

Tatjana Vlaskalin · Christine J. Wong ·  
Catherine Tsilfidis

## Growth and apoptosis during larval forelimb development and adult forelimb regeneration in the newt (*Notophthalmus viridescens*)

Received: 17 March 2004 / Accepted: 13 May 2004 / Published online: 21 August 2004  
© Springer-Verlag 2004

**Abstract** Many of the genes involved in the initial development of the limb in higher vertebrates are also expressed during regeneration of the limb in urodeles such as *Notophthalmus viridescens*. These similarities have led researchers to conclude that the regeneration process is a recapitulation of development, and that patterning of the regenerate mimics pattern formation in development. However, the developing limb and the regenerating limb do not look similar. In developing urodele forelimbs, digits appear sequentially as outgrowths from the limb palette. In regeneration, all the digits appear at once. In this work, we address the issue of whether regeneration and development are similar by examining growth and apoptosis patterns. In contrast to higher vertebrates, forelimb development in the newt, *N. viridescens*, does not use interdigital apoptosis as the method of digit separation. During adult forelimb regeneration, apoptosis seems to play an important role in wound healing and again during cartilage to bone turnover in the advanced digits and radius/ulna. However, similar to forelimb development, demarcation of the digits in adult forelimb regeneration does not involve interdigital apoptosis. Outgrowth, rather than regression of the interdigital mesenchyme, leads to the individualization of forelimb digits in both newt development and regeneration.

**Keywords** Apoptosis · Forelimb development · Forelimb regeneration

Edited by R.P. Elinson

T. Vlaskalin · C. Tsilfidis (✉)  
Ottawa Health Research Institute, University of Ottawa Eye  
Institute,  
501 Smyth Road,  
Ottawa, ON, Canada, K1A 8L6  
e-mail: ctsilfidis@ohri.ca  
Tel.: +1-613-7378233  
Fax: +1-613-7396070

C. J. Wong  
Department of Zoology, Ramsay Wright Zoological  
Laboratories, University of Toronto,  
25 Harbord Street,  
Toronto, ON, Canada, M5S 3G5

### Introduction

Urodele amphibians such as the red spotted newt (*Notophthalmus viridescens*) possess the ability to regenerate limbs lost through injury or amputation. During limb regeneration, the cells beneath the wound epithelium dedifferentiate, which involves the reversion of differentiated cells to an unspecialized “embryonic-like” state (Butler 1933; Hay 1959; Brookes and Lo 1994). These cells re-enter the cell cycle and rapidly proliferate until a mesenchymal mass, the blastema, is formed. The undifferentiated blastema cells subsequently re-differentiate and undergo morphogenesis to give rise to the new limb. Molecular studies have shown the regeneration process to involve many of the genes originally expressed during development of the forelimb (Simon et al. 1995; Gardiner and Bryant 1996; Imokawa and Yoshizato 1997; Simon et al. 1997). It has been suggested that many of these developmental genes are expressed at low levels in intact adult tissues, enabling their rapid upregulation in the regeneration process. This has led to the conclusion that forelimb regeneration is a recapitulation of forelimb development.

Forelimb development in the newt differs from other vertebrates in the mechanisms that set up the initial patterning of the limb. In urodeles, digits 1 and 2 are the first to appear, followed by digits 3 and 4 (Jarvik 1980). Each digit grows out sequentially from the limb palette. This contrasts with higher vertebrates, in which a flattened palette grows outward from the apical epidermal ridge, and all the digits appear at once. Growth and interdigital apoptosis leads to the individualization of digits (Sanders and Wride 1995; Salas-Vidal et al. 2001; Zuzarte-Luis and Hurle 2002). This interdigital apoptosis appears to be absent in amphibians (Cameron and Fallon 1977), although no study has specifically examined this process in *N. viridescens* forelimb development.

The current study was prompted by our observation that the morphology of the newt limb during embryonic forelimb development and adult forelimb regeneration appears to be quite different, even though regeneration has

been called a recapitulation of development. Since the later stages of regenerate differentiation and morphogenesis more closely resemble the palette observed during limb development in higher vertebrates, we compared growth and apoptosis patterns in forelimb development and regeneration to determine whether the two processes are the same. To date, only one study has investigated the role of apoptosis during urodele regeneration. Mescher et al. (2000) performed bilateral amputations with unilateral denervations (transections of brachial nerves 3, 4, and 5) in larval axolotl forelimb regenerates and concluded that denervation results in limb regression through apoptosis. The role of apoptosis during digit individualization in the newt forelimb regenerate has yet to be determined.

## Materials and methods

### Embryo collection and fixation

*N. viridescens* embryos/larvae at various limb developmental stages were collected after natural or induced spawning (Khan and Liversage 1995). They were maintained in Holtfreter's solution and allowed to develop to various stages of limb development (limb field, limb bud, cone, palette, early digit 2 to advanced digit 4). Embryos or larvae were fixed overnight at room temperature (RT) in MEMFA (0.1 M MOPS, pH 7.4, 2 mM EGTA, 1 mM MgSO<sub>4</sub>, 3.7% formaldehyde) with gentle agitation, dehydrated with 25, 50, 75, and 100% methanol in PBS containing 0.1% Tween 20 (PBT), and stored in 100% methanol at -20°C. Early-stage embryos were dissected from their jelly coat prior to fixation.

### Adult forelimb amputations and fixation

Adult newts were obtained from Charles Sullivan (Tennessee) and housed in aerated aquaria containing dechlorinated tap water. Newts were kept at RT on a 12L/12D photoperiod and fed ground beef heart or live blackworms weekly. All amputations were performed following anaesthesia with 0.05% 3-aminobenzoic acid ethyl ester (MS-222-Sigma) adjusted to pH 7.0 with NaHCO<sub>3</sub>. Regenerating limbs were staged according to Iten and Bryant (1973). Forty-eight animals were amputated through the right mid-zeugopodium, and the protruding bone was trimmed back to the level of the soft tissue. Limbs were allowed to regenerate to different stages post-amputation (24 h, 3 days, and 1 week up to the sixth week). Six limbs were re-sampled through the mid-stylopodium for each time point and fixed overnight in 4% buffered paraformaldehyde (PFA). Tissues were cryoprotected in 30% sucrose O/N, embedded in 1:1 sucrose/OCT (Tissue Tek) compound, quick frozen in liquid nitrogen, and stored at -80°C for cryosectioning. Sections (10–16 µm) were adhered to positively charged slides (Superfrost Plus, Fisher Scientific), dried at room temperature for 2 h, and stored at -20°C prior to being used for terminal

deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labelling (TUNEL) analysis.

### TUNEL analysis of embryos

A TUNEL protocol modified from Gavrieli et al. (1992) and a *Xenopus* whole-mount TUNEL protocol adapted from Veenstra et al. (1998) were used. Embryos were rehydrated in PBT, treated with proteinase K (25 µg/ml) at 37°C for 20 min, and washed twice with PBT. After incubation, embryos were rinsed in 1× PBS and pre-incubated in 1× TdT buffer (30 mM Tris, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride, GIBCO-BRL) for 30 min at RT. End labelling was carried out overnight at RT in TdT buffer containing 150 U/ml TdT and 0.5 mM digoxigenin-dUTP (Boehringer Mannheim). The following day, embryos were washed 2×45 min in 1× PBS/1 mM EDTA at 65°C, followed by 4×45 min in 1× PBS at RT. Embryos were equilibrated in MAB (100 mM maleic anhydride, 150 mM NaCl) with 0.2 mg/ml BSA for 30 min at RT and incubated in 1 ml blocking solution (20% sheep serum in MAB with BSA) overnight at 4°C. Anti-digoxigenin antibody coupled to alkaline phosphatase (Anti-DIG-AP, 150 U/ml, Boehringer Mannheim) was diluted 1:400 in blocking solution containing 10 mg/ml newt powder and mixed overnight at 4°C. The following day, the newt powder was spun down, and the anti-DIG containing supernatant was further diluted up to 1:1000 in blocking solution. Embryos were transferred to this solution and gently agitated overnight at 4°C. Embryos were then washed 10×15 min in MAB at RT and equilibrated in alkaline phosphatase (AP) buffer (100 mM Tris-Cl, pH 9.5, 100 mM NaCl, 50 mM MgCl<sub>2</sub>) 2×5 min at RT. The chromogenic reaction was carried out in AP buffer containing 100 mg/ml NBT and 50 mg/ml BCIP. The reaction was monitored (10–30 min) and stopped by transferring the embryos to Bouin's fixative overnight at 4°C. Embryos were cleared in several changes of 70% ethanol and 80% MeOH/20% H<sub>2</sub>O<sub>2</sub>. Embryos were viewed following dehydration in methanol and photographed under a dissecting scope. Positive controls were obtained by pre-treating embryos in DNase buffer (50 mM Tris-Cl, pH 7.5, 10 mM MnCl<sub>2</sub>, 50 µg/ml BSA) containing 0.1 U/µl DNase I (Sigma) for 30 min at 37°C immediately after the proteinase K treatment. Negative controls were obtained by omitting the TdT enzyme.

### TUNEL analysis of adult tissue cryosections

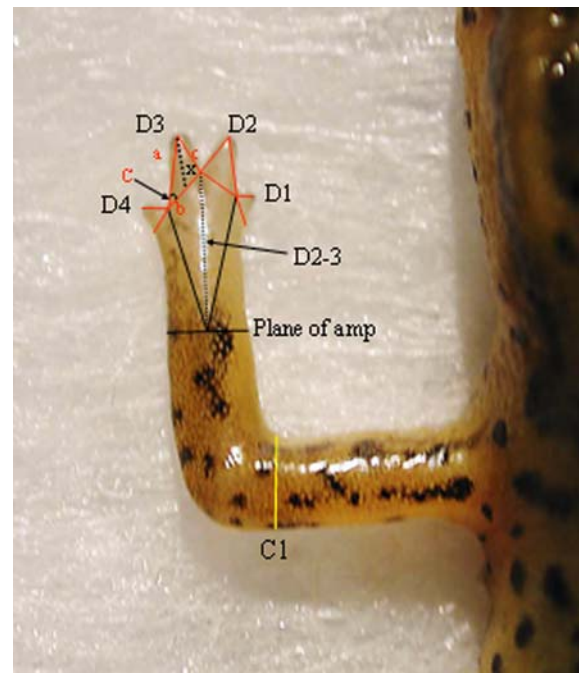
Tissue-section immunohistochemistry protocols were adapted from Cadinouche et al. (1999) and Jensen and Wallace (1997). Frozen tissue sections of regenerates at different stages were re-hydrated in tap water, fixed in 1% PFA for 10 min at RT, and washed 2×5 min in 1× PBS. Sections were post-fixed in pre-cooled ethanol/acetic acid (2:1) for 5 min and washed 2×5 min in PBS followed by a

5-min wash in PBT. In a humidified chamber, sections were incubated for 20 min in PBT containing 20  $\mu\text{g}/\text{ml}$  proteinase K at RT and rinsed  $3 \times 1$  min in PBS. Slides were rinsed in PBS, equilibrated in 1x TdT buffer for 5 min and incubated in TdT buffer containing 150 U/ml TdT and 0.5 mM digoxigenin-dUTP for 1 h at 37°C. End labelling was stopped by transferring the sections into 1x PBS/1 mM EDTA for 10 min at RT, followed by  $2 \times 2$  min rinses in PBS. Cryosections were equilibrated for 5 min in MAB-T (MAB, 0.1% Tween 20), and incubated in blocking solution [20% sheep serum, 20% blocking reagent (Boehringer Mannheim) and 60% MAB-T] for 1 h at RT. Tissues were incubated overnight at 4°C in blocking solution containing 1:1500 dilution of anti-dig-AP. The following day, sections were rinsed in MAB-T and equilibrated 5 min in AP buffer. The chromogenic reaction was carried out in AP buffer containing 100 mg/ml NBT and 50 mg/ml BCIP. The reaction was monitored for up to 2.5 h and stopped by several washes in 1x PBS. Slides were dried and mounted in 1x PBS/glycerol (1:1). Images were taken on a Zeiss Axioskop 2 microscope equipped with an AxioCam HRc camera. Negative controls were obtained by omitting the TdT enzyme. Positive controls were obtained by pre-treating sections with DNase I in DNase buffer for 10 min at RT.

### Growth analysis

Six animals were amputated through the mid-zeugopodium, and the protruding bone was trimmed back to the level of the soft tissue as previously described. Limbs were photographed before and after amputation, using a Nikon Coolpix 990 digital camera. Limbs were allowed to regenerate to the palette stage when the formation of digits became visible (~5 weeks post-amputation). At this time, and every 3–4 days thereafter, animals were re-anaesthetized and photographed until the digits were visibly separated (9 weeks post-amputation). Digital measurements were taken for each animal at each stage (seven time points). The law of cosines was used to obtain the length of each digit at the midline (Fig. 1). Measurements were also taken from the most proximal interdigital point between two digits to the plane of amputation. In order to eliminate variability in length values due to small magnification changes during the photography or superimposition of the images, all measurements were normalized to control values in the intact, non-regenerating region of the limb (Fig. 1). Growth rate changes were statistically analyzed using multiple analysis of variance at  $P < 0.05$  confidence interval.

Images were superimposed and aligned, based on the position of the elbow joint and the location of pigment spots proximal to the original plane of amputation. Contour outlines of each regenerate stage were drawn, and images were analyzed for interdigital tissue elongation or regression (see Salas-Vidal et al. 2001 for similar studies in mouse).



**Fig. 1** Digital lengths are calculated for digit 2 ( $D2$ ) and digit 3 ( $D3$ ), using the law of cosines. Measurements are taken from the distal tip to either side of the interdigital grooves ( $a$ ,  $c$ ), and along the base of the digit between the interdigital grooves ( $b$ ). These three values are used to calculate angle  $C$ , using the law of cosines:  $c^2 = a^2 + b^2 - 2ab \cos C$ . The calculated value for  $\cos C$  is applied to the formula  $x^2 = a^2 + (b/2)^2 - 2a(b/2) \cos C$  to obtain the digital length ( $x$ ) of  $D3$ . Length for  $D2$  is similarly obtained. Length  $D2$ – $D3$  is measured as the distance from the interdigital groove between digits two and three to the midline at the plane of amputation. Control length,  $C1$ , is measured and used to normalize values for digital and interdigital lengths at each time point for each animal

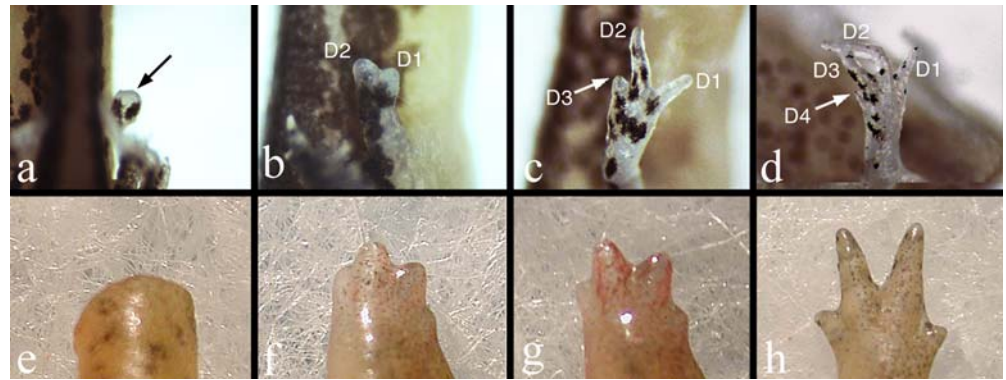
## Results

Although newt forelimb regeneration has been called a recapitulation of development, the morphology of the limb appears to be different in the two processes (Fig. 2). In developing larval forelimbs, outgrowth of the individual digits occurs. During regeneration in the adult, however, all the digits form concomitantly from the palette, a pattern reminiscent of that which occurs in higher vertebrates. Since interdigital apoptosis is a major characteristic of morphogenesis and patterning in the higher vertebrate limb, we examined patterns of apoptosis in *N. viridescens* forelimb development and adult forelimb regeneration.

### The role of apoptosis during *N. viridescens* development

Developing newt forelimbs from limb-bud to four-digit stages were examined, and there was no clear evidence of apoptosis that could be involved in pattern formation. Individual apoptotic cells were occasionally observed in the interdigital mesenchyme (Fig. 3a), but these were not present in the contralateral limb, suggesting these were isolated events resulting from cell damage prior to tissue

**Fig. 2** Morphological comparison between forelimb development and adult forelimb regeneration. The *top panel* shows forelimb development from the limb bud (a, *arrow*) through to the four-digit stage (d), and clearly illustrates that digits grow out sequentially from the limb palette. During regeneration (e–h), all the digital projections are visible simultaneously, and digits grow out from the palette throughout the regeneration process



fixation. Internal positive controls for TUNEL staining were provided by the detection of TUNEL labelling in both the embryonic gills (Fig. 3a) and the dorsal tailfin (data not shown). For additional verification of protocol specificity, a TUNEL assay was performed on E13.5-day mouse forelimbs. TUNEL-positive staining was found in the interdigital mesenchyme cells of mouse embryonic limb palettes (Fig. 3b).

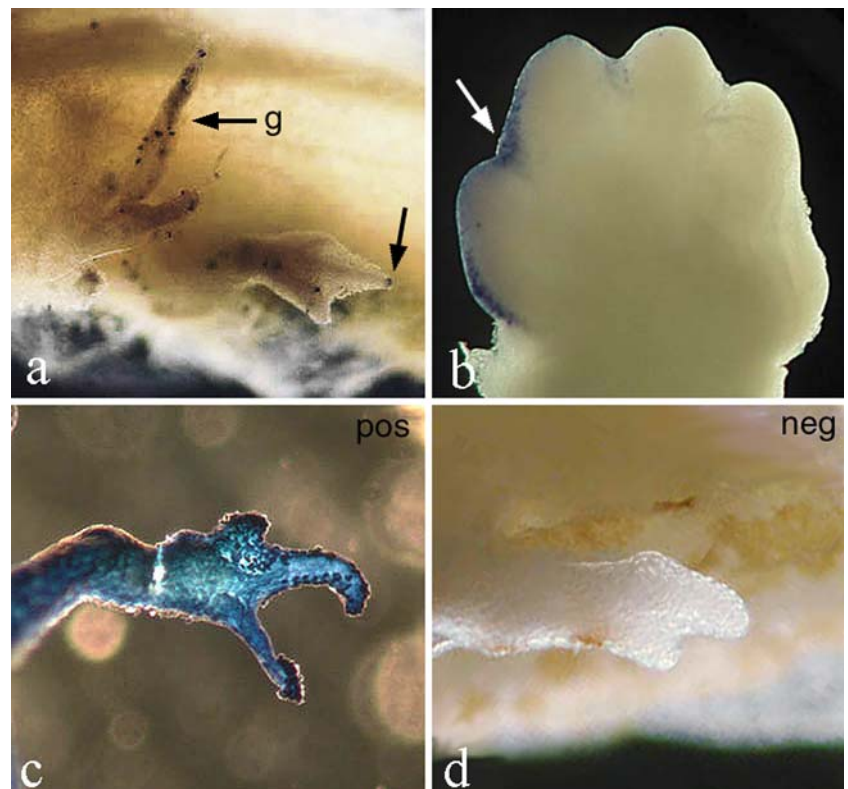
The role of apoptosis during *N. viridescens* forelimb regeneration

Cryosections of adult forelimb regenerates from 24 h to 6 weeks post-amputation were stained using a TUNEL protocol adapted for newt tissue. At 24 h post-amputation, the cut surface is covered by a thin, transparent wound

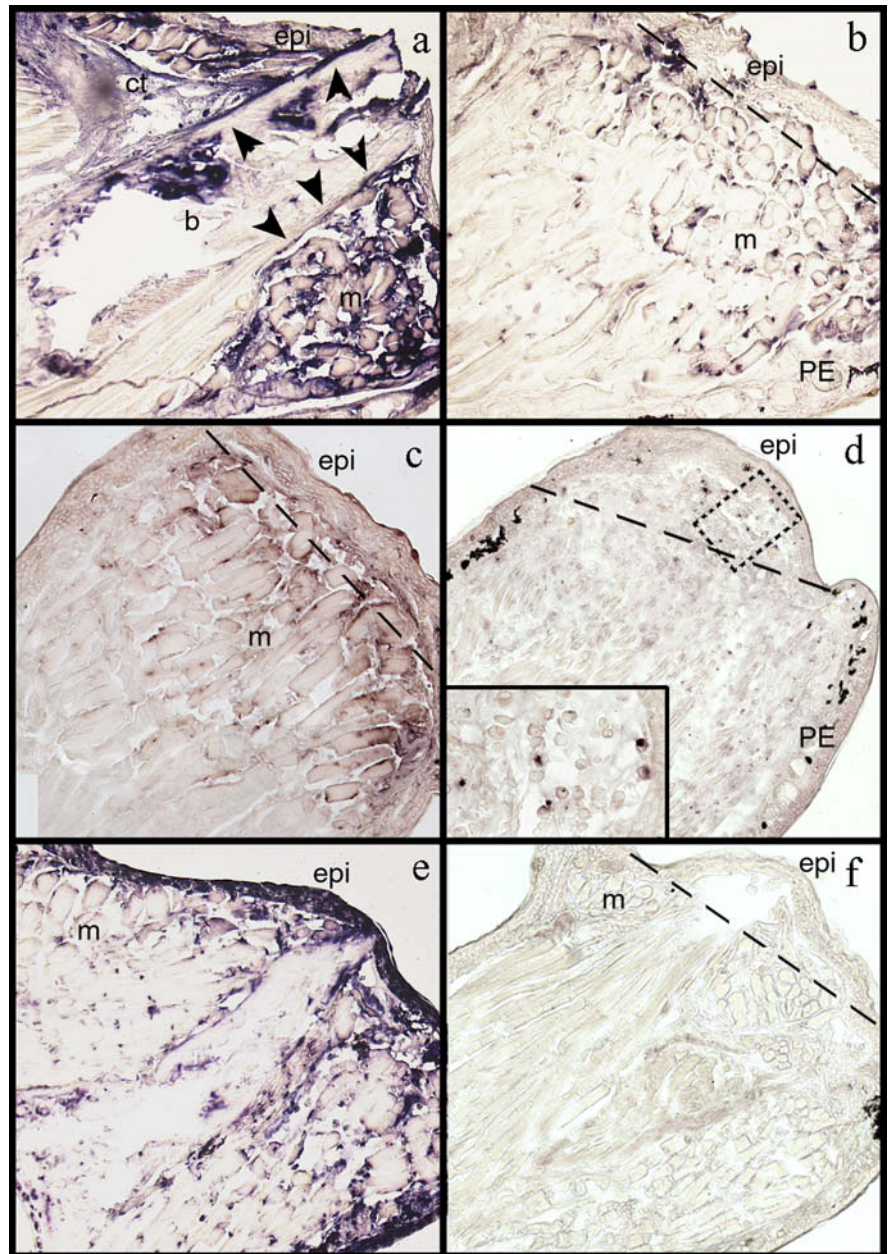
epithelium. At this stage, weak TUNEL-positive staining is seen throughout the epidermal and dermal cell layers at the plane of amputation (Fig. 4a, b). Proximal to the epithelial layer, individual positively staining cells are seen. Strong TUNEL staining is present in the periosteum along the edges of the intact stump bone (Fig. 4a). Degenerating muscles surrounding the bone are also strongly stained. Dorsal and ventral to the radius and ulna, individual TUNEL-positive muscle fragments have separated from the surrounding longitudinal muscle fibres (Fig. 4b).

By 3 days post-amputation, the wound epithelium thickens. Further degeneration of the muscle is evident. At this stage, TUNEL-positive cells are clearly found in areas of muscle breakdown, but fewer positive cells are found in the epidermis and dermis (Fig. 4c).

**Fig. 3** Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labelling (TUNEL) staining of a three-digit embryo shows a single positive cell at the tip of digit 2 (a, *arrow*) and in the interdigital mesenchyme. However, the contralateral limb was negative. Positive controls are provided by the gills (g) in the developing embryo (a), by staining of a 13.5-day mouse embryonic limb palette (b, *arrow*), and by DNase treatment (c). Negative controls were generated by omitting the TdT enzyme (d)



**Fig. 4** TUNEL staining in the early regenerate. At 24 h post-amputation (a), extensive TUNEL-positive staining is seen throughout the distal stump of the wound. Weak staining is found in the wound epithelium (*epi*). Fragmented muscle (*m*), bone (*b*), and connective tissue (*ct*), are seen in the cellular debris. Apoptotic cells are found along the periosteum outlining the bone (*arrowheads*). The staining inside the bone (a) is most likely artefactual, because it is seen in the negative controls as well. Dorsally from the plane of the radius/ulna (b), there is extensive staining in the fragmenting muscle. At 3 days post-amputation (c), there is persistent apoptotic activity in the muscle. At 7 days post-amputation (d), there are a few TUNEL-positive cells (1–2%) in the blastema (see *inset*). In d, the pigmentation in the skin proximal to the plane of amputation should not be confused with TUNEL-positive staining. Positive controls (e) were obtained by treating sections with DNase. Negative controls (f) omitted the TdT enzyme. *PE* pigmented epithelium



At 1-week post-amputation, the apical wound epithelium thickens to become the apical epithelial cap (AEC). The dermis has not yet formed beneath the AEC, and less debris is evident in the distal stump. Sparse, individual TUNEL-positive nuclei are identified throughout the regenerating tissue. The labelled cells represent approximately 1–2% of the total cells within the regenerate (Fig. 4d).

By 2-weeks post-amputation, the blastema has become established at the distal tip of the stump, and the regenerate begins to appear cone-shaped. At 3-weeks post amputation, the regenerate begins to flatten at the apex and appears palette-shaped. Cartilage condensation is beginning at the tip of the radius/ulna, just proximal to the blastemal mass. At both 2 weeks and 3 weeks post-

amputation, there are no detectable levels of TUNEL-positive cells in the regenerate (data not shown).

At 4-weeks post-amputation, chondrogenesis continues, and the beginning of patterning of the autopodium (hand) becomes visible (Fig. 5a). Four digital projections are evident at the distal tip separated by three interdigital grooves. At this stage, longitudinal sections through the condensing cartilage of the regenerating distal tip contain TUNEL-positive chondrocytes within the distal digital elements of the limb, suggesting the beginning of turnover of cartilage to bone (Fig. 5a). This positive staining of chondrocytes within the regenerating hand becomes more abundant in tissue sections at both 5- and 6-weeks post-amputation, when patterning and the separation of the digits is evident (Fig. 5c, d). No TUNEL-positive cell staining is seen to contribute to the demarcation of either

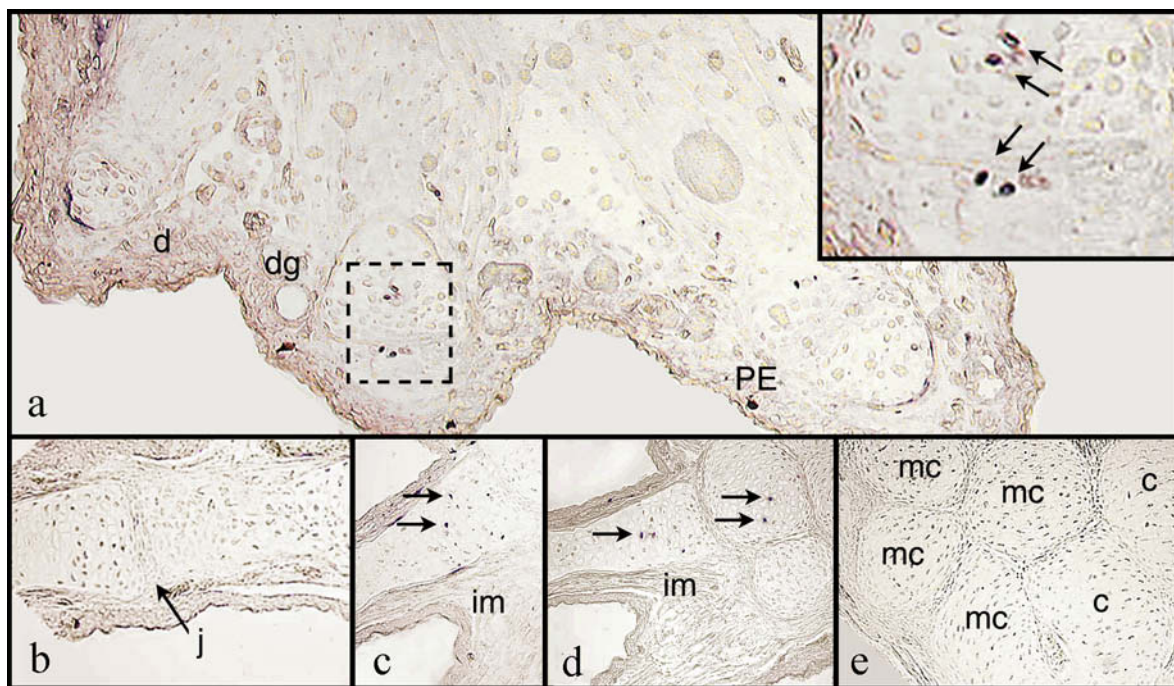
the phalangeal joints or the individualized carpal and metacarpal bones at this stage of morphogenesis (Fig. 5b–e). Individual digits are clearly separating from their neighbours and elongating, but apoptosis in the interdigital mesenchyme does not seem to contribute to this process at any stage of early or late digit formation (Fig. 5a–d).

### Growth analysis

Overlapping image contours of the regenerating limbs at various stages post-amputation do not intersect in the interdigital regions, suggesting that interdigital apoptosis is not contributing to the individualization of the digits (Fig. 6). This is further supported by growth measurements of digits 2 and 3 and the distance from the amputation site to the proximal interdigital region between them (Fig. 7). Measurements of digital length were only calculated for digits 2 and 3 (D2 and D3), since the lateral borders of digits 1 and 4 were difficult to ascertain. Digital lengths for D2 and D3 increase up to 9 weeks post-amputation, at which time they begin to plateau (Fig. 7). Length D2–D3 (see Fig. 1) increases up to approximately 8 weeks and then plateaus. This suggests that up until 8 weeks post-amputation, the digits and the carpals/metacarpals elongate to contribute to the growth of the entire hand, and after this time, the palm stops growing but the digits continue to grow.

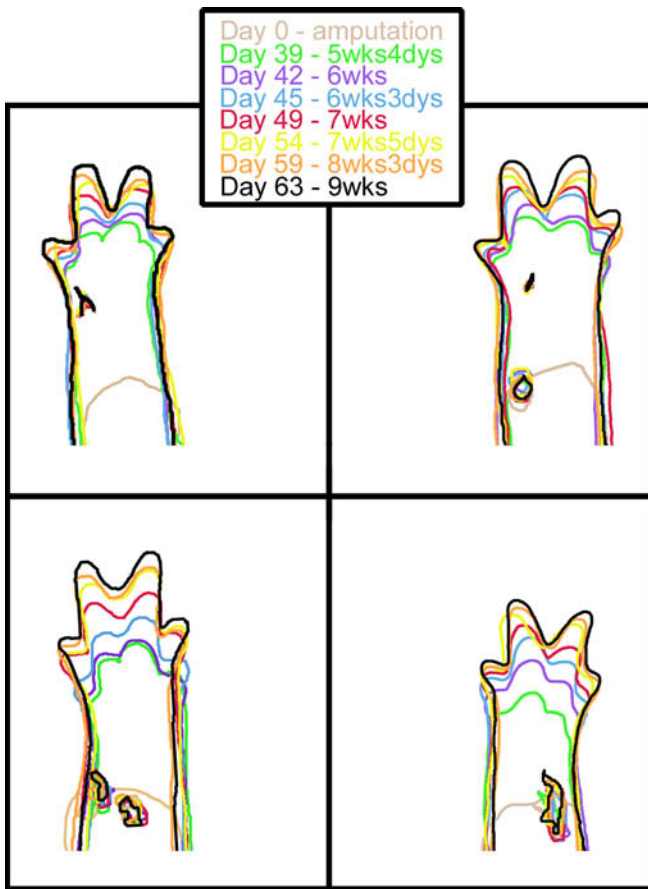
### Discussion

Is adult newt forelimb regeneration really a recapitulation of embryonic newt development? Numerous studies seem to suggest that the answer to this question is yes, since many of the genes expressed in development of the forelimb appear to be re-expressed in a similar manner during regeneration (Simon et al. 1995; Gardiner and Bryant 1996; Imokawa and Yoshizato 1997; Simon et al. 1997). However, these conclusions are often based on an extrapolation of developmental studies in mammals to regeneration studies in newt. Until recently, developmental gene expression studies were difficult to do in the newt because of an inability to reliably breed newts in captivity. However, more recent studies of the newt have identified differences between embryonic forelimb development and adult forelimb regeneration, which suggest that in some ways, adult forelimb regeneration more closely resembles forelimb development in higher vertebrates than development in urodeles. These studies show that although the same genes are often expressed during development and regeneration, the expression patterns in these genes is not always conserved between the two processes. *Tbox-4* and *Tbox-5* are notable examples. In higher vertebrates, *Tbox-5* expression determines limb identity, with *Tbox-5* being exclusively expressed in the forelimb, and *Tbox-4* being exclusively expressed in the hindlimb (Gibson-Brown et al. 1996; Gibson-Brown et al. 1998; Isaac et al. 1998; Logan et al. 1998). A similar pattern is seen during hindlimb and forelimb regeneration in the adult newt. However, *Tbox-4* and *Tbox-5* are expressed in both the

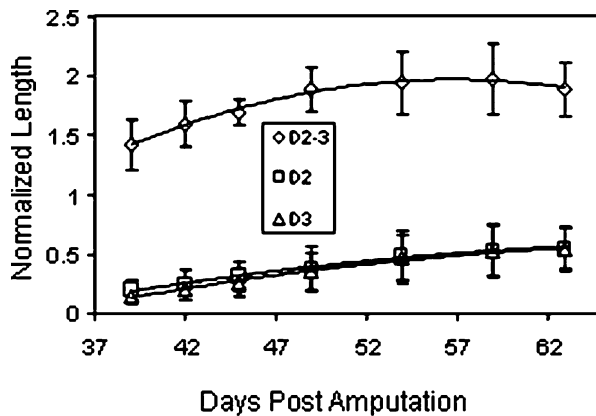


**Fig. 5** TUNEL activity in the 4-week (a) and 6-week (b–e) regenerates. There is no apoptotic activity in the interdigital mesenchyme (c, d), in the forming phalangeal joint (b) or between the developing metacarpals or carpals (e). The occasional TUNEL-

positive cells are found in regions of cartilage to bone turnover (a, inset and arrows in c and d). d Dermis, dg dermal gland, PE pigmented epithelium, j phalangeal joint, im interdigital mesenchyme, mc metacarpal, c carpal



**Fig. 6** Superimposition of contour images of the regenerates at various stages post-amputation shows no overlap in the interdigital regions, suggesting that digit outgrowth and not interdigital apoptosis is leading to elongation of the digits. Each of the four panels represents a different regenerate



**Fig. 7** Graphical presentation of growth (see also Fig. 1) in digits D2 and D3 and in the distance from the plane of amputation to the proximal interdigit ( $D2-D3$ ). All values were normalized by dividing by control length C1. Digits continue to elongate throughout the 9 weeks of the experiment, but appear to slow down as the weeks progress. The length from the amputation plane to the proximal interdigit ( $D2-D3$ ) increases up to week 8, and then plateaus. This suggests that up until 8 weeks post-amputation, the digits and the carpals/metacarpals elongate to contribute to the growth of the entire hand, and after this time, the palm stops growing but the digits continue to elongate

hindlimb and forelimb during embryonic newt development (Simon et al. 1997; Khan et al. 2002). *HoxC6* provides another example in which developmental expression patterns do not mimic regeneration expression patterns. *HoxC6* is expressed in newt forelimbs during development, but is absent in the hindlimb and tail, within the limits of detection of the in situ hybridization assay (Khan et al. 1999). In contrast, regeneration studies show highest expression of *HoxC6* occurs in hindlimbs, followed by tails and then forelimbs (Savard et al. 1988; Savard and Tremblay 1995).

Not only are there differences in gene expression between larval forelimb development and adult forelimb regeneration, but there are differences in morphology as well. We clearly show that the morphology of developing larval newt forelimbs is different from the morphology of adult forelimb regenerates (see Fig. 2). During forelimb development, digits emerge from the limb palette in a sequential manner; digits 1 and 2 are the first to grow out, followed by digits 3 and 4 (Jarvik 1980). Apoptosis does not appear to play a role in this process. The only apoptotic cells identified were individual cells at probable sites of injury (see Fig. 3). Our findings for the absence of interdigital apoptosis in development are not surprising, based on the results of previous studies. Although it has been long proposed that interdigital apoptosis is the major contributor during digit individualization in birds (Saunders 1966) and mammals (Ballard and Holt 1968; Mori et al. 1995), it has also been shown that programmed cell death does not occur during limb development in several amphibians (Cameron and Fallon 1977).

During adult forelimb regeneration, all the digits seem to appear simultaneously, a pattern which more closely resembles the developing forelimb palette in higher vertebrates such as mouse or man. Due to the similarities with higher vertebrates, we studied whether individualization of the digits during newt forelimb regeneration also occurs through a process involving growth in combination with apoptosis of the interdigital mesenchyme. Salas-Vidal et al. (2001) used TUNEL analysis and image superimposition of developing mouse forelimbs at various stages to show that interdigital regression occurs during forelimb development in higher vertebrates. We used the same strategy to examine digital elongation in regenerating forelimbs of adult *N. viridescens*. Individual regenerates were followed for a period of 9 weeks, and pictures were taken at various stages in the regeneration process. Our results show that there is extensive apoptosis in the early stages of regeneration (especially in the first 3 days), when wound healing and the elimination of cellular debris from the injury site are underway (see Fig. 4). We also observe apoptotic cells in the advanced regenerate at sites of cartilage to bone turnover (see Fig. 5). It is known that during embryonic cartilage development, mesenchymal cells condense and differentiate into chondrocytes. During the final stages of chondrocyte maturation, the chondrocytes undergo apoptosis and are replaced by the formation of bone through the action of osteoblasts (Hickok et al. 1998). Our identification of these apoptotic cells indicates

the beginning of ossification in the advanced regenerate. This observation is supported by a recent study showing the beginnings of mineralization of the distal regenerate by day 37 post-amputation (Stock et al. 2003). It should be noted that there is one previous study examining apoptosis in regenerating forelimbs of larval axolotls: Mescher et al. (2000). Although both newts and axolotls have the ability to regenerate forelimbs, direct comparisons cannot be made between our study and that of Mescher et al. (2000), because one examines adult regenerating tissues and the other studies larval regeneration. The massive apoptosis seen in our adult newt forelimbs within the first 3 days post-amputation does not appear to be present in the axolotl forelimbs, probably due to the fact that cells in the forelimb of the larval axolotl are not fully differentiated. Consequently, the inflammation and wound-healing response may be moderate in these forelimbs. Nevertheless, our findings are similar at 1-week post-amputation. TUNEL-positive mesenchymal cells are found in small clusters or singly distributed near the tip of the stump beneath the wound epidermis and represent approximately 1–2% of the cells of the dedifferentiating tissue. Mescher et al. (2000) only studied the role of apoptosis within 11 days post-amputation, whereas our study examines apoptosis throughout the regeneration process. At later stages of regeneration, we show that apoptosis is not involved in pattern formation and is mainly involved in cartilage turnover to bone.

Notably, TUNEL analysis of larval developing forelimbs and adult regenerating forelimbs, as well as the examination of superimposed images of growing adult regenerates, identified no interdigital mesenchymal apoptosis. The individualization of both developing and regenerating digits appears to result from growth and elongation of the digits rather than by the apoptosis of the preformed interdigital tissue. This result differs from limb development in higher vertebrates such as chick and mouse (Salas-Vidal et al. 2001).

It is interesting to note that the progression of digit morphogenesis during regeneration is very much dependent on the developmental stage of the animal. In amputated larvae of *N. viridescens*, the forelimb regenerate follows the developmental pattern, where individual digits emerge sequentially from the limb palette (Khan et al. 1999, and personal observations). However, the adult newt forelimb regenerate differs morphologically from the newt larval forelimb (and the larval regenerate) and appears to more closely resemble the palette in higher vertebrates. Even so, apoptosis and growth patterns suggest that the newt forelimb regenerate and the developing-limb palette of higher vertebrates are also different. It appears that the adult newt regeneration mechanism is unique, sharing both similarities and differences with developing newt forelimbs and developing higher vertebrates. Care must be taken and further research is warranted before we can truly say that regeneration is or is not a recapitulation of development.

**Acknowledgements** We thank Adam Baker for technical expertise, help with the figures, and critical reading of the manuscript. We thank Sandy Vascotto and Richard A. Liversage for helpful discussions. This work was supported by the Natural Sciences and Engineering Council of Canada (C.T.) and by the Canadian Institutes of Health Research (C.T.).

## References

- Ballard KJ, Holt SJ (1968) Cytological and cytochemical studies on cell death and digestion in the foetal rat foot: the role of macrophages and hydrolytic enzymes. *J Cell Sci* 3:245–262
- Brookes JP, Lo DC (1994) Reversibility of the mononucleate-to-multinucleate myogenic transition during amphibian limb regeneration. *Eye* 8:151–154
- Butler EG (1933) The effects of x-radiation on the regeneration of the forelimb of *Amblystoma* larvae. *J Exp Zool* 65:271–315
- Cadinouche MZ, Liversage RA, Muller W, Tsilfidis C (1999) Molecular cloning of the *Notophthalmus viridescens* radical fringe cDNA and characterization of its expression during forelimb development and adult forelimb regeneration. *Dev Dynam* 214:259–268
- Cameron JA, Fallon JF (1977) The absence of cell death during development of free digits in amphibians. *Dev Biol* 55:331–338
- Gardiner DM, Bryant SV (1996) Molecular mechanisms in the control of limb regeneration: the role of homeobox genes. *Int J Dev Biol* 40:797–805
- Gavrieli Y, Sherman Y, Ben-Sasson SA (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 119:493–501
- Gibson-Brown JJ, Agulnik SI, Chapman DL, Alexiou M, Garvey N, Silver LM, Papaioannou VE (1996) Evidence of a role for T-box genes in the evolution of limb morphogenesis and the specification of forelimb/hindlimb identity. *Mech Dev* 56:93–101
- Gibson-Brown JJ, Agulnik SI, Silver LM, Niswander L, Papaioannou VE (1998) Involvement of T-box genes *Tbx2-Tbx5* in vertebrate limb specification and development. *Development* 125:2499–2509
- Hay ED (1959) Electron microscopic observations of muscle dedifferentiation in regenerating *Amblystoma* limbs. *Dev Biol* 1:555–585
- Hickok NJ, Haas AR, Tuan RS (1998) Regulation of chondrocyte differentiation and maturation. *Microsc Res Tech* 43:174–190
- Imokawa Y, Yoshizato K (1997) Expression of Sonic hedgehog gene in regenerating newt limb blastemas recapitulates that in developing limb buds. *Proc Natl Acad Sci USA* 94:9159–9164
- Isaac A, Rodriguez-Esteban C, Ryan A, Altshuler M, Tsukui T, Patel K, Tickle C, Izpisua-Belmonte JC (1998) *Tbx* genes and limb identity in chick embryo development. *Development* 125:1867–1875
- Iten LE, Bryant SV (1973) Forelimb regeneration from different levels of amputation in the newt, *Notophthalmus viridescens*: length, rate and stages. *Wilhelm Roux Arch* 173:263–282
- Jarvik E (1980) Basic structure and evolution of vertebrates. Academic, London
- Jensen AM, Wallace VA (1997) Expression of Sonic hedgehog and its putative role as a precursor cell mitogen in the developing mouse retina. *Development* 124:363–371
- Khan PA, Liversage RA (1995) Spawning of *Notophthalmus viridescens* and rearing of embryos under laboratory conditions. *Herpetol Rev* 26:95–96
- Khan PA, Tsilfidis C, Liversage RA (1999) Hox C6 expression during development and regeneration of forelimbs in larval *Notophthalmus viridescens*. *Dev Genes Evol* 209:323–329
- Khan P, Linkhart B, Simon HG (2002) Different regulation of T-box genes *Tbx4* and *Tbx5* during limb development and limb regeneration. *Dev Biol* 250:383–392



- Logan M, Simon HG, Tabin C (1998) Differential regulation of T-box and homeobox transcription factors suggests roles in controlling chick limb-type identity. *Development* 125:2825–2835
- Mescher AL, White GW, Brokaw JJ (2000) Apoptosis in regenerating and denervated, nonregenerating urodele forelimbs. *Wound Repair Regen* 8:110–116
- Mori C, Nakamura N, Kimura S, Irie H, Takigawa T, Shiota K (1995) Programmed cell death in the interdigital tissue of the fetal mouse limb is apoptosis with DNA fragmentation. *Anat Rec* 242:103–110
- Salas-Vidal E, Valencia C, Covarrubias L (2001) Differential tissue growth and patterns of cell death in mouse limb autopod morphogenesis. *Dev Dynam* 220:295–306
- Sanders EJ, Wride MA (1995) Programmed cell death in development. *Int Rev Cytol* 163:105–173
- Saunders JWJ (1966) Death in embryonic systems. *Science* 154:604–612
- Savard P, Tremblay M (1995) Differential regulation of Hox C6 in the appendages of adult urodeles and anurans. *J Mol Biol* 249:879–889
- Savard P, Gates PB, Brockes JP (1988) Position dependent expression of a homeobox gene transcript in relation to amphibian limb regeneration. *EMBO J* 7:4275–4282
- Simon HG, Nelson C, Goff D, Laufer E, Morgan BA, Tabin C (1995) Differential expression of myogenic regulatory genes and Msx-1 during dedifferentiation and redifferentiation of regenerating amphibian limbs. *Dev Dynam* 202:1–12
- Simon HG, Kittappa R, Khan PA, Tsilfidis C, Liversage RA, Oppenheimer S (1997) A novel family of T-box genes in urodele amphibian limb development and regeneration: candidate genes involved in vertebrate forelimb/hindlimb patterning. *Development* 124:1355–1366
- Stock SR, Blackburn D, Gradassi M, Simon HG (2003) Bone formation during forelimb regeneration: a microtomography (microCT) analysis. *Dev Dynam* 226:410–417
- Veenstra GJ, Peterson-Maduro J, Mathu MT, van der Vliet PC, Destree OH (1998) Non-cell autonomous induction of apoptosis and loss of posterior structures by activation domain-specific interactions of Oct-1 in the *Xenopus* embryo. *Cell Death Differ* 5:774–784
- Zuzarte-Luis V, Hurlle JM (2002) Programmed cell death in the developing limb. *Int J Dev Biol* 46:871–876