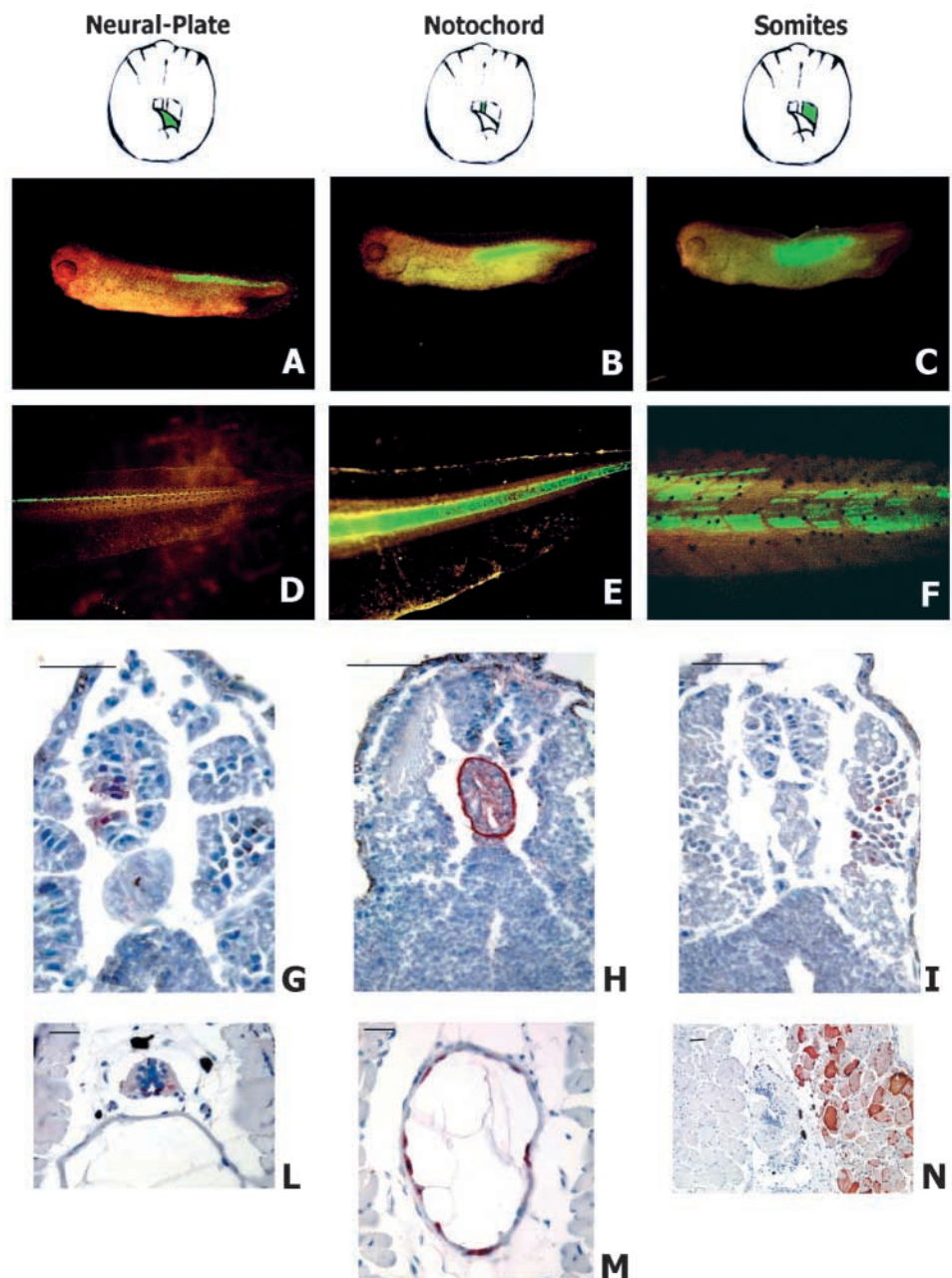


Fig. 4. GFP-labelled grafts of the three embryonic tissues to the presumptive tail region. (A-C) Embryo stage NF37. (D-F) Tadpole stage NF49. (G-I) Transverse sections of caudal region of host at tailbud embryo stage NF37. (L-N) sections of tail at tadpole stage NF49. The sections of the three different graft types are immunostained with anti GFP (red/brown) and counterstained with Haematoxylin (blue) showing specific labelling of each of the three tissue types. Scale bars: 100 μ m.

contained a CMV promoter driving *GFP*, with a *lox-STOP-lox* sequence at the beginning of the coding region (see Materials and methods). The effect is to label permanently with GFP a proportion of the cells that have activated the *Car* promoter at any time. These specimens were compared with others that were simply transgenic for *Car-GFP*, which will show label in all cells which currently have the *Car* promoter active.

Ten transgenic tadpoles expressing *Car-Cre* and *CMV-lox-STOP-lox-GFP* were created with a sufficient proportion of

myofibres labelled (Fig. 6A,B,E,F). Unlike the *Car-GFP* transgenics, not all the myofibres are labelled but the proportion gradually increases with time because of new recombination events occurring in cells where Cre recombinase is expressed. Three days post-amputation, the regeneration bud contained no fluorescent cells at all for either of the two transgenic models (Fig. 6C,G). This shows that there is no de-differentiation of multinucleate fibres to mononuclear cells. In the *Cre/lox* transgenics, any cells dedifferentiated from green fibres would still be making GFP. In the *Car-GFP* transgenics, although production of GFP would cease on de-differentiation, the protein itself should persist for a few days.

After 5 days from the tail amputation a substantial number of green myofibres appeared in the regenerated region of the *Car-GFP* transgenics (Fig. 6H). This indicates the normal tempo of muscle regeneration. By contrast in the *Cre/lox* transgenics, there were just a few isolated green fibres (Fig. 6D). We believe that the time scale and position of appearance of the few fluorescent fibres seen in the regenerating *Cre/lox* transgenics indicates that they are due to new recombination events and not to de- and re-differentiation of muscle cells previously expressing GFP. The absence of any short-term labelling of mononuclear cells in the regeneration bud, and the slow appearance of the labelled myofibres in the *Cre/lox* tadpoles suggests that the myofibres themselves contribute no cells to the regeneration bud. This is consistent with the morphological picture, which shows large scale degeneration

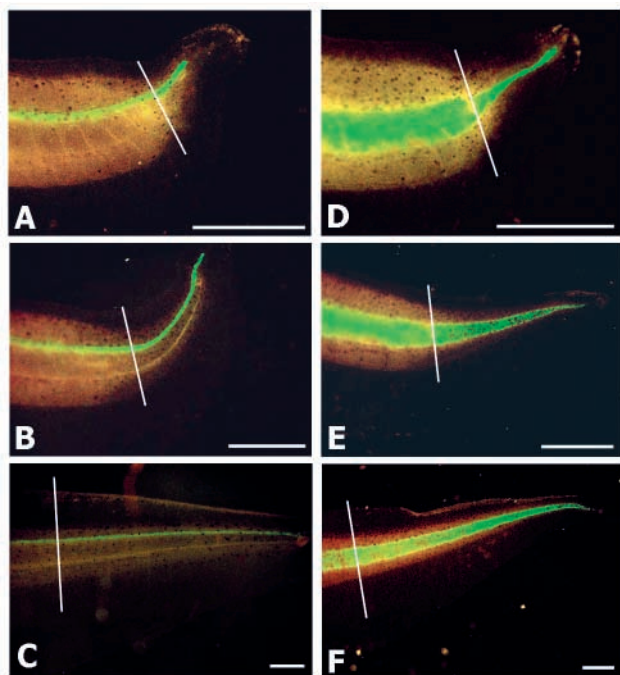


Fig. 5. (A-C) Spinal cord-labelled regenerating tadpole tails showing localisation of the GFP exclusively in the spinal cord of the stump and the regenerate. (A) Five days after amputation. (B) Ten days after amputation. (C) Twenty days after amputation. (D-F) Notochord-labelled regenerating tadpole tails showing localization of the GFP exclusively in the notochord of the stump and the regenerate. (D) Five days post-amputation. (E) Ten days post-amputation. (F) Twenty days post-amputation. White bar indicates the amputation level. Scale bars: 500 μ m.

of myofibres in the vicinity of the cut surface, and it suggests that the new myofibres must come from some other cellular source than pre-existing myofibres.

Presomite plate grafts

The most informative data relating to this question was that from the grafts of neurula-stage presomite mesoderm (PSM), although the behaviour was somewhat more complex than that of neural plate and notochord grafts as it depends on the stage of the donor and on the position of origin of the graft within the PSM. Three different types of graft were performed: PSM adjacent to the notochord at stage 13 (early medial); PSM adjacent to the notochord at stage 17 (late medial); and PSM from a more lateral position at stage 13 (early lateral).

In all the three cases, the labelling of the host tadpoles was exclusively in the somites and later in the myotomes (Fig. 7).

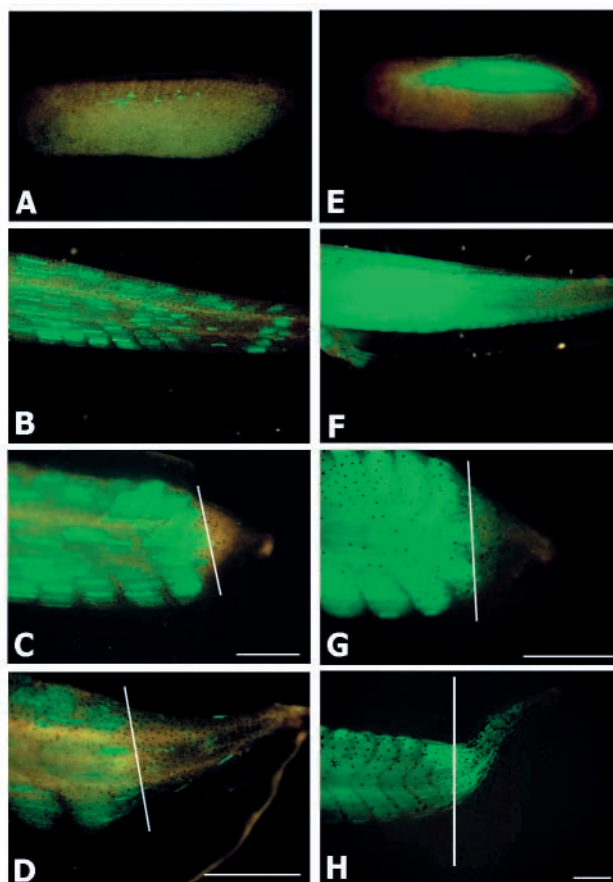


Fig. 6. (A-D) *Car-Cre/Lox-GFP* transgenics expressing GFP in the myofibres after intrachromosomal recombination. (E-H) *Car-GFP* transgenics expressing GFP in all myofibres. (A) Stage 28 NF embryo showing a few somitic cells starting to express GFP. (B) Stage 49 tadpole, before tail amputation, expressing GFP in numerous myofibres. (C) Regenerating tail 3 days after amputation showing no GFP-labelled cells in the blastema. (D) Five-day regenerating tail starting to express GFP in a few new myofibres. (E,F) Stage 28 embryo and stage 49 tadpole expressing GFP in the muscle under control of the cardiac actin promoter (*Car*). (E) Three-day regenerating tail showing no GFP-labelled cells in the blastema. (F) Five-day regenerating tail showing substantial new formation of labelled myofibres. White bar indicates amputation level. Scale bars: 200 μ m.

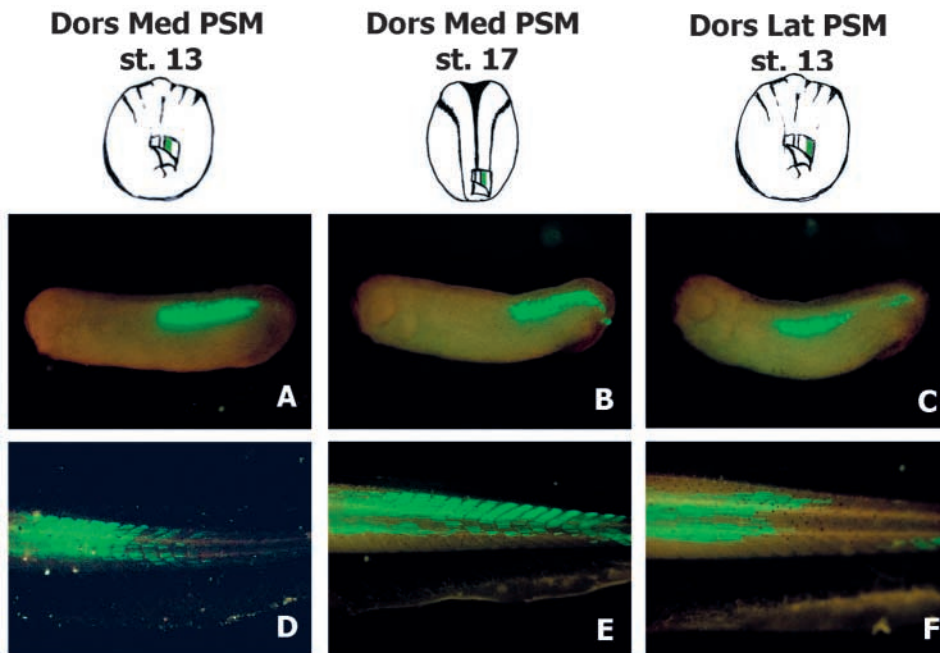


Fig. 7. Presomite mesoderm (PSM) grafts from different stages and positions. In all cases, GFP expression is visible in the somites then in the myotomes. (A-C) Transplanted embryos at stage 28 NF. (D-F) Transplanted larvae stage 49 NF.

However, the behaviour during tail regeneration was completely different.

(1) For early medial PSM grafts, at 3 and 5 days post-amputation the blastema region was completely negative for GFP expression, even if the adjacent stump muscles showed strong fluorescent labelling (Fig. 8A,B). As the tail regenerated, the re-forming muscle fibres remained negative for GFP expression and no fluorescence was seen when the tail was completely regenerated, except for the previously labelled muscle fibres in the stump (Fig. 8C,D).

(2) For the late medial PSM grafts, by 3 days after amputation the blastema contained some undifferentiated mononuclear cells expressing GFP, and by 5 days the labelling of the regenerating muscles became apparent. In general

following a PSM graft only some of the muscle fibres at the level of the amputation are labelled, as there is some mixing of donor-derived with host-derived fibres. The position of the GFP positive mononuclear cells in the blastema was adjacent to the labelled myofibres in the stump and the proportion of labelled to non-labelled cells roughly similar to that in the adjacent stump (Fig. 9A). This suggests that the mononuclear cells were migrating from previously labelled muscle tissue to colonise the regenerating tail and give rise to new muscle fibres. 4-5 days from amputation the GFP-labelled cells in the blastema began to elongate and by 5 days some GFP-positive multinucleate myofibres could be seen (Fig. 9B). In these specimens, no other tissue in the regenerate apart from the myofibres showed GFP expression (Fig. 9C,D).

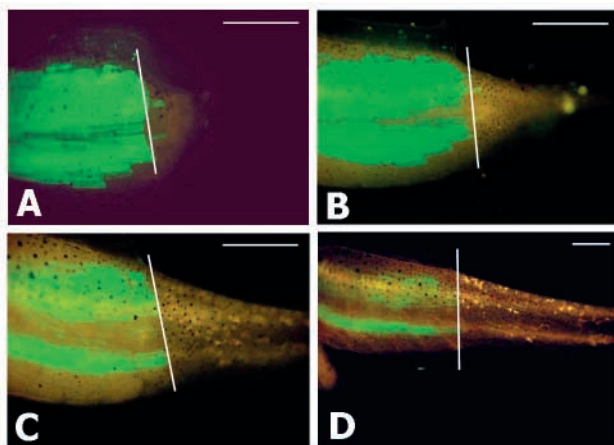


Fig. 8. Regenerating tadpoles labelled in the myotomes by early medial PSM graft. (A) Three days post-amputation. (B) Five days post-amputation. (C) Ten days post-amputation. (D) Twenty days post-amputation. White line indicates amputation level. There is no labelling of myofibres, or any other cells, in the regenerate. Scale bars: 200 μ m.

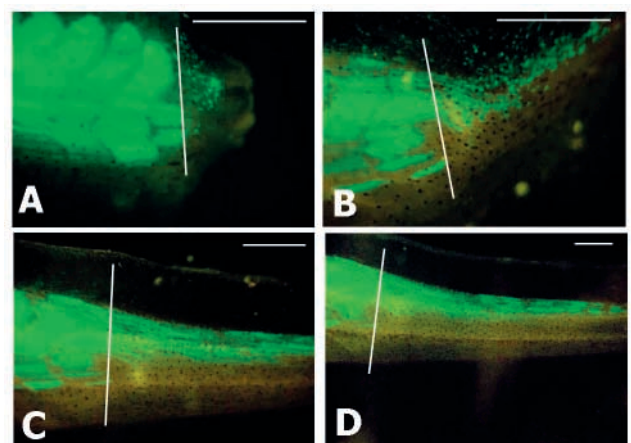


Fig. 9. Regenerating tadpoles labelled in the muscles by late medial PSM graft. (A) Three days post-amputation. (B) Five days post-amputation. (C) Ten days post-amputation (D) Twenty days post-amputation. White line indicates amputation level. There is substantial labelling of myofibres in the regenerate. Scale bars: 200 μ m.

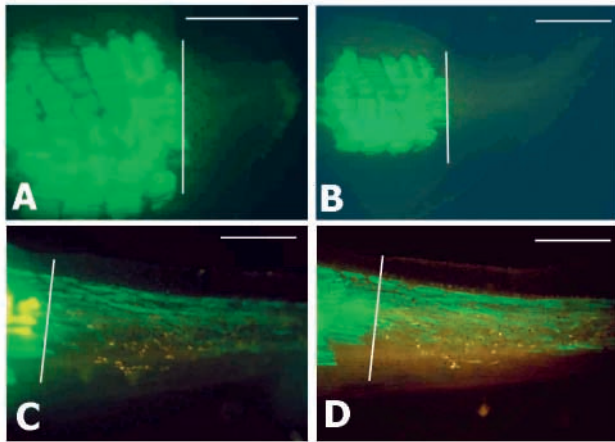


Fig. 10. Regenerating tadpoles labelled in the muscles by early lateral PSM graft. (A) Three days post-amputation. (B) Five days post-amputation. (C) Ten days post-amputation (D) Twenty days post-amputation. White line indicates amputation level. There is slight labelling of myofibres in the regenerate. Scale bars: 200 μm .

(3) For the grafts of early lateral PSM at 3 and 5 days, no fluorescent cells could be seen in the regenerating blastema (Fig. 10A,B). Nevertheless, by 10 days, some fluorescent myofibres had appeared in the regenerate, although fewer and fainter than in the stump (Fig. 10C). As for the late medial PSM graft, the labelled fibres were in the same part of the tail cross-section as the labelled cells in the stump. The GFP signal from these regenerating muscle fibres was faint compared with that of the stump muscle. This is presumably due to the fact that only one or a few fluorescent cells participate in the formation of each myofibre, so the overall GFP intensity within the fibres is diluted. When the tail regeneration was complete, the GFP-positive fibres persisted, and no fluorescent label was present in any other tissue (Fig. 10D).

The different behaviour of the three types of PSM graft confirm that the presence of labelled myofibres in the stump, regardless of their position in the cross-section of the tail, does not necessarily guarantee the formation of any labelled myofibres in the regenerate. The myofibres must therefore originate from some other cell type derived from the presomite mesoderm, and present in the myotomes, the precursors of which are found in the lateral rather than the medial PSM. The presence of even more precursors in the late PSM grafts is fully consistent with this idea, as there is a massive dorsal convergence of mesoderm during neurulation, so a late medial graft will necessarily contain cells that were in a substantially more lateral position at earlier stages.

GFP expression in different muscle cell populations

In order to characterise the difference between the three different PSM graft types in terms of cell population, the grafted tadpoles were analysed by immunostaining against GFP (see Materials and methods). Both longitudinal and transverse sections were used although the transverse sections proved most informative. The nuclear localisation signal means that GFP protein is concentrated in nuclei, although highly expressing cells show some in the cytoplasm as well.

(1) Early medial PSM graft sections showed the labelling of

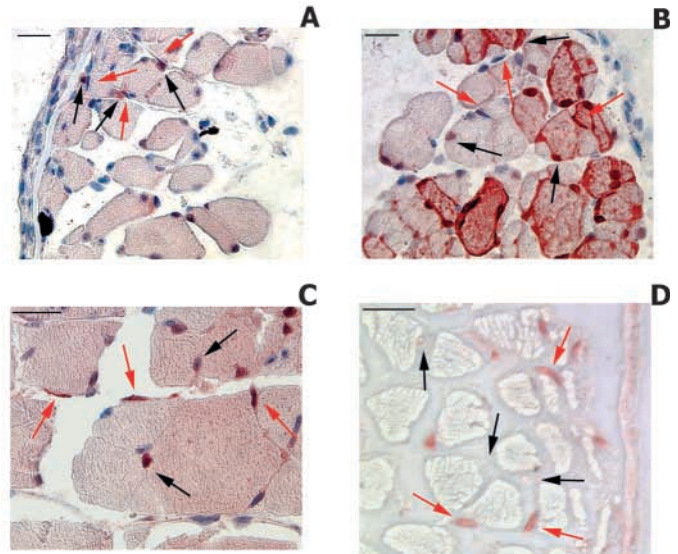


Fig. 11. (A–C) GFP expression in myotomes after PSM grafts performed at different stages and level. Transverse sections were immunostained with antibody against GFP (brown) and counterstained with Haematoxylin (blue). (A) Early medial PSM grafted muscles expressing GFP in the myofibre nuclei (black arrows), but not in the satellite cell nuclei (red arrows). (B) Late medial PSM grafted muscles showing GFP expression in both myofibre nuclei and in satellite cells. (C) Early lateral PSM grafted muscle showing GFP expressed in myofibre nuclei and in a few satellite cells. (D) Muscle transverse section immunostained with Pax7 antibody (brown) (not counterstained). Pax7 expression is detected in the flat nuclei (red arrows) and not in the large round myofibre nuclei (black arrows), suggesting that the flat nuclei are those of satellite cells. Scale bars: 10 μm .

large nuclei, appearing round in transverse section, within the segmented muscle of the grafted side of the tadpole (Fig. 11A). Those are the nuclei of the muscle fibres, called myonuclei, expressing GFP. In the same sections many GFP-negative nuclei, stained blue by the Haematoxylin counterstain, can be seen. Two different types of negative nuclei were visible. The most numerous were the round myonuclei similar to the GFP-positive ones, representing the population of negative muscle fibres not expressing GFP. The second type of nucleus showed a different morphology, being flat, placed only in the edges of myofibres, but intimately associated with them.

(2) Late medial PSM transplant sections showed a subtly different pattern of labelling. They also showed immunoreaction with the round myonuclei, but here some flat nuclei were also GFP positive (Fig. 11B). Where the muscle fibres showed GFP expression, both types of nuclei tended to be positive. In some cases flat nuclei could be labelled in isolated regions away from the positive myonuclei.

(3) Early lateral PSM graft sections immunostained for GFP showed an intermediate appearance. Mostly the GFP-positive nuclei were the round myonuclei, as in the early medial PSM transplants; nevertheless, a very small number of flat nuclei also showed GFP expression (Fig. 11C). The proportion of GFP-positive flat nuclei was much lower than for the late medial PSM grafts.

We scored in the three different types of PSM transplant the

Table 2. Ratio of GFP-positive flat nuclei

	PSM graft		
	Early medial	Late medial	Early lateral
GFP flat nuclei (%)	0	58	5

proportion of labelled flat nuclei over 100 cells in several GFP positive areas and the results are summarised in Table 2.

The flat nuclei have the appearance of muscle satellite cells (Mauro, 1961; Muntz, 1975). These are mononuclear cells lying within the basement membrane of the myofibres that can re-enter mitosis and contribute to growth and regeneration of the muscles. A monoclonal antibody against the transcription factor Pax7, a molecular marker for muscle satellite cells in mammals (Seale et al., 2000), was used in order to confirm this identification. The cell type labelled by Pax7 mAb was morphologically identical to the type labelled by GFP immunostaining in the types of graft that give labelled muscle in regenerates (Fig. 11D).

Further proof of the identity of these cells as satellite cells is given by the incorporation of BrdU. It has been demonstrated that the only cell type proliferating in mammalian differentiated muscles are the satellite cells (Walsh and Perlman, 1997). Unamputated tadpoles were injected with BrdU and processed 24 hours later. Many cells all over the tadpoles are labelled, but in the muscle the labelling is predominantly in the flat peripheral nuclei of the type expressing Pax7 (Fig. 12A,B). Very few, if any, of the myonuclei are labelled, although a few fibroblasts or other cell types within the muscle are labelled.

Finally, we investigated whether the regeneration behaviour of the different types of PSM graft correlated with the presence of satellite cells in the grafts. This was done by co-immunostaining sections of grafted tadpoles for Pax7 and for GFP (Fig. 13). Single staining (Fig. 13A,B) showed once again the different pattern of reaction between Pax7 and GFP: Pax7 was expressed in the elongated nuclei and exclusively nuclear, while GFP was expressed mostly in the round nuclei and cytoplasm of the muscle fibres. In the double immunostaining it could clearly be seen that the two labels co-localized only in the late medial PSM graft type, which yields labelled muscle

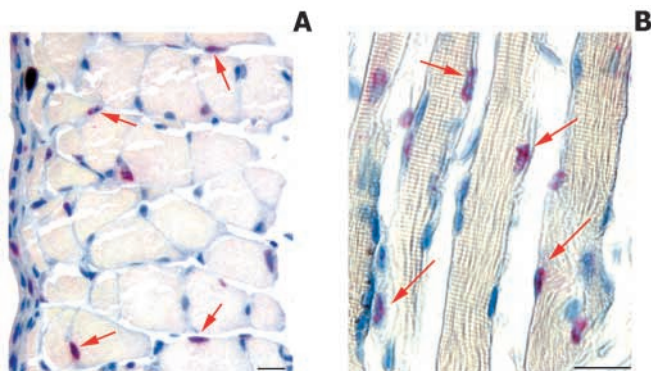


Fig. 12. BrdU incorporation in *Xenopus* tadpole muscle shown by immunostaining and counterstained with Haematoxylin (BrdU in red). (A) Transverse section. (B) Longitudinal section. Positive cells are indicated by red arrows, and are mostly the flat nuclei at the edges of myofibres, indicative of satellite cells. Scale bars: 5 µm.

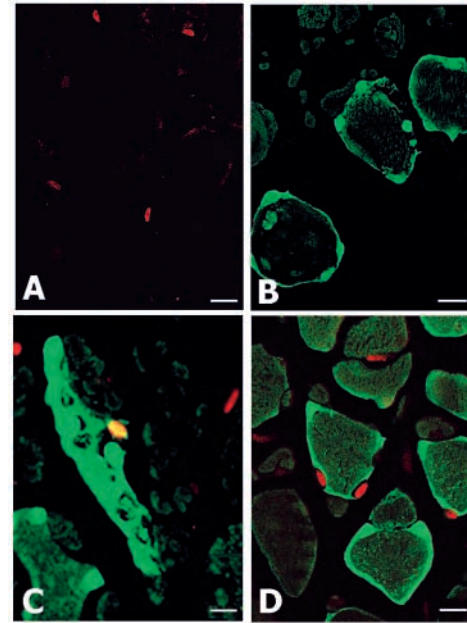


Fig. 13. Immunofluorescence with anti-Pax7 (red) and anti-GFP (green) on transverse sections of tadpole myotomes. (A) Pax7 in ungrafted tadpole. (B) GFP immunoreaction on myotome in PSM grafted embryo. (C) Double immunostaining of Pax7 and GFP on muscles from late medial PSM graft. (D) Double immunostaining of Pax7 and GFP on muscles from early medial PSM graft. Co-localization is seen only in the late grafts. Scale bars: 5 µm.

in the regenerate (Fig. 13C). No co-localization is shown in the early medial PSM transplanted tadpoles in which muscle is not labelled in the regenerate (Fig. 13D).

In summary, our results suggest that the flat nuclei belong to muscle satellite cells and that their precursors are not uniformly distributed in the presomitic mesoderm. They are not formed from the early medial PSM, some arise from the early lateral PSM, and most from the late PSM, which will include tissue from an extreme lateral position in the earlier mesoderm because of the process of dorsal convergence during neurulation. We thus believe that the satellite cell precursors originate from the lateral region of the PSM.

As there is an excellent correlation between the extent of labelling of satellite cells by the three types of graft, and the extent to which the grafts contribute to myofibres in the regenerate, we believe that the new myofibres of the regenerated tail arise from satellite cells and not by de-differentiation of pre-existing myofibres.

Discussion

Morphology of the regeneration bud

From the results obtained in this paper we think that the term 'blastema' is not appropriate for the whole of the regenerating tissues during *Xenopus* tail regeneration. A 'blastema' is usually defined as a mass of undifferentiated pluripotent proliferating cells associated with the epimorphic process of regeneration. In this study, we refer to the whole of the regenerating region as a 'regeneration bud' and only the visibly undifferentiated tissue within this as a 'blastema'. The

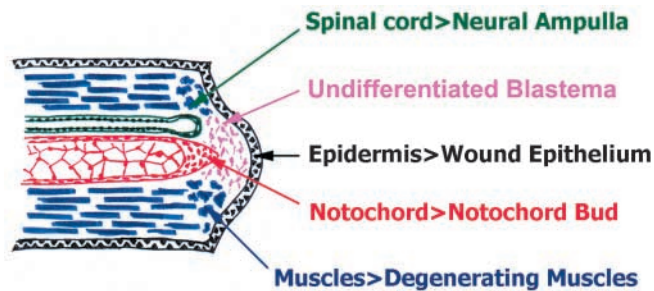


Fig. 14. The composition of the regeneration bud during *Xenopus* tadpole tail regeneration.

morphological study showed that spinal cord and notochord do not appear to de-differentiate. After amputation, they keep their tissue identity, and their own specialised cell populations proliferate to give rise the regenerating spinal cord and notochord respectively. The spinal cord regenerates from a terminal ‘ampulla apicalis’, formerly described by Stefanelli (Stefanelli, 1951) in both amphibians and reptiles. This structure was explained as the result of the enlargement of the ependymal canal because of the intense proliferation in the regenerating inner layer of the spinal cord. The notochord regenerates from a bullet-shaped mass of cells that forms at its tip after amputation. The blastema proper is presumably derived from the fin mesenchyme and dermis, as well as from the muscle satellite cells, although we do not have direct evidence for this from the present study. Of the regenerated axial tissues that have been studied here, only the myofibres of the muscle derive from cells in the blastema. (A schematic view of the regeneration bud is shown in Fig. 14.)

Transgenesis and graft

Our technique of grafting into normal hosts from neurulae of transgenic embryos ubiquitously expressing *CMV-GFP* showed specific labelling in the tissue targeted. The transplanted tissue, which expressed GFP, retained fluorescence throughout regeneration and enabled us to follow any processes of cellular de-differentiation and re-differentiation.

The results for the spinal cord and notochord are straightforward and are consistent with the morphological appearance. Our results show that these two tissues each regenerate as a self-contained compartment with no export or import of cells, and without de-differentiation or metaplasia. The labelling of spinal cord was carried out in such a way that only the ventral part was labelled. We did not, therefore, examine the potential of the neural crest cells that are derived from the dorsal part of the neural tube. Our results are in marked contrast to a recent cell labelling study on axolotl tail regeneration in which radial glial cells were shown to give rise to neurons, melanocytes, myofibres and chondrocytes in the regenerate (Echeverri and Tanaka, 2002). We believe that both sets of data are correct and that they point to radically different modes of regeneration in the urodele and anuran amphibians.

Origin of muscle in the regenerate

Our results suggest that the regenerated muscle comes from satellite cells in the myotomes, and not from pre-existing myofibres. The evidence is as follows. First, the original myofibres can be seen to be degenerating in the vicinity of the

cut surface. Second, myofibres labelled by the Cre-Lox technique do not contribute to myofibres in the regenerate in the short term. The small numbers of labelled fibres that do appear are late and consistent with new recombination events following the new differentiation of muscle fibres whose time course can be seen in the *Car-GFP* transgenics. Ryffel et. al. (Ryffel et. al., 2003) also used the Cre-Lox method to label myofibres, using a protocol involving breeding, and also showed that labelled cells did not enter the muscle of the regenerate. Third, myofibres labelled by the early medial PSM grafts never contribute to myofibres in the regenerate. Fourth, the tadpole myotomes contain cells, identified by Pax7 expression, with the morphology of muscle satellite cells. Fifth, these cells are the proliferating cells within the myotomes as shown by the BrdU incorporation. Sixth, for the three types of PSM graft, the labelling of myofibres in the regenerate is correlated with the number of satellite cells labelled by the graft.

The different behaviour of the PSM grafts is fully consistent with the idea that the satellite cell precursors lie in the far lateral part of the PSM. During somitogenesis there is a strong dorsal convergence of cells from the ventral side of the embryo (Pourquié, 2001). The PSM in the early neurula is not much thicker than the ventral mesoderm, but by the late neurula it has become markedly thicker, at the expense of the ventral mesoderm, and segmentation has commenced at the anterior end (Nieuwkoop and Faber, 1967). We believe that our early lateral grafts capture some of the satellite cell precursors but that the late medial grafts capture more because of the substantial migration of tissue towards the dorsal midline that occurs in this stage interval.

Again, our result on the origin of regenerated muscle is discordant with previous results from urodeles. In newt, limb regeneration it has clearly been shown that the nuclei of myofibres can re-enter S phase following a nearby injury, that the fibres can break up into viable mononuclear cells, and that these cells can re-differentiate to form new muscle fibres and also make some contribution to other cell types (Kintner and Brockes, 1984; Echeverri et al., 2001; Echeverri et al., 2002).

Muscle satellite cells were first described by Mauro (Mauro, 1961), who provided EM pictures of cells from the frog very similar in appearance to the flat nucleus cells that we find labelled by the late PSM graft. Subsequent work has all been on birds and mammals (reviewed by Seale and Rudnicki, 2000), although it is likely that amphibian satellite cells have similar properties. Following muscle damage, satellite cells re-enter mitosis and start to express myogenic transcription factors, and the resulting myoblasts fuse with each other to generate new myofibres (Schultz, 1996; Cooper, et. al., 1999). The formation of mammalian satellite cells, but not primary or secondary myofibres, is now known to depend on the transcription factor Pax7 (Seale et. al, 2000). They have been previously shown to originate from the somites in birds, using quail-chick grafting (Armand et al., 1983), although more recent work suggests that they may arise from the aorta (De Angelis et al., 1999). As the dorsal aorta is now known to be colonised by a population of somitic angioblasts (Pardanaud et al., 2000), these findings are not necessarily inconsistent. Our results indicate that *Xenopus* has satellite cells similar to those in birds and mammals, that they express Pax7, and that they arise from the lateral region of the PSM.

Conclusions

The regeneration of the *Xenopus* tadpole tail operates through mechanisms that are completely different from those found in the appendage regeneration of urodeles. In urodeles functional cells de-differentiate to form a blastema. This proliferates and re-differentiates to form the regenerate. There is a certain amount of metaplasia shown, indicating that at least some of the blastema cells show pluripotency. In *Xenopus* the spinal cord regenerates through proliferation of an apical ampulla continuous with the ependymal layer of the more proximal spinal cord. The notochord regenerates by proliferation at the tip. The muscle regenerates by the multiplication, differentiation and fusion of satellite cells to form new myofibres.

Overall, *Xenopus* tail regeneration seems much more akin to the normal tissue renewal mechanisms found in mammals than to the specialised regeneration mechanisms found in the urodeles (Carlson, 2003). This may make *Xenopus* a more useful model organism than formerly suspected for experimental work in regenerative medicine.

We thank Caroline Beck and Bea Christen for the helpful advice and communication of results before publication. This work was supported by a Wellcome Trust Prize studentship to C.G.

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