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Single Cell Analysis of Tail Regeneration in
Ambystoma mexicanum

A Dissertation Presented

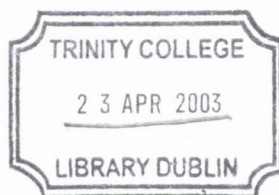
By

Karen Echeverri

This thesis is submitted in fulfilment of requirements for the degree
of Doctor of Philosophy to Trinity College, University of Dublin.

Department of Zoology
University of Dublin
Trinity College, Dublin
Ireland

September 2002

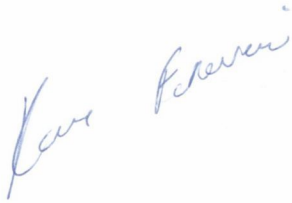


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September 2002

Acknowledgements

First and foremost I would like to thank Elly Tanaka who gave me the opportunity to work on such a fascinating problem and has guided me through the exciting world of regeneration with great expertise and patience and has kept me laughing on days when it seemed nothing would ever work, and gave me a long leash to explore many unrelated avenues. I especially want to thank you Elly for encouraging me to do so many 'afternoon experiments' and for the fun I had in doing them with you.

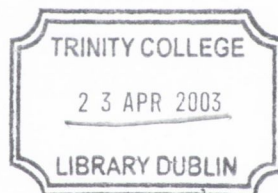
Special thanks must go to Tony Hyman for taking me into his lab as an inexperienced summer student and introducing me to the wonderful world of basic research and of how to keep a proper lab notebook, a skill I will be forever grateful for!

To Prof. Roberts for giving me helpful advice and suggestions from his vast experience working on the fascinating problem of regeneration in another system.

To all the Tanaka lab members over the years, many thanks for the good times and for listening to me complaining during numerous coffee and lunch breaks.

I must express my thanks to many people at the MPI-CBG Dresden, especially to Kurt Anderson for so much help with microscopy throughout my PhD.

To the members of my thesis committee, Marcos, Suzanne and Michael for many useful discussions, continued encouragement and advice for my future. To various

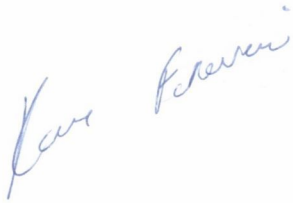


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Karen Echeverri

September 2002

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To everybody : Thank you

Go raibh maith agut

Merci beaucoup

Vielen Dank

Summary

This present study examined tail regeneration in the Mexican salamander *Ambystoma mexicanum*, on a single cell level *in vivo*. The primary aim of this study was to understand where the cells were coming from to replace all the lost structures when a tail is amputated.

Tail regeneration in *Ambystoma mexicanum* more commonly referred to, as the axolotl was first described in the 1950's. The axolotl was chosen for this study as the larval animals are optically clear with very large cells ideally suited to microinjection and imaging. Regeneration is also rapid in these animals, taking about 14 days until the first morphological signs of differentiation are apparent. The process of regeneration was first documented using differential interference contrast microscopy (DIC). This allowed the tail to be optically sectioned and the cells at the level of muscle and the underlying notochord and spinal cord could be imaged. Regeneration occurs initially by healing of the wound, the skin crawls over from all sides and a wound epidermis is formed within six hours. A mound of proliferating undifferentiated cells then forms which is referred to as the blastema. The cells of the blastema go on to differentiate to replace all lost structures. At the same time as a blastema is formed the spinal cord is growing out as a separate entity.

The first major question to be addressed in this thesis was, where do the cells from the blastema come from?

Classical histological studies suggested that terminally differentiated muscle fibres dedifferentiate and form mononucleate cells that populate the blastema. As this

theory was inferred from static images many argued that what was observed was actually fusion of mononucleate cells to form new muscle fibres. To address this issue of dedifferentiation I developed a method to inject single muscle fibres in the axolotl tail with a fluorescent tracer shortly before amputation and to follow the fate of the muscle fibre *in vivo*. Using this technique it was established that amputation of the tail alone was not enough to induce dedifferentiation but that clipping of the end of the fibre was also required along with a signal released in response to severe tissue damage. Muscle fibres were found to initially fragment, forming mononucleate cells which then proliferate and contribute to the blastema, making up approximately 20% of cells in the blastema.

The second question to be addressed was, does cell mixing occur between the blastema and the spinal cord? The spinal cord was originally thought to grow out as a separate entity to the blastema and that no cell mixing occurred between the two. By developing a method of single cell electroporation to label a small number of cells within the spinal cord and combining it with a promoter which is specifically turned on in radial endodermal cells in the axolotl, these cells and their descendents could then be followed throughout regeneration. First, a zone of approximately 400µm rostral to the plane of amputation in the spinal cord was identified as the source of the new cells in the regenerating spinal cord. These cells migrate out and proliferate to rebuild the new spinal cord. The radial endodermal cells were found to be very plastic cells capable of crossing cell fate boundaries. These cells not only have the ability to migrate out of the spinal cord to form neural crest derivatives but can also transdifferentiate to form muscle and

cartilage, both cell types of mesodermal lineage. These data show that cell mixing occurs by spinal cord cells migrating out into the blastema.

Overall the findings from this study demonstrate the remarkable ability of the axolotl to replace lost structures via dedifferentiation of mature muscle fibres and cell lineage switching of radial ependymal cells.

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Abbreviations

CMV	Cytomegalovirus
BrdU	5-Bromo-2-Deoxyuridine
DIC	Differential Interference Contrast
DsRed	<i>Discomoma sp.</i> Red fluorescent protein
EGF	Epidermal Growth Factor
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
FCS	Foetal Calf Serum
N-CAM	Neural cell adhesion molecule
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline plus 0.1% Triton X-100
Pen/Strep.	Penicillin plus Streptomycin
PFA	Paraformaldehyde
PSA	Polysialylated
RA	Retinoic acid
Rb	Retinoblastoma protein

Chapter 1

General Introduction

Historical Perspective

Many larval and adult animals have the amazing ability to regenerate large parts of their body after transection or amputation (Goss, 1969). This unique phenomenon was first described scientifically in 1740 by Abraham Trembley who discovered that a tiny almost microscopic animal decorated at its cephalic end by an array of tentacles could completely regenerate its head after transection. The unexpected ability of these animals to regenerate their head combined with its morphological appearance prompted Trembley to name them after the mythical Hydra (Lenhoff and Lenhoff, 1986).

Trembley's work was the beginning of an intense phase of regeneration studies in Europe. Peter Simon Pallas described in 1766 the unique regenerative ability of a 'new and obscure' species of animal known today as the planarians (Dinsmore, 1991). Then in 1768 Lazzaro Spallanzoni described the ability of the amphibian tadpole to regenerate their tails and that salamanders could regenerate limbs, tails, eyes and jaws (Spallanzani, 1768, Dinsmore, 1991).

The discovery that several different species have the ability to regenerate prompted the question of how widely distributed is the phenomenon among metazoans? Examination of the proposed phylogenetic trees of the Metazoa clearly revealed that regeneration is widespread amongst the phylum (Brusca, 1990, deRosa, 1999). Almost every phylum contains one or more species capable of some form of regeneration. Urodele amphibians are capable of regenerating complete appendages while planarians can regenerate entire organisms from tissue fragments of $1/127^{\text{th}}$ of their body size (Morgan, 1898). This stark contrast in

regenerative abilities then lead scientists to wonder whether there are different modes of regeneration? Did every phylum 'develop' its own way to regenerate or did all phyla inherit one common ancestral program with which to carry out their own individual route of regeneration?

Morgan was the first to begin to classify types of regeneration in Metazoa. He noted that regeneration can be grouped into two categories based on the following criteria: 1) regeneration occurring in the absence of cell proliferation, and 2) regeneration which requires cell proliferation. The first type is referred to as *morphallaxis*, a term coined by Morgan (Morgan, 1898, Morgan, 1901).

Morphallaxis means the re-creation of missing body parts solely by remodelling of the pre-existing cells, it requires no cell proliferation. An example of this type of regeneration is seen in Hydra, as first shown by Trembley who noted its ability to completely regenerate its head (Lenhoff and Lenhoff, 1986) and the later demonstration that differentiation of new structures occurs in the absence of cell proliferation (Park, 1970, Holstein, 1991).

The second type of regeneration that requires cell proliferation was originally termed *epimorphosis* (Morgan, 1901). Today epimorphosis is subdivided into two categories: non blastemal and blastemal based regeneration. Blastema is derived from the Greek word meaning sprout or bud, a blastema is defined in terms of regeneration as being a mound of proliferating undifferentiated cells that eventually differentiate and replace all lost structures. The cone shaped structure that we today refer to as a blastema was originally called a cone by

Spallanazi. The blastema very strongly resembles a limb or tail bud as seen during development (Gilbert, 1985).

Non-blastemal regeneration occurs via transdifferentiation of the remaining tissue into the missing tissue, by limited dedifferentiation and proliferation of surviving cells in the organ after injury or amputation or by the proliferation and differentiation of stem cells already present in the damaged tissue.

Examples of these mechanisms of epimorphic regeneration can be seen in lens regeneration in urodele amphibians whereby remaining cells transdifferentiate to replace lost structures (Reyer, 1954), in human liver the hepatocytes start dividing and reform the lost structures (Michalopoulos and DeFrances, 1997) and bone marrow is known to contain stem cells which can be differentiated in culture into osteoblasts, chondrocytes and even myoblasts (Prockop, 1997).

Blastema regeneration occurs via formation of a mound of proliferating undifferentiated cells that eventually redifferentiate. The structure of the blastema is very similar in shape and composition to the limb bud. Both limb buds and blastemas are made up of two defined compartments: a superficial sheet of epithelial cells that covers the bud and an underlying mass of cells of mesenchymal origin (Alvarado, 2000). Depending on the age and species of the animal, the blastema may form within a matter of hours or days, but in all cases the missing structures are replaced by eventual redifferentiation of cells

in the blastema. This type of epimorphic regeneration is seen in planarian (Brondsted, 1969), echinoderms (Candia Carnevali *et al.*, 1995), urochordates (Huxley, 1921) and in limbs (Brockes, 1997) and tails (Iten and Bryant, 1976) in vertebrates.

It is very interesting that such diverse phyla such as Planaria and salamanders both require blastemas to regenerate and that all these blastemas are so structurally similar (Alvarado and Newmark, 1998). In 1927 Korschelt was the first to actually note this morphological similarity between blastema from different species and suggested that regeneration may have been a primordial attribute of all metazoans (Korschelt, 1927). But this theory was not widely accepted, as it was difficult to reconcile an idea of an evolutionary conserved structure for regeneration with the fact that many related species within the same phyla cannot regenerate. Later Goss proposed a solution to this problem, he proposed that for the Korschelt theory to be true and to explain the irregular distribution of regenerative ability throughout species of a phylum then regeneration must have been selected against rather than for, throughout the evolution of the Metazoa (Goss, 1991). From Goss's theory all blastemas would share a common evolutionary origin and hence the conservation of blastemal structures between distant phyla would be explained. Also negative-selection would explain the random distribution of

epimorphic regeneration between different species, as they would have been subjected to different selective pressures.

To date there is no evidence to suggest a common ancestral origin for regeneration. Evidence so far is correlational and comes from the study and comparison of vertebrate regeneration versus the better understood developmental process. In 1952 Needham described the characteristics shared by development and regeneration, he noted that there is little difference in the end product derived during embryonic development of a limb versus a regenerated limb of an adult animal (Needham, 1952). The implication from Needham was that both events must share evolutionary conserved mechanisms. Therefore an obvious extension of this assumption would be that the high degree of conservation across different phyla between their regeneration blastemas and their related embryonic structures would imply the existence of shared molecular mechanisms controlling epithelial to mesenchymal interactions that are required for their respective structures.

Historical theory of the origin of blastema cells

To date most research has focused on understanding where the cells come from in the limb blastema of salamanders like the newt or axolotl. Spallanzani was the first to actually describe and sketch a urodele blastema in the late 1760's and wondered if the blastema was simply the product of 'expanding germs' (Spallanzani, 1768). From the end of the nineteenth century to date various theories have been put forth on the origin of blastema cells, with evidence to support them, four main origins can be considered: 1. blood cell origin, 2. reserve cell source, 3. wound epithelial or epidermal cell origin and 4. dedifferentiation of mesodermal stump tissue source.

Colucci described the role of blood cells in regeneration in 1884. He described how blastema cells arose from the white blood cells by emigration of cells from the blood vessels surrounding the wound and from the bone marrow or cartilage (Colucci, 1884). Hellmich later also described two major classes of cells in the adult urodele regenerate: hematogenic cells and histiogenetic, or blood and mesenchymal cells. He emphasised in his work that in fact hematogenic cells make a greater contribution to the 'regeneration cell' population than do the various tissues of the mature stump (Hellmich, 1930). He later described how regeneration is similar in *Plethodon cinereus*, *Eurycea bislineata* and *Ambystoma mexicanum*, in that he found cells of hematogenic origin in all blastemas (Hellmich, 1931).

Originally it was assumed that each tissue in a stump generated more of the same cells following amputation. Later it was shown that when skeletal elements are

removed from a stump, new bone forms in the regenerate, distal to the plane of amputation from cells in the blastema (Fritsch, 1911, Weiss, 1925).

Weiss postulated that the blastema forms due to the presence in the limb of undifferentiated reserve cells, of connective tissue origin, which only differentiate when the need arises. His theory was that these reserve cells give rise to new bone tissue in a limb where the bone has been removed prior to amputation (Weiss, 1939). Potential reserve cells have been described for muscle in some urodele species, like the newt (Cameron, 1986), but the extent of their role in regeneration is still unclear.

In 1928 Godlewski proposed that wound epithelium may actually contribute cells to the mesenchymal blastema (Godlewski, 1928). Godlewski and Rose noticed a close contact between the apical epithelium and the blastema, they observed tongue like projections composed of inner basal epithelium cells projecting into the blastema cell mass. They maintained that these cells underwent metaplasia, entered the blastema and became blastema cells (Rose, 1948). Later others also observed prominent projections from the apical epithelium into the blastema (Scheuing and Singer, 1957, Schmidt, 1958) and noted that the wound surface was different from the epidermis at the lateral edges of the wound. Based on cell counts, Rose concluded that there was considerable loss of epithelial cells covering the wound at the same time as an increase in the blastema cell population (Rose, 1948).

The term 'dedifferentiation' was first used by Driesch in 1902 and later Schultz, in experiments describing the regeneration of the branchial chamber in *Clavellina*

(Ascidians). Their idea was that differentiated cells reverted to an embryonic state; hence the term 'dedifferentiation' was used to describe the phenomenon by which cells became pluripotent again (Driesch, 1902, Schultz, 1907). In 1914 Schaxel disputed the capability of cells to dedifferentiate and was convinced that once development was complete it was not reversible. He considered 'dedifferentiated cells' to be merely dying cells. He postulated that a regenerate was composed of reserve cells which could be found throughout the body and these cells proliferated in response to injury and formed the regenerate (Schaxel, 1914). Classical experiments by Butler again brought forward the use of the term 'dedifferentiation'; in 1935 he provided evidence that the blastemal cells of the regenerating urodele limb arose from local mesodermal tissues immediately proximal to the amputated limb (Butler, 1935). Later studies confirmed this initial evidence, Butler and O' Brien lead shielded an entire salamander larvae (*Eurycea bislineata*) except for its left knee which it exposed to 2.5-5.0 kilorads of x-irradiation. When the shielded ankle was amputated a perfect regenerate formed. Amputation through the knee of irradiated limb resulted in no regenerate. A later amputation through the lead protected thigh of the irradiated animal resulted in a perfect regenerate, thereby demonstrating that the cells for regeneration blastema had to be of 'local origin' (Butler and O' Brien, 1942). The authors concluded that the cells dedifferentiated from the mesodermal cut stump tissues (Figure A).

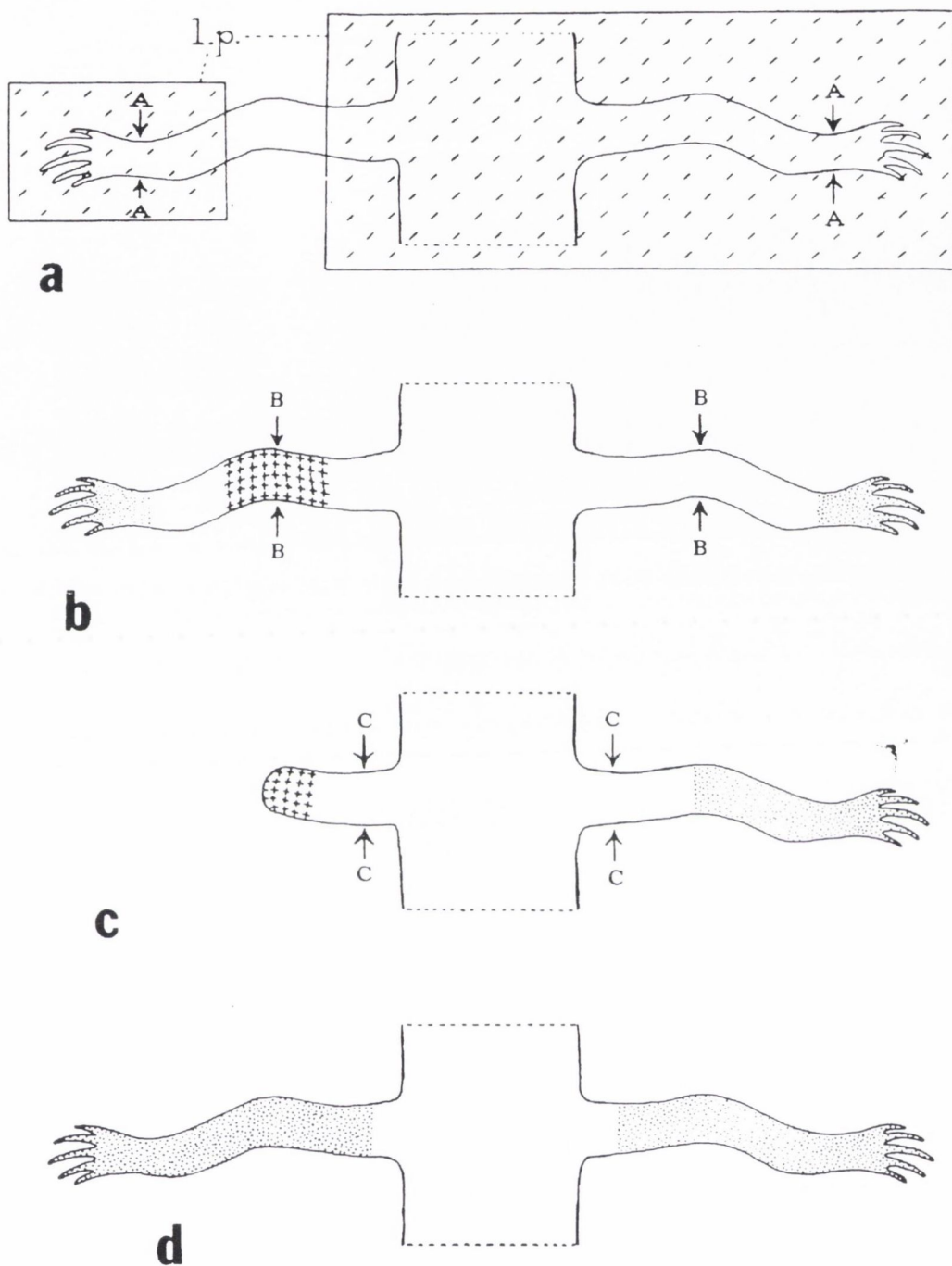


Fig. A X-irradiation of limbs. Diagram for localized radiation (a). The entire larva was shielded with lead plate (l.p.), with the exception of the region of the knee joint of the left hind leg. Arrows (A-A), indicate level of first limb amputation. Same larva 3 weeks after irradiation and first limb amputation (b). Stippled areas represent regenerated structures; crosshatching indicates region originally irradiated; arrows

(B-B) show level of second amputation. Larva 9 weeks after second amputation (c). Stippled areas represent regenerated structures, cross hatching indicates nonregenerating irradiated regions, arrows (C-C) show level of third amputation. Same larva 4 weeks after amputation (d). Stippled areas indicate regenerated limbs. (Butler and O'Brien 1942, Anat. Rec.)

Studies from Chalkey in the early 1950s noted that one of the main events in blastema formation is actually the initiation of tissue dissociation and cellular dedifferentiation in mesodermal tissues at the amputation plane (Chalkley, 1954). From cell counting experiments he concluded that periosteum, muscle and different layers of connective tissue probably provide up to 85% of the cells in the blastema. He also concluded, contrary to previous work by Rose, that the wound epithelium does not contribute significant numbers of cells to the mesenchymal mass of the blastema (Chalkley, 1954, Rose, 1948). Although Chalkey's study clearly showed that the blastema cells originated from mesodermal dedifferentiation in the stump, it showed no evidence or speculated about a mechanism by which this phenomenon could occur.

Hay was one of the first to postulate and show some preliminary evidence for how a mature differentiated cell could actually dedifferentiate.

Observations from detailed electron micrographs of muscle fibres at the plane of amputation suggested that muscle fibres lost their myofibrillar structure, their nuclei became enlarged, and then mononucleate cells were budded off into the blastema (Hay, 1959a; Hay and Fischman, 1961)(Fig. B). The way Hay described

the process of muscle fibre dedifferentiation was that many vesicles form in the cytoplasm, and the mitochondria move to one end of the cell, adjacent to the nucleus. While enclosing a small area of the inner cytoplasm and the nucleus, the vesicles align; they then fuse with the outer membrane, thus freeing the nucleus and a small amount of cytoplasm from the rest of the still intact muscle mass (Hay, 1974). Aggregates of these cells would then form the progenitor cells in the blastema and would proliferate. This idea for the mechanism of how differentiated muscle fibres would dedifferentiate was deduced from static images, so the possibility still remained that the mechanism was different or that images in fact showed fusion of mononucleate cells to form new muscle fibres.

Images of Blastema Formation

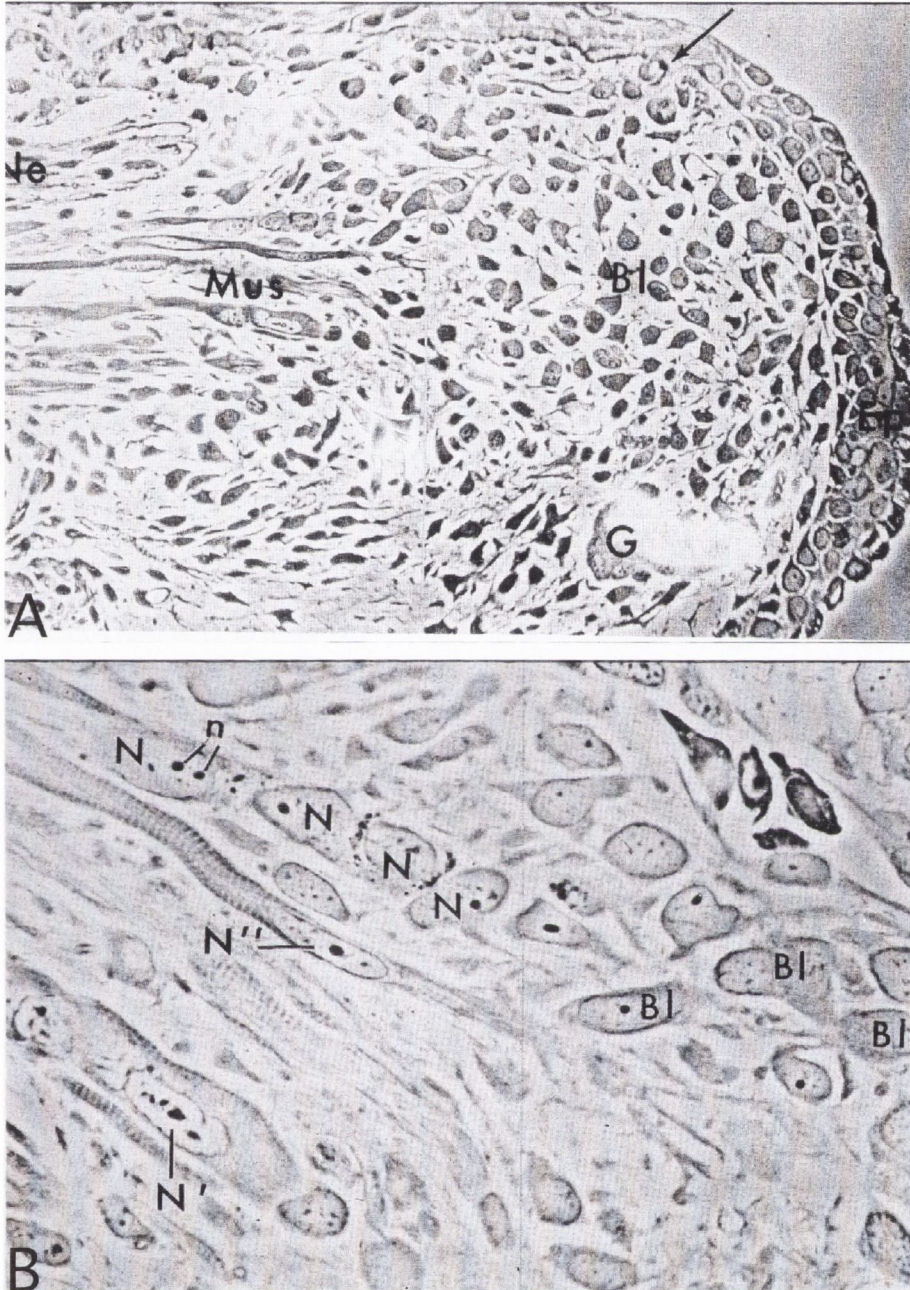


Fig B. Phase contrast photomicrograph of an unstained section of a regenerating limb 5 days after amputation (A). This section passes lateral to the humerus and illustrates a dedifferentiating muscle (Mus). Numerous blastema cells (Bl), most of which derived from muscle, appear distal to the intact muscle fibres. The tip epidermis (Ep) is slightly thickened. A multinucleate giant cell (G) is associated

with a remnant of cartilage and part of a nerve (Ne) appears in the proximal portion of the section. Magnification: x 140.

Enlarged photomicrograph of the tips of the dedifferentiating muscle fibres illustrated in (A). Typical blastema cells with relatively little cytoplasm appear on the right (Bl). A series of nuclei (N) belonging to muscle units that have almost completed the transformation to blastema cells appears on the left. During the transformation, nuclei change from the elongate shape typical of differentiated muscle (N'') to a more oval shape (N') and finally become rounded (N). Nucleoli (n) become prominent. Myofibrils have partly disappeared from the cytoplasm of the cell the nucleus of which is labelled N' and are no longer detectable with the light microscope in the cytoplasm of the cells labelled N. Magnification: x 500.

(Hay, ED, 1959, Dev. Biology).

To further confirm these electron micrograph studies Hay went on to use tritiated thymidine which incorporates into dividing cells as a method to follow the migration of dividing cells from the stump into the blastema (Hay and Fischman, 1961). In the first series of experiments they injected the isotope into animals between 1-28 days after amputation and fixed on the same day. From this they established that DNA synthesis begins 4-5 days post amputation in dedifferentiating mesodermal stump tissues approximately 1mm proximal to the amputation plane. They observed increased rates of DNA synthesis 10-20 days after amputation in cells of the stump but that the epithelium that migrates over and covers the wound discontinues DNA synthesis two days after amputation (Hay and Fischman, 1961).

In another set of experiments they injected tritiated thymidine prior to limb amputation and found that the epidermis incorporated the label but the internal tissues did not. After amputation labelled epidermal cells migrated over the wound, an apical epidermal ridge formed and remained labelled throughout blastema formation (Hay and Fischman, 1961).

The tritiated thymidine studies carried out by Hay also revealed that DNA synthesis occurred in muscle fibres at the same time as the nuclear enlargement and budding (Hay and Fischman, 1961). This result suggested that cell cycle re-entry was initiated prior to formation of the mononucleate cells, which would then proliferate to populate the blastema.

The limb mesenchyme is known to contain a number of cell types, especially cartilage, muscle and interstitial fibroblasts and although there are anatomical

descriptions of reversal of the differentiated state (Thornton, 1938, Chalkley, 1954, Hay, 1959), it is also necessary to introduce cell markers or lineage tracers to follow the fate of differentiated cells. This was carried out by Steen by implanting a piece of labelled cartilage beneath the wound epidermis, and sectioning at various time points after amputation the label was found in mononucleate cells of the blastema and eventually in connective tissue and cartilage of the regenerate (Steen, 1968).

Of the potential origins of blastemal cells put forth from the old literature, there is very little evidence to support the idea of blood cells giving rise to blastemal cells, although the blood most likely contributes macrophages and lymphocytes. Reserve or stem cells remains a possibility as they have been identified in newts, but their contribution to the blastema has not been accurately calculated and in other salamanders like axolotls they have not been found. At this point the most convincing evidence to support the theory of dedifferentiation comes from electron micrographs, tritiated thymidine labelling and tissue implantation experiments.

Recent experimental evidence to support dedifferentiation

The role of dermis in regeneration has remained uncertain for many years. In recent times Muneoka *et al* have taken advantage of the triploid/diploid cell marker system in the axolotl and analysed to what extent cells derived from dermis and skeletal elements contribute to the blastema (Muneoka *et al.*, 1986). Using this method they could successfully calculate that 43% of the blastemal cell population comes from dermis while only 2% of the cells come from skeletal elements. When

they compared this to the availability of cells at the plane of amputation, dermal cells over contributed by greater than ten fold whereas skeletal elements under contributed by several fold (Muneoka *et al.*, 1986). Their data correlates very nicely with previous research showing the effects these two tissues types have on pattern formation of the limb pattern during regeneration. Dermis has been shown to have dramatic effects such that a small implant of skin strips of dermis can induce supernumerary limbs (Tank, 1981). Skeletal tissue on the other hand appears to have no influence over patterning, when the skeleton of the limb stump was rotated 180° (Carlson, 1974; Carlson, 1975) or when a limb stump was supplied with an extra skeletal element (Goss, 1956), no major effect on the pattern of the subsequent regenerate was observed.

As dermis is known to contribute 43% of the cells in the blastema it is important to consider which cell types may be contributing to the blastema. The dermis of the axolotl is composed of two morphologically distinct cell types, fibroblasts and pigment cells arranged in a network of extracellular fibres (Holder and Glade, 1984). Pigment cells can be eliminated as being important for regeneration process as it is known that limbs of the axolotl mutant *d/d*, which lack these cells, regenerate perfectly (Wallace and Wallace, 1973). Fibroblasts are by far the most abundant cell type in the dermis and are almost certainly responsible for the dermal contribution to the blastema, as well as for the patterning role of the dermis. The underlying molecular mechanisms by which dermis cells actually influence the patterning of the regenerate is still poorly understood.

Dermis contributes 43% of the cells in the blastema, so where do the other 47% come from? Lo showed the first experimental evidence to support the 'budding off' theory of progenitor cell production (Lo *et al.*, 1993). When newt limb cells are propagated in culture they are able to divide for at least two hundred generations without any evidence of crisis or senescence (Ferretti and Brockes, 1988). When these newt myoblasts are placed in low serum they can form myotubes, which express known markers of muscle differentiation like myosin heavy chain (Lo *et al.*, 1993). Lo implanted size selected multinucleated newt myotubes, which had been formed in culture and injected with rhodamine dextran, into a regenerating newt limb (Fig. C & D). After one week, the lineage label was found in mononucleate cells (Fig. D). The number of labelled cells was such that at least 15-20% of implanted muscle fibres must have undergone dedifferentiation. The average number of mononucleate cells increased over time, suggesting that cell division had occurred. As the regenerate began to differentiate the lineage label was thought to be found in differentiating cartilage, an interesting observation as this would imply that mononucleate cells derived from multinucleated muscle fibers become very plastic after entering the environment of the blastema and can form other non-muscle cell types.

Recently these implantation experiments were repeated using an integrated retroviral marker, reducing the possibility that mononucleate cells were derived from cytoplasmic transfer of the lineage tracer, and similar results were found (Kumar *et al.*, 2000). Using BrdU injections, Kumar *et al.* also showed that some nuclei of the retrovirally labelled implanted myotubes re-entered S-phase,

although it was unknown if these same nuclei after replicating their DNA would then 'bud off' and populate the regeneration blastema. Although Lo and Kumar's experiments strongly support the theory of dedifferentiation, the question still remains whether endogenous muscle fibres actually dedifferentiate to produce a large number of cells of the blastema, and is this event actually a significant contributor to the blastema.

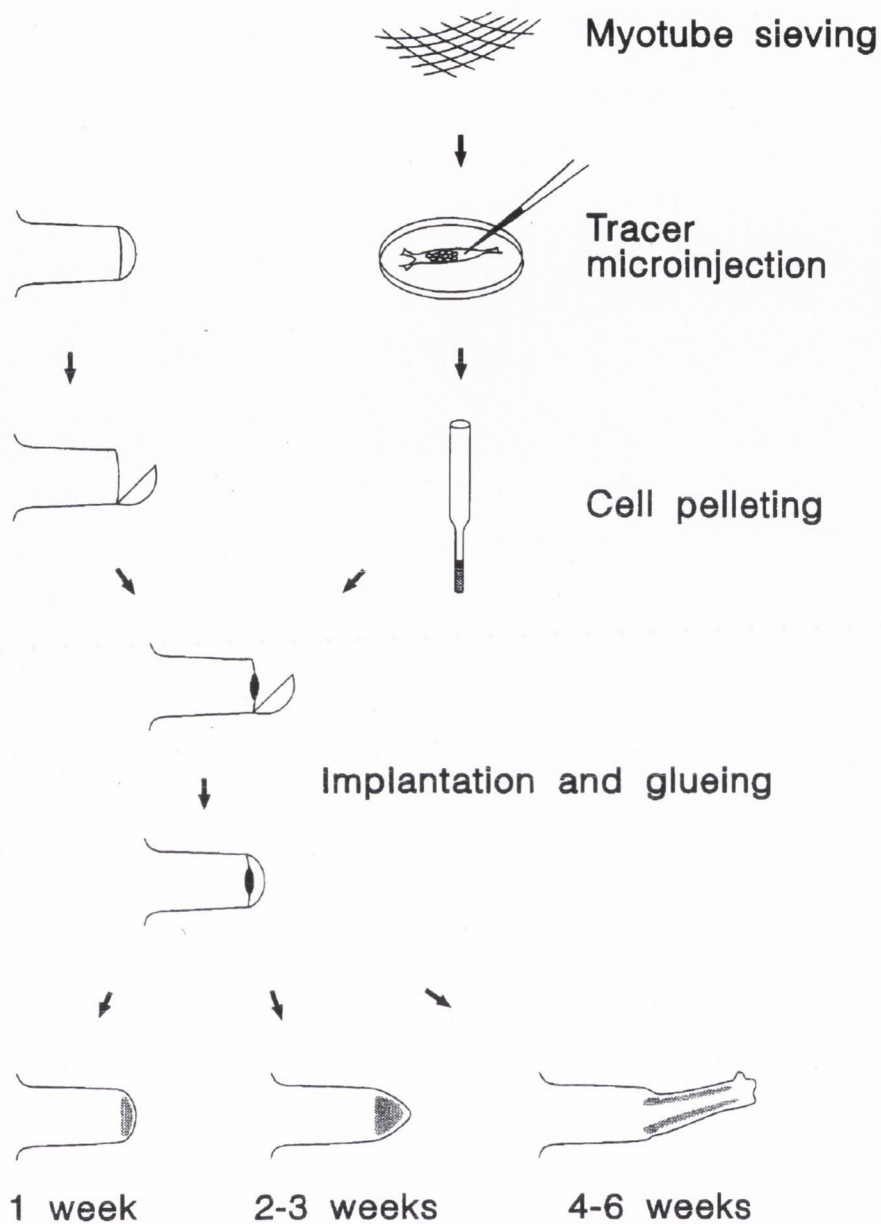


Fig. C Schematic representation of an implantation experiment. Lineage tracer was injected into individual myotubes previously induced in culture and purified; labelled myotubes were pelleted and implanted beneath the wound epidermis. Limbs were allowed to regenerate and blastemal tissue was collected after three periods shown. (Lo *et al*, 1993, PNAS)

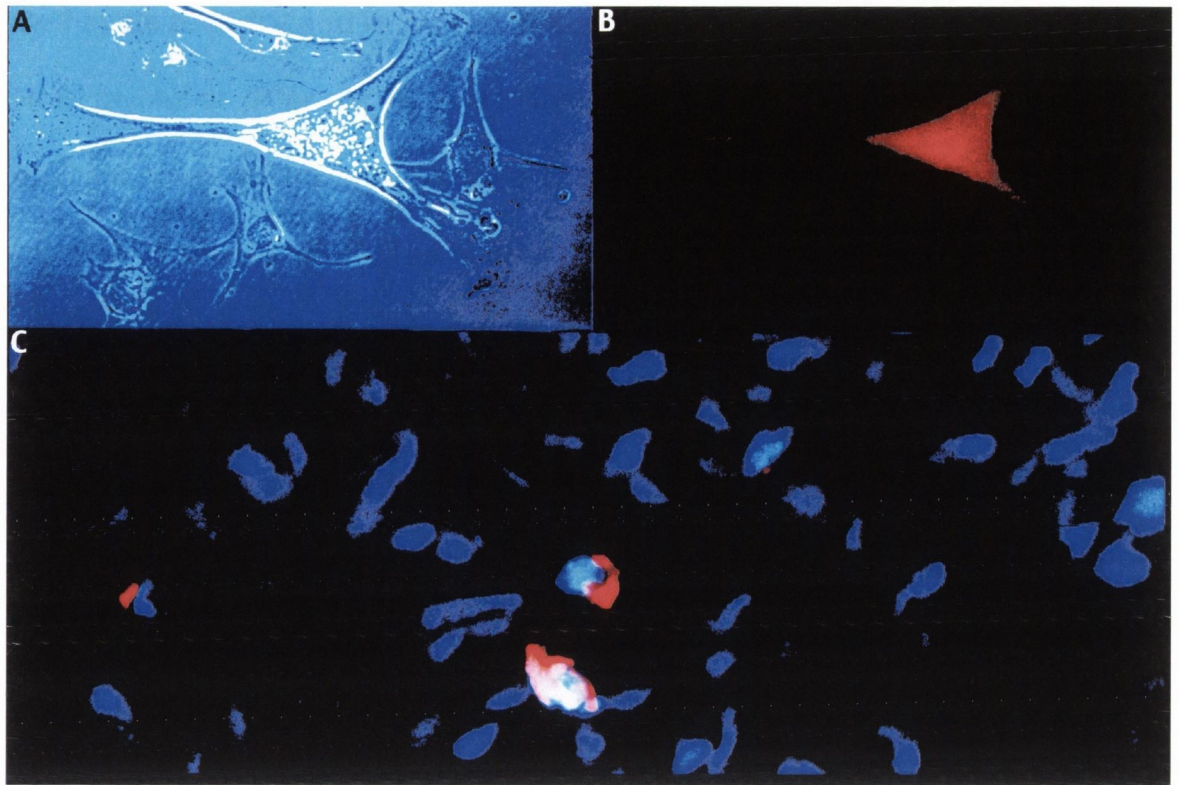


Fig. D Labelling of cultured newt myotubes by injection with a fluorescent lineage tracer. Myotube that received the injection of rhodamine lysinated dextran was surrounded by mononucleate cells. (A) Phase contrast microscopy. (B) Fluorescent optics. (C) Example of labelled mononucleate cells in sections of regenerating limbs 9-10 days after implantation of labelled myotubes, two mononucleate cells with rhodamine labelled cytoplasm and Hoeschst stained nucleus (Lo *et al*, 1993, PNAS).

Cell cycle re-entry and regeneration

Newt myotubes differ significantly to their other vertebrate counterparts in their ability to enter and traverse S-phase after serum stimulation in culture (Tanaka *et al.*, 1997, Tanaka, 1999). The nuclei double their DNA and become arrested in G2. The response to serum is not observed in other vertebrate myotubes, with the exception of mouse cells lacking both copies of the retinoblastoma gene (Rb), pRb has an essential role in regulating the G1-S transition (Schneider *et al.*, 1994). Other data suggests that pRb is important for maintenance of the differentiated state in vertebrate myotubes, not only for the stable arrest from the cell cycle (Schneider *et al.*, 1994), but also for transcription from certain muscle promoters that depend on activation of members of the myocyte enhancer factor 2 (MEF2) family of transcription factors (Novitch *et al.*, 1999). Newt myotubes express pRB but serum stimulates a pathway that leads to its inactivation by phosphorylation and hence triggers the transition from G1-S phase (Tanaka *et al.*, 1997). If a plasmid encoding mammalian p16, a specific inhibitor of the cyclinD-cdk4 , a protein kinase that inactivates RB is injected into newt myotubes, they are effectively blocked from entering S-phase after serum stimulation (Tanaka *et al.*, 1997). This data shows the first evidence that newt myotubes are intrinsically different from their mammalian counterparts. The fact that newt myotubes can activate a pathway that leads to phosphorylation of pRB and re-entry to the cell cycle is suggestive that the regulation of this pathway is important to regeneration. The activity found in serum which is active on newt myotubes is not due to the presence of typical protein growth factors like platelet derived growth factor

(PDGF) or epidermal growth factor (EGF); these factors act on mononucleate myoblasts but not on myotubes (Tanaka *et al.*, 1997b, Tanaka, 1999). This activity in serum has no effect on mouse myotubes. Serum is the soluble fraction of clotted blood and results from the activation of prothrombin to generate the serine protease thrombin. Thrombin activates the clotting cascade and various other events that mediate the response to injury. When crude prothrombin is activated *in vitro* , the resulting thrombin preparations contain a distinct activity that acts directly on newt myotubes in serum free medium (Tanaka *et al.*, 1999). Tanaka . postulate that this ligand is generated downstream of prothrombin activation both in regeneration and in culture and it is this factor that acts on the myotubes and other differentiated cells to promote re-entry (Tanaka *et al.*, 1999).

The identity of the serum factor is unknown, but it could be a signal related to wounding or clotting that acts on myotubes and other differentiated cells. The response in culture is inhibited by contact with other cells and it may be significant that blastema formation is associated with increased induction of proteolytic activity (Grillo *et al.*, 1968). An interesting example of this is the expression of a matrix metalloprotease in the mesenchyme 3 to 4 hours after amputation (Yang and Bryant, 1994). As well as permitting cells to escape from their matrix and migrate into the blastema, these activities may also facilitate reversal by disrupting cell contacts and allowing responses to soluble factors.

Very little is known to date about the molecular mechanisms that cause myotubes to fragment and form mononucleate cells. An interesting study was carried out in

mouse myotubes, whereby mononucleate myoblasts were stably transfected with the homeobox gene *Msx-1* linked to a conditional promoter and shown to dedifferentiate and form mononucleate cells (Odelberg *et al.*, 2000). Previous studies had indicated that *Msx* genes promote cell proliferation and that their expression is inversely correlated with differentiation (Song *et al.*, 1992, Woloshin P, 1995). Odelberg *et al* showed that after fusion of the stably transfected myoblasts, expression of *Msx-1* in myotubes was induced and this lead to a decrease in the expression of myogenic regulatory genes. About 50% of the myotubes were induced to cleave into viable fragments (smaller myotubes), and another 5% fragmented into mononucleate cells which proliferated. In some cases the clonal progeny of a single myotube were isolated, propagated and shown to be capable of either chondrogenic, adipogenic or myogenic differentiation depending on the culture conditions (Odelberg *et al.*, 2000). As *Msx-1* has been found upregulated in axolotl limb regeneration, it has been proposed that *Msx-1* is a master regulator of the programme for fragmentation that is expressed in urodele regeneration (Koshiba *et al.*, 1998) and that it can also induce this programme in mammalian myotubes.

Positional identity of the Blastema

Epimorphic regeneration provides an accessible context in which to study how positional identity is encoded and is manifested in cellular properties that are important for tissue patterning such as proliferation, adhesion and migration. Research has shown that juxtaposition of blastema cells from different axial levels suggests that these cells have different intrinsic properties (Nardi and Stocum, 1983, Crawford, 1988, Pescitelli, 1980). When two blastema mesenchymes from different proximal-distal levels are juxtaposed in a hanging drop culture, the more proximal member engulfs the distal one, whereas two from the same position maintain a stable boundary. This behaviour is suggestive of a graded difference in the surface adhesivity of blastema cells along the axis. These differences are also observed when a distal blastema is grafted to the dorsal surface of a proximal blastema *in situ* so that the mesenchymal cells are in contact. Under these conditions it moves during regeneration to the distal level that is identical to its level of origin. In another assay, a distal blastema from the wrist level for example, is transplanted onto a shoulder stump so that the wrist and shoulder are juxtaposed, a classic experiment referred to as intercalary regeneration. The result is that a normal limb in which the structure between the shoulder and wrist are regenerated predominantly by growth from the proximal partner, whereas the cells from the wrist blastema give rise to the hand (Nardi and Stocum, 1983, Crawford, 1988, Pescitelli, 1980, Pecorino, 1996). These assays suggest that proximal distal identity in urodele regeneration is encoded as a graded property, probably in some part at the cell surface and that cell behaviour relevant to the axial

specification like growth, movement and adhesion is a function of the expression of this property relative to neighbouring cells.

It is not clear to what extent blastema cells may inherit their positional identity, maybe from differentiated precursor cells or to what extent they are they are subject to signals that induce expression. The role of Hox genes in positional identity during limb regeneration is not clearly understood, but the expression of HoxA9 and A13 in the mesenchyme is thought to be an early indication of local specification as early as 1 to 2 days post amputation in newt limbs (Savard *et al.*, 1988, Brown and Brockes, 1991, Gardiner *et al.*, 1995, Simon *et al.*, 1997). The early dedifferentiation stages of regeneration have been shown to be exposed to a variety of signals from wound epidermis and from the general injury response, retinoic acid (RA) (Maden, 1982), hedgehog protein (Roy *et al.*, 2000) and FGF (Boilly *et al.*, 1991). It is unclear yet if these signalling molecules are involved with establishing positional identity, the most realistic models for pattern formation in limb regeneration stress the critical role of local interactions between blastema cells (French *et al.*, 1976, Gardiner, 1989). For example, in the early stages of regeneration, cells migrate into the centre of the blastema and make contact allowing such interactions to happen (Gardiner and Bryant, 1989). It is also a possibility that after amputation at a particular proximal distal location, cells arise with the appropriate level specific identity and then generate more distal positions by successive local interactions (French *et al.*, 1976, Gardiner, 1989, Gardiner, 1995).

Retinoic acid (RA) is known to respecify the proximal distal (PD) axis during limb regeneration (Maden, 1982) and under some circumstances to respecify the dorsoventral and anterioposterior axes (Stocum, 1991), furthermore it can switch the identity of a tail blastema in some anuran tadpoles so that it gives rise to limbs (Mohanty - Hejmadi *et al.*, 1992). If a distal limb blastema is exposed to RA for 48 hours, the blastema cells are respecified to a more proximal level resulting in a serial duplication of certain proximal structures. The mechanism of proximalization has been clarified by experiments in which the RA response pathway has been activated in individual distal blastema cells, rather than by global application of RA (Pecorino *et al.*, 1996). The proximalizing activity is mediated by activation of RA receptors (RARs), which are ligand dependant transcription factors of the nuclear receptor family. Five different newt RARs have been cloned and shown to be expressed in cells of the limb and limb blastema (Ragsdale *et al.*, 1989, Ragsdale, 1992, Ragsdale, 1993). One receptor RAR β 2 has been shown to be necessary and sufficient to proximalize the distribution of transfected cells in intercalary regeneration (Pecorino *et al.*, 1996). The effect of RA on PD identity is mediated by RAR β 2, the urodele equivalent of mammalian RAR γ 2 (Ragsdale *et al.*, 1989, Ragsdale, 1992, Ragsdale, 1993). During regeneration RA acts directly on cells to respecify their identity, in contrast to its effect on limb development, in which local application to the bud is thought to induce a signalling centre that acts locally with the epidermis to respecify the underlying mesenchyme. It seems unlikely that RA might act indirectly on PD identity by promoting dedifferentiation or inhibiting proliferation; the effect on proliferation is known to be mediated by a

different receptor, RAR1 (Schilthuis *et al.*, 1993). This research also shows that PD identity is an isoform specific target of RAR β 2 activation, an observation which promotes approaches to identify target genes of β 2 in blastemal cells that are not common to activation of the closely related β 1 as a means to further understand the role of RA in specifying PD identity during limb regeneration.

Spinal Cord Regeneration

Fish and urodele amphibians have the amazing ability to successfully regenerate their mature spinal cords following either complete cord transection or following tail amputation. Some degree of regeneration is also possible in reptiles and metamorphosing anuran amphibians. Spinal cord regeneration occurs in teleost fish (Anderson *et al.*, 1986; Anderson *et al.*, 1994), in anuran amphibians before metamorphosis (Beattie *et al.*, 1990), in the tail spinal cord of lizards (Simpson, 1968, Duffy, 1992), and during embryonic and fetal development in birds and mammals (Shimizu *et al.*, 1990, Hasan, 1993, Iwashita, 1994). Table A summarises the various regeneration capabilities across species. Changes in regenerative ability are most likely due to combinations of several factors such as cellular and molecular changes acquired during evolution, environmental differences between embryonic and adult central nervous system (CNS) and a decrease in the number of neural progenitors, or the ability to recruit them *in vivo* in the adult central nervous system of higher vertebrates. Recent evidence of the existence of multipotent neural progenitors in adult mammalian CNS and of much higher neural cell plasticity, at least *in vitro* than previously thought makes the understanding of how adult urodeles can spontaneously regenerate a fully functional spinal cord an important question to be answered (Gage, 2000, Morshead, 2001, Vescovi, 2001, Song, 2002)

In anuran amphibians, regeneration following spinal cord transection fails after metamorphosis (Beattie *et al.*, 1990), and virtually all central nervous system

regeneration fails in higher vertebrates, leaving urodele amphibians as the only tetrapod vertebrates that can regenerate all regions of the spinal cord as adults. Lampreys are primitive agnathan fish capable of considerable anatomical and functional recovery following complete transection of the spinal cord both as larvae (Rovainen, 1976, Selzer, 1978) and as adults (Cohen *et al.*, 1989, Lurie, 1991). Eight weeks after a spinal transection locomotor movements and the coordination of muscle activity across the transection have returned to normal (Davis *et al.*, 1993). Retrograde labelling studies have shown that almost all brainstem and spinal descending axons are capable of long distance axon regeneration (Davis and McClellan, 1994) and axonal regrowth follows appropriate pathways (Yin *et al.*, 1984, Mackler, 1986).

In anurans successful axonal regeneration following spinal cord transection depends on developmental stage and species. There is no regrowth across a transection in adult spinal cord (Piatt and Piatt, 1958, Forehand, 1982). Generally axon repair is good in young larvae and becomes increasingly poor in older larvae (Hooker, 1925; Clarke *et al.*, 1986), but the capabilities of different axon tracts can change at different times during development. For example, ascending sensory tracts in the dorsal funiculus of *Rana temporaria* lose their ability to grow across a transection before the descending tracts in the ventral funiculus (Clarke *et al.*, 1986). Also in the diencephalons neighbouring axon tracts can display quite different regenerative responses, which may be explained by intrinsic differences or differences in their microenvironment (Lyon and Stelzner, 1987).

Metamorphosis seems to be a very permissive time for spinal repair as both the

number of axons crossing a transection and the length of axonal growth past a transection are improved during metamorphosis compared to late larval stages (Forehand and Farl, 1982).

In contrast in lizards the regenerated structure contains only ependymal cells and many axons but no neurons (Egar *et al.*, 1970). Axons come largely from local spinal neurons just rostral to the plane of amputation and only 4% of the normal population of supraspinal axons regenerate into the new structure (Duffy *et al.*, 1992). The axons that grow down into the regenerated cord structure do not arise from newborn neurons and at least 28% of the axons in the regenerate are regenerated after axon transection, but most may be sprouts from local neurons that previously had no projections down into the tail cord (Duffy *et al.*, 1992). Duffy *et al* suggest that descending axons may be prevented from entering the regenerate by 'synaptic capture' in the segments rostral to the transection. Neurons in this region lose their synaptic input from ascending axons because of the transection, they replenish these synaptic sites with new contacts from descending axons that are stimulated to sprout by the transection; and it is the formation of these synapses that stabilizes the growth of these axons and thus prevents them from growing into the regenerate (Duffy *et al.*, 1992).

Adult urodele amphibians can fully regenerate their spinal cords following a lesion, crush or full transection. Retrograde labelling studies with horseradish peroxidase (HRP) have demonstrated substantial axonal growth across mid-trunk spinal transactions in urodeles. In the axolotl the number of brainstem neurons with axons that reach to a position 1.5cm caudal to the transection site is almost 70%

of normal number at 23 months post transection (Clarke *et al.*, 1988). In the newt brainstem 40% of normal neuronal numbers have axons at least 1 cm caudal to the transection after 60 days (Davis *et al.*, 1989). In both studies axons from all the normal brainstem nuclei have crossed the transection. It seems likely that most of this axon growth is from transected and regenerated axons rather than from a sprouting from uninjured axons in the vicinity of the transection, but direct evidence of this is till lacking. Fig. E shows a schematic of how spinal cord transections are thought to be repaired in the larval urodele.

Table A Summary of repair processes across various species (Holder, N and Clarke, JDW, 1988, TINS)

Animal	System	Developmental state of tissue
Type 1 repair: Axons grow through damaged area, negotiate a normal path and make appropriate connections with their normal targets.		
Goldfish	Retinotectal connection	Neurogenesis of retinal ganglion cells
<i>Xenopus laevis</i>	Retinotectal connection	Neurogenesis of retinal ganglion cells
<i>Rana pipiens</i>	Retinotectal connection	Neurogenesis of retinal ganglion cells
<i>Rana pipiens</i>	VIIIth nerve	Neurogenesis of VIIIth ganglion not demonstrated
Axolotl	Limb motor system	Neurogenesis of motoneurons
<i>Rana catesbeiana</i>	Limb motor system	Early tadpole stages during neuron birth
Rat	Facial and intercostal motor systems	Neonatal stages only
Rat	Olfactory nerve and vomeronasal nerve	Continuous neurogenesis by basal stem division in olfactory epithelium
Hamster	Lateral olfactory tract	Repair restricted to period of normal axon growth
Rat	Preganglionic fibres to superior cervical ganglion	Neurogenesis ceases before birth
Probable type 1 repair:		
Axolotl	Spinal cord	Neurogenesis demonstrated in cord and hindbrain; specific pathways and connectivity not yet shown
<i>Rana temporaria</i>	Spinal cord	Repair restricted to period of normal axonal growth in tadpole; normal terminal specificity not shown
Type 2 repair: Axons grow across damaged area, fail to re-establish their normal path, yet make synapses that allow functional recovery.		
Larval lamprey	Spinal cord	Continued neurogenesis not established, but animal is a continuously growing larva
Goldfish	Spinal cord	Specific connectivity not yet shown; neurogenesis demonstrated in CNS of some fish (e.g. guppy and stingray) but not yet in goldfish
Hamster	Corticospinal tract	Repair restricted to period of normal axon growth in neonate
Type 3 repair: Axons grow across damaged area, fail to locate their normal paths and make synapses resulting in paralysis.		
All higher vertebrates examined	Peripheral motor nerves	Neurogenesis ceases before birth

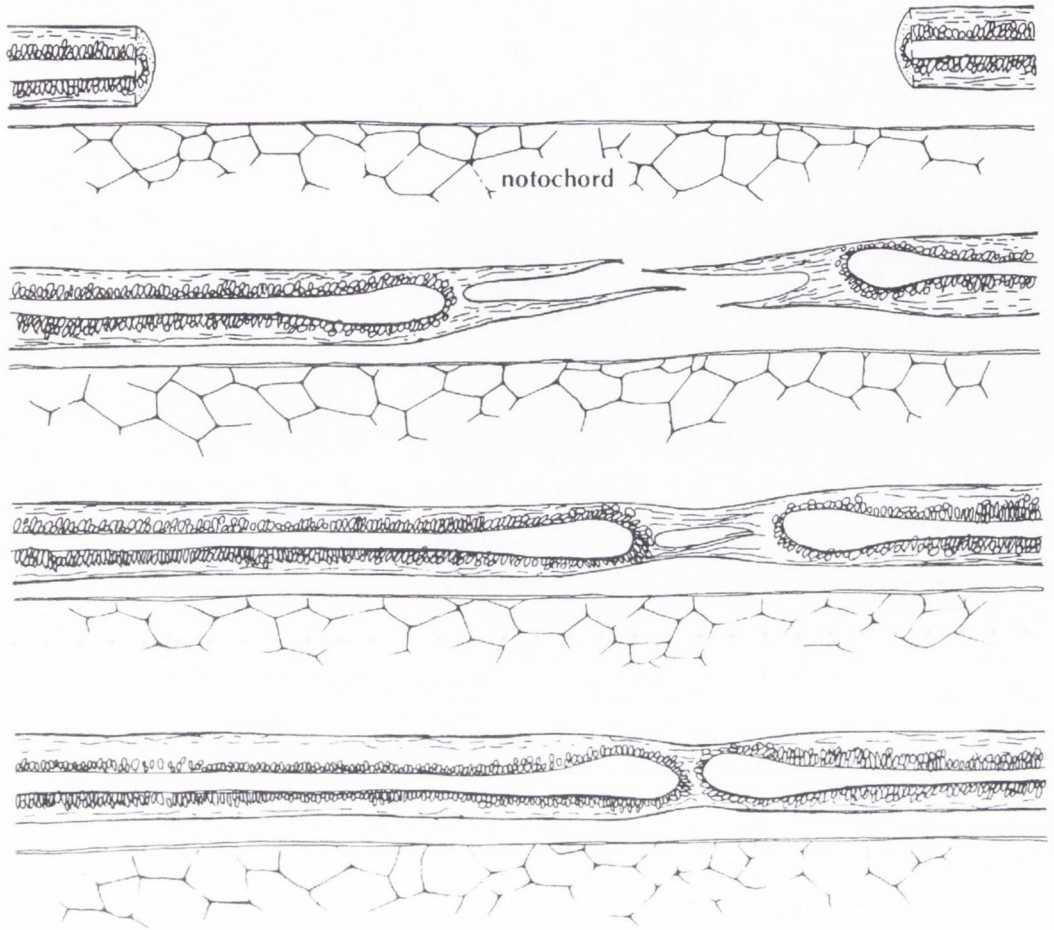


Fig. E Stages in the regeneration of a 2mm length of spinal cord in the larval salamander. Neural tube grows faster from the anterior than the posterior end. Regenerating nerve fibres grow out from either end and establish first contacts. Outgrowing neural canals swollen into terminal vesicles later meet and fuse. (Butler and Ward, 1965, J. Exp. Zoology)

Structure of the Normal Axolotl Spinal Cord

The urodele spinal cord consists of a neural canal surrounded by a layer of radial glial that extend processes out towards the pial surface. The radial glial cell layer is surrounded on the outside by an astrocyte layer and outside that lie the neuronal cell bodies and axons (Zamora, 1978).

The mature urodele spinal cord distinguishes itself from that of birds and mammals in that it maintains a network of radial glial cells that extend from the pial surface to the ventricular surface throughout life. The cells typically have one process that stretches from the grey matter, into the white matter where it branches. Holder *et al* have shown that the branching pattern achieved actually depends upon the radial glial cells dorsoventral position within the spinal cord (Holder *et al.*, 1990). Whether these radial glial cells, which are interchangeably referred to as ependymal cells are really homologous to mammalian ependymal cells is unknown. Structurally axolotl ependymal cells are more similar to the mammalian embryonic ependymal cells than to the adult and histological evidence indicates that there are differences in antigenic reactivity between the two cell types.

Radial glial cells are widespread throughout the developing CNS of vertebrates. They are best known for their role in guiding migrating neurons (Rakic, 1972, Hatten, 1999). There is good evidence to show that radial glial cells divide during neuro- and gliogenesis, since they incorporate S-phase markers such as tritiated thymidine (Levitt *et al.*, 1981, Mission, 1988). Radial glial cells are characterized by their morphology with a characteristic long radial process reaching the basal

surface of the brain as well as by their astroglia characteristics. For example, radial glial of the developing primate cortex contain the glial fibrillary acidic protein (GFAP) (Levitt and Rakic, 1980, Choi, 1981). It has also been shown that radial glial cells transform into astrocytes at later stages after neurogenesis and neuronal migration are completed (Voigt, 1989, Edwards, 1990, Culican, 1990). This is interesting in light of recent research suggesting that astrocytes are stem cells in the adult telencephalon (Doetsch *et al.*, 1999)

Urodele ependymal cells contain glial fibrillary acidic protein (GFAP) (Arsanto *et al.*, 1992). GFAP is an intermediate filament found in mature astrocytes, which is thought to be important for astrocyte – neuronal interactions. In mammals GFAP is not normally expressed in mature radial glial cells but is upregulated during glial cell maturation and in response to central nervous system injury (Chen and Liem, 1994). Vimentin, another interfilament protein is expressed in mammalian radial glial cells and is co-expressed with GFAP during astrocyte maturation but is not found in axolotl radial glial cells (O'Hara *et al.*, 1992). Axolotl radial glial cells also express simple epithelial keratins 8 and 18 (Holder *et al.*, 1990; O'Hara *et al.*, 1992), keratin expression has also been seen in the ependymal cells of other adult vertebrates which have the ability to regenerate their central nervous system such as fish and lamprey. A relationship between intermediate filament expression and the ability to regenerate has been postulated but no direct evidence has been found.

The ependymal cells of the spinal cord of the developing salamander, *Pleurodeles waltl* seem to synthesize the extracellular matrix molecule tenascin

and tenascin immunoreactivity is observed around the ependymoglia cells and in the axon tracts of the spinal cord (Caubit *et al.*, 1994). Tenascin expression remains high in the axon tracts in the adult spinal cord but is low in the ependymal zone. Expression of the adhesion molecules of the neural cell adhesion molecule (N-CAM) family is observed in the developing ependymoglia and in the nervous tracts of the spinal cord. An antibody to all N-CAM family members shows strong reactivity also in the adult spinal cord, an antibody specific to the embryonic isoforms polysialylated(PSA)-NCAM shows that this molecule is significantly down-regulated in the normal adult spinal cord where it becomes restricted to the ependymal cells (Caubit *et al.*, 1993).

Ependymal cells of the newt spinal cord have been suggested to be a heterogeneous population based on observations from electron microscopic observations (Schonbach, 1969, Egar, 1972). This observation is important in view of the search to identify which cells are responsible for the regenerative potential of these animals. Non –radial astrocytes are thought to be also present in the newt spinal cord (Egar and Singer, 1972).

An unusual feature of the urodele and fish central nervous system is their ability to continue to generate new neurons at a low level throughout life. There is an age-related increase in the number of spinal neurons in several fish (Leonard *et al.*, 1978, Birse, 1980) and a tritiated thymidine study has shown neurogenesis in the mature axolotl spinal cord (Holder *et al.*, 1991). These data suggest that in contrast to mammals, some cells within the mature spinal cord of fish and

urodele spinal cords retain a neurogenic role even in the absence of any injury and this may in fact be related to their ability to regenerate.

Initiation of Spinal Cord Regeneration

The steps of spinal cord regeneration appear to be increased proliferation of the cells adjacent to the plane of amputation, formation of a terminal vesicle to seal off the end of the cut spinal cord and the migration of cells towards the terminal vesicle (Piatt, 1955, Holtzer, 1956; Egar and Singer, 1972).

The first proliferative response of the radial glial cells has been reported to be in the cells adjacent to the plane of amputation but not directly at it (Holtzer, 1956; Nordlander and Singer, 1978). A terminal vesicle forms at the end of the spinal cord within 4 days post amputation and these cells are described as having a more cuboidal shape instead of the normal columnar shape of ependymal cells (Fig. F). The function of the terminal vesicle is not completely clear but its function is thought to be simply to seal off the end of the cut spinal cord (Holtzer, 1956). Cells then migrate out from the remaining mature spinal cord to re-establish a neural tube and once this occurs mitotic figures are reported to be seen in equal numbers along its length suggesting that growth occurs by intercalation and not from one specific growth zone (Holtzer, 1956).

Interestingly Holtzer also showed that the zone of mature spinal cord lying next to the plane of amputation is in fact essential for regeneration to occur, if the mature segments adjacent to the plane of amputation are removed after transection, the wound heals over but no blastema forms until the spinal cord re-

grows to reach the original position (Holtzer, 1956). This suggests that the cells closest to the plane of amputation play an important undefined role in directing blastema formation.

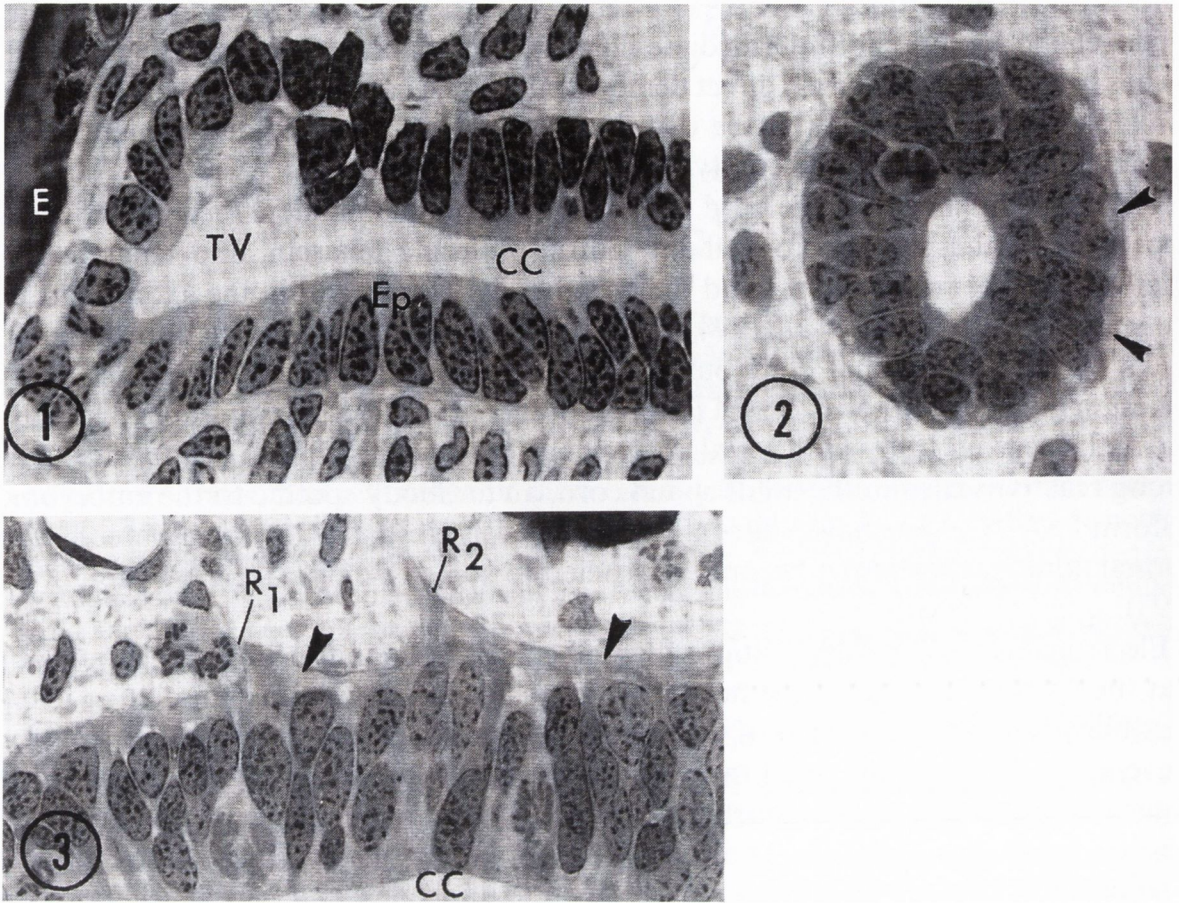


Fig. F (1) Longitudinal section through a 62 day regenerating newt tail. The simple neural tube is composed of ependymal cells (Ep) surrounding the central canal (CC). The terminal vesicle (TV) is very close to the epidermis (E) covering the regenerate. (2) Transverse section just behind the terminal vesicle: the regenerating neural tube closely resembles that seen during embryonic development. Channels between ependymal processes at the periphery of the tube contain axons (arrows). (3). Longitudinal section of a 21-day regenerate cord: note the presence of mitotic figures near the central canal (CC) and of ventral roots (R1 and R2) leaving the neural tube. Arrows indicate longitudinal channels along tube perimeter. (Nordlander and Singer, 1978, J. Comp. Neur.)

In urodele limb regeneration, the formation of the wound epidermis is a critical step in the regenerative process and this may also be the case for the formation of the tail blastema. If the wound epidermis is crucial for the success of spinal cord regeneration within the tail blastema is not known but the fact that the spinal cord regeneration is also successful following transection within the vertebral column instead of within a tail blastema suggests that this may not be the case. The structural processes of cord regeneration following resection or transection may be quite similar to those in tail regeneration since the success in both cases is based on the migration of ependymal cells and their processes away from their cut stumps (Butler, 1967). Retraction of cut stumps following transection means that considerable bridge building is required to bring the stumps back together again and this process can in fact rebuild the deficit caused by removal of several segments of thoracic cord (Butler, 1967).

Changes in the phenotype of ependymal cells after tail amputation or spinal cord transection have been reported, but their significance is still unknown and a lot of discrepancies occur in the literature which may be explained by different types of surgery, species difference or the specific reactivity of the antibodies used.

In axolotl's keratins, vimentin and GFAP all remain expressed in radial glial cells during regeneration. Up regulation of vimentin is observed in axolotl resected cords and in newt tails after amputation (O'Hara *et al.*, 1992, Ferretti, 1991), although with slightly different timecourses. In contrast, Margotta *et al* find that in *Triturus carnifex* vimentin is first down regulated for 10 days after amputation but then reexpressed (Margotta *et al.*, 1991). Co-expression of simple epithelial

keratins 8 and 18 and vimentin has been observed in many regenerating systems and it has been suggested that in axolotl changes in the expression of these filaments reflects an epithelial to mesenchymal transformation (O' Hara CM *et al.*, 1992, Chernoff, 1996). Vimentin expression in the regenerating ependymal cells may not reflect a real epithelial to mesenchymal transition but rather reflects a physiological change in the state of the cells themselves which allows them to divide at a higher rate than in the normal cord.

It has also been shown that molecules whose expression is tightly regulated during spinal cord development are reexpressed during regeneration. Tenascin expression during regeneration parallels that during development (Caubit *et al.*, 1994), and fibronectin which is expressed at a very low level in normal spinal cord has been shown to be upregulated between two to three weeks after spinal cord transection in the axolotl ependymal cells (O'Hara *et al.*, 1992). Also, the polysialylated embryonic form of neural adhesion molecule N-CAM, PSA-N-CAM, which is weakly expressed in the ependymal cells of normal cord, is highly up regulated in regenerating spinal cords (Caubit *et al.*, 1993). The fact that molecules expressed in the developing spinal cord are again expressed during regeneration suggests a close similarity between the molecular events underlying both processes.

Wnt-10, a member of a family of secreted glycoproteins which play an important role in the development of many body structures and particularly of the nervous system has been found to be slightly upregulated during regeneration (Caubit *et al.*, 1997). In the future it will be interesting to establish if this also occurs in other

salamander species and whether it is causally related to the regenerative ability of these animals.

Where do the cells come from to form the New Spinal Cord?

It is thought that the radial glial cells of the mature spinal cord have the ability to generate all cell types in the new regenerate, the possibility that even neurons at the plane of amputation may dedifferentiate has not been completely ruled out. The ependymal tube that forms after tail amputation in urodeles is thought to originate from the radial glial cells of the cord stump (Egar *et al.*, 1970, Nordlander, 1978), although it is not known whether all these cells or only a subset of them contribute to spinal cord regeneration. The radial glial cells are likely to be a heterogeneous cell population on the basis of morphologic and molecular differences (Holder *et al.*, 1990) and it is not known whether they are all multipotent or some of them are already committed to different lineages. The potential of radial glial cells has been investigated in several ways *in vivo* and *in vitro*. Clonal cell lines have been obtained from non-regenerating *Pleurodeles* spinal cord which expressed markers of glial cells like GFAP and vimentin. These cells were transfected with the lineage markers rhodamine dextran and a Lac Z construct. When the cells were implanted into the regenerating blastema they were found to have differentiated into melanocytes or Schwann cells (Benraiss *et al.*, 1996). Although it seems quite likely that the cells implanted are radial glial cells the possibility still remains that the cell cultures were contaminated with other cell types.

This study opened up the question of whether neural crest derivatives migrate out of the ependymal tube during regeneration like during development. To address this issue Benraiss . developed a technique of using biolistics to transfect cells at the cut surface of the spinal cord with a plasmid marker. The regenerates were later sectioned and the label was found in Schwann cells and melanocytes, suggesting that neural crest-like cells migrate out of the spinal cord during regeneration and partake in regeneration of the peripheral nervous system (Benraiss *et al.*, 1997). Their data also suggested that the route of exit was most likely to be via the terminal vesicle, which to them appeared to be a very loosely organised structure contrary to Holtzer's previous description of it (Holtzer, 1956; Benraiss *et al.*, 1997).

These experiments still lacked the ability to actually follow the cells *in vivo* and the possibility that cells lying just outside the spinal cord were labelled also remains. But these experiments again reinforce the idea that a lot of events, which occur during development, are recapitulated during regeneration. They also bring forth again the role of the terminal vesicle and whether the end of the spinal cord is really a tightly closed structure that prevents cell mixing from occurring between the cells of the spinal cord and the blastema. Also if cells come out of the spinal cord are they restricted to become cells of the ectoderm lineage only?

Neuron Regeneration

The main source of neurons in the regenerating spinal cord is thought to be from the radial glial cells that form the simple neural tube that first extends down into the regenerating tail blastema. Here, these cells continue to divide and some generate new neurons. The evidence for this comes from studies that follow the fate of 5' bromo 2' deoxyuridine (BrdU) positive ependymal cells in the regenerating neural tube (Benraiss *et al.*, 1999) and from *in vitro* studies of ependymal cells derived from regenerating fish spinal cord (Anderson *et al.*, 1994). The possibility that neurons at the site of injury may be able to contribute to the cellular makeup of the regenerating cord has not been ruled out. Zhang *et al.* have recently shown that spinal cord cells immediately adjacent to the amputation plane die and are removed by phagocytic cells and that post mitotic neurons adjacent to these cells translocate into the regenerating spinal cord (Zhang *et al.*, 2003). The mechanism by which the neurons translocate is not known but one possibility would be that they are attracted into the regenerate by a chemical signal and then actively migrate by chemotaxis. Or they may be pushed or carried into the regenerate by the proliferation, cellular rearrangement and migration of the radial glial cells that surround them. It is quite likely that, in the normal spinal cord, the radial ependymal cells play a similar role to the mammalian system in supporting the migration of new born neurons (Hatten, 1999). If so, the cell surface of radial ependyma is likely to express molecules that promote their association with neurons, thus, supporting their migration into the regenerating spinal cord. Once the regenerating neural tube is established,

mitotic figures are found along the entire length, suggesting that elongation is by intercalation of new ependymal cells rather than from a focal growth zone (Holtzer, 1956, Iten, 1976). This intercalation of new cells would then tend to spread any translocated neurons evenly along the length of the new spinal cord as observed by Zhang. They also propose that the translocated neurons form a relatively distributed framework of mature neurons between which locally born neurons derived from the ependymal cells are intercalated as the regenerate grows (Zhang *et al.*, 2003, Benraiss, 1999). A schematic diagram suggesting the process of neuron translocation during spinal cord regeneration as seen in Fig. G below.

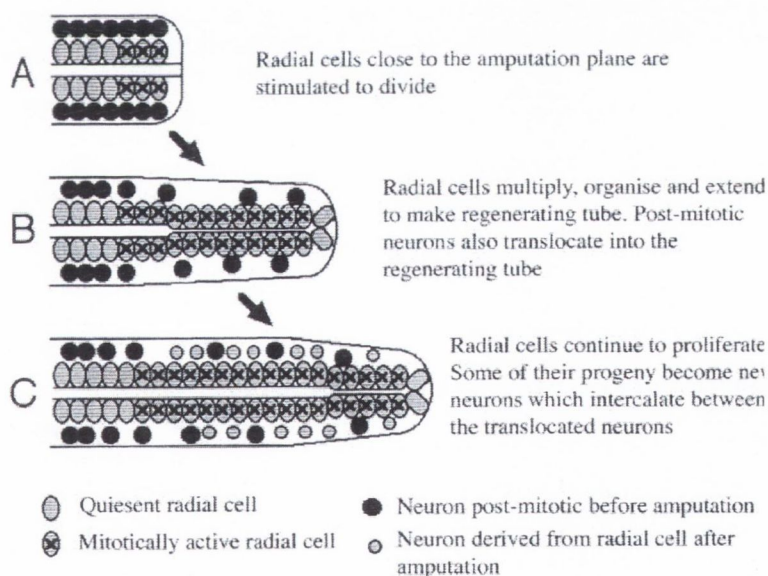


Fig. G A model suggesting the three stages in spinal cord regeneration that follow tail amputation. (Zhang ., 2003, Dev. Dyn.)

Spinal Ganglia Regeneration

After resection of a few spinal segments as well as after tail amputation, not only the spinal cord but also the associated ganglia regenerate. These structures, like Schwann cells myelinating the peripheral nerves and melanocytes develop from neural crest. It is known however that simple ablation of a spinal ganglion is not enough to initiate regeneration of this structure. Ablation of part of the spinal cord together with the ganglion is necessary for regeneration of all the missing structures to occur (Bernardini *et al.*, 1992, Filoni, 1995). Under these circumstances, an ependymal response with formation of an ependymal tube occurs and this occurrence is believed to be fundamental to ganglion regeneration (Filoni *et al.*, 1995).

It is not understood if regeneration of the peripheral nervous system is a close recapitulation of development or whether a neural crest like structure forms within the dorsal ependymal tube during spinal cord regeneration in adult animals. This possibility seems unlikely on the basis of cellular and molecular analysis suggesting that dorsal root ganglia precursor cells migrate from the ventral aspect of the regenerating spinal cord (Arsanto *et al.*, 1992, Nicolas, 1996). However it has recently been proposed that the discontinuity of the basement membrane observed in the dorsal region of the terminal vesicle is indicative of the existence of neural crest like cells in the dorsal region that emigrate as during development (Benraiss *et al.*, 1997). It will take more development of *in vivo* labelling and imaging techniques to obtain a better understanding of the exact source of cells for ganglion regeneration.

Molecules that play a role in Spinal Cord Regeneration

Very little is known about the signals that initiate and maintain the proliferative response of the cells that give rise to the regenerated spinal cord. It is probably initiated by the up-regulation or *de novo* expression of one or more growth factors but repression of inhibitory activities may also play an important role in it. In culture, factors such as epidermal growth factor (EGF) and fibroblast growth factor (FGF) have been shown to keep neural stem cells from both embryonic and adult mammalian central nervous system in the cell cycle (Craig *et al.*, 1996). *In vitro* studies have indicated that a number of factors including EGF are mitogenic for urodele ependymal cells in culture (O'Hara and Chernoff, 1994), but nothing is known about their role during spinal cord regeneration *in vivo*. Expression of FGF receptors has also been shown in the CNS of developing *Pleurodeles walt* (Launay *et al.*, 1994), suggesting that FGF's may also play a role in the growth of amphibian neural stem cells. FGF2 is up regulated after spinal trauma in mammals (Mocchetti *et al.*, 1996) and endogenous FGF2 has been shown to improve neuronal survival and functional recovery in injured spinal cords (Rabchevsky *et al.*, 2000). The mechanism of action possibly involves apoptosis as FGF2 has been shown to prevent caspase 3 activation in neuronal cells *in vitro* (Miho *et al.*, 1999).

In the urodele, *Pleurodels waltl*, there is an early up regulation of the levels of FGF2 in the spinal cord after amputation (Zhang *et al.*, 2000). Expression of FGF2 parallels that of markers of cell proliferation in the regenerating cord. This finding, together with an increase in cell proliferation within the ependymal tube

seen after application of FGF coated beads *in vivo* , suggests a role for FGF2 in sustaining proliferation of progenitor cells during regeneration (Zhang *et al.*, 2000). This idea is consistent with a FGF2 requirement for proliferation of mammalian neural progenitors in several *in vitro* systems, and with the reduction in neurone number seen in cortex and hippocampus of FGF2 knockout mice (Ortega *et al.*, 1998, Raballo, 2000). The importance of FGF signalling is further supported by evidence that FGF receptors are differentially regulated during spinal cord regeneration (Zhang *et al.*, 2002). Their pattern of expression suggests that while FGFR1 may mediate the mitogenic effects of FGF2 in the regenerating spinal cord, FGFR4, whose level peaks at later stages of regeneration, may play a potential role in neural differentiation. This effect may be regulated by other FGF's also, supporting the idea of multiple different roles for FGF signalling during spinal cord regeneration (Zhang *et al.*, 2002).

Many growth factors, either permissive or inhibitory are likely to play a role in spinal cord regeneration and it will be important to begin to characterize the milieu of factors involved in initiating and maintaining the proliferative activity in the progenitor cells in the spinal cord *in vivo* . It will also be important to understand exactly which cells are the progenitors for the regenerate, how plastic these cells are and which signals direct them through proliferation and eventual differentiation.

Patterning during Regeneration

The positional cues required for axonal regrowth to appropriate targets in spinal cord regeneration studies may vary depending on the species. In the mouse cord for example, distances are small and only short range axonal migration cues may be required. Glial bridges and channels surrounding axons could support Ephrin/Eph interactions, cadherin, extracellular matrix components and neurotrophic factor influences. In more extensive rodent cord injury models , successful axonal regrowth across implanted bridge materials may not result in appropriate synaptic connections (Bamber *et al.*, 2001). In these situations, necessary patterning or positional information may be lacking. For complete regeneration to occur it may be necessary to reexpress anterior (A), posterior (P) and dorsal (D), ventral (V) axis cues which are normally only active during development. These patterning mechanisms may be what is active during amphibian spinal cord regeneration. As the embryonic sources of signalling molecules and the original progenitor cell populations involved in AP or DV patterning are no longer present or active, the source of signals and the mechanism of activation may differ. The cranial and caudal stump tissue flanking the lesion site are strong candidates for the source of patterning information in the regenerate. AP patterning of the vertebrate embryonic central nervous system starts by additive expression of Hox-a and Hox-b cluster genes and progresses to establishing the identities of subgroups of spinal neurons (Altmann and Brinanolou, 2001). No direct correlation has been made so far between AP patterning during regeneration and the early steps of embryonic AP

patterning, although patterning genes are reexpressed. Research on caudal regeneration of the urodele, *Pleurodeles waltl*, spinal cord show that two Nkx3 related genes establish positional information along the AP axis during regeneration (Nicolas *et al.*, 1999). A distal less gene *dlx3* (*Pwdlx30*) is expressed in the lateral endodermal tube in association with remaking of the dorsal root ganglia. This gene is not found expressed in uninjured tissue and may not be part of the embryonic development of the dorsal root ganglia (Nicolas *et al.*, 1996).

Dorsal ventral (DV) patterning of the embryonic spinal cord is also a complex process involving many different regulatory pathways and transcription factors. Sonic hedgehog (*shh*) is a key regulator of the ventralizing process during development, while Bone Morphogenic Proteins (BMPs) are important for dorsalization during development (Altmann, 2001, Eggenschwiler *et al.*, 2001). To date very little is known about DV patterning during amphibian spinal cord regeneration. In the intact larval *Xenopus laevis* spinal cord *Shh* and *nrp1* are coexpressed in the floor plate but during regeneration *Shh* is expressed ventrally in the mesenchymal outgrowth but in a location complementary to *nrp1/musashi1* expression, suggesting differences in regulation of DV patterning during regeneration and embryonic development (Chernoff *et al.*, 2001)

To date very little is known about the patterning during spinal cord regeneration, it does appear that all connections are made correctly but how this occurs and is controlled at a molecular level still remains a mystery. Much work still needs to be done to fully understand where the cells are coming from, what tells them to

migrate, how plastic these cells are and how functionality is reestablished so perfectly on a molecular level before we can begin to understand the differences between why some species can regenerate and others can't, or why this is something which was selected against during evolution.

Chapter 2

***In vivo* imaging indicates muscle fibre dedifferentiation is
a major contributor to the regenerating tail blastema**

Introduction

Urodele amphibians have the impressive ability to perfectly regenerate their tails throughout adulthood. Amputation initially results in wound healing, followed by the formation of a blastema of proliferating cells that go on to form the complete array of tissue types. It is still unknown how this diversity of cell types is reformed with such precision during regeneration. Two major questions need to be addressed, firstly the number and type of mature cells in the remaining stump that contribute to the blastema. Though tissue grafting experiments have shown that nerve, dermis, bone and muscle all contribute cells to the blastema, the grafted tissue represented a complex mixture of cell types so such experiments have not completely resolved whether cells arise from the reactivation of a resident, interstitial stem cell, or through dedifferentiation (Stocum, 1995). Secondly, it is difficult to distinguish between different populations of cells within the blastema to determine whether it contains segregated progenitor cells specific for muscle, bone and nerve as opposed to a pluripotent progenitor.

Studies on limb regeneration suggest that there is considerable dedifferentiation and cell plasticity during the formation of the blastema. Detailed histological studies of the fate of muscle fibres at the plane of amputation suggested that muscle fibres lose their myofibrillar structure, their nuclei enlarge and then bud off as mononucleate cells into the blastema (Thornton, 1938; Hay, 1959b). In these studies however, the process of dedifferentiation was inferred from static images, and as a result, the true relationship between the muscle cells and blastema cells remained unproven. It has been argued that the observations actually represented

the fusion of myoblasts into newly forming muscle fibres instead of budding off of mononucleate cells. The first experimental evidence to support this unusual 'budding off' mechanism of producing progenitor cells was provided by Lo *et al* (Lo *et al.*, 1993b). Multinucleate myotubes formed in culture were purified by size selective sieving, injected with lineage tracer and then implanted into a regenerating newt limb. After one week the lineage label was found in mononucleate cells. The average number of mononucleate cells increased over time suggesting that the cells were dividing. The implantation experiments were repeated using an integrated retroviral marker, thus reducing the possibility that mononucleate cells were derived from cytoplasmic transfer of lineage tracer (Kumar *et al.*, 2000).

Many outstanding questions remain concerning dedifferentiation that cannot be addressed by histological experiments or implantation experiments. Since Lo's experiments relied on the use of cultured myotubes, it remains an open question whether endogenous muscle fibres dedifferentiate and then trans-differentiate to form other cell types. Secondly, the quantitative contribution of muscle dedifferentiation to the blastema is unknown. It is unclear whether only a small subpopulation of relatively immature myotubes is capable of reversing their differentiation or whether fully formed, large calibre muscle cells are also capable of this transition. Finally, it is unknown what signals generated from amputation may induce muscle cell dedifferentiation.

To begin addressing these questions I have established a system to trace the fate of single cells during tail regeneration of the axolotl (*Ambystoma mexicanum*).

The use of *Ambystoma mexicanum* as a system allows us to address the questions of cell fate during regeneration with satisfactory precision. Axolotls 2-3cm in length have a considerable amount of mature tissue but the animals are still optically clear (Fig. 1), allowing easy visualisation of fluorescent lineage tracers. Furthermore, larval regeneration occurs in a relatively short period so it should be possible to trace cells from beginning to late stages of tail regeneration. Using this system I first focused on visualising muscle cell dedifferentiation during regeneration. It was found that 3-5 days post - amputation the muscle cells fragment into mononucleate cells. All nuclei from a single multinucleate muscle fibre 'bud off' from the muscle cell, not just the nuclei closest to the amputation site. The mononucleate cells that are generated are morphologically indistinguishable from blastema cells. This study also shows that direct injury of the muscle fibre as well as an unknown signal released in response to severe tissue damage around the fibre is required to trigger dedifferentiation.

The Axolotl

Ambystoma mexicanum

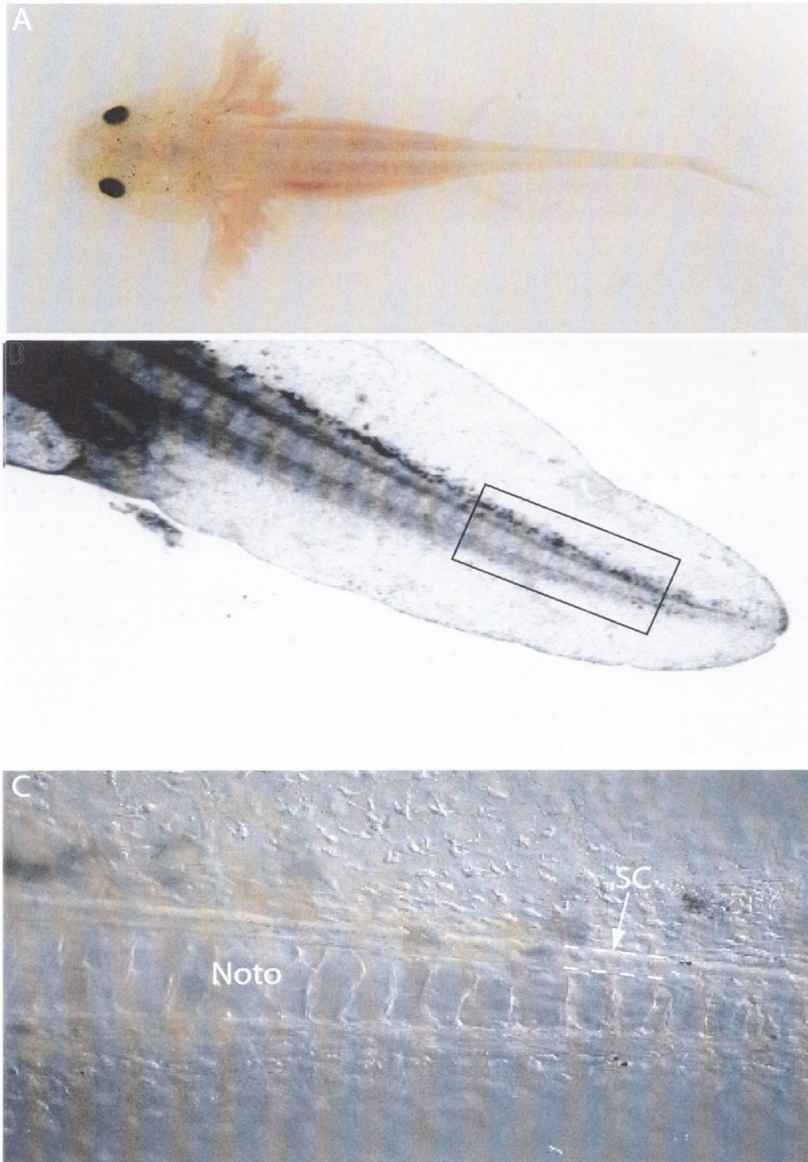


Fig. 1 Panel A, photograph of a larval axolotl 2-3 cm in length. Panel B, dissecting microscope image of a lateral view of the larval axolotl tail. Panel C, 10x differential interference contrast (DIC) image of the lateral view of the marked portion of the tail from panel B, the notochord (noto) and spinal cord (SC) are clearly visible.

Materials and Methods

Animals and Surgery

All experiments were carried out on larval urodele amphibia *Ambystoma mexicanum* (axolotl), supplied by the Indiana Axolotl colony (Bloomington, USA). Axolotls were maintained at 17°C in 40% Holtfreter's (NaCl 80mM, KCL 0.1mM, CaCl₂ 0.1mM and MgSO₄ 0.2mM) and fed daily with artemia. Tail amputations and microinjections were performed on axolotls anaesthetised in 0.01% Ethyl p-Aminobenzoate (Sigma).

Microinjection of Fluorescent Lineage Tracers

For all microinjection experiments anaesthetised animals were stabilised on an optically clear polymer matrix: sylgard (Dow Corning, Wiesbaden Germany) using insect pins and then covered in 0.01% anaesthetic. The first layer of skin was surgically peeled back creating a replaceable skin flap. Rhodamine or fluorescein dextran 10,000 kDa lysine fixable (Molecular Probes), dissolved in sterile H₂O at 25 mg/ml was pressure injected into single muscle fibres. The pressure injections were carried out using a Fine Science Tools Pressure Injector mounted along side of an Olympus Stereo SZX12 dissecting microscope with fluorescence attachment. The animals were then allowed to recover in water containing 0.1mg/ml Penicillin/Streptomycin (Gibco) for 2-3 days before amputation was performed.

The tail amputations were performed on anaesthetised animals placed on a slide that was taped to the stereomicroscope stage. The tail was amputated either close to the labelled fibre leaving it untouched or just clipping off the end of the fibre. All

imaging of the labelled fibre was carried out using a Zeiss Axioplan 2 microscope controlled by a Metamorph image acquisition system (Visitron, Munich, Germany).

Nuclear Localisation Signal (NLS) Dextran

In order to allow visualisation of the nuclei with the muscle fibre together with the cytoplasm of the fibre, a nuclear localisation signal was conjugated to a fluorescein dextran molecule. This guides the dextran molecule into the nucleus and directly labels the nuclei within the muscle fibre. The nuclear localisation signal sequence (CGYGVSRRKRPRPG-CONH₂)

was conjugated to the dextran as follows. 10 kD lysine fixable fluorescein dextran (Molecular Probes, Eugene, Oregon, USA) was dissolved at 25 mg/ml in 1ml DMSO. 10µl triethylamine was added. Iodacetic acid succinimidyl ester was dissolved in DMSO at 50mg/ml. 160µl of IANHS (Sigma) was added to each of 4 tubes containing 250µl fluorescein dextran and the mixture was incubated for 30mins RT. This mixture was then loaded onto equilibrated Nap 5 gel filtration columns (Pharmacia) and eluted in 1ml 100mM Hepes pH7.0. The eluate was then loaded onto a Nap10 column and eluted in 1.5ml of Hepes pH7.0. The peptide was dissolved at 50mg/ml in H₂O and the following volumes were added to one of the four tubes containing 250µl fluorescein dextran: 30, 10, 2.5 and 0 µl. This mixture was then reacted over night at RT. β-mercaptoethanol was then added at 5% and the resulting mixture incubated for 20mins RT. This solution was passed through an equilibrated Nap 10 column and eluted in 1.5ml 100mM Hepes pH7.0. The conjugated NLS-Dextran was subsequently concentrated using a centricon (Millipore).

Microinjection of Blastema Cells

The posterior 5-10mm of the tail was amputated using a sterile scalpel; the animals were then allowed to recover in H₂O containing Pen/Strep. The blastema cells were injected in 2, 4 and 6 day regenerates. The animals were anaesthetised, pinned onto the sylgard matrix and then a small hole was made in the first layer of skin to allow easy access of the injection needle. Rhodamine dextran 25mg/ml was injected using a pressure injector, as described for the muscle fibre injections. The animals were allowed to recover for two days and then the blastemas were fixed in 4% paraformaldehyde(PFA)(Sigma), washed extensively in Phosphate buffered saline (PBS) and then mounted for analysis using either a Zeiss Axioplan 2 microscopes or a Leica confocal microscope.

Antibody Staining

The last 5-10mm of 2-3cm axolotl tails were amputated with a clean scalpel, the tails were allowed to regenerate and the samples were fixed at various time points. The tails were fixed in 4% PFA for 20 minutes at room temperature and then washed extensively in PBS, followed by PBS plus 0.1% Triton X-100 (PBST). The tails were then blocked for 1 hour in PBST plus 10% goat serum. They were then incubated over night at 4 °C in primary antibody, monoclonal against muscle specific myosin heavy chain (clone A4-1025, gift from Dr. S.Hughes), diluted 1:100 in PBST plus 1% goat serum (Gibco).

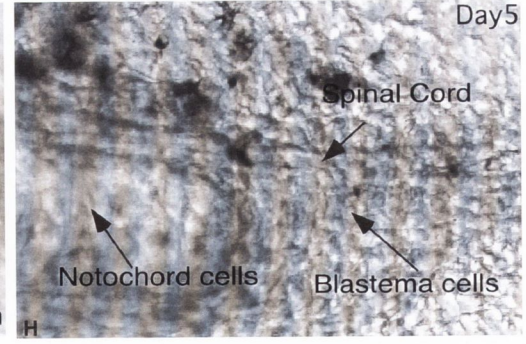
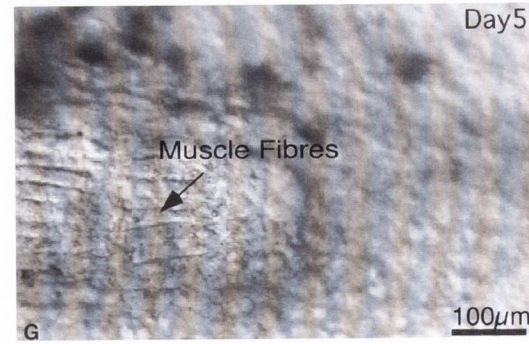
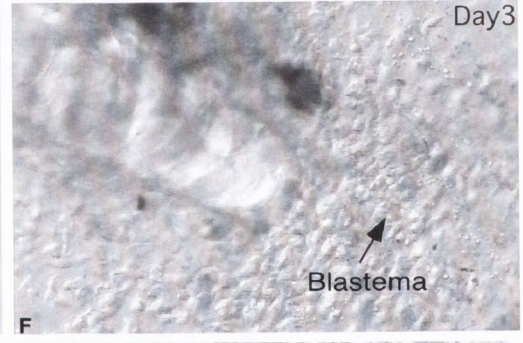
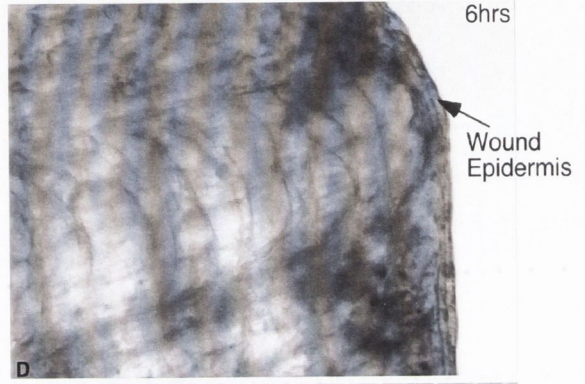
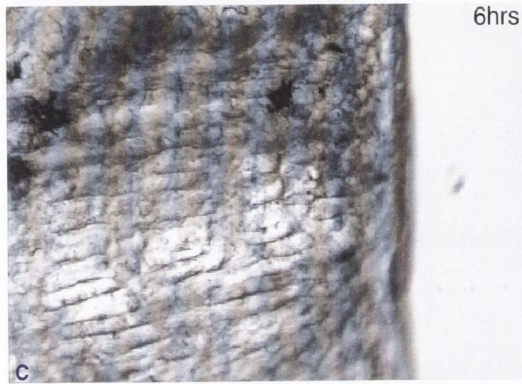
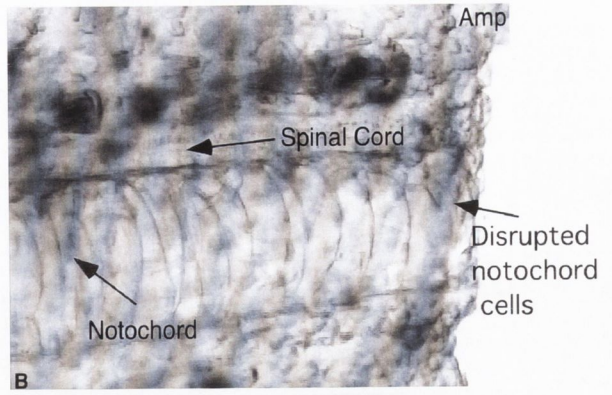
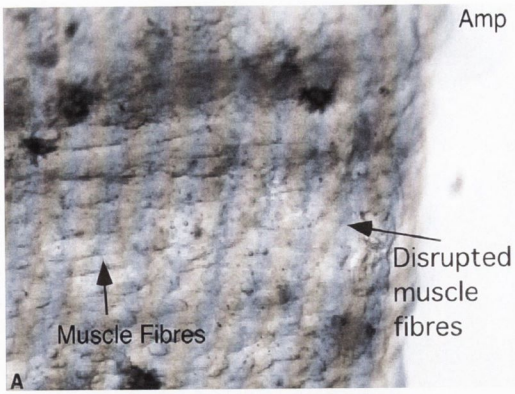
The tails were then washed in PBST for several hours and then the secondary anti-mouse TRITC, (Dako) was added diluted 1:200 in PBST plus 1% goat serum for 1 hour at room temperature. The samples were then washed in PBST and mounted for analysis.

Results

Differential Interference Contrast (DIC) Imaging of Axolotl Tail Regeneration

Larval Axolotls 2-3cm in length have a very flat, optically clear tail, which although being approximately only 10 cells deep, has considerable differentiation of mature tissue. DIC imaging allowed me to optically section through the tail to observe regeneration at all tissue levels. In the larval tail I was able to discern muscle (Fig.2A), notochord (Fig.2B), and the spinal cord (Fig.2B). At this stage, the larval animal still had its notochord. Amputation was performed by surgically removing the last 5-10mm of the tail and initially resulted in tissue disruption. The muscle fibres contracted and the large rounded cells of the notochord became distorted as shown in Fig.2 B. In the first few hours of regeneration the epithelial skin cells from the sides crawl over the wound forming the wound epidermis (Fig.2 C, D). Between 2 and 5 days I observed a mound of proliferating cells between the end of the mature tissue (muscle and notochord) and the wound epidermis, this is the regeneration blastema (Fig.2 F, H). The exact origin of all of the cells in the blastema remains unknown. At the same time as blastema formation an independent outgrowth of the spinal cord can also be observed (Fig.2 H). Classical studies suggest that the spinal cord is essential for regeneration to occur and it plays an important role in patterning the tissue around it (Holtzer, 1956).

Fig. 2. Characterisation of axolotl tail regeneration using DIC imaging. The left panels show the focal plane of the muscle and the right panels show the focal plane of the central axis. A&B show the plane of amputation after the last 5-10mm of tail was removed with a sterile scalpel. Initially wounding results in contraction of the muscle fibres as shown in A and distortion of the regular oval morphology of the notochord cells, B. Within 6hrs the skin crawled over the wound, forming a wound epidermis as shown in C&D. After a wound epidermis (E) has formed the next important stage of regeneration is blastema formation (F). The blastema consists of a mound of proliferating undifferentiated cells that will eventually give rise to the lost tissues. In panels G&H we see the end of the muscle fibres and notochord cells mark the plane of amputation. A gap now exists between the mature tissue and the wound epidermis. The cells filling this gap are the blastema cells.



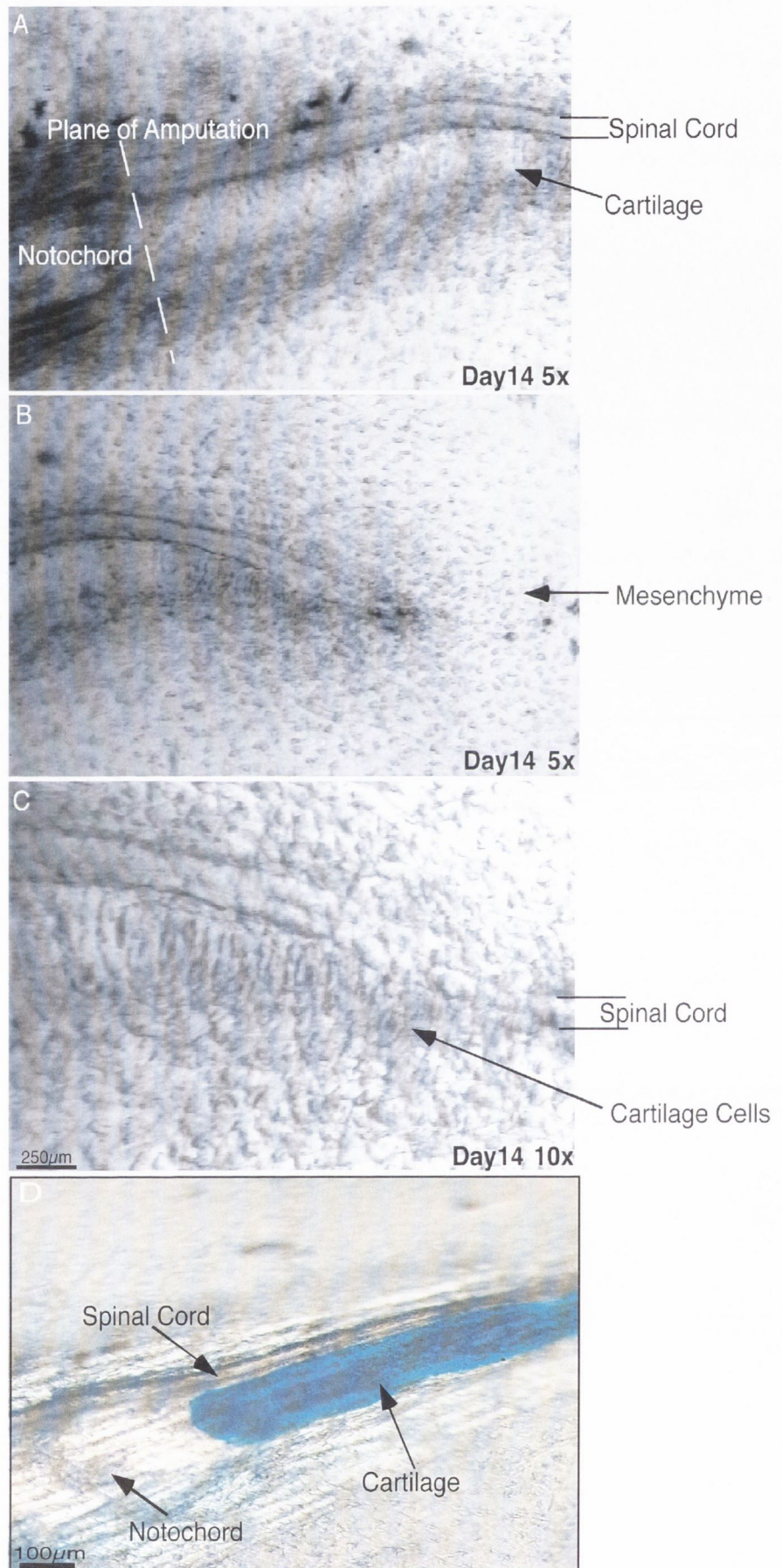
Blastema proliferation and outgrowth continues over several days. The first morphological evidence of tissue re-differentiation are seen 14 days post-amputation (Fig.3). The central axis of the regenerating tissue continuing from notochord is in fact a central rod of cartilage as seen in the tail regeneration of other species of salamander but not the anuran *Xenopus laevis* which regenerates a notochord (Holtzer, 1956). I verified that the regenerate was indeed cartilage using the histological stain, alcian blue (Fig.3D).

Fig.4 shows a montage of the regenerated tail at 30 days at which point the muscle close to the amputation plane is almost fully regenerated and organisation into myotomal segments can be observed. A rod of cartilage has formed in place of the notochord; the boundary between the two marks the original plane of amputation.

Wholemout staining with muscle-specific myosin heavy chain reveals that there is also considerable muscle differentiation in the distal regenerate that has not yet organized into myotomes (Fig.5E). The blastema is initially negative for myosin heavy chain staining (Fig.5 B&C). At later time points the myosin positive muscle fibres begin to organise themselves into regular myotomes from proximal to distal (Fig.5 D&E).

Fig. 3. The morphology of the 14-day regenerating tail as viewed by DIC optics.

Panel A&B give an overall 5x view of the regenerate. In A, a clear boundary is visible between the notochord cells of the mature tissue and the regenerate where cartilage is formed in place of notochord. Panel B, shows the tip of the regenerate. Panel C, illustrates the first morphological signs of differentiation – the round cartilage cells are morphologically distinguishable beneath the regenerating spinal cord. Fig.3D, alcian blue staining confirms the identity of regenerating cartilage cells. The staining clearly shows the boundary between the regenerated cartilage cells (blue) and the notochord cells of the stump that do not take up the stain. Although not shown here, the alcian blue stains the cartilage cells along the length of the regenerate to the tip of the tail.



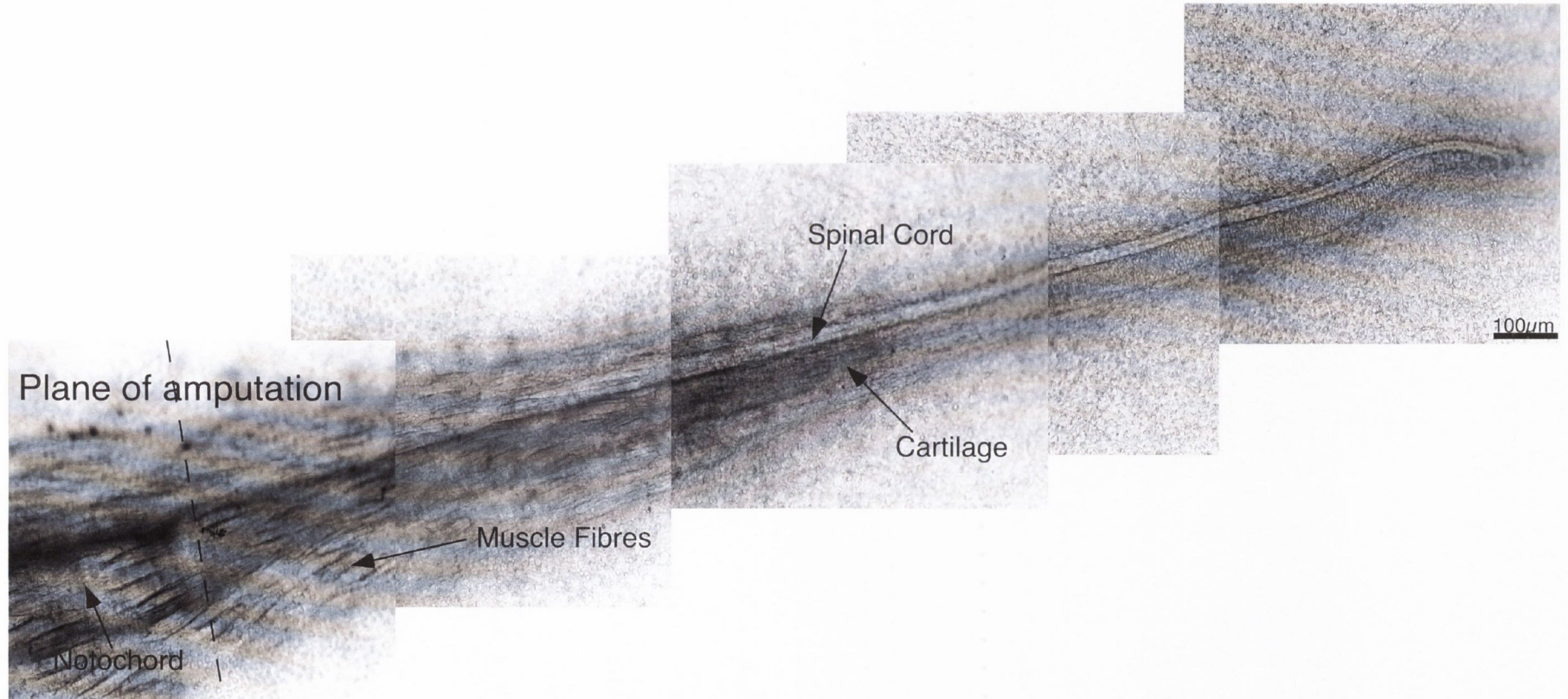


Fig. 4. A montage of the overall tail regenerate after 30 days. The plane of amputation is marked by the boundary between notochord and cartilage cells. The differentiation of muscle was observed by the reformation of myotomes close to the amputation plane. The mesenchymal fin cells also fully regenerate although this is not clearly shown in this image.

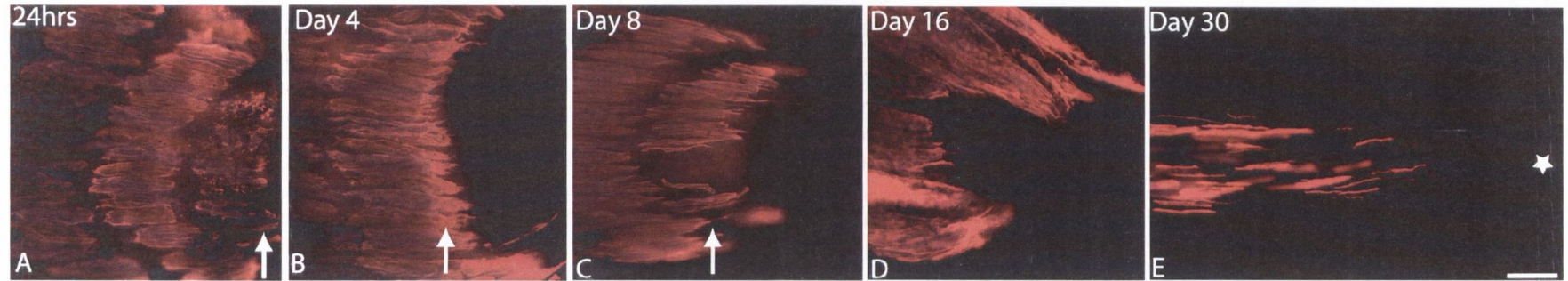


Fig. 5 Timecourse of wholemount myosin staining during axolotl tail regeneration. Panel A, the muscle fibers closest to the plane of amputation, which is indicated by the arrow, become disorganised in response to amputation. By 4 days post amputation the area distal to the plane of amputation is negative for myosin staining. At Day 8 the first myosin positive cells beyond the plane of amputation are visible on the dorsal and ventral sides of the tail, a similar effect is seen again on Day 16. By Day 30 the muscle bundles are still not completely organised into distinct myotomes and the tip of the new tail (star) is still myosin negative. The muscle appears to differentiate and organise themselves from proximal to distal in the tail. In Panels D & E the tail has fully regenerated, so the plane of amputation is not visible in these images. Scale bar = 100 μ m.

In Vivo Injection of Muscle Fibres

Having documented regeneration using static images, I now wanted to address the role of endogenous muscle fibres during regeneration. To assay this, muscle fibres were pressure injected with rhodamine dextran, NLS-FITC dextran or a mixture of both into anesthetized animals. It was found that fibres injected with rhodamine dextran in a control unamputated animal (Fig.6) remained stable and bright for 10 days, after which the fluorescent signal began to gradually decrease.

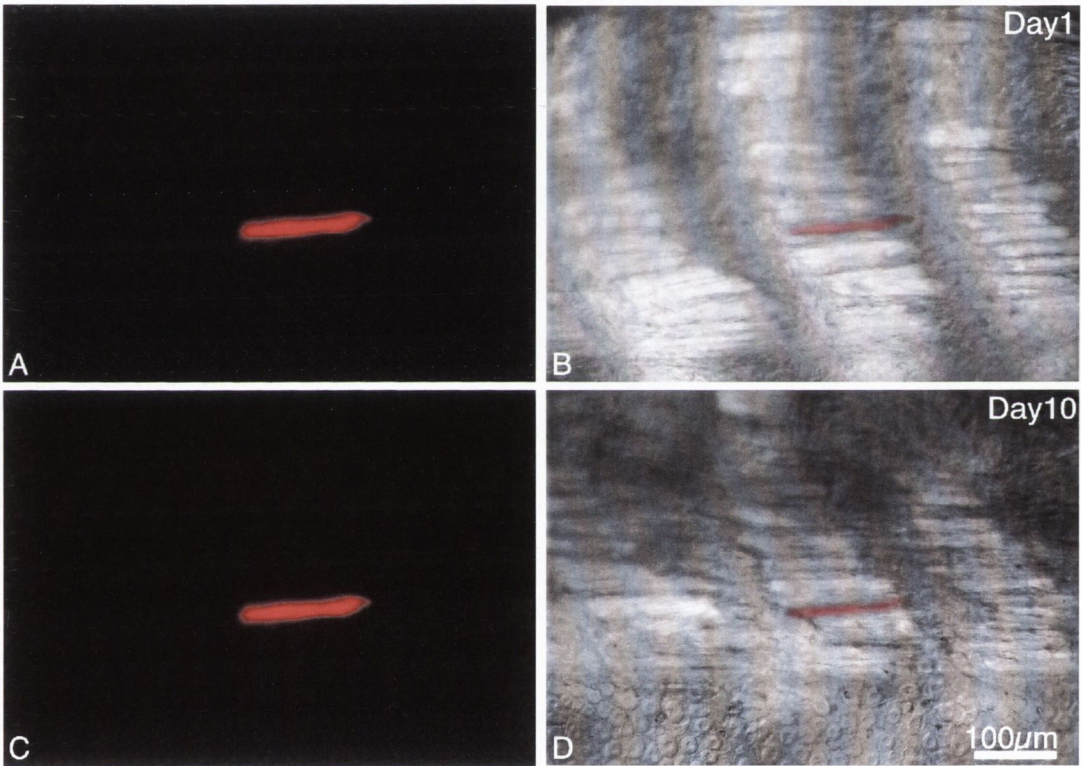


Fig. 6. A rhodamine-dextran-injected muscle fibre remains stable over 10 days in an uninjured animal. Panel A, rhodamine dextran injected-fibre in the mature tail on Day 1. Panel B, corresponding DIC image of the tail with fluorescent overlay of the muscle fibre. Panel C is the same injected-fibre 10 days later; Panel D shows the matching DIC image with fluorescent overlay.

In labelling fibres *in vivo* with both a cytoplasmic and nuclear marker I observed a wide variety of fibre types differing greatly in size and number of nuclei. I have grouped them into three categories (Fig.7). Figure 7A. shows a small fat fibre usually containing 2-3 nuclei, B&D show long thin fibres with on average 5-8 evenly spaced nuclei and C&E illustrate fibres of medium length and width, usually containing in the range of 6-10 nuclei which are located close to the surface of the fibre. The distribution of the different fibre types appeared to be completely random within the tail.

Compared to the cytoplasmic dextran the frequency of successful injections with the NLS-dextran was quite low since it appeared to stick to the extracellular matrix and it did not always accumulate in the nucleus. Therefore, though I was able to use the nuclear label to characterize the different fibre types in the tail, it was not practical to use it when following individual fibres during regeneration.

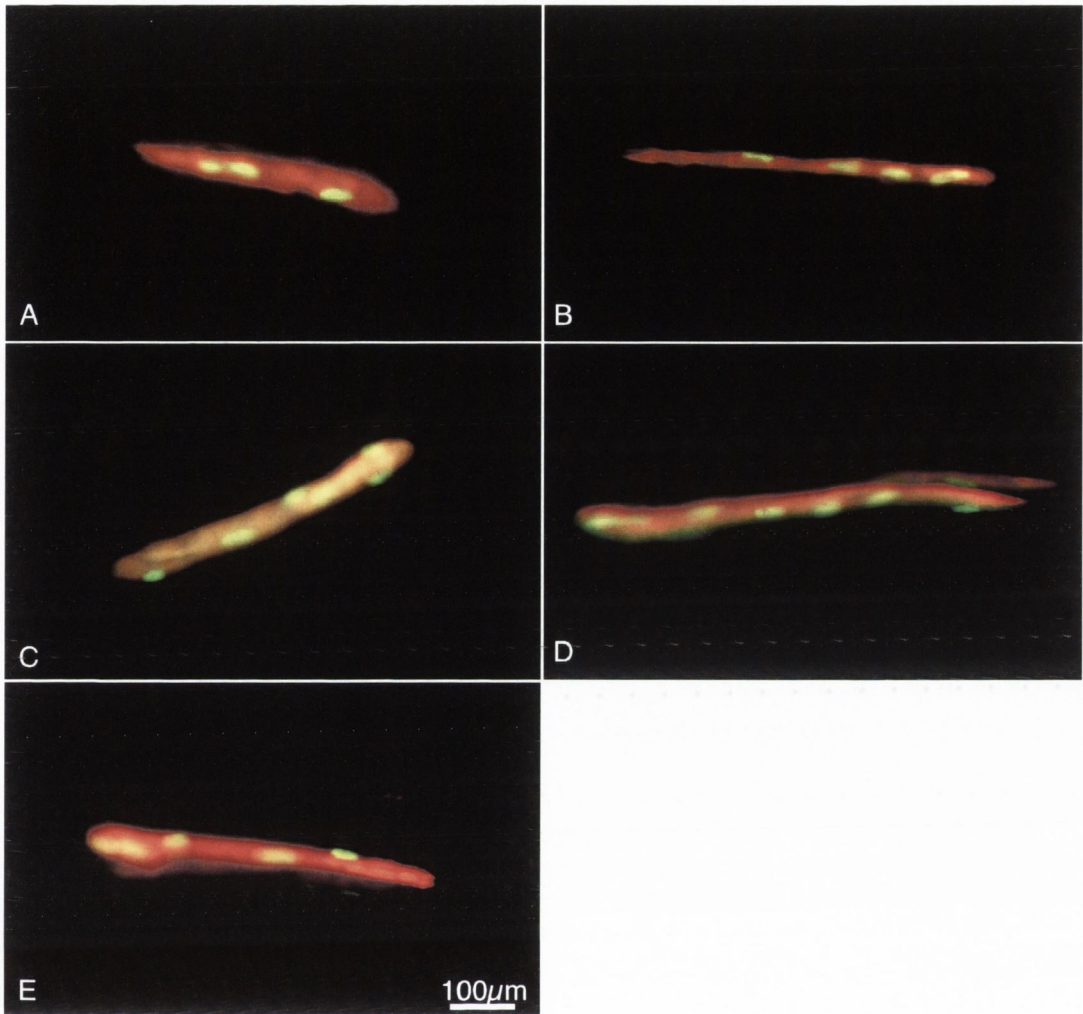


Fig. 7. Injection of single muscle fibres with cytoplasmic rhodamine dextran and a nuclear fluorescein dextran reveal the different types of fibres found in axolotl tails. Panel A. shows a short fat fibre with 3 nuclei, B. shows a long thin fibre with 4 nuclei, while C. shows a medium length mature fibre with multiple nuclei close to the surface. D. shows a very long fibre with 8 nuclei evenly spaced along its length and E. shows a medium length, medium width fibre with four nuclei randomly dispersed but also quite close to the surface of the fibre.

Dedifferentiation of mature muscle fibres *in vivo* requires direct cell injury

To understand the extent of wounding necessary to induce dedifferentiation, different amputation conditions were examined. Three general classes of muscle cell behaviour were found depending on the specific amputation conditions. The results are summarised in Table 1.

Class I: When the tail was amputated close to the labelled fibre (50-100 μm) but no damage was done to the fibre, the injected cell remained stable throughout the course of regeneration. The fibre did not dedifferentiate and it did not become incorporated into the regenerate (n=25).

Class II: When amputation resulted in removal of approximately fifty percent of the labelled fibre, the fibre initially retracted, followed by vesiculation of the label and within 3hrs the labelled fibre had disappeared. This likely represents death of the cell (n=25).

Class III: When tail amputation resulted in 'clipping' of the labelled fibre (Fig.8) I observed the generation of mononucleate cells from the muscle fibre. 'Clipping' involved removing a small portion of cytoplasm at the end of the fibre so that its terminal attachment to the myoseptum was disrupted during amputation.

Immediately after clipping the fibre retracted followed by re-elongation on day 2 after amputation (Fig.8C). Three to five days post amputation, the fibre had fragmented into 6-10 mononucleate cells, as seen in Fig.8G. The number of mononucleate cells formed correlated with the number of nuclei I saw in my labelling of larger calibre, unamputated fibres. The mononucleate cells initially appeared round or oval shaped

(Fig.8G) but within one day after the initial dedifferentiation the cells extended processes in various directions (Fig.8 I & Fig.10). Dedifferentiation occurred in 15 out of 58 cases of clipping amputation. Taken together these results indicate that direct injury to the muscle is required to trigger dedifferentiation.

Table 1. Summary of the ‘Injury Conditions’ necessary to Induce Muscle Dedifferentiation

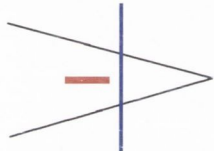
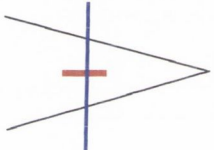
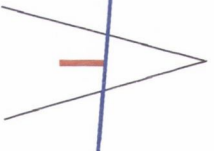
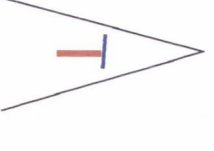
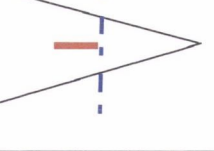
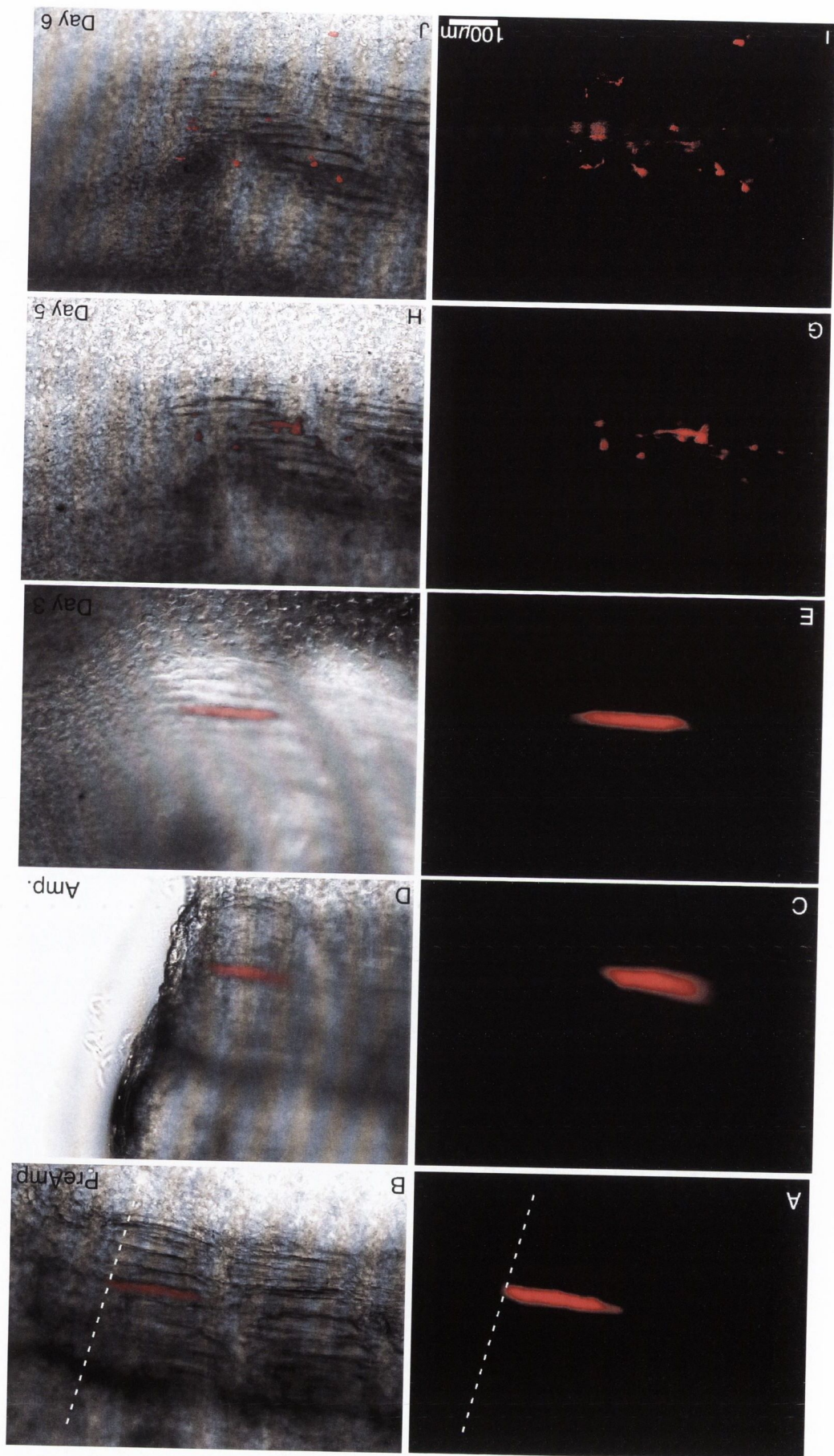
	Type of Surgery	Result	No. of Examples
	Amputation plane close to fibre	Fibre stable	25 out of 25
	Amputation plane cuts through the middle of the fibre	Fibre degenerates	25 out of 25
	Amputation plane clips off the end of the fibre	Fibre Dedifferentiates	15 out of 58
	Clipping of the muscle fibre with minimal tissue injury	Fibre stable	15 out of 15
	Severe injury clipping the end of the fibre, the tail remains loosely attached. 'Gap Regeneration'	Fibre Dedifferentiates	10 out of 25

Fig. 8. Tail amputation accompanied by clipping of the fibre results in dedifferentiation of a mature muscle fibre. A single fibre in the tail was labelled with rhodamine dextran (A). Panel B shows the matching DIC image with overlay of the labelled fibre in the tail. The tail was amputated along the dotted line (A&B), clipping the end of the labelled fibre. Panel C shows the retraction of the fibre in response to amputation, the overlay in Panel D illustrates the position of the injected fibre in relation to the amputation plane. The fibre then re-elongated as the wound healed over and as the blastema formed (E&F). At five days post amputation the labelled fibre fragmented giving rise to multiple mononucleate cells (G); the position of the mononucleate cells can be seen clearly in the overlay in Panel H. During the next 24hrs the initially round mononucleate cells spread out having long processes associated with them (I). The stellate morphology is shown in higher magnification in Fig.10.

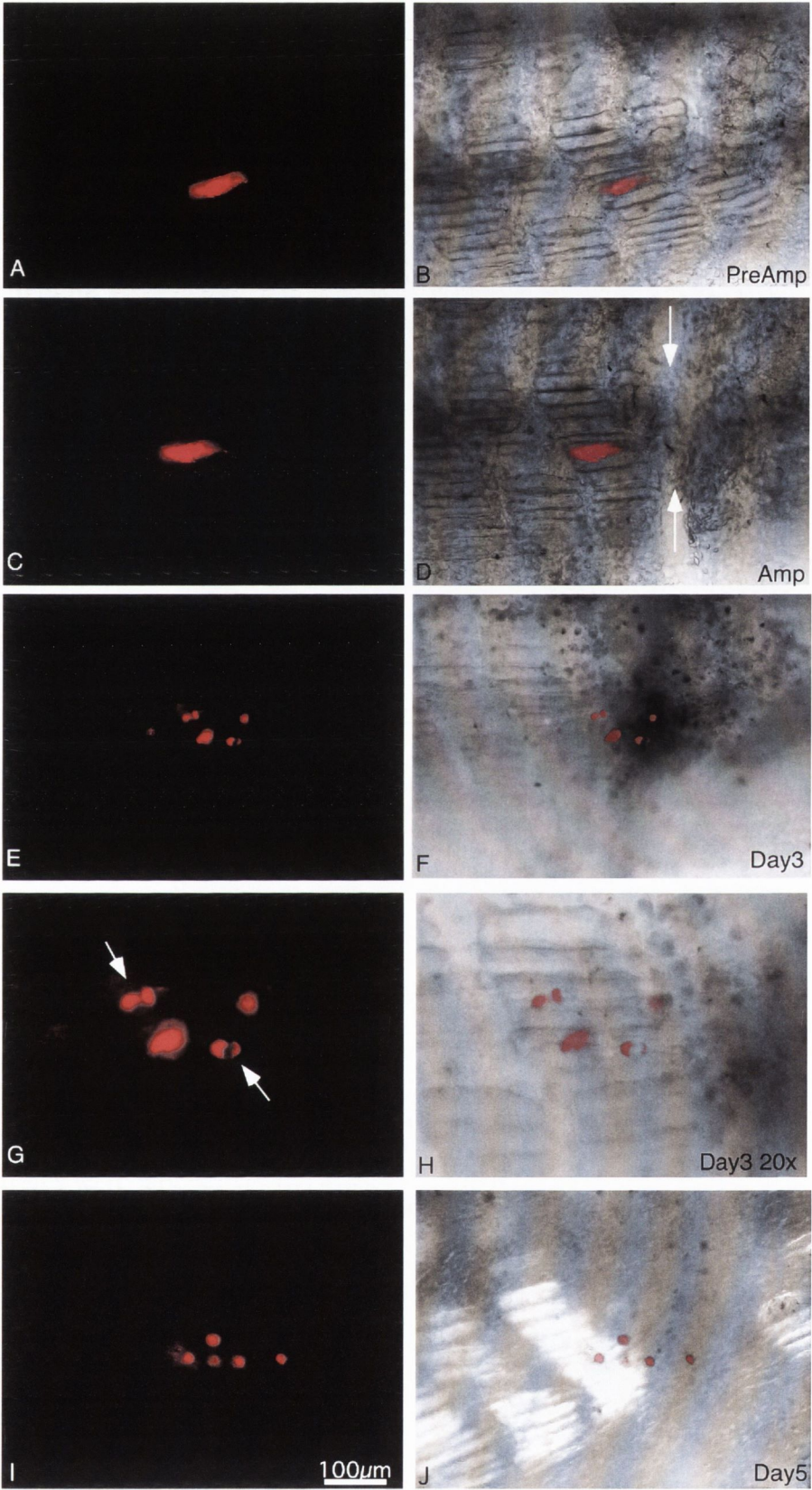


Tissue damage is also required to initiate dedifferentiation

To test whether clipping of the fibre is a sufficient signal to induce dedifferentiation in the absence of tail amputation I used either a sharp forceps or hypodermic needle to release the end of the muscle fibre from its neighbours in an unamputated tail. Under these circumstances the muscle fibres re-elongated within 2 days and remained stable over the 15 days for which it was followed. Furthermore, creating a small tear in the tissue adjacent to the labelled fibre while clipping the fibre did not result in dedifferentiation. The fibre remained stable and unchanged in all cases (n= 15).

This left the question of whether complete transection of the tail was required to induce dedifferentiation. To test this I induced significant injury by cutting halfway through the tail but leaving the distal portion attached so that the two parts healed back together. This process is referred to as 'gap regeneration'. During gap regeneration a zone of cells resembling a blastema was formed in the middle of two mature pieces of tail. Under these circumstances the labelled fibre dedifferentiated (10/25 cases) and contributed cells to the forming "blastema" (Fig.9).

Fig. 9. Muscle dedifferentiation during “gap regeneration”. A single fibre was labelled by microinjection with rhodamine dextran (A); Panel B shows an overlay with the DIC image to show its position within the myotome. The tail was severely injured, creating a gap in the tissue as shown by the arrows in Panel D. During injury the end of the fibre was clipped (C), but the whole tail was not amputated. At three days the labelled fibre fragmented forming mononucleate cells (Panel E), the matching DIC image with overlay shows that a blastema-like structure has formed joining the two pieces of mature tissue together (Panel F). Panel G & H are high magnification views of Panel E & F to show the mononucleate cells which appear to be just finishing cytokinesis (see arrows). The matching DIC image (H) shows the position of the fragmented cells within the tail. Over subsequent days these cells increased in number (I) but all the cells that arose from dedifferentiation are not visible in Panel I as the gap blastema is a rounded structure and the cells are on different focal planes. Some cells have also migrated away from the site of the original fibre, as can be seen from the overlay with the DIC image in Panel J. In the DIC image (Panel J) the zone of cells that forms between the two mature uninjured parts of the tail is clearly visible.



The comparison of dedifferentiated cells to blastema cells

Several observations indicate that the mononucleate cells generated from muscle fibre dedifferentiation are blastema cells. First, I observed cells whose morphology indicated that they were undergoing cytokinesis one day after they formed mononucleate cells (Fig.9G). The total number of visible mononucleate cells had increased, doubling within a 24-hour period (Table II). During the time of cell doubling, cells moved laterally and down into the tissue so that they no longer resided in one field of view, and rested in multiple focal planes (Fig9I). Therefore it is difficult to capture all cells in one picture. Also, this makes it difficult to accurately count 100% of the cells derived from dedifferentiation.

Table 2. Mononucleate cells generated from dedifferentiation of a labelled muscle fibre

Sample	<u>Number of Mononucleate Cells Generated</u>	
	24hrs*	48hrs
Animal 1	8	15
Animal 2	5	11
Animal 3	9	15

Muscle fibres were labelled *in vivo* in the tail and the fate of the cells in response to amputation was monitored. 24hrs* indicates the number of mononucleate cells counted 24hrs post-fragmentation of the labelled fibre. 48hrs indicates the number of labelled mononucleate cells 48hrs post-fragmentation

Several days after dedifferentiation, the mononucleate cells that derived from the muscle cells extended numerous processes towards their neighbours

(Fig.8I&Fig.10). To determine if this striking morphology was characteristic of blastema cells in general, I used rhodamine dextran to label blastema cells *in vivo*. Cells were labelled in dorsal, ventral and axial positions within the blastema and at the tip of the blastema adjacent to the wound epidermis. This allowed assessment of whether sub-populations of morphologically distinct blastema cells exist. All of the labelled blastema cells that I observed in 10 different blastema samples were morphologically similar and were indistinguishable from dedifferentiated muscle cells (Fig.10).

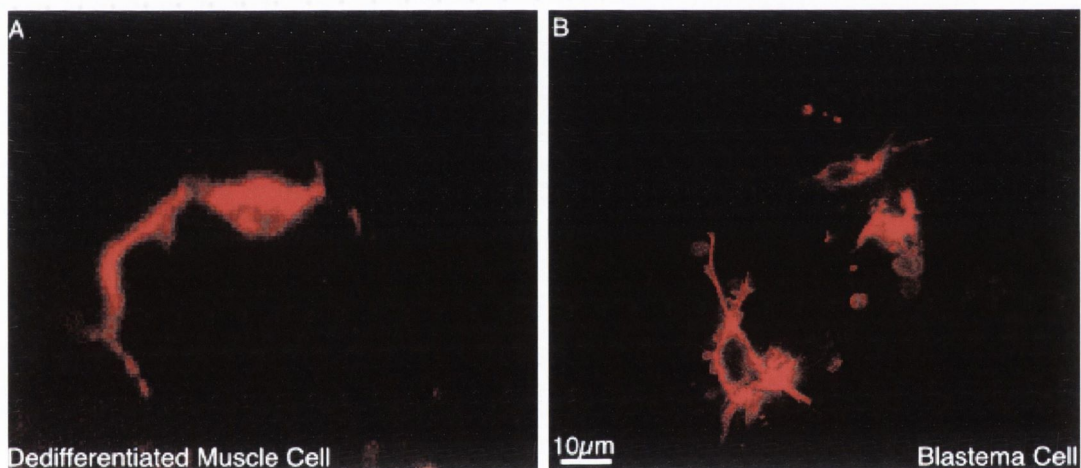


Fig. 10. Cells of dedifferentiated muscle fibres morphologically resemble blastema cells. Panel A, a high magnification image of a mononucleate cell with a long thin process derived from a dedifferentiated muscle fibre. Panel B shows *in vivo* labelled blastema cells showing similar morphology.

I attempted to confirm the identity of the mononucleate cells generated from dedifferentiation as blastema cells by using the blastema cell marker, RGE 53

(Ferretti *et al.*, 1991). It was found that in these preparations this anti-cytokeratin antibody was not a specific marker as it also stained notochord, cartilage and spinal cord (data not shown). I do not know if this difference from previous descriptions is due to the fact that I am looking in younger animals, tails versus limbs or because I am looking in a different species. The other blastema marker 22/18 (Kintner and Brockes, 1984) can only be used under specific fixation conditions that are not compatible with the lineage tracing experiments.

Discussion

Endogenous muscle fibres dedifferentiate during regeneration

For many years it has been presumed that endogenous muscle fibres undergo dedifferentiation in response to wounding but there was no experimental evidence *in vivo* to support the theory. The results presented here are the first definitive evidences that dedifferentiation of endogenous muscle fibres occurs during regeneration. Previous work has concentrated on illustrating the possibility of dedifferentiation using histological studies (Thornton, 1938; Hay, 1959b) or implantation of cultured myotubes (Lo *et al.*, 1993a; Kumar *et al.*, 2000). Here I have followed the changes in a single fibre over time, within the physiological context of the regenerating axolotl tail.

The features of *in vivo* muscle fibre dedifferentiation

Dedifferentiation of mature muscle fibres occurred between three days and five days after an amputation or severe wounding that clipped the end of the muscle fibre *in vivo*. Contrary to Hay's observations in the limb I did not observe budding of mononucleate cells at the end of the fibre closest to the amputation plane. Rather the muscle fibre appeared to fragment giving rise to mononucleate cells approximately equal in number to the original number of nuclei in the mature fibre. This difference may arise from the different morphology of limb muscle fibres, which are very long and thin versus tail muscle fibres, which are relatively short and thick.

Three days post-amputation the cells derived from the dedifferentiated muscle fibre appeared to rapidly undergo cytokinesis, (as seen in Fig.9) and the mononucleate cell number increased within the first 48hrs after formation of mononucleate cells. The timing of the cytokinesis that I observe during dedifferentiation suggests that the nuclei in the mature muscle fibre re-entered S-phase before breaking apart into mononucleate cells since S-phase entry and completion of DNA synthesis takes at least 3 days in urodele myoblast cells under standard *in vitro* culture conditions (Tanaka *et al.*, 1997b). This observation is also consistent with experiments showing that urodele myotubes can re-enter S-phase from the multinucleate, differentiated state (Tanaka *et al.*, 1997b; Kumar *et al.*, 2000). The combined detection of a DNA synthesis marker with our lineage tracing method to confirm this still poses technical challenges.

I found that the mononucleate cells assumed a striking stellate morphology with multiple processes that wrapped around their neighbours. Direct labelling of blastema cells showed that this is a characteristic morphology of all blastema cells. This unusual morphology of blastema cells having multiple processes has also been observed by EM in preparations from limb blastemas (Egar, 1988). It is interesting to speculate that these blastema cell processes are involved in the patterning of the regenerating structure since previous work has shown that cell migration is activated if blastema cells of different positional identities are juxtaposed (Nardi and Stocum, 1983; Pecorino *et al.*, 1996). The stellate morphology of the blastema cells could allow blastema cells with different positional identities to recognize and move past each other.

Direct cell injury is necessary but not sufficient to induce dedifferentiation

The ability to follow a single fibre over time has allowed me to define the conditions that trigger dedifferentiation and the formation of mononucleate cells. These results clearly show that damage to a single muscle fibre though required, is not sufficient to induce dedifferentiation. The signal, which causes mature differentiated fibres to fragment and produce mononucleate cells, is only released in response to severe tissue damage. This suggests that at least 2 signals are needed to initiate the process of dedifferentiation: direct cell injury and tissue damage.

In culture it has been shown that urodele myotubes must lose contact inhibition and must be exposed to a component of clotted blood in order to initiate one aspect of dedifferentiation, S-phase re-entry (Maden, 1976, Tanaka, 1997).

However these culture conditions are not sufficient to induce mononucleate cell formation as occurs *in vivo*. In the future it will be important to define the molecular nature of the injury-related signals that induce mononucleate cell formation and to determine whether the signals that initiate mononucleate cell formation and cell cycle re-entry overlap.

Dedifferentiation was induced in 25% and 40% of fibres after complete or partial amputation, respectively. It is not clear whether muscle dedifferentiation is only observed in a proportion of fibres because all fibres did not get the equivalent signal, or whether different subpopulations of fibres exist, some of which are capable of dedifferentiating and others that are not. Previous histological descriptions of early stages in limb regeneration found that the outer

circumference of muscle undergoes larger morphological changes consistent with dedifferentiation compared to inner layers of muscle (Dinsmore, 1977). It is possible that fibres dedifferentiate from discrete locations within the stump to preserve the structure of the mature tissue while still contributing to the blastema. It should however also be noted that when Lo . implanted cultured myotubes into the regeneration blastema, they estimated that 25% of implanted nuclei were found in dedifferentiated mononucleate cells (Lo *et al.*, 1993b).

Estimating the number of blastema cells generated through muscle dedifferentiation

The significance of dedifferentiating muscle as a contributor to the blastema has long been disputed (Hay, 1959a, Dinsmore, 1977). Does dedifferentiation of muscle fibres produce a significant proportion of the founding population of blastema cells or is another mechanism, such as activation and proliferation of a resident stem cell the primary mechanism for forming the blastema? From my data I can estimate the number of blastema cells that have come from the dedifferentiation of muscle. This calculation is based on the number of muscle fibres present in a cross-sectional area of the tail, the frequency at which I see dedifferentiation occurring, and the number of nuclei per muscle cell. Myosin heavy chain staining of muscle fibres in the tail (Fig.5) revealed approximately 60 muscle fibres or small muscle bundles within the tail cross-section that could contribute to blastema formation. My results showed that 25-40% of muscle fibres

give rise to mononucleate cells when a labelled fibre is clipped during tail amputation. Taking cell death into consideration, I estimate that probably closer to 25% of the muscle fibres at the plane of amputation would dedifferentiate. These figures imply that about 15 muscle fibres dedifferentiate during larval tail regeneration. Since about 10 nuclei were generated from a single dedifferentiating fibre, approximately 150 cells would be generated directly from muscle dedifferentiation, before cell proliferation begins.

Does this number represent a significant proportion of the founding population of the regeneration blastema? Precise quantitative data on the number of blastema cells during early stages of larval tail regeneration is not available but Chalkley (Chalkley, 1954) carefully documented the number of cells present in a newt limb blastema. Applying a modified version of Chalkley's method to the tail, I estimate that the 4-day axolotl tail blastema contains 907 cells. Gardiner *et al* (1986) found that 43% of limb blastema cells come from dermis. Therefore, approximately 516 cells of the tail blastema are likely to be derived from other tissues. My *in vivo* data shows approximately 150 cells, which is 29% of non-dermis-derived blastema cells come from dedifferentiating muscle. From these calculations it can be argued that muscle cell dedifferentiation makes a significant contribution to the makeup of the regeneration blastema.

This data suggests that 29% of the cells in the blastema come from dedifferentiation of muscle fibres. When the muscle fibre fragments and forms mononucleate cells, the cells begin to migrate away from the site of the original fibre and towards the blastema (Fig.8 J, Fig. 9J). As the blastema is a very

rounded structure it was very difficult to image all of the cells once they entered into that area, but as no labelled cells were observed in the mature tissue it is thought that once fragmentation occurs all mononucleate cells generated from the fibre enter the blastema.

The role of dedifferentiation during regeneration

It is striking that muscle dedifferentiation already occurs efficiently in animals that are still rapidly growing and must still contain many myogenic progenitor cells. The question arises - why do two systems for tissue growth co-exist? Commitment to the myogenic lineage occurs during embryonic development and the actual number of uncommitted stem cells may be very small even in these growing animals. Dedifferentiation of muscle and other cell types is likely to be an important mechanism for re-programming cells to form a multipotent population of progenitor cells that are capable of being re-patterned. This idea is supported by studies on *msx1*, a gene that is re-expressed during regeneration (Crews *et al.*, 1995; Simon *et al.*, 1995), (Koshiba *et al.*, 1998). When *msx1* is expressed in cultured myotubes it induces at a low frequency the formation of mononucleate cells (Odelberg *et al.*, 2000). Strikingly, the mononucleate cells that are formed are capable of differentiating into multiple cell types such as adipose tissue, cartilage, and osteoclasts—a spectrum of cell types not formed by the myogenic cells that formed the myotubes in the first place.

Although this system allows me to follow the process of dedifferentiation *in vivo*, it is clear that it will require further technical development to determine the exact fate

of the dedifferentiated muscle cells. In this experiments the limiting factor has been the fact that the lineage label becomes diluted over time, in the future the use of plasmids encoding green or red fluorescent proteins which have longer life spans that dextrans may overcome this technical limitation and allow cells to be followed to their final fate. Extending the lineage analysis studies presented here should eventually allow the process of regeneration to be followed on the single cell level *in vivo* from the dedifferentiation step to patterning and re-differentiation.

Chapter 3

Electroporation as a Tool to Study Spinal Cord

Regeneration *In Vivo*

Introduction

Spinal cord regeneration is one of the most amazing but least understood characteristics of the axolotl. Where the cells come from to form the new structure is not clearly understood nor whether the cells from the spinal cord can also populate the blastema. Regeneration of the spinal cord occurs in the context of tail regeneration. It is initiated by the skin crawling over the wound from all sides to form a wound epidermis. Then a mound of proliferating undifferentiated cells called the blastema is formed via dedifferentiation of mature cells. The blastema then grows and eventually differentiates to form the lost structures (Holtzer *et al.*, 1955; Echeverri *et al.*, 2001). At the same time as the blastema is forming the spinal cord is regrowing as a separate entity, initially the end seals over and at 4-6 days a bulbous structure is visible called the terminal vesicle, the function of which remains unclear (Holtzer, 1956).

Past studies have suggested that the first events in spinal cord regeneration are the induction of proliferation and migration of the cells close to the plane of amputation ((Egar and Singer, 1972; Benraiss *et al.*, 1999). It is thought that the ependymal cells lining the neural canal can generate all the cell types to form a new fully functional spinal cord, implying that in urodeles ependymal cells retain or acquire upon injury the characteristics of embryonic neuroepithelial cells. It is not known how many of the spinal cord cells behind the amputation plane are induced to participate in regeneration. Furthermore, it is unknown what diversity of cell types a single progenitor cell forms. To address such issues Benraiss *et al* used

biolistics to transfect the human alkaline phosphatase gene DNA into spinal cord cells at the plane of amputation and could later identify cells positive for the marker outside of the spinal cord mainly, in Schwann cells and melanocytes (Benraiss *et al.*, 1996). Their data indicated that the cells probably left the spinal cord from the dorsal side of the terminal vesicle but their studies were limited by the inability to actually visualize the movement of the cells.

The technique of rhodamine dextran injection, developed to follow the fate of muscle fibres during regeneration was initially used to try to label the spinal cord cells, but the injections proved technically difficult due to the inaccessibility of the spinal cord and the much smaller cell size and also as the cells were dividing the label was quickly diluted out, so a more stable longer lasting labelling technique was needed.

Here we describe a technique which overcomes the technical difficulties experienced in the past of precisely labelling cells *in vivo* and actually allows the movement and identity of the cells to be followed in live animals during the course of regeneration. Electroporation is a well-established method for introducing charged molecules like DNA, RNA, dyes and proteins into cells (Neumann *et al.*, 1982; Potter, 1988). Electroporation works by the formation of temporary pores in the cell membrane by the application of electrical pulses of short duration, allowing charged macromolecules like DNA to actively enter the cell. Non-charged molecules may also enter cells by passive diffusion using this method (Neumann *et al.*, 1982). One difficulty had been that the application of electric pulses often

damaged cells. A breakthrough in this regard was the implementation of a rapid series of short pulses that highly reduced cell death (Muramatsu *et al.*, 1996). Since then, in ovo electroporation has become a technique widely used by chick embryologists (Itasaki *et al.*, 1999; Swartz *et al.*, 2001), and now this technique has become extensively applied to other systems. With traditional electroporation flat, broad electrodes were used, limiting the ability to accurately target plasmid expression into single cells in defined tissues. Recently, micropipette electrode techniques were used to transfect single neurons within the intact brain of live *Xenopus* (Haas *et al.*, 2001). Here I describe how this technique has been adapted to label single cells or groups of cells within the spinal cord of an axolotl close to the plane of amputation, which then allows the fate of individual spinal cord cells to be visualized during the course of regeneration.

Materials and Methods

Animals and Surgery

These experiments were carried out using larval axolotl urodele amphibia, *Ambystoma mexicanum* (axolotl), bred in captivity in our facility. Axolotls were maintained at 17°C in 40% Holtfreter's (NaCl 80mM, KCL 0.1mM, CaCl₂ 0.1mM, MgSO₄ 0.2mM) and fed daily with artemia. All experiments were carried out on axolotls anesthetized in 0.01% Ethyl-p-Aminobenzoate (Sigma).

Tail Amputation and Electroporation

Anaesthetized animals were immobilised on an optically clear polymer matrix: sylgard (Dow Corning, Wiesbaden, Germany) and the tail was amputated under an Olympus Stereo Sz microscope. A kimwipe soaked in PBS was placed over the axolotl and the ground electrode, which was placed next to the animal (Fig.11). The micropipettes were pulled using a Sutter Flaming Brown P-97 puller to a tip size of approximately 1-2µm. A silver wire was placed inside the micropipette containing 10 µl of 0.5µg/µl of plasmid DNA purified using a Quiagen Maxi Prep kit. This was then inserted into the neural tube and 5 trains of pulses of 50V, 200Hz and pulse length 100ms were applied using a SD9 Stimulator (Grass Telefactor, USA). The animals were then allowed to recover in water containing Penicillin/Streptomycin (Gibco).

The plasmids used in these experiments were, nuclear - pEGFP-N1 and cytoplasmic pDsred2-N1, both were obtained from Clontech. The plasmids were

diluted in PBS at 0.5µg/µl and 0.05µg/ul respectively and were mixed 1:1 for co-electroporation experiments.

Cryostat Sectioning

The tails of the axolotls containing labelled cells were fixed in 4% paraformaldehyde (PFA, Sigma) containing 5% sucrose(Merck) at room temperature for 20 minutes. Washed 3x5 minutes in PBS + 5% sucrose, followed by 4x20 minutes washes in the same wash solution.

The tails were then embedded in 1.5% agarose(Gibco) plus 5% sucrose. The agarose was allowed to set and then the blocks containing the tails were cut out and placed in a solution of 30% sucrose overnight at 4°C.

For sectioning the agarose blocks were frozen in Tissue Tec (Sakura) and the sections were collected on Histobond Adhesion microslides (Marienfeld). The sections were allowed to air dry for 2-3 hours and were then covered with glycerol and a coverslip placed on top. The samples were then imaged using a Zeiss Axiovert 2 microscope controlled by a Metamorph image acquisition system (Visitron, Germany).

Imaging of Labelled Cells

The animals containing labelled cells were imaged every day, by anaesthetizing the animals in 0.01% Ethyl-p-Aminobenzoate (Sigma), placing on a coverslip and imaging using a Zeiss Axiovert 2 microscope controlled by a Metamorph image acquisition system (Visitron, Munich, Germany). The pigment cells on the dorsal side of the spinal cord were used as an aid for correct orientation.

Results

Single Cell Electroporation of Spinal Cord Cells

The goal was to develop a technique to label single or a few ependymal cells in the spinal cord and follow their fate in live animals during regeneration.

Axolotls of 2-3cm were used as regeneration is rapid, and the tail tissue is transparent allowing good visualization of the spinal cord. Micropipette electroporation was carried out by amputating the tail and then inserting the glass electrode containing the plasmid DNA into the neural canal through the cut end of the spinal cord (Fig.11). By tilting the axolotl at an angle as shown in Fig.11b I could easily insert the needle into the lumen of the spinal cord under the stereomicroscope. A rapid series of pulses (50V, 200Hz) was then applied.

With this method, I found on average 10-30% of animals contained labelled cells. Animals with labelled cells contained an average of 2-3 cells labelled. I found that the change in efficiency of transfection when varying the number and frequency of pulses applied were very similar to those described by Haas *et al* (2001). The optimum efficiency of transfection was obtained at 50V, 200Hz, higher voltages caused tissue damage while lower voltages gave a very low frequency of transfection (Table 3). To transfect single cells a tip diameter of 1-2 μ m was used with a resistance of 10-15Mohms. Micropipettes with a larger tip diameter resulted in a higher number of transfected cells that were usually labelled as clusters.

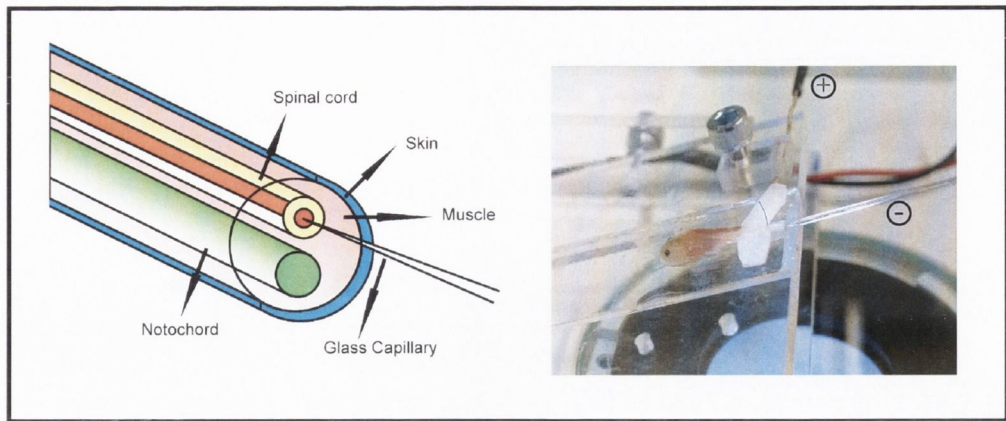


Fig.11 Schematic diagram of single cell electroporation set-up and a photo of the experimental set-up. First the tail is amputated, a needle electrode containing the DNA solution and the negative electrode is then inserted into the neural tube and a pulse of 50V, 200Hz and pulse length of 100ms is applied five times. 48hrs later the distance of the labelled cell from the amputation plane is measured and the fate of the labelled cell is recorded in the regrowing spinal cord.

Table 3 Parameters Tested to Obtain Maximum Transfection Efficiency

	Voltage (V)	Pulse length (ms)	Number of Pulses	Trains (50Hz, 1 sec.)	Trains (200Hz, 1sec.)	No. of labelled animals	Total no. of animals transfected
A.							
	20	1	5			1	100
	20	100	5			2	100
	50	1	5			5	100
	50	100	5			6	60
B.							
	50	10		5		9	60
	50	100		5		6	60
	50	10			5	9	60
	50	100			5	18	60
	80	10			5	1	25

Animals were electroporated in the spinal cord as described in the text and checked for transfected cells after 2 days. **A.** Electroporation was performed with a BTX Electro Square Porator 830. **B.** Electroporation was performed with a Grass SD9 stimulator. Trains: 200 or 50 pulses per second with an interstimulus interval of 5 ms.

When co-transfection of two plasmids was used I found that nuclear GFP driven by the CMV promoter was first visible after 12-24 hours while DsRed2 under the same promoter became visible at 36-48 hours. Bright GFP fluorescence could be detected for 7-14 days, while DsRed2 lasted 14-22 days, the length of visualization is also dependent on the number of cell divisions that the labelled cells underwent.

Labelled Cells lie within the Spinal Cord

The use of ubiquitous promoters means that cells outside the spinal cord can theoretically also be labelled. The accuracy of the labelling technique depends on the optical clarity of the axolotl tail allowing one to visualize the diameter and

length of the spinal cord as the micropipette is being inserted into the spinal cord. To assess the accuracy of the technique I cryostat sectioned the labelled tail and found that in all tail samples that were cryosectioned (n=12), the labelled cells lay within the spinal cord (Fig.12).

Given the efficiency of this method to deliver DNA plasmids into cells within the spinal cord and also into other cell types of the tail, I imagine that this technique can be further developed to deliver other charged macromolecules into specific cell types to allow us to further elucidate the molecular signalling mechanisms underlying regeneration.

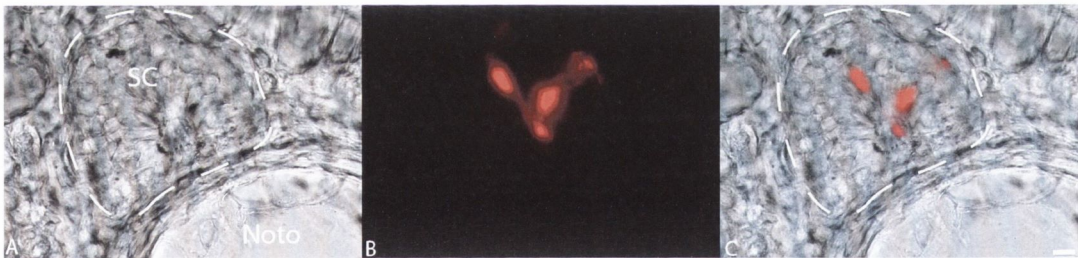


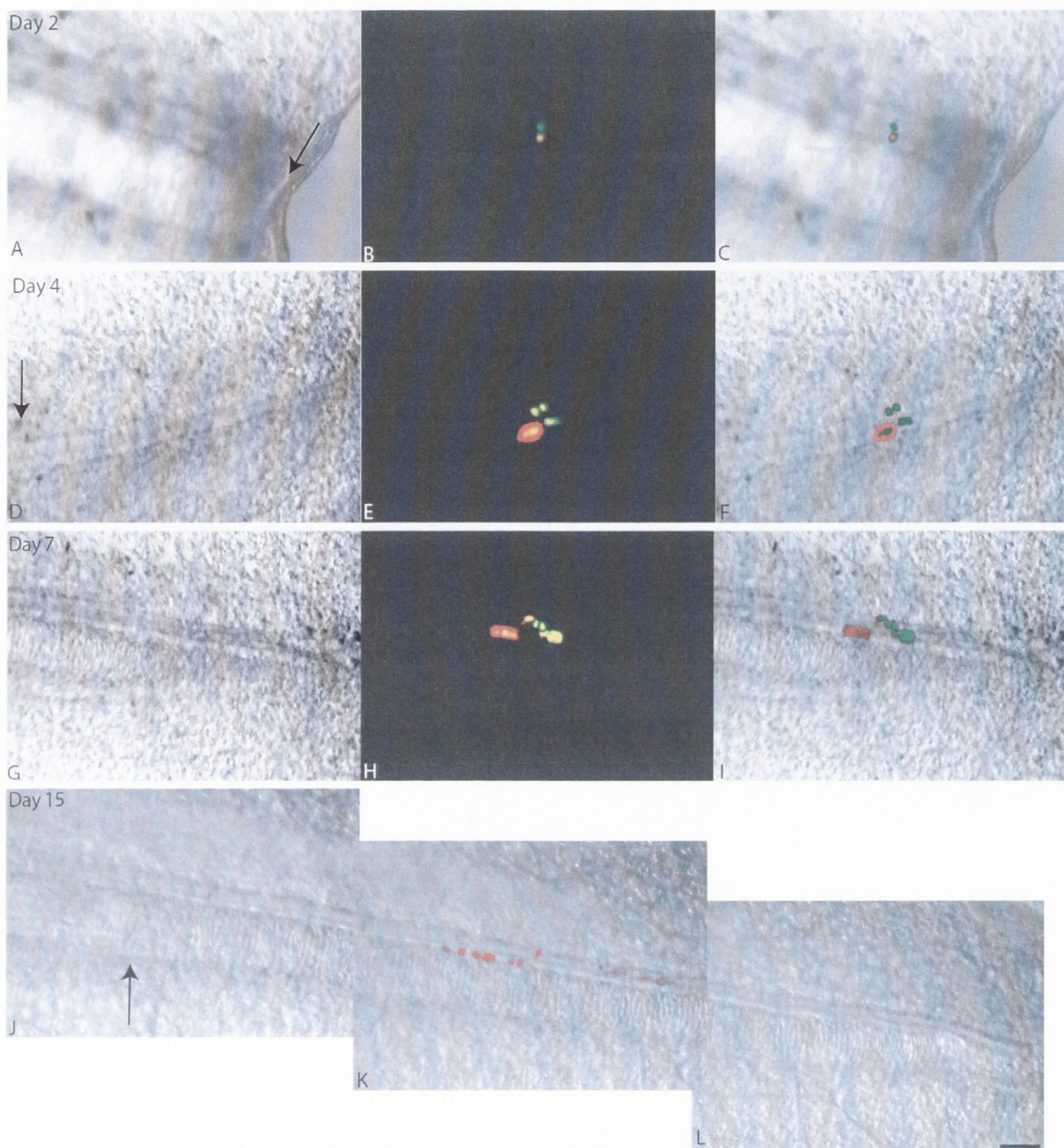
Fig.12 100 μ m transverse cryosections of the regenerating axolotl tail. Panel A is the DIC image of the spinal cord (SC) and part of the notochord (Noto). Panel B shows a fluorescent image of cells labelled by SCE with CMV-DsRed2-N1. Panel C, overlay of DIC image and the fluorescent image, showing that the labelled cells are cells of the spinal cord. Scale bar = 50 μ m

Spinal Cord Regeneration is not affected by Electroporation

Electroporation techniques used in the past have caused considerable tissue damage. I found that the use of a rapid succession of short pulses to transfect the cells limited tissue damage, and the electroporated tail tissue underwent normal regeneration. In the axolotl, tissue damage caused by the electric pulse can often be observed as contraction of the cells close to the tip of the needle.

Tail regeneration in 2-3 cm axolotls occurs in about 14 days at which point blastema cells begin differentiating into structures such as the rod of cartilage ventral to the spinal cord (Echeverri *et al.*, 2001). In Fig. 13 two cells in the spinal cord have been co-transfected with the CMV-nuclear GFP and cytoplasmic CMV-DsRed2 plasmids and the cells are lying approximately 300µm anterior to the plane of amputation (C). On day 2 the DsRed2 is expressed at a low level in the cells and becomes brighter over the next days. As the spinal cord grows (D), both cells begin to divide rapidly (E, F) and have moved out of the mature tissue to form part of the regenerating spinal cord (F, I). By day 15 of regeneration, the nuclear GFP is no longer visible but the DsRed protein is still easily visible (K). The cells, which were initially 300µm rostral to the plane of amputation, have proliferated and migrated to give rise to cells on both the dorsal and ventral sides of a segment of the mid-portion of the newly formed spinal cord. Neither cell has left a descendent in the mature spinal cord (J-L). Also at this time point it can be seen that the cells of the blastema have begun to differentiate and have formed a rod of cartilage. These results illustrate that electroporation of single cells in the spinal cord does not affect their ability to proliferate or migrate.

Fig. 13 Cells were labelled with cytoplasmic DsRed2-N1 and nuclear GFP (B, C). The fluorescent overlay with DIC image at 2 days post amputation shows both cells lying within the spinal cord about 250-300µm away from the plane of amputation (C). Over the following two days the cells divide and contribute to the regenerating spinal cord (E, F). The cells continue to rapidly divide as the new spinal cord is growing (G, H, I). Panels J, K, L show a composite of the DIC images overlayed with the fluorescent image at 15 days. At this point the original two cells have given rise to approximately 10 cells on both the dorsal and ventral sides of the mid-portion. The arrow indicates the original plane of amputation. Scale bar=100µm.



Single Cell Electroporation can be used to study the fate of spinal cord cells during regeneration

In Fig.13 I showed that SCE can be used to label the ependymal cells in the mature spinal cord that participate in spinal cord regeneration. Fig. 14 demonstrates that it can also be used to label neurons (Fig.14 B&C). In the same animal an ependymal cell lying close to the plane of amputation has also been labelled (B&C), so here I can follow the fate of two cells approximately 250µm apart. Over time the neuron remains unchanged but the other cell begins to divide (E&F). The cells then begin more rapid cell division and also begin to migrate into the regenerating portion of the spinal cord (H&I). By Day 10 of regeneration cell division appears complete, the initial cell has given rise to approximately 10 cells which now begin to spread out to populate the ventral side of the newly forming regenerate (J&K). The neuron does not dedifferentiate or migrate, it remains behind the plane of amputation but stretches its axons out towards the newly forming muscle (J). In the cases where neurons have been electroporated (n=6), based on morphology alone the cells have not been observed to dedifferentiate or to migrate into the regenerate. However from this limited data I cannot conclude that all classes of neurons exhibit this behaviour. In these experiments it is possible that a sub-set of neurons that do not participate in regeneration may have been selectively labelled

Again it can be seen that electroporation, even of neurons does not cause damage to the cells and does not inhibit either cell division or cell migration, two key aspects of regeneration.

But not all ependymal cells at the plane of amputation contribute to the regenerating spinal cord. In Fig. 15, a cell lying approximately 250 μ m back from the plane of amputation (B, C) does not contribute to the regenerate. As regeneration progresses the cell remains constant (E, F) and eventually as the cells of the blastema differentiate, the labelled cell begins to divide but all cells remain rostral to the plane of amputation (H, I).

Using this technique for labelling and documenting the fate of cells during regeneration, cells have also been observed which migrate with the tip of the newly forming regenerate (Fig.16 B, C). The cluster of cells remains at the tip of the regenerate and at Day 5 start dividing (H, I) and these cells migrate out into the surrounding fin mesenchyme (K, L). The ultimate fate of these cells could not be determined as the Dsred and GFP proteins were undetectable soon after the cells began to exit the spinal cord.

This method of single cell electroporation allows the fate of cells during regeneration to be followed live in the animal. By recording the distance of the labelled cell from the plane of amputation and recording its fate in the regenerate (n=38), this allowed a growth zone to be defined (Table 4). From this data it can be seen that cells labelled more than 400 μ m back from the plane of amputation never contribute to the regenerate. Cells within a zone of 400 μ m anterior to the plane of amputation appear to have an equal chance of staying close to the plane of amputation or contributing to form the tip of the regenerate.

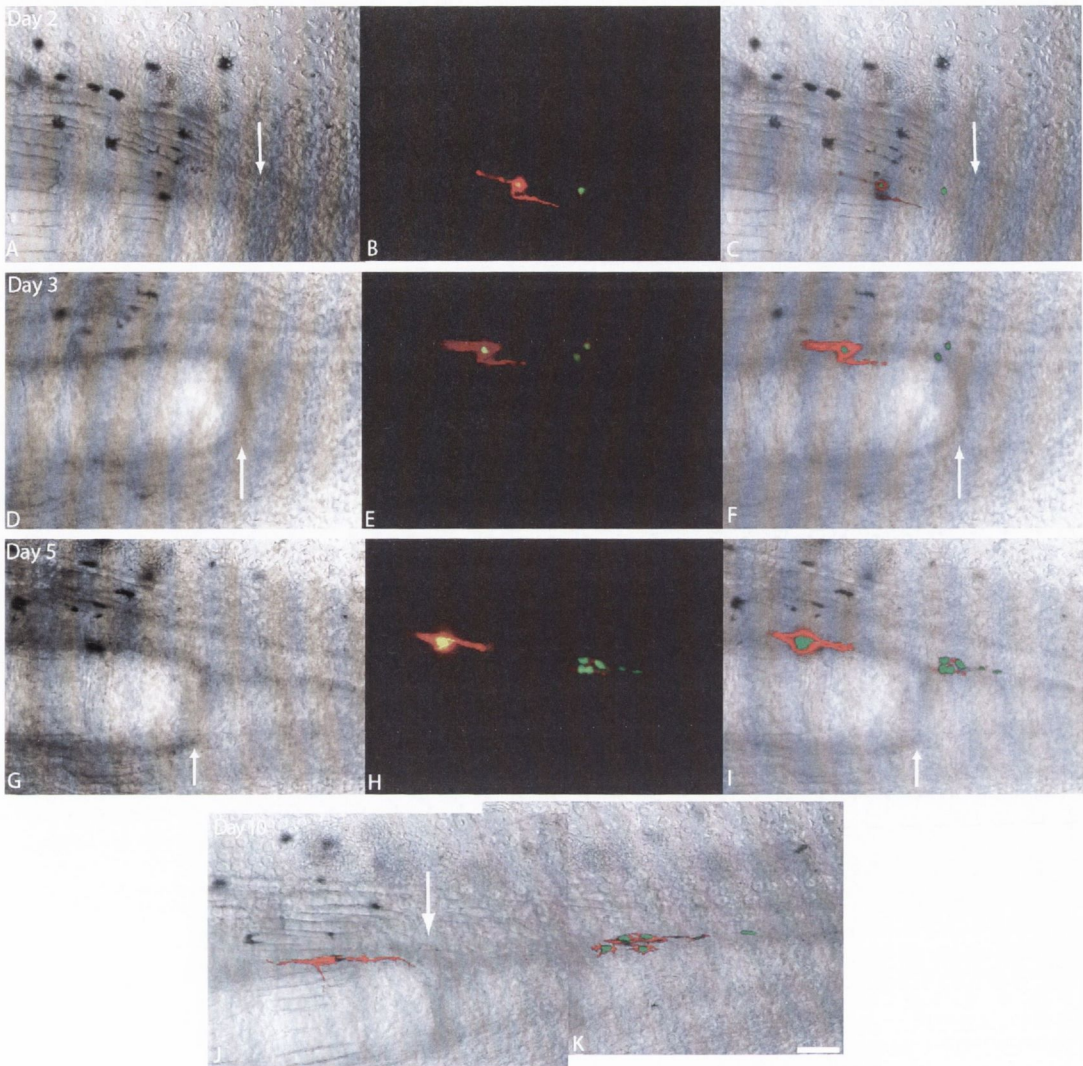


Fig.14 Ependymal cells and neurons can be labelled using the single cell electroporation technique. Panel B, C show each cell type expressing the Dsred and nuclear GFP two days after electroporation. As regeneration progresses the ependymal cells divide rapidly (E&H), while the neuron remains unchanged (E&H). By 10 days post amputation the rapidly dividing ependymal cells have migrated out to contribute to the regenerating spinal cord (J&K), while the neuron remains behind the amputation plane putting out axons to probably innervate the muscle lying above it (J&K). The arrow indicates the plane of amputation. Scale bar = 100µm.

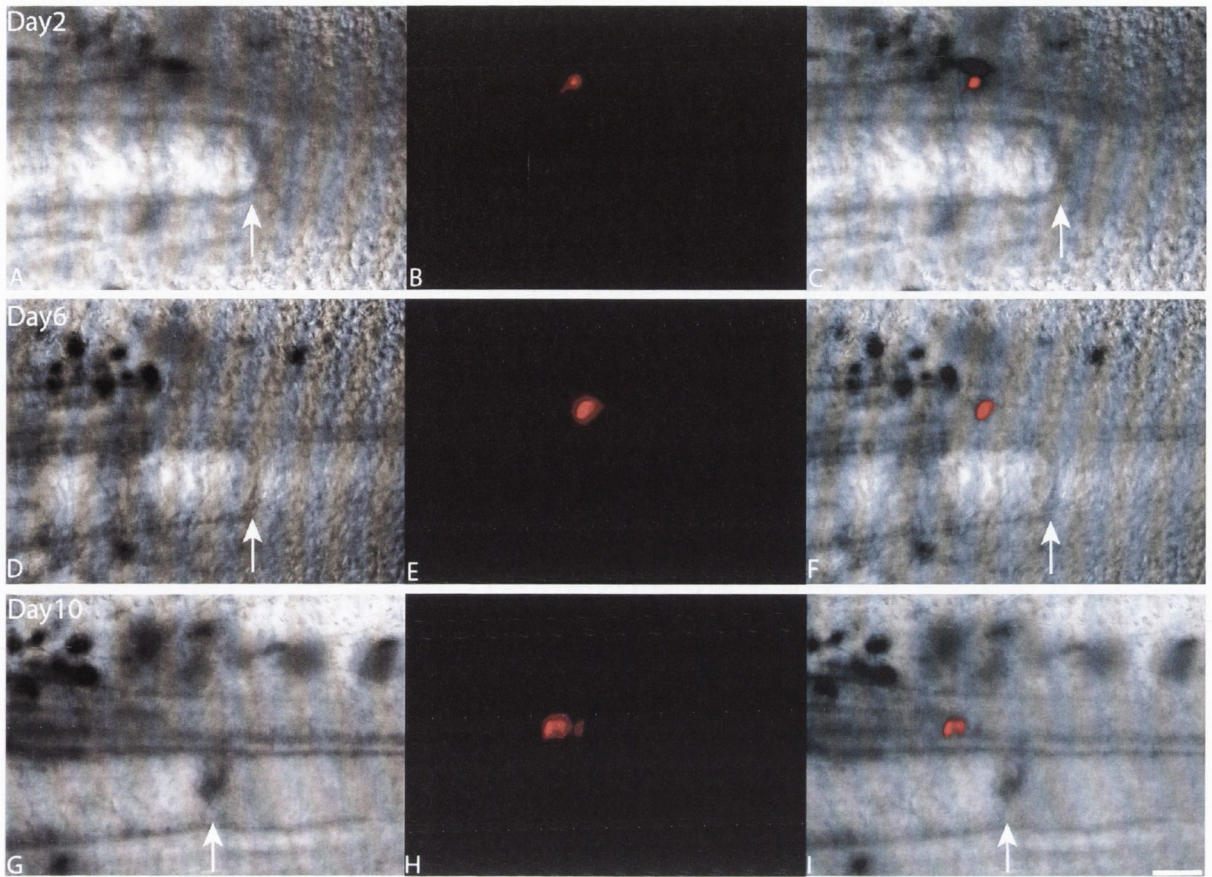


Fig. 15 Labelled cells stay close to the plane of amputation. Panel A, D, G show the DIC images of the regenerating tail over time. Panels B, E, H are the corresponding fluorescent images of the cell in the spinal cord labelled by single cell electroporation with Dsred-N1. The overlay of 2 days post amputation DIC image with the fluorescent image shows that the labelled cells lie within the spinal cord, approximately 250 μ m away from the plane of amputation (C). Over time the labelled cells stay close to the plane of amputation but never contribute to the regenerate (F&I). The arrows indicate the original plane of amputation. Scale bar= 100 μ m.

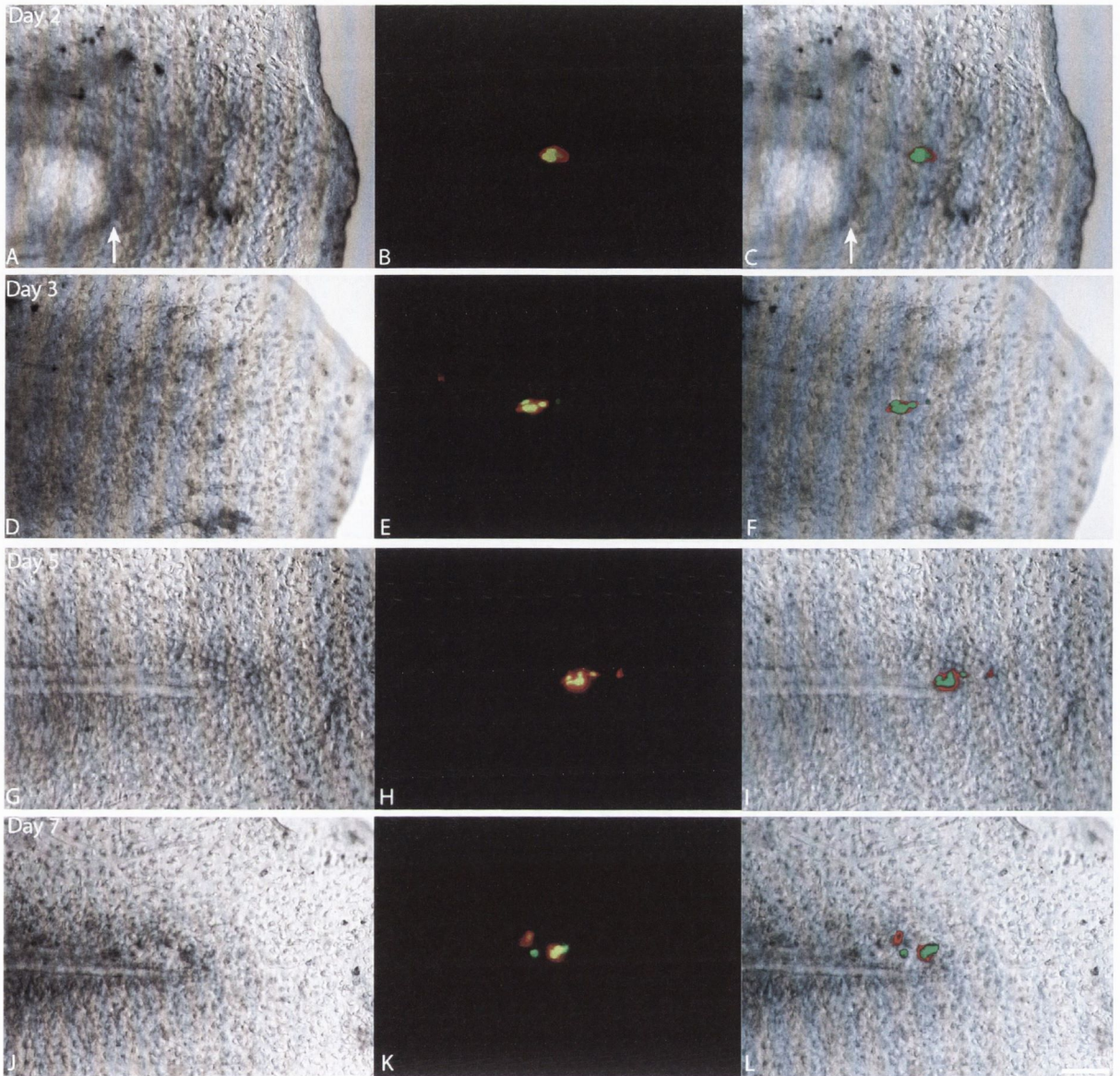


Fig. 16 Cells that form the tip of the new spinal cord also contribute to the blastema. Panels on the left show the DIC image of the tail, middle panels show the fluorescent image of the labelled cells and panels on the right show the overlay. Arrows indicate the original plane of amputation. Cells were labelled with cytoplasmic Dsred2-N1 and nuclear GFP (B&C). The labelled cells that were about 50µm away from the plane of amputation stay at the tip of the re-growing spinal cord (D, E, F). The cells begin to divide and migrate out of the blastema into the surrounding tissues (I, L). Scale bar =100µm.

Electroporation as a Method to Define a Growth Zone for Spinal cord

Regeneration

This method of cell electroporation allows the fate of cells during regeneration to be followed live in the animal. By recording the distance of the labelled cell from the plane of amputation and recording its fate in the regenerate (n=38), this allowed a growth zone to be defined. From this data in Table 4 it can be seen that cells labelled more than 400µm back from the plane of amputation never contribute to the regenerate. Cells within a zone of 400µm anterior to the plane of amputation appear to have an equal chance of staying close to the plane of amputation or contributing to form the tip of the regenerate. Also observed is that cells can come from the dorsal, ventral or middle of the spinal cord to contribute to the regenerate and from these preliminary observations it would appear that the progeny of one cell can populate either the dorsal or ventral side of the regenerate.

It is not known how the cells migrate to form the new spinal cord but from the data in Table 4 it could be interpreted that a lot of cell mixing occurs in this 'Growth Zone' and that cells are pushed out to form the new spinal cord and hence it is random chance as to where you end up in the regenerate.

Table 4: Cells from within a zone of 400µm rostral to the plane of amputation give rise to the new spinal cord.

Name of Sample	Distance from plane of amp. µm	Position: dorsal, ventral , middle	Final Position in Regenerate.
1	450	ventral	Behind plane of amputation
2	500	middle	Behind plane of amputation
3	400	middle	At plane of amputation
4	550	ventral	Behind plane of amputation
5	600	dorsal	Behind plane of amputation
6	450	middle	At plane of amputation
7	500	ventral	At plane of amputation.
8	550	dorsal	Behind plane of amputation
9	300	dorsal	Mid portion of regenerate
10	250	dorsal	Tip of regenerate
11	380	middle	Close to plane of amputation
12	100	dorsal	Close to tip of regenerate
13	50	ventral	Close to plane of amputation
14	400	dorsal	Close to plane of amputation
15	20	dorsal	Close to plane of amputation
16	50	middle	At tip of regenerate
17	200	dorsal	At tip of regenerate
18	250	ventral	Mid portion of the regenerate
19	50	ventral	At tip of regenerate
20	200	ventral	At tip of regenerate
21	50	dorsal	Mid portion of the regenerate
22	10	ventral	At plane of amputation
23	10	ventral	Mid portion of the regenerate
24	10	middle	Tip of the regenerate
25	140	ventral	Close to the plane of amputation

26	50	ventral	Close to plane of amputation
27	100	middle	Tip of the regenerate
28	200	middle	Tip of the regenerate
29	340	ventral	Mid portion of the regenerate
30	10	middle	Tip of the regenerate
31	250	dorsal	Plane of amp.
32	250	ventral	Tip of regenerate
33	10	ventral	Tip of regenerate
34	70	ventral	Tip of the regenerate
35	10	ventral	Tip of the regenerate
36	100	dorsal	Mid portion of the regenerate
37	10	middle	Mid portion of the regenerate
38	10	ventral	Tip of the Regenerate

Note: The final position in the regenerate, dorsal, ventral or middle is not listed as at the end of regeneration the initial cell had always divided several times and in all cases the progeny were not restricted to only dorsal, ventral or a middle position.

Discussion

In summary, the development of this technique allows transfection of DNA into the axolotl spinal cord efficiently and with no detectable cell damage via electroporation with a DNA-filled microelectrode. Coupled with the use of small optically clear animals, we are beginning to understand how neural precursors within the mature tissue are recruited to form the regenerating spinal cord. From this study and work from other labs it is clear that in response to injury cells in the mature spinal cord proximal to the plane of amputation increase cell division and begin to migrate out to form the regenerate.

This initial data suggests that some spinal cord cells may actually exit the spinal cord and contribute to the blastema but in these experiments the limiting factor has again been the length of expression of the lineage label, so the ultimate fate of the cell has not been determined but this is a technical obstacle, which can be eventually overcome.

An interesting aspect of spinal cord regeneration is the question of where do the cells come from to form the new spinal cord and can cells far away from the plane of amputation contribute to the regenerate? By measuring the distance of the labelled cell from the plane of amputation and recording its fate I have been able to define a growth zone at the end of the mature spinal cord. This data then allows us to think about models of how the spinal cord may actually regenerate.

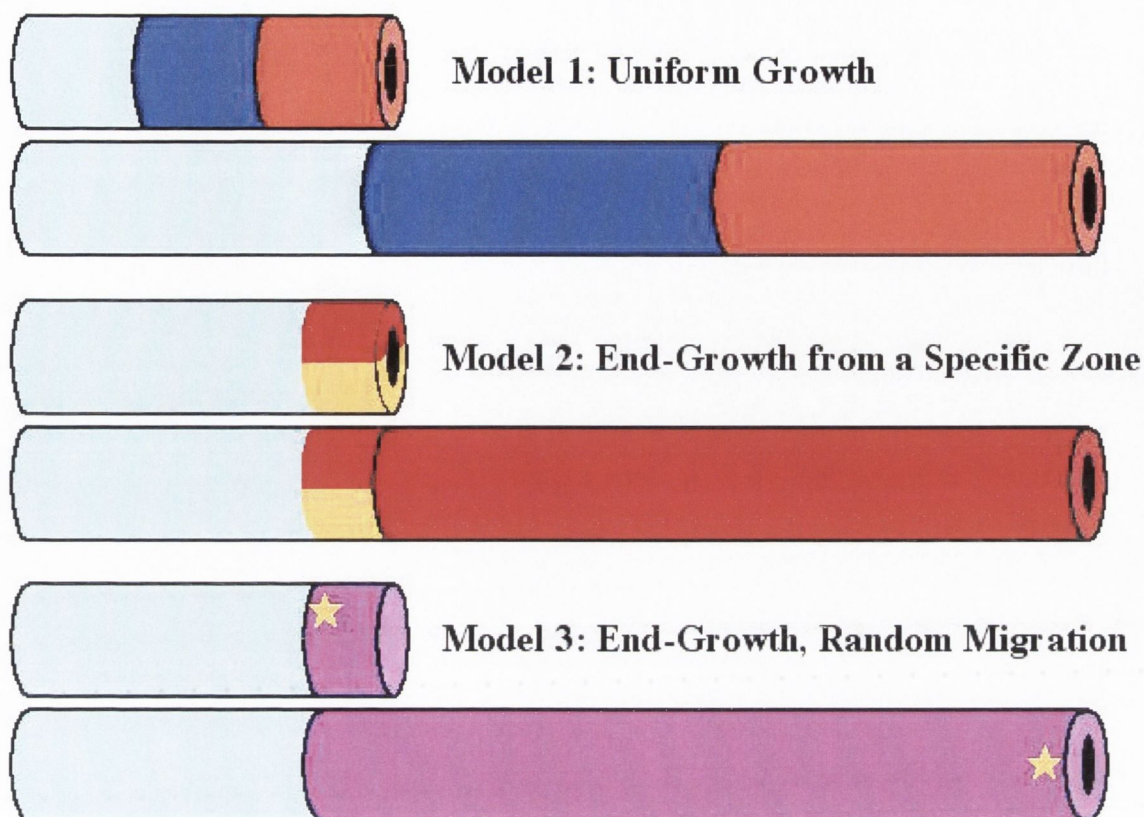


Fig. 17 Models of Spinal Cord Regrowth

The models proposed were generated prior to the collection of the data while trying to envision how such an essential structure like a spinal cord could be regrown so quickly and precisely.

The first model proposed is of uniform growth as illustrated above, the underlying idea being that the mature remaining spinal cord would regenerate by equal growth along the whole length of the remaining tissue. This idea would imply that in response to wounding that all cells within the mature spinal cord, regardless of their distance away from the plane of amputation would rapidly start dividing again and regrow to it's original length. Initial experiments of looking at the nuclei within

the spinal cord by Dapi staining would argue against this model as increased mitotic indices were not observed along the length of the mature spinal cord.

The second model suggests that regeneration would occur from a defined zone on the dorsal or ventral side of the remaining stump, that cells in this specific zone would increase cell division and migrate to form the new spinal cord. This model was proposed as a very organised manner in which multipotent progenitors could increase cell division and then migrate to form whichever cell types were necessary. This would have been an interesting scenario to observe as it would imply that cells from this area are still undetermined or that they can dedifferentiate and can be respecified in response to a signal generated by injury.

The data I have collected suggests that this is not the case as I have observed cells coming from both the dorsal and ventral sides of the mature stump participating in regeneration (Table 4).

My data, shown in Table 4 favours the third model of 'End-growth, Random Migration'. In this model the regenerate would form from cells of a specified zone, from my data the zone would be approximately 400µm long anterior to the plane of amputation, and a lot of cell mixing would occur in this zone such that cells have an equal chance of being pushed out to the tip or of staying at the plane of amputation and that once the position has been decided then the ultimate cell fate decision is made. This model would explain why some cells stay at the plane of amputation (Fig.15), others move a small distance into the regenerate (Fig.14) while others move out to the tip and eventually into the blastema (Fig.16).

From this initial data it would appear that the progeny of a dorsal or ventral cell can populate both positions in the regenerate, implying that the cells are quite plastic.

So far little is known about the signalling molecules involved that tell the cells to divide and migrate or about the interactions between the spinal cord and surrounding tissue. Almost certainly this application can also be applied to labelling other defined cell types to understand tissue dynamics and remodelling on a single cell level.

The development of this technique will hopefully aid the mechanism of building a new spinal cord to begin to be elucidated on both a mechanistic and molecular level by being able to combine following cell fate with eventually testing gene function in cells undergoing regeneration.

Chapter 4

Multilineage Potential of Radial Glial Cells during Axolotl

Spinal Cord Regeneration

Introduction

Lineage restriction into ectodermal, mesodermal and endodermal germ layers was classically considered a unidirectional process that occurred during development. Recent data, however, indicates that adult cells from various sources including, brain, skin and bone marrow can form cell types of other lineages when exposed to novel or foreign environments (Bjornson *et al.*, 1999; Brustle *et al.*, 1999; Clarke *et al.*, 2000; Rietze *et al.*, 2001; Toma *et al.*, 2001). It is still under debate whether such examples represented true cases of cell-type switching and whether lineage switching represents a rare or frequent event. (Anderson *et al.*, 2001; Morshead *et al.*, 2002). The issue of neural cell plasticity has been particularly controversial. Cells derived from the ventricles of the adult brain have been documented to form muscle in transplantation and co-culture experiments, and bone marrow when infused into irradiated mice (Bjornson *et al.*, 1999; Galli *et al.*, 2000; Rietze *et al.*, 2001; Shih *et al.*, 2001). Furthermore when transplanted into chick embryos these neural progenitors populated tissues arising from all three embryonic germ layers (Clarke *et al.*, 2000). Questions have been raised as to whether 1.) the cell that gave rise to other cell types was truly of neural origin rather than a contaminating cell type, 2.) long-term culture may have selected cell characteristics not present in the original neural cell itself (Morshead *et al.*, 2002) and 3.) whether the cells merely fused with other cells and thereby their fate was determined by fusion to a predetermined cell type (Terada *et al.*, 2002; Ying *et al.*, 2002). In addition it is unclear if such lineage switching represents a rare or frequent event. Such ambiguities are largely due to the cell assays that involve placing populations of

cultured cells into unnatural environments and examining the outcome after numerous cell doublings have occurred. Understanding cell plasticity is important, however, because of its potential in creating progenitor pools for tissue regeneration but since most mammalian tissues do not naturally regenerate it has been difficult to assess the relevance of this phenomenon for tissue repair.

To determine if lineage switching is an integral part of tissue regeneration I developed a method to follow individual neural precursor cells live during axolotl tail regeneration. In this system tail amputation results in perfect regeneration of all tail structures including the spinal cord, muscle, cartilage, dermis and skin in several weeks (Clarke .1988, Chernoff 1996). Previously I showed that the entire process of tail regeneration could be visualized in the light microscope with single cell resolution in 2 cm long animals (Echeverri *et al.*, 2001). Figure 18 A-C provides a schematic overview of the three major stages of tail regeneration. Immediately after amputation epidermal cells migrate over the cut end to form the wound epidermis (Figure 18A). Over the next few days, a zone of proliferating progenitor cells called the blastema forms (Figure 18B). The blastema cells largely derive from muscle and dermis dedifferentiation and in the late stages of regeneration re-differentiate into muscle, cartilage and dermis (Muneoka *et al.*, 1986; Echeverri *et al.*, 2001). The regenerating spinal cord (blue) constitutes a tube of neuroepithelial cells separate from the blastema. As regeneration proceeds the tube elongates, and differentiation of spinal cord cells such as neurons occurs.

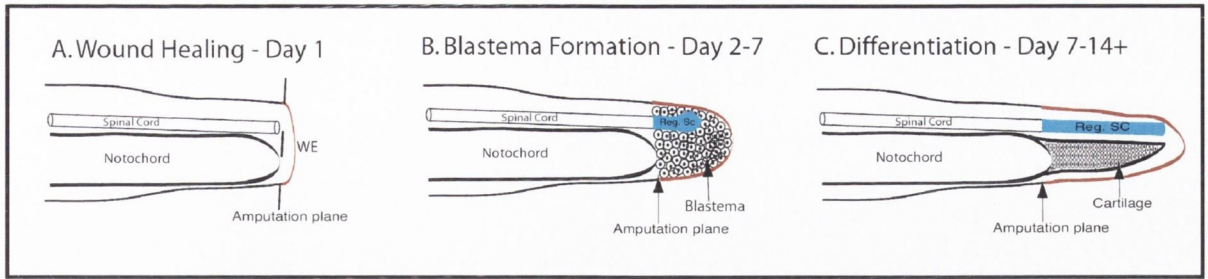


Fig. 18 Schematic overview of tail regeneration. Initially the wound heals by crawling of epidermal cells from all sides to form a wound epidermis (red, WE) (A). A mound of proliferating undifferentiated cells is then formed called the blastema (B). The main source of these cells is from dermis and muscle dedifferentiation. The first morphological signs of differentiation are seen by the formation of a cartilage rod at approximately Day 14 (C). At this time point the muscle and other cell types are also differentiating, (not illustrated).

Classical histological studies indicated that lineage restrictions were likely maintained during regeneration; the wound epidermis derived from mature epidermis, the regenerating spinal cord and neural crest formed from the spinal cord while mesodermal tissues such as muscle and cartilage formed from dedifferentiation of tissues originating from mesoderm (Holtzer *et al.*, 1955; Hay and Fischman, 1961; Geraudie *et al.*, 1988; Benraiss, 1999). However, these observations depended on the reconstruction of events from static images of cell populations within regenerating tissue. Therefore it remained an open question whether cells cross lineage boundaries during regeneration.

Materials and Methods

pGFAP-GFP plasmid

The plasmid used contained a glial fibrillary acidic protein (GFAP) promoter driving expression of the GFP protein via the enhancer construct Gal4VP16UAS. The human GFAP 2.2kb promoter was cloned into the Bgl 11/Sal 1 sites of the multiple cloning site of the Clontech EGFP-1 vector. The construct was checked by restriction digest for the insert of correct size.

The Gal4VP16UASGFP fragment was then cloned into the Sac 11 site in the multiple cloning site. The construct was then checked again by restriction digest to ensure that the fragment of the correct size and orientation was inserted. The whole construct was then sequenced by the DNA sequencing facility. The GFAP promoter and the Gal4VP16-UASGFP constructs were kind gifts from Marina Matyash (MPI-CBG, Dresden) and Reinhard Koster (Caltech, USA).

Testing of GFAP-Gal4VP16-UAS-GFP Construct

The specificity of the GFAP-GFP construct was tested by initially transfecting the construct into U87 human glioma cells by lipofection, the muscle cell lines, newt A1 cells and C2C12 mouse cells were used as controls.

The U87 cells were grown in Basal Medium (Eagles)(Gibco) plus 10% Foetal Calf Serum(Gibco) (FCS), 10mM Hepes, 20mM L-Glutamine(Gibco) and 1% Penicillin/Streptomycin (Gibco), at 37°C with 5%CO₂.

The newt A1 cells were grown in Minimal Essential Medium with Earles Salts (Gibco) plus sterile double distilled water, 15%FCS, 1% Pen/Strep, 1% Glutamine and 1% Insulin (Gibco), the cells were grown at 25°C with 2%C02.

The C2C12 cells were grown in Dulbecco's Minimal Essential Medium (Gibco) with 10% FCS, 1%Pen/Strep and 1%Glutamine. The cells were grown at 37°C with 10%C02.

For testing the plasmids, dishes of confluent cells were transfected using Lipofectamine Plus (Invitrogen), following the supplied protocol. In all cell lines the pGFAP-GFP plasmid was co-transfected with the CMV-DsRed2-N1 plasmid. Both plasmids were also transfected on their own to ensure mixing didn't affect transfection efficiency.

As a further control the mixture of both plasmids was randomly injected by hand using a syringe and needle into the body of the axolotl and a voltage of 50V was applied five times using flat square electrodes of approximately 1cm x 1cm, placed on either side of the axolotl's body. The animals were allowed to recover in water containing Penicillin/Streptomycin and two days later they were imaged as previously described in chapter 2.

Single Cell Electroporation

Single cell electroporation and cryostat sectioning were both carried out as described in chapter 3.

Antibody Stainings

Fixation and staining of the tails was carried out as previously described in Chapter 2. For the GFAP staining a monoclonal anti-GFAP Clone G-A-5 (Sigma), diluted 1:400 was used.

For staining the cell lines, the cells were fixed in 4%paraformaldehyde (PFA) at room temperature for 20 minutes and then washed in PBS. The cells were then blocked in PBS plus 10% goat serum for 20 minutes and the rest of the staining procedure was the same as for the tails as described in Chapter 2.

HNK-1 and 1E8, neural crest antibodies were obtained form the Developmental Studies Hybridoma Bank as supernatants which were tested undiluted, 1:10, 1:50, 1:100 and 1:200 on fixed axolotl tails and cryostat sections of the tail.

Anti-collagen type 11 tested was from Research Diagnostics and anti-GFP polyclonal was from Molecular Probes.

Results

GFAP Promoter is Specific to Radial Glial Cells

To follow spinal cord cells during regeneration I implemented single cell electroporation to express the green fluorescent protein in the neural precursor cells of the spinal cord. These precursor cells are radial glial cells similar to radial glial cells that act as neural precursors in the mammalian brain (Noctor *et al.*, 2002). I targeted GFP expression to radial glial cells by inserting the DNA-filled microelectrode into the lumen of the spinal cord through the cut end of the tail and by driving GFP expression with the Glial Fibrillary Acidic Protein promoter (GFAP promoter)(Nolte *et al.*, 2001) via the GAL4-VP16 enhancer (Fig.21)(Koster and Fraser, 2001). The GFAP promoter was chosen because GFAP protein is expressed solely in the radial glial cells of the mature axolotl spinal cord and expression is maintained in the regenerating spinal cord cells (Arsanto *et al.*, 1992). The expression of the GFAP protein was tested on axolotl by antibody staining on cryosections (Fig. 19) and through a timecourse of tail regeneration by wholemount stainings (Fig. 20). By both methods it confirmed that GFAP is expressed only in the cells of the spinal cord.

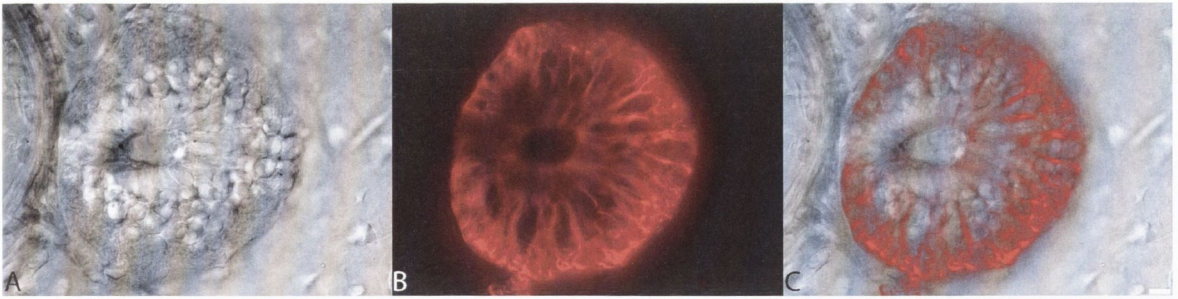


Fig. 19 Panel A shows a 50µm DIC image of a cryosection of a mature axolotl tail. Panel B, immunofluorescent staining with an antibody against the Glial fibrillary acidic protein (GFAP), the antibody specifically stains the processes of the radial glial cells. Panel C shows the overlay of the DIC image with fluorescent image. Scale bar = 50µm

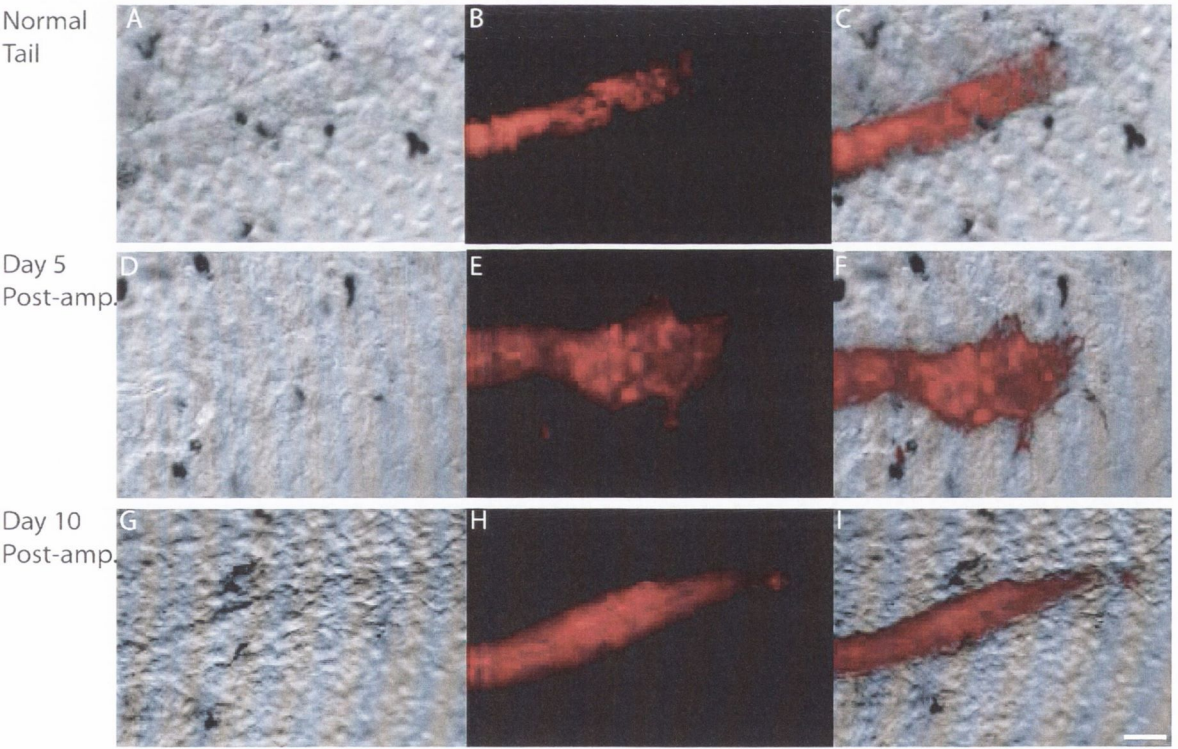


Fig. 20 Wholemount GFAP staining of axolotl tails. Panel A, D, G show the DIC image of normal non-regenerating tail, 5day regenerate and a 10 day tail regenerate. GFAP staining of a normal tail shows the end of the spinal cord to be

a closed structure (C). At 5 days post amputation a bulb like structure, termed the terminal vesicle is clearly visible by GFAP staining at the end of the regenerating spinal cord (E, F). At 10 days post amputation the terminal vesicle is no longer visible, the end of the spinal cord is now a more regular is shape similar to the non regenerating state (C), although the ventral side appears by GFAP staining to be longer than the dorsal side (H, I), suggesting that the end of the spinal cord may remain open during regeneration. Panels C, F, I show the overlay of the DIC image with the fluorescent image. Scale Bar = 100 μ m.

The use of an endogenous promoter often means the protein is not expressed at a high level in cells, to avoid this issue I used the enhancer construct designed by Koster (Koster and Fraser, 2001) based on the *Drosophila* Gal4-UAS system (Brand and Perrimon, 1993), in which stable transgenic lines expressing the transcriptional activator Gal4 under control of an endogenous promoter (Gal4 driver/ activator lines) can be crossed to transgenic lines (Gal4 effector lines) which carry a transgene of interest under the control of Gal4 binding sites (UAS, upstream activating sites). The progeny of the cross express the transgene strongly in a Gal4 dependent manner (Brand and Perrimon, 1993). Therefore expression is amplified via Gal4 and in principal more than one effector can be expressed at the same time. In this system the GFAP promoter drives expression of the Gal4-VP16 gene and the GFP is driven by the UAS-Gal4. The inclusion of the Gal4-VP16 enhancer system ensured that GFP persisted even if cells turned

off the GFAP promoter and transdifferentiated, due to high levels of the Gal4-VP16 protein being initially produced.

The promoter was tested by transfecting human glioma cells, newt and mouse muscle cells with the construct. I found that only the glioma cells expressed GFP or stained positive with an anti-GFAP antibody (n=4). As a further control, when I targeted plasmid transfection to cells outside of the spinal cord, no expression of the GFAP-GFP construct was observed despite strong expression of the co-transfected ubiquitously expressed plasmid, CMV-DsRed2-N1 (n=38).

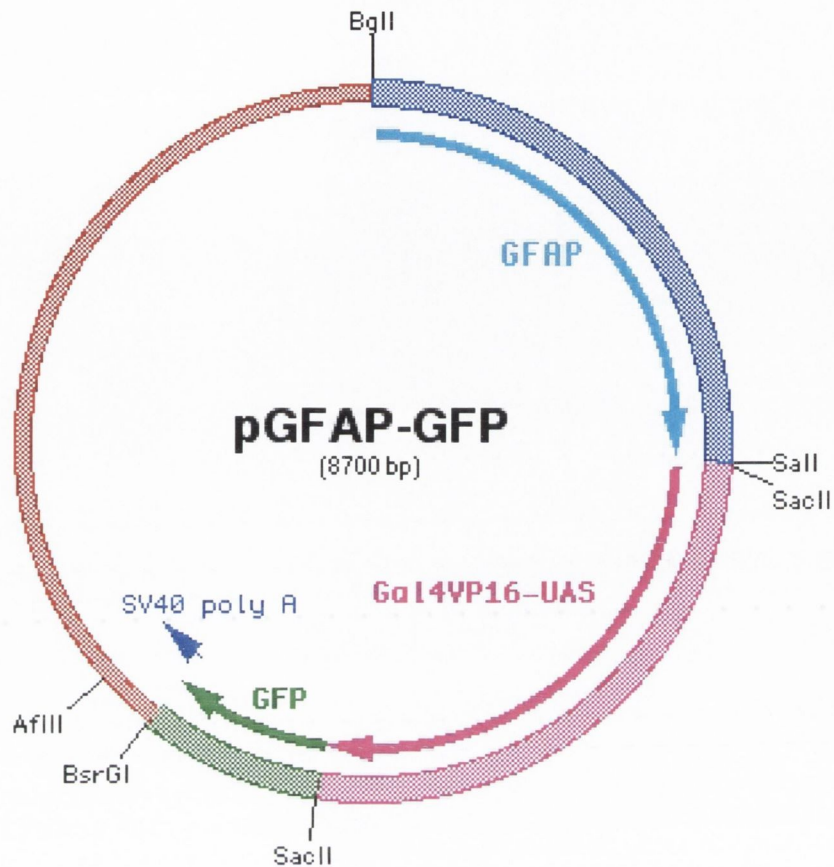


Fig. 21 A 2.2kb fragment of the human GFAP promoter was cloned at the Bg11, Sal1 sites and the enhancer construct was inserted at the Sac11 site. The vector backbone is pEGFP-1 from Clontech.

I confirmed the specificity of the labelling method in cryosections of the transfected tails (n=10) where the labelled cell was always found in the cell layer lining the neural canal (Fig.22 A-C). Figure 22A shows a transfected cell in a cross section

of the spinal cord. The cell has the characteristic fine radial processes of a GFAP-positive spinal cord cell while the overlay with a DIC image of the cross section confirms that the cell is in the layer lining the spinal cord lumen. Figure 22C illustrates the overall layout of the cross section and the positions of the GFAP-positive radial glial cells.

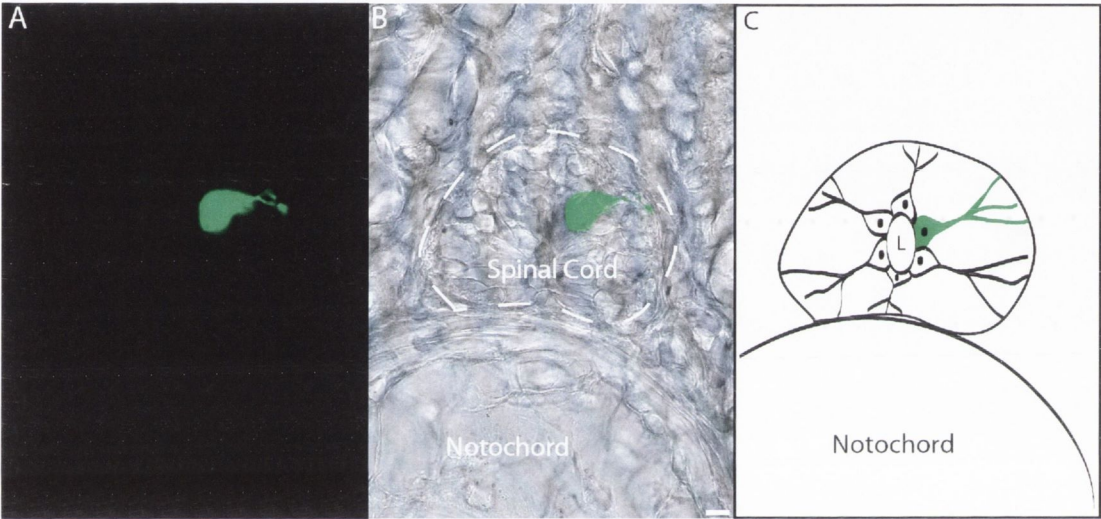


Fig. 22 100µm transverse cryosection of the axolotl tail containing a transfected radial glial cell. Fluorescent image of a GFP-expressing cell where the radial cell processes are clearly visible (A). The DIC image overlaid with the fluorescence image shows one labelled cell lying in the spinal cord and extending a process towards the outer pial layer of the spinal cord (B). Drawing to illustrate the morphology of the spinal cord. The radial glial cells that make up the ependymal layer are lying closest to the lumen (L), they extend processes out towards the pial edge, one labelled cell is illustrated in green (C). The ependymal layer is

surrounded on the outside with a layer of astrocytes and followed by neuronal cell bodies and axons (not illustrated). Scale bar = 50µm.

Radial Glial Cells form New Neurons during Spinal Cord Regeneration

Figure 23 illustrates labelled cells whose descendants formed expected neural cell fates including a differentiated neuron. A cell is initially visible at the end of the cut spinal cord two days post-transfection (Fig. 23 A-C, cell 2). By day 6 the cell, which exhibited increased signal intensity, was positioned at the end of the regenerating spinal cord that had grown to 350 µm in length (D-F). By Day 13, the cell had differentiated into a neuron with an axon that extended 1.4 mm back towards the body (arrowheads, G). In this example, a second labelled cell further back from the amputation plane (cell 1), divided to produce a row of radial glial cells (Fig. 23G). This behaviour was typical of many examples, where I observed that cells generated 8-10 descendants within the regenerating spinal cord. In the axolotl, the cell division cycle lasts on average 72 hours, the maximum number of cells expected at 14 days post-amputation is 16. In most examples the labelled cells migrated out to a certain point in the regenerate and then started dividing 2-4 days post amputation. In many examples during spinal cord regeneration I observed one GFAP-positive radial glia cell giving rise to between 8-10 progeny. These examples where labelled cells contributed to neural cell fates indicated that I was indeed labelling the neural cell precursors of the spinal cord.

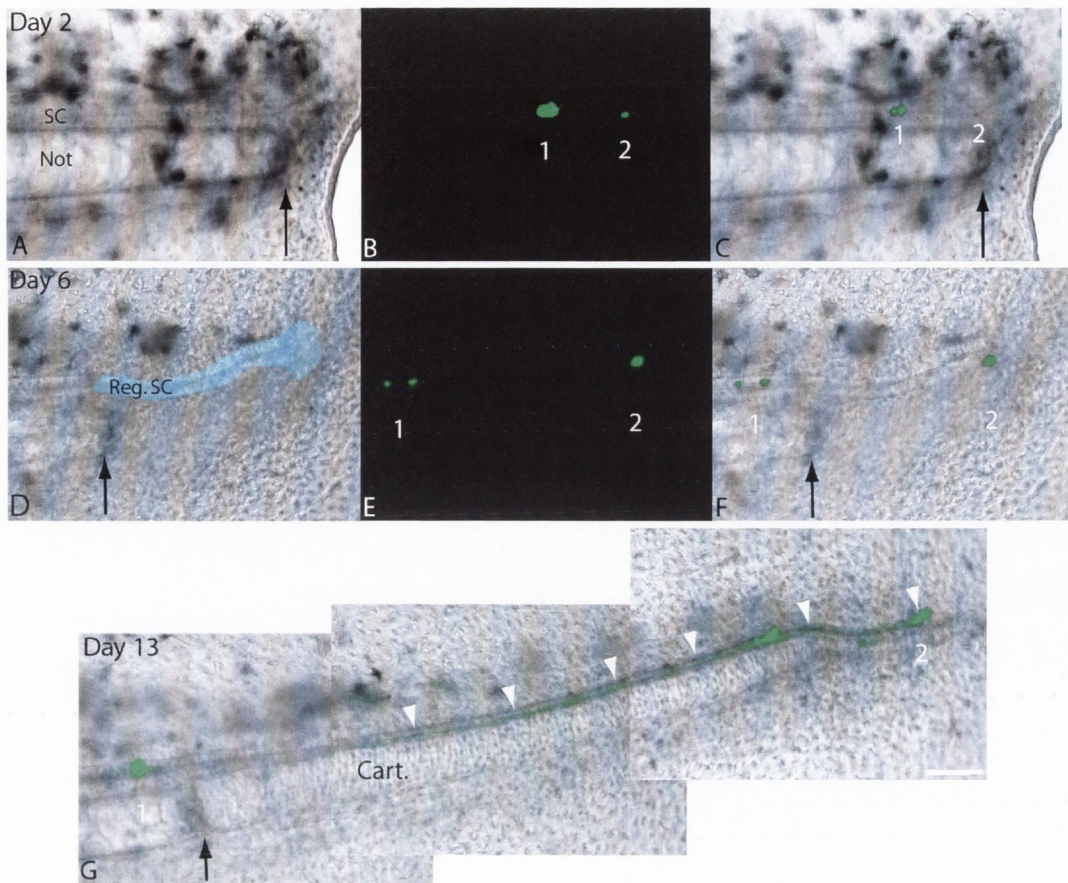


Fig.23 GFAP-positive radial glial cells give rise to neurons during regeneration. Left panel shows the DIC image of the tail, middle panel: fluorescent image of the transfected cells and right panel shows the overlay. Two days post amputation the wound epidermis has formed (A) and cells labelled with GFAP-GFP are seen lying within the spinal cord (B&C). In Panel B the brightness of the image has been increased to aid visualization of cell 2. By six days a regenerating spinal cord (D, reg. SC, highlighted in blue) is clearly seen and Cell 1 has divided and stays behind the plane of amputation (E&F) while Cell 2 has migrated to the tip of the newly forming spinal cord (F). At Day 13 differentiation has started, a rod of cartilage (Cart.) is now visible and Cell 2 has extended an axon back towards the

body (arrowheads), while Cell 1 remains behind the plane of amputation and has generated a row of radial glial cell descendents (G). In Panels A, D and G the arrow indicates the original plane of amputation. Scale bar = 100 μ m.

Radial Glial Cells Contribute to Cells of the Ectoderm and Mesodermal Lineage

Strikingly when I followed individual radial glial cells during regeneration, I found that their descendents were not all restricted to an ectodermal cell fate. While many labelled cells formed the expected neural cell types, at high frequency the descendents of labelled spinal cord cells migrated out of the regenerating spinal cord and contributed to the regenerating mesodermal structures; muscle and cartilage (Table 4). Three of the major classes of cell fates are illustrated in Figures 23-27. The most striking and frequent cases of cell type switching were to muscle (Figure 24.). Two days post-transfection, four cells were labelled in the mature spinal cord (Fig. 24 A-C). By day six the cells had divided and spread into four clusters in the regenerating spinal cord (D-F). On Day eight, cell cluster three was no longer lying within the spinal cord (G). By Day 13, cells of cluster three and four (three cells) had clearly moved outside of the spinal cord and displayed an elongated shape resembling nascent muscle fibres (H, I). In contrast the cells from clusters one and two remained in the spinal cord (H, I). To confirm that the cells of cluster three and four had transdifferentiated into muscle, the regenerating tail was fixed and processed for whole-mount immunohistochemistry using muscle-specific myosin heavy chain. Fig 24J shows that the cells were positive for the myosin marker and were lying within the area of the tail undergoing extensive muscle differentiation. This illustrates that radial glial cells originally derived from the ectoderm can lineage switch during tail regeneration and contribute to muscle, a cell type originally made from mesoderm.

Whether these radial glial cells were predetermined to form muscle before exiting the spinal cord and fusing with other cells around is not yet known. However, I also observed labelled cells contributing to the cartilage rod that forms ventral to the regenerating spinal cord (Fig.25). Differentiation into cartilage does not involve cell fusion. In this example, two cells were initially transfected in the mature spinal cord (A-C). At Day four, cell one remained at the amputation plane while cell two lies close to the tip of the regenerating spinal cord (D-F). By Day 8 cell two had divided within the regenerating spinal cord (G-I) but then these cells exited out of the spinal cord, rapidly changed shape (Day 9, J-L), and became morphologically distinct cartilage cells by Day 11 (N-O, arrowheads).

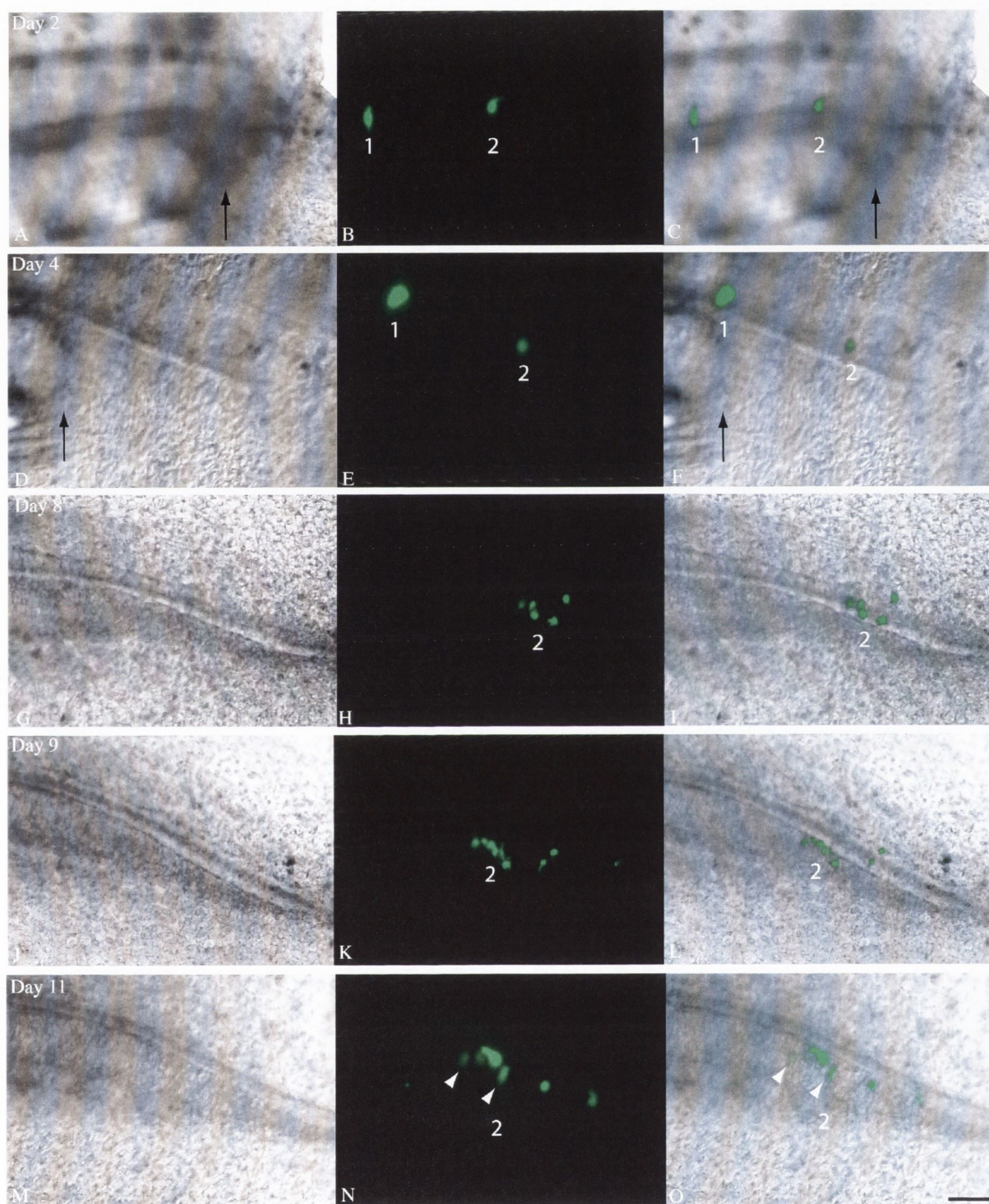
The identity of the rod of cartilage formed during regeneration was previously confirmed using the histological stain Alcian Blue (chapter 2, figure 3). Here I tried to confirm that the green cells were definitely cartilage cells using an anti-collagen 11 antibody, unfortunately the antibody did not cross react but the unique morphology and position of the cells within the tissue leaves indicates that they are cartilage cells.

Once the cells formed cartilage the GFP protein was rapidly degraded making further imaging difficult, I attempted to amplify the signal using an anti-GFP polyclonal antibody but was unsuccessful.

Cartilage cells are mononucleate thereby their fate could not be determined by fusion with other predetermined cells.

Fig. 24 Radial glial cells exit the spinal cord and contribute to muscle. Two days post amputation 4 cells labelled by GFAP-GFP are visible in the spinal cord (B&C). In Panel B the brightness of the fluorescent image has been increased to allow visualization of cells 1 & 3. The arrow in Panels A, D, G and H indicates the plane of amputation. By Day 6 the cells have begun to proliferate (E&F). The montage in Panel G shows that by Day 8 the cells of cluster 3 have exited the spinal cord. At Day 13 the cells of cluster 4 have also exited the spinal cord, while cells from 1&2 have migrated into the regenerate but remain within the spinal cord (H). Scale bar = 100µm. At higher magnification it is clear that the exited cells display an elongated shape resembling nascent muscle fibres (I). Panel J shows that the exited cells (green) are positive for the differentiated muscle marker, myosin heavy chain (red). Scale bar = 50µm.

Fig. 25 GFAP positive radial glial cells can also form cartilage. Two cells are initially labelled in the spinal cord (B&C). The cell closest to the plane of amputation, cell 2, migrates out into the regenerating spinal cord (E&F) and begins to proliferate (H&I). By day 8, cell 1 is no longer visible in this image. It remained close to the plane of amputation and gave rise to new radial glial cells. By Day 9 cells from cell cluster 2 begin to exit the spinal cord (K&L) and by Day 11 all labelled cells have left the spinal cord, rapidly changing shape to become morphologically distinct cartilage cells, see arrowheads, (N&O). Scale bar = 100µm.



Radial Glial Cells Have Two Exit Routes From the Spinal Cord

Figures 24 and 25 show clear examples of radial glial cells exiting the spinal cord from the side to form cells of the mesodermal lineage. But not all cells which exit the spinal cord form cells of the mesodermal lineage, other radial glial cells have also been seen to exit the spinal cord from the side and form cells of the neural crest lineage as previously described by Benraiss *et al* (Benraiss *et al.*, 1997). In Fig.26 one cell can be initially seen lying within the spinal cord (Fig.26C), by 5 days post amputation the cell began to divide (E, F). At 9 days four cells are visible in the mid-portion of the regenerate and these are already beginning to exit the spinal cord (H, I). By 17 days post amputation eight cells are seen which have exited the spinal cord and appear to be lying above the level of the cartilage rod (M, N). In some incidences cells in this position, when followed for around 30 days formed melanocytes. The cells were no longer GFP positive at this point but were identified as being the same cells based on their position within the tissue. The exact identity of the cells is not clear, from their position they are thought to be neural crest cells but the antibodies tested including HNK-1 and 1E8 (Developmental Studies Hybridoma Bank) failed to cross react.

The other exit route observed by these cells was from the tip of the regenerating spinal cord. The cells migrated with the tip of the newly forming spinal cord, divided and then some of the cells began to exit the tip and contributed to the fin mesenchyme. Figure 27 is an example where initially a clump of cells is labelled in the mature spinal cord (C), the cells begin to divide and migrate with the newly

forming regenerate (E, F). The cells segregate into two distinct populations (H, I), cluster 1 remains in the mid-portion of the regenerate and cluster 2 eventually goes to the tip (K, L). The cells in cluster 2 can be seen to be entering the area of the fin mesenchyme (L) and by higher magnification it can be clearly seen that radial glial cells which exit the spinal cord from the tip form fin mesenchyme (N, O).

Fig. 26 Radial glial cells exit from the side of the spinal cord. Panels on the left show the DIC image, middle panels show the fluorescent image of the labelled cells and on the right the overlay. The arrows indicate the original plane of amputation. Initially one cell is labelled with GFAP-GFP (B, C); the cell begins to divide and migrates into the newly forming spinal cord (E, F). By 9 days post-amputation 4 cells are visible in the mid-portion of the spinal cord (H, I). These cells rapidly exit the spinal cord and move away from each other (K, I), the cells continue to divide overtime and appear to reside in a layer of cells lying above the rod of cartilage (N, O). Scale bar = 100µm.

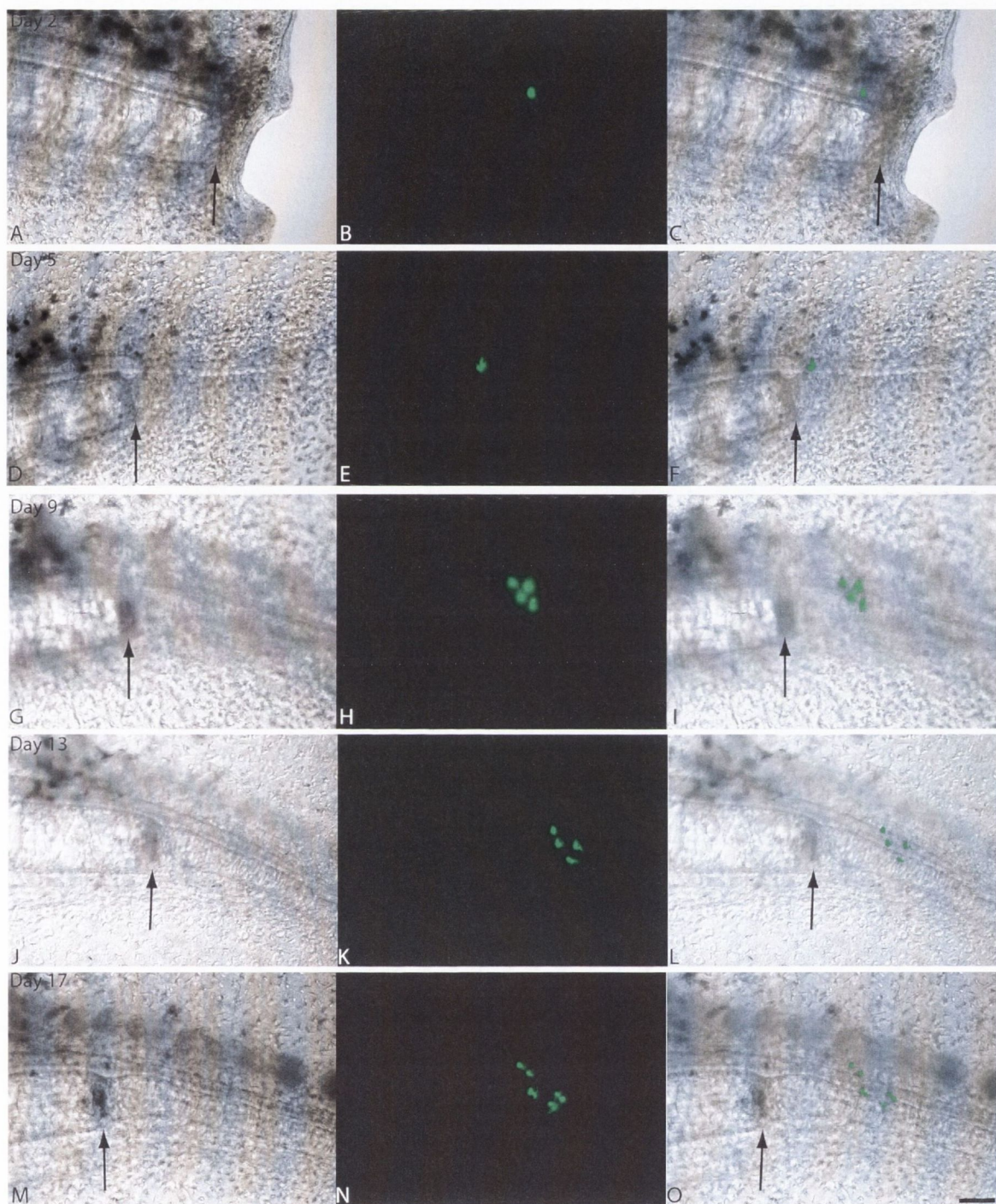
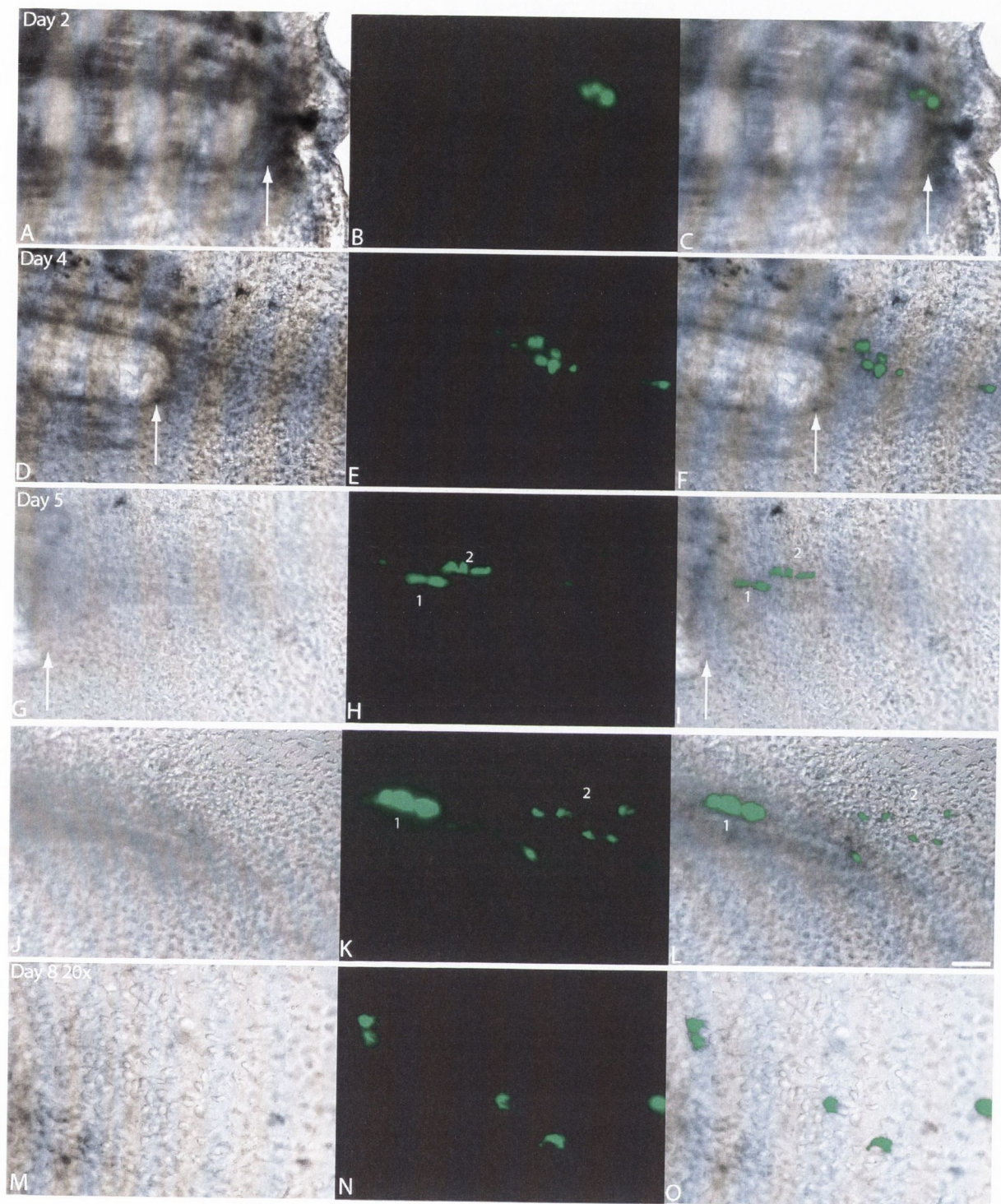


Fig. 27 Radial glial cells exit from the tip of the newly regenerating spinal cord.

Panels on the left show the DIC image, middle panels show the fluorescent image of the labelled cells and on the right the overlay. The arrows indicate the original plane of amputation. Initially a group of cells has been labelled in the mature spinal cord behind the plane of amputation (B, C). These cells rapidly divide and begin migrating into the regenerating spinal cord (E, F). By day 5 two spatially distinct clusters of cells are visible (H, I). Two days later cluster 2 has moved to the tip of the regenerating spinal cord and cells are beginning to exit (L), while cluster 1 stays in the mid-portion of the spinal cord. By higher magnification it can be seen that the exited cells actually partake in forming the new fin mesenchyme at the end of the tail (N, O). Scale bar = 100µm.



Radial Glial Cells Switch Lineage at a Significant Frequency

Figures 24 and 25 show clear examples of cells from the ectoderm lineage switching to the mesodermal lineage during regeneration. Table 5 summarizes the frequency and distribution of all cell fates observed. In 24 percent of animals I observed glial cells that exited the spinal cord to form muscle and in 12 percent of animals cells exited to form cartilage. Since animals contained multiple labelled cells, this represents 20 and 8 percent respectively of the starting number of labelled cells. Other radial glial cells formed the expected cell types, including glia, neurons and neural crest derivatives such as fin mesenchyme and melanocytes.

Table 5. Different cell types deriving from GFAP-positive cells during tail regeneration

<u>Cell Type</u>	<u>Frequency(%)</u>
Muscle	24 (6/25)
Cartilage	12 (3/25)
Neurons	16 (4/25)
Neural Crest-side	28 (7/25)
Neural Crest-end	24 (6/25)
Melanocytes	8 (2/25)
Glial Cells	28 (7/25)

Frequency is calculated based on the number of animals followed (25). Animals contained multiple labelled cells, and some labelled cells gave rise to descendents that had different fates so the total frequency is greater than 100%. "Neural Crest-side" denotes neural crest cells that exited through the wall of the regenerating spinal cord. "Neural Crest-end" denotes neural crest cells that exited out of the end of the regenerating spinal cord.

Discussion

Foreign environments induce adult stem cells to switch lineages and populate multiple tissue types but it has been an open question if this mechanism is used for tissue repair. To determine if lineage switching is an integral feature of regeneration I utilized the urodele amphibian system that can regenerate fully functional, multi-tissue structures including the limb and tail. I have followed individual spinal cord cells live during tail regeneration in the axolotl. Strikingly, spinal cord cells migrate at high frequency into surrounding tissue to form regenerating muscle and cartilage.

These results show that cells switch lineage during a real example of regeneration.

This is the first time that radial glial cells have been observed *in vivo* to naturally give rise to cells of other lineages without being placed in an artificial environment. In the axolotl I have observed that GFAP-positive radial glial cells have the ability not only to re-form a functional spinal cord but also to contribute to regenerating tissues outside of the spinal cord such as muscle and cartilage. These experiments establish the relevance of neural cell plasticity to regenerating functional tissue. Furthermore they imply that tail injury generates signals that induce radial glial cells to migrate and replace cells of both ectodermal and mesodermal lineages.

Surprisingly the radial glial cells exited the spinal cord and formed cells of the mesodermal lineage, from these experiments it is not clear if muscle formed due

to fusion with adjacent pre-determined myoblasts or if they were already determined before exiting the spinal cord. As these radial glial cells also formed cartilage at a significant frequency, and cartilage cells are mononucleate this would suggest that the cells were either predetermined or responded to signals from the area they migrated to and differentiated to form that cell type.

Interestingly when the cells exited to form neural crest derivatives they took two exit routes, from the side and the tip of the regenerate. Cells exiting from the terminal vesicle area have been previously described by Benraiss . They described the tip of spinal cord regenerate which forms the terminal vesicle which had previously been thought to be a closed structure (Holtzer *et al.*, 1955; Holtzer, 1956), to be in fact very loosely organized and possibly to be an open structure, hence allowing cells to exit via this route (Benraiss *et al.*, 1997). In these experiments I observed a similar occurrence, whereby cells migrated to the tip of the regenerate, proliferated and exited the spinal cord to contribute to the surrounding fin mesenchyme. Also in these experiments I have been unsuccessful in finding an antibody to specifically identify these cells as true neural crest cells and have chosen to use this term to describe them as they are morphologically similar, and are found in the same position as cells previously described during amphibian regeneration by Benraiss *et al* as being neural crest (Benraiss *et al.*, 1997). But in the future it would be better to develop an axolotl specific marker for cells of the neural crest lineage.

In classical terms neural stem cells have been described as having the potential to differentiate into neurons, astrocytes and oligodendrocytes and to undergo self-renewal through the process of asymmetric cell division. Although individual cells may harbour such potential, my observations suggest that in practice, a single neural progenitor may generate a limited number of cell types and may not even undergo self-renewal. I have observed that some cells proliferate to replenish the pool of radial glial cells, while others produce progeny, all of which differentiate. As seen in figures 24 and 25, all of the visible progeny of a marked cell apparently exit the spinal cord leaving no self-renewing cell behind in the spinal cord. It is not yet known if this observation reflects differences in innate potential, differences in the extracellular cues encountered or that it suggests a new definition of the term 'neural stem cell'. Further experiments will be required to verify this intriguing observation.

These experiments establish cell lineage switching as a mechanism that is used during a true example of tissue regeneration. But to date nothing is known about the signalling that causes cells to change fates. For example when radial glial cells exit and form muscle, it is not known whether the cells already begin to express markers of muscle myoblasts like myoD before leaving the spinal cord or whether this is an identity they assume upon exit.

Axolotls have this amazing ability to regenerate and use cell lineage switching as a mechanism for tissue repair, the remaining questions is, do mammalian cells

also have the ability to lineage switch if given the right signals and could this be used as a mechanism to enhance or induce endogenous tissue repair?

Chapter 5

General Discussion

Part 1: Muscle Dedifferentiation

The work presented in Chapter 2 of this thesis clearly shows how differentiated muscle fibres are capable of dedifferentiating to form mononucleate cells when confronted with the right combination of signals. Dedifferentiation only occurs when the end of the muscle fibre is 'clipped' and a signal is released in response to severe tissue damage. The mononucleate cells then go on to proliferate and populate the blastema. Here a technique has been established which allows *in vivo* visualisation of the process of dedifferentiation and it provides a model for further elucidation of the molecular signalling and cyoskeletal rearrangement necessary to induce dedifferentiation. Importantly this work shows for the first time that dedifferentiation of mature muscle fibres is actually a significant contributor to the blastema, approximately 20% of the cells in the blastema actually come from muscle, but the question of how plastic these cells really are remains, it is not known how many cells types they can contribute to in the regenerate.

The question of whether a cell re-enters S-phase before fragmentation into mononucleate cells still remains but much work has been done on this issue *in vitro*. In these *in vivo* studies technical difficulties were experienced in maintaining the fluorescent dextran label, with staining for BrdU incorporation, hopefully in the future these issues can be overcome and the cell cycle progression and actual fragmentation process can be monitored at higher resolution to determine whether mononucleate cells are formed by cytokinesis or by a budding off mechanism.

The relationship between cell-cycle progression and formation of mononucleate cells.

At least two distinguishable events, cell cycle re-entry and fragmentation into mononucleate cells, are required to create blastema cells from a muscle fibre *in vivo*. When do these events occur during dedifferentiation, and are they mutually dependent? Hay and Kumar's DNA-labelling experiments indicated that DNA synthesis occurs in the multinucleated myotube before fragmentation (Hay and Fischman, 1961; Kumar *et al.*, 2000). These findings raised the possibility that the nuclei proceed through to mitosis and bud off the fibre at cytokinesis as 2N nuclei. Alternatively, the nuclei may bud prior to mitosis as 4N nuclei and subsequently undergo cell proliferation. To examine the relationship between cell cycle progression and the fragmentation process Velloso . blocked S-phase re-entry either by X-irradiation or by transfection of the CDK4/6 inhibitor p16 in cultured myotubes that were then implanted into the regenerating newt limb blastemas (Velloso *et al.*, 2000). These arrested myotubes could still form mononucleate cells even though the cells could not subsequently proliferate. This data showed that the fragmentation process is independent of cell-cycle progression and thus strongly suggested that mononucleate cells were not formed through mitosis. This means that two parallel pathways are likely involved in dedifferentiation: a pathway to form mononucleate cells, and a separate pathway to re-enter the cell cycle with rapid proliferation occurring after fragmentation.

In the future, it will be fascinating to determine the relationship between the extracellular signals and the intracellular pathways. For example, is one

extracellular signal dedicated to triggering cell cycle re-entry, and a separate extracellular signal for forming mononucleate cells, or is there overlap in the signalling pathways? Work described below suggests that the regenerating tissue may contain multiple overlapping cues to initiate the process of dedifferentiation.

Toward a molecular understanding of dedifferentiation

Progress toward unravelling the signalling mechanisms for dedifferentiation has come from the development of functional assays that reproduce aspects of muscle dedifferentiation *in vitro*. Mouse myotubes have served as a paradigm for a cell type that becomes stably withdrawn from the cell cycle and refractory to growth factors upon differentiation. In contrast, newt myotubes in culture have been shown to re-enter S-phase in response to serum stimulation (Tanaka *et al.*, 1997a). Using this assay two primary lines of experimentation have been directed at identifying the intracellular pathways that mediate cell cycle re-entry, and the extracellular cues that trigger these pathways. The first line of work was initially stimulated by the report that mammalian myotubes derived from mice lacking the retinoblastoma (Rb) gene re-enter S-Phase in response to serum stimulation (Schneider *et al.*, 1994). This suggested that in newts the cell cycle response pathway might regulate Rb, a known key regulator of the G1 -S transition that is inactivated to allow transcription of S-Phase re-entry genes by the E2F transcription factors. Tanaka *et al* showed that serum stimulation of newt myotubes leads to the inactivation of the Rb protein via phosphorylation. (Tanaka *et al.*, 1997a). In contrast, Rb in wild-type mouse myotubes remains

unphosphorylated, and the cells do not re-enter S-phase. The serum dependent phosphorylation of Rb in newt myotubes was the first molecular, intracellular difference to be identified between newt and mammalian myotubes related to a regeneration phenotype.

The question, however, remains: what is the intracellular block to S-phase entry that exists in mouse myotubes and is somehow relieved by the serum factor in newt myotubes? Is it a direct regulator of the Rb pathway or does it block an earlier step in cell cycle re-entry such as the G0-G1 transition? Again, work forcing cell cycle re-entry in mouse myotubes may provide a clue to this question. Tianen *et al* demonstrated that stimulation of mouse myotubes with serum induces the expression of immediate early response genes such as c-fos and c-myc, indicating that the mouse myotubes were transiting from G0 to G1, even though the myotubes never entered S-Phase (Tiainen *et al.*, 1996). This suggests that the G0 to G1 transition is not the major block to cell cycle re-entry in mouse myotubes. Consistent with this finding, Latella *et al* demonstrated in mouse myotubes that forced expression of cyclinD and cdk4, a kinase that phosphorylates Rb at the G1 to S phase, was sufficient to induce S-Phase re-entry in mouse myotubes cultured in high serum conditions (Latella *et al.*, 2000). These results raise the possibility that inhibition of the cyclinD/cdk4 kinase at G1 to S may be the critical block to S-phase re-entry in mouse myotubes. It will be interesting to determine whether this kinase pathway is the critical point of regulation during newt myotube cell-cycle re-entry.

Extracellular signals leading to cell cycle re-entry

A second line of work to understanding cell cycle re-entry has involved analysing the extracellular signals that trigger the process. Although cultured myotubes enter S-phase in response to a factor in serum, normal serum growth factors like PDGF or EGF are incapable of inducing the response (Tanaka *et al.*, 1997). Furthermore newt myoblasts that proliferate in response to PDGF and EGF are unresponsive to the partially purified extracellular factor, suggesting that sensitivity is acquired during the myogenic differentiation process (Tanaka and Brockes, 1998). Additional characterization of the factor revealed that it is activated by thrombin proteolysis to generate a ligand, which acts directly on newt myotubes (Tanaka *et al.*, 1999). An attractive aspect of this factor is that during wound healing, the first phase of regeneration, thrombin activity has been shown to be upregulated at the end of the limb stump (Tanaka *et al.*, 1999).

Contact inhibition of dedifferentiation

Another interesting aspect of this work is that serum stimulation of the cultured myotubes is inhibited by contact between the myotubes and mononucleate cells (Tanaka *et al.*, 1997). This *in vitro* characterization suggests that cell cycle re-entry requires at least two extracellular conditions: loss of cell-cell contact and a soluble extracellular factor derived from clotted blood. This phenomenon is reminiscent of what I have observed *in vivo* whereby release of cell-cell contact and tissue injury is necessary to induce dedifferentiation during axolotl tail regeneration.

So far this extracellular factor has been found in all animal sera but only newt myotubes are responsive to it. Tanaka . suggest that mammalian myotubes have lost the receptor to respond to the factor (Tanaka *et al.*, 1999). Interestingly both nuclei of mouse/newt hybrid myotubes re-enter S-Phase upon stimulation with serum, suggesting that the ability to respond is somehow conferred from the newt cytoplasm to the mouse nucleus (Velloso *et al.*, 2001).

Can mammalian myotubes respond to dedifferentiation factors?

Based on the work described above it seems that mammalian myotubes may have either lost the receptors for responding to dedifferentiation signals or their downstream signalling pathways. However work by McGann *et al* provides evidence to the contrary. A protein extract made from the newt limb blastema could stimulate 25% of mouse myotubes to re-enter S-Phase and 9% of the myotubes to subsequently form mononucleate cells (McGann *et al.*, 2001). Newt myotubes displayed a similar response to the blastema extract. The mouse myotubes also exhibited a reduced expression of muscle differentiation markers like MyoD and myogenin. The authors speculate that the factors found in their extract are soluble extracellular proteins, which are capable of acting as ligands to induce receptors that can carry dedifferentiation signals to receptive cells. It is unclear why only a proportion of the myotubes formed mononucleate cells in response to the extract. Are there sub-populations of receptive myotubes, or is some feature of cell culture such as adhesion to the substrate simply blocking most myotubes from fragmenting?

Nonetheless the McGann *et al* results imply that mammalian cells may not have lost the receptor, but simply that they never receive the correct signals in the mouse. So how can this data be reconciled with the previous results from Tanaka *et al* with the serum factor? There are several interesting possibilities. The blastema factor and the serum factor could be completely different molecules that act through distinct pathways. Alternatively, the blastema extract may contain the serum factor plus an additional factor. For example, the blastema extract may represent a more “complete” protein complex that is more potent than the serum factor. Although it may be appealing to think of one extracellular factor being responsible for inducing dedifferentiation, in reality perhaps several pathways remain to be elucidated for us to really understand how this unique phenomenon is induced and to allow us to try mimick it in non-regenerative systems.

Is Msx1 a Master Transcriptional Regulator of Dedifferentiation?

Little is known about the intracellular signalling pathway of dedifferentiation but one component appears to be the Msx-1 gene. Msx genes are a family of homeobox-containing transcription factors that are known to be expressed during embryogenesis in areas of epithelial to mesenchymal transitions. Interestingly, their expression is also associated with areas containing proliferating progenitor cells. During development, Msx-1 is expressed in the growing end of the limb bud, where cells are maintained in an undifferentiated state. In fetal and neonatal mouse, Reginelli *et al* found that the zone of Msx-1 expression correlates with a zone in the fingertip that retains regeneration ability (Reginelli *et al.*, 1995).

During urodele limb regeneration, the *msx* family members, *Msx-1* and *Msx-2* are expressed in the blastema (Carlson *et al.*, 1998; Koshiba *et al.*, 1998). Data from Carlson *et al.* suggests that *Msx-2* is one of the first known genes to be expressed during regeneration and in wound healing, and therefore it may be involved in a pathway common to both processes (Carlson *et al.*, 1998). During limb regeneration *Msx1* is found to be upregulated later at mid-bud, when all cells in the blastema are already believed to be in an undifferentiated state (Simon *et al.*, 1995; Koshiba *et al.*, 1998).

Functional studies of the *Msx-1* gene have shown that it can keep cells in an undifferentiated state. For example, expression of *Msx-1* in cultured mouse myoblasts prevented formation of myotubes in response to normal differentiation signals (Sassoon *et al.*, 1989). On a molecular level, expression of *Msx-1* results in down regulation of the myogenic regulator factor, *MyoD*, consistent with the cells transforming into a less differentiated state (Woloshin P *et al.*, 1995).

The role of *Msx1* in dedifferentiation

In addition to its role in maintaining cells in an undifferentiated state, *Msx-1* may also be involved in causing differentiated cells to dedifferentiate. Odelberg *et al.* used ectopic expression of *Msx-1* to force dedifferentiation of mouse myotubes (Odelberg *et al.*, 2000). Early after *Msx-1* induction, the levels of genes associated with muscle differentiation, *MRF4*, *p21* and *myogenin*, were reduced in the myotubes, followed by a down regulation in *MyoD* levels. Approximately 9% of the myotubes produced smaller myotubes or proliferative mononucleate cells,

which were capable of being forced to redifferentiate into several lineages including osteoblasts, chondrocytes and adipocytes.

Since ectopic expression of Msx-1 acts directly in the nucleus, it bypassed the need for a receptor to respond to an external signal so the upstream activators of the process that are normally used during regeneration still remain to be elucidated. One potential candidate molecule found by Shimizu-Nishikawa and colleagues that is upregulated during newt limb regeneration is Nrad, a ras protein associated with diabetes that is thought to act as a switching molecule with GTPase activity (Shimizu-Nishikawa *et al.*, 2001). It is the first factor found to be upregulated, 4hrs post amputation specifically in the nuclei of muscle fibres at the plane of amputation. This upregulation is found in different types of regenerating structures such as the limb, the tail and heart muscle in response to amputation. The question arises, is rad part of the upstream pathway which eventually turns on Msx-1 or might it be involved in the cell cycle re-entry pathway?

Open questions about the role of Msx-1 in dedifferentiation

The Msx-1 data gives us an important clue to the intracellular pathway of dedifferentiation but a number of key questions remain. It is not known whether the Msx-1-expressing myotubes enter S-Phase before dedifferentiating. Furthermore it is not clear if ectopic expression of Msx-1 alone is sufficient to cause dedifferentiation, or if a combination of Msx-1 overexpression with the serum that was present in the medium is required to cause mouse myotubes to

form mononucleate cells. Therefore, it is also not yet clear if Msx-1 is involved in both myotube fragmentation and cell cycle re-entry or just one of these pathways. How do Odelberg's molecular results in cultured mouse myotubes relate to *in vivo* dedifferentiation during urodele regeneration? In their experiments, Odelberg *et al* found that Msx-1 had dedifferentiating activity but not Msx-2. Although ectopic expression of Msx-1 appears to lead to dedifferentiation *in vitro*, Msx-1 upregulation has not been observed in myotubes next to the plane of amputation by *in situ* hybridization *in vivo*. In the regenerating limb Msx-2 is expressed during the dedifferentiation phase while Msx-1 is upregulated at later stages of regeneration, after dedifferentiation is finished (Koshiba *et al.*, 1998). The significance behind the differences between the *in vivo* gene-expression patterns and the *in vitro* dedifferentiation activities is not clear. In their experiments Odelberg used the mouse *msx* genes, which may have different activities, compared with the amphibian homologues. Alternatively, other genes that have similar activities to Msx-1 may be the true inducers of dedifferentiation *in vivo*. Clearly it will be important to identify other genes in the dedifferentiation pathway. It will also be necessary to test the role of Msx-1 *in vivo*. For example, one test would be to ectopically express Msx-1 in a subset of fluorescently labelled myofibres *in vivo* in the mouse and to determine if they would then dedifferentiate in response to wounding. It would also be interesting to inactivate Msx-1 function in dedifferentiating muscle during regeneration.

In the *in vitro* cell assays, the mononucleate cells derived from Msx-1-expressing myotubes could differentiate into other mesenchymal lineages such as adipocytes

and chondrocytes. It will be interesting to determine whether the dedifferentiated muscle cells found *in vivo* in a urodele limb or tail blastema display the same range of potential as those created *in vitro*.

The molecular pathway of muscle dedifferentiation

Fig. 26 indicates what is currently known about the mechanism of muscle dedifferentiation. This figure represents a consolidation of observations from *in vivo* and *in vitro* experiments, as well as experiments in urodele and mouse cells. Therefore, while the cellular events of muscle dedifferentiation are reasonably well defined, the relationships between various molecular events are speculative. After injury, the muscle fibre loses contact with its neighbours and contracts before re-elongating. Soluble factors in serum and/or the blastema induce the muscle cell nuclei to undergo S-phase. Concomitantly extracellular blastema extract and/or intracellular Msx-1 expression causes the gene transcriptional program of the muscle cell to change with down-regulation of myogenic genes and cell cycle regulators such as p21. *In vivo* data indicates that the Nrad gene would be upregulated in these early stages. The formation of mononucleate cells from the syncytium appears to occur prior to mitotic division.

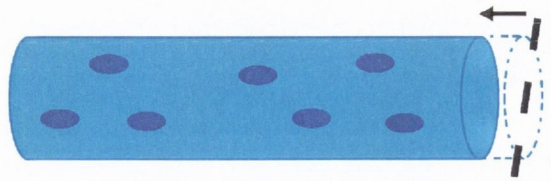
There are many open questions about the relationship between the currently known players in a pathway. Msx-1 appears to play a key role in establishing and maintaining cells in an undifferentiated state. Yet it is still unclear exactly at which step in the dedifferentiation pathway it acts. Furthermore, what is its relationship

to the signals to initiate dedifferentiation? For example, does the blastema extract induce dedifferentiation through Nrad and the Msx-1 gene?

Fig.28 Schematic diagram of muscle fibre dedifferentiation. (1.) *In vivo* muscle fibres retract in response to injury. Around the fibre, a blood clot is formed and the wound heals over. The retracted muscle fibre then re-elongates. (2.) *In vitro* an extracellular factor found in both serum and newt limb blastema extract is capable of pulling the cells out of G0 and allowing them to progress to S-phase, where they become arrested in a 4N state. The G1-S transition is mediated via the phosphorylation of the Rb protein in newt myotubes. (3.) Ectopic expression of Msx1 or the presence of newt blastema extract causes mammalian myotubes to down regulate markers of terminally differentiated muscle. Newt myotubes upregulate Nrad in the nucleus of muscle fibres at the plane of amputation. Steps 2 and 3 are likely to occur concomitantly. (4. and 5.) *In vivo*, axolotl tail muscle fibres fragment and form mononucleate cells in response to clipping the end of a muscle fibre in combination with a signal released in response to severe tissue damage. *In vitro* a newt blastema extract can stimulate a response in both newt and mammalian myotubes, causing 9% of the myotubes in culture to fragment and form mononucleate cells. The factors responsible for inducing the cells to fragment, divide and eventually re-differentiate are still unknown.

1. Injury

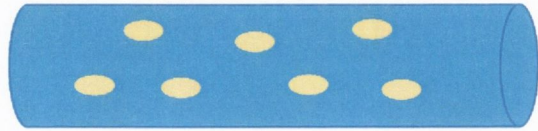
Loss of cell to cell contact
Fiber contraction



Serum Factor
or
Blastema Extract Factor

2. Cell Cycle Re-entry

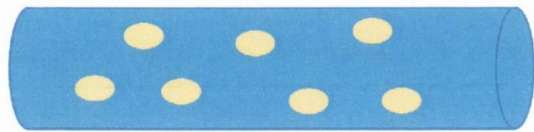
G0 - G1 = ?
G1 - S = Rb[Ⓟ]



Msx1[↑]
or
Blastema Extract Factor

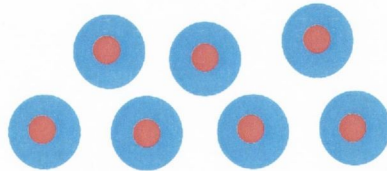
3. Nuclear Reprogramming

MRF4
p21
Myogenin
MyoD
Nrad

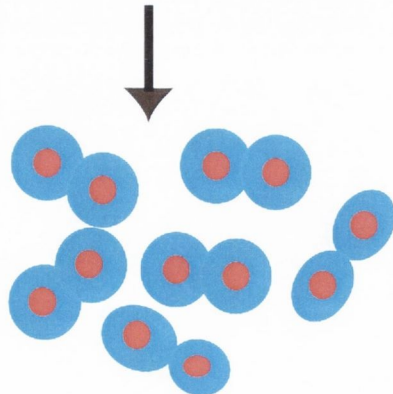


4. Fragmentation

?



5. Cell Division



Future Perspectives on Muscle Dedifferentiation

By focusing on muscle cell dedifferentiation as a unique cell process that distinguishes urodeles from other non-regenerating species, the fascinating but complex problem of regeneration has just begun to be understood on the cellular and molecular level. The technical advances in following dedifferentiating cells in regenerating tissue, as well as *in vitro* functional assays have been a crucial starting point for delineating the underlying regulatory pathways.

Clearly many more molecular players must be identified to understand how dedifferentiation occurs in urodele amphibians, and why it does not occur in mammals. Importantly, although the extracellular activities have been characterized, their molecular identity is still unknown. Further down the line, the identity of cell surface receptors and the intracellular pathways that these factors activate will play a crucial role in our understanding of dedifferentiation.

The work in Chapter 2 of this thesis establishes a technique which has the potential to be further developed into a functional assay to study the process of dedifferentiation *in vivo* by combining the ability to label single cells and image them *in vivo* with methods to specifically disturb gene function. This work clearly illustrates that direct injury to the fibre along with a signal induced by injury is needed to induce dedifferentiation, the identity of this factor remains unknown but work from other groups shows that it is probably a factor found in serum which can also be found in blastema extract but the exact molecular identity remains to be elucidated.

As gene candidates are beginning to be identified which are involved in the dedifferentiation process, this *in vivo* dedifferentiation assay combined with single cell electroporation allows a functional system to actually test ectopic expression of these gene candidates in selected muscle fibres at the plane of amputation. Whether genes like Msx-1 alone can really induce dedifferentiation can then be tested *in vivo*, and eventually single cell electroporation may also be used to deliver inhibitors of different genes specifically to single cells or groups of cells and will help the molecular mechanism underlying dedifferentiation to be elucidated.

Part 2: Spinal Cord Regeneration

The development of the single cell electroporation technique has allowed single cells or groups of cells to be labelled within the axolotl spinal cord and combining this with *in vivo* imaging techniques has allowed me to begin to understand where the cells are coming from to form the regenerate and to understand the contribution of these cells to the surrounding structures. Surprisingly the GFAP positive radial glial cells which form the inner layer of the spinal cord have the ability to migrate out to form the new regenerate but also to switch lineage and give rise to cells of the mesodermal lineage. This is the first time that radial glial cells have been observed in an *in vivo* situation to cross cell fate boundaries but the molecular mechanism underlying this remarkable event is completely unknown.

How is a New Spinal Cord Built?

Work from several groups in the past describes how the radial glial cells of the mature stump proliferate and then migrate to form the regenerate (Egar *et al.*, 1970; Egar and Singer, 1972; Singer *et al.*, 1979). Not only is a new ependymal tube formed, but unlike in lizard, fully functional axons are regrown in newt and axolotls (Singer *et al.*, 1979, Clarke *et al.*, 1988, Benraiss, 1999). Physically how the cells migrate out is not known, but by developing the technique of spinal cord cell electroporation it has allowed me to define a growth zone at the end of the mature stump. By labelling cells, recording their distance from the end of the notochord, which is used to mark the plane of amputation and then recording the

fate of the cells during regeneration, has allowed me to postulate on a model for spinal cord regeneration which is described in more detail in Chapter 3. From these data recordings it seems that all cells come from a growth zone of about 400µm at the end of the mature spinal cord, the cells in this zone seem to have an equal chance of ending up at the tip of the regenerate or of staying close to the plane of amputation. Therefore it would seem that the cells in this area increase cell division and are then pushed out to form the new spinal cord. But a lot of very careful timelapse studies need to be done to really understand how the cells actually migrate, whether it is really migration over cells or if the cells are being physically pushed out. Another interesting question to contemplate is whether cells retain their dorsal/ventral identity when they form the new spinal cord?

Do Neurons Dedifferentiate?

One of the interesting questions in spinal cord regeneration has always been whether neurons can dedifferentiate? Since the theory of muscle dedifferentiation became widely accepted then the idea that neurons might also dedifferentiate has also been postulated. Holder and Clarke have shown that even in adult axolotls axons can regrow across the lesion site (Clarke, 1988) and they also have suggested that the regeneration capacity of the spinal cord is correlated to the permanent neurogenesis exhibited in adult urodele amphibians (Holder and Clarke, 1988). Work from Benraiss *et al* shows that the new neurons in the regenerate derive from the proliferative ependymal layer of the neural tube (Benraiss *et al.*, 1999), this data correlates with the idea of neuroplasticity in adult

urodeles as indicated by the continued expression of embryonic markers in the adult like cytokeratins 8 and 18 (Holder *et al.*, 1990; Benraiss *et al.*, 1996), and the transient re-expression of embryonic forms of adhesion molecules like polysialylated neuronal adhesion molecule (Caubit *et al.*, 1993). Although this data is suggestive that all new neurons come from radial glial cells it does not rule out the possibility that neurons themselves dedifferentiate and redifferentiate into other cells types.

Using electroporation I have been able to label neurons within the spinal cord and follow their fate over time in the regenerate, in these limited experiments described in chapter 3 I found that neurons which were being identified only on the basis of morphology remained close to the plane of amputation, changed shape slightly by rearranging the axons to probably innervate the new muscle formed above them, but they did not appear to migrate into the regenerate or to dedifferentiate which is in contrast to data from Zhang, Ferretti and Clarke who describe how DiL labelled neurons migrate into the regenerate to form the neurons of the regenerating spinal cord (personnel communication). The possibility remains that different sub-populations of neurons behave differently and as in both sets of the experiments the specific type of neurons labelled is not yet known this may prove to be the cause of the discrepancy in the results.

Can stem cells really switch lineage?

In recent times many papers have begun to describe examples of lineage switching when cells are injected into different places or are cultured and then implanted into new environments. The issue of neural stem cell plasticity is still a very controversial issue. But several groups have shown that cells derived from the ventricles of adult brain have the potential to form muscle in transplantation and co-culture experiments and to form bone marrow when injected into irradiated mice (Bjornson *et al.*, 1999; Galli *et al.*, 2000; Rietze *et al.*, 2001; Shih *et al.*, 2001). Neural stem cell plasticity is not unique to mice, Clarke *et al.* demonstrated that when these neural stem cells are transplanted into chick embryos they can give rise to cells of all three germ layers (Clarke *et al.*, 2000).

But it is not only neural stem cells which exhibit this plasticity, stem cells from adult skin and bone marrow have also been reported to switch cell fate in response to placement into a new environment (Bjornson *et al.*, 1999; Brustle *et al.*, 1999; Clarke *et al.*, 2000; Rietze *et al.*, 2001; Toma *et al.*, 2001). But these reports have raised many questions as to whether long term culture may have selected for characteristics not originally present in the neural stem cells, whether the implanted cells were really homogenous and whether these were examples of true cell lineage switching or if the identity assumed was due to a simple fusion event? If cells can switch lineage how do they do it and is it a frequent or rare event? Does lineage switching occur by transdifferentiation or dedifferentiation followed by redifferentiation to a cell type of another lineage?

Lineage Switching and Regeneration

To date it has been difficult to follow the fate of endogenous stem cells *in vivo* in response to wounding. In chapter 3 of my thesis I developed a technique to label cells of the spinal cord and as described in chapter 4 the technique was combined with a cell type specific promoter to label the radial glial cells of the axolotl spinal cord, these cells are thought to be the neural stem cells of the axolotl. By following the response of these cells to tail amputation I was able to observe true examples of lineage switching during real regeneration. These radial glial cells appear to have the intrinsic ability to form a new ependymal tube but also to migrate out and contribute to cells of the mesodermal lineage like muscle and cartilage. Since these radial glial cells form multinucleate muscle but also mononucleate cartilage cells, it is thought that they truly switch lineage and do not obtain their fate by fusion to other cell types. Another interesting aspect of these studies was that when the radial glial cells exited the spinal cord, all labelled cells in that area exited, no one cell was seen to be left behind to replenish stocks.

Lineage switching during spinal cord regeneration appears to occur at a significant frequency, in approximately 24 % of animals for muscle and in 12 % of animals for cartilage, although these frequencies may be underestimated if not all daughter cells received the plasmid. Another interesting question is, why would cells exit the spinal cord and switch lineage? Since the origin of all the cells in the blastema is not known, one could imagine that migration of cells out of the spinal cord into the region of the blastema may be the source of a significant proportion of cells in the blastema.

This is the first time that cell lineage switching has been observed *in vivo* in the context of urodele amphibian tail regeneration and is contrary to recent data presented by Prof. Jonathan Slack at a Regeneration meeting (Stem Cells and Regeneration, Italy Sept. 2002). Slack and colleagues addressed the issue of metaplasia in the regenerating *Xenopus* tail by transplanting chunks of specific GFP labelled tissues into other animals and recording their fate in the regenerate. They found no evidence of metaplasia when examining muscle, notochord and spinal cord. This opens up the question of how similar regeneration is in *Xenopus* and Axolotls; do they use the same mechanisms to rebuild lost structures? Are different mechanisms for regeneration used in *Xenopus* as they only acquire the ability to regenerate after Stage 47 and lose this ability again after metamorphosis?

The observation of true lineage switching in response to injury raises many difficult questions. It is not known if the cells which decide to exit the spinal cord have already become determined to another cell fate before they leave the environment of the spinal cord, or if they dedifferentiate in response to injury, exit the spinal cord and then redifferentiate to a different cell fate? Also nothing is known about the signals that actually tell the cells to migrate out of the spinal cord. In the future it will be important to begin to understand the molecular mechanisms underlying cell lineage switching and hopefully the technique of cell electroporation will provide a useful method to specifically overexpress genes in specific cells and to specifically inhibit gene function in certain cell types.

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