Expression of FGF2 in the limb blastema of two Salamandridae correlates with their regenerative capability

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Limb regenerative potential in urodeles seems to vary among different species. We observed that *Triturus vulgaris meridionalis* regenerate their limbs significantly faster than *T. carnifex*, where a long gap between the time of amputation and blastema formation occurs, and tried to identify cellular and molecular events that may underlie these differences in regenerative capability. Whereas wound healing is comparable in the two species, formation of an apical epidermal cap (AEC), which is required for blastema outgrowth, is delayed for approximately three weeks in *T. carnifex*. Furthermore, fewer nerve fibres are present distally early after amputation, consistent with the late onset of blastemal cell proliferation observed in *T. carnifex*. We investigated whether different expression of putative blastema mitogens, such as FGF1 and FGF2, in these species may underlie differences in the progression of regeneration. We found that whereas FGF1 is detected in the epidermis throughout the regenerative process, FGF2 onset of expression in the wound epidermis of both species coincides with AEC formation and initiation of blastemal cell proliferation, which is delayed in *T. carnifex*, and declines thereafter. *In vitro* studies showed that FGF2 activates MCM3, a factor essential for DNA replication licensing activity, and can be produced by blastemal cells themselves, indicating an autocrine action. These results suggest that FGF2 plays a key role in the initiation of blastema growth.

Keywords: apical epithelial cap; fibroblast growth factor; FGFR2; proliferation; regeneration; urodele amphibians

1. INTRODUCTION

At larval stages, both urodeles (e.g. newt and salamander) and anurans (e.g. frog, *Xenopus*) can regenerate their limbs, but in the adult complete limb regeneration can occur only in urodeles (Wallace 1981; Tsonis 1996). Limb regeneration is a typical example of epimorphic regeneration as it occurs via the formation of a growth zone, the blastema, and the cellular and molecular basis underlying this striking capability are starting to be unravelled (Gardiner & Bryant 1998; Géraudie & Ferretti 1998; Brookes & Kumar 2002). The main events leading to the regeneration process are wound healing in response to amputation, accumulation of a mass of undifferentiated mesenchymal cells (blastemal cells) by dedifferentiation, and formation of a specialized apical epidermal cap (AEC) lacking a basement membrane that, together with the nerve, is required for blastemal cell proliferation (Singer & Salpeter 1961; Thornton 1968; Singer 1974). The absence of a basement membrane between the AEC and the underlying mesenchyme is characteristic of epimorphic regeneration. In mammalian wound healing the basement membrane of the skin is fully reconstituted by 72 h after injury (Johnson & Pierce 1970) impeding the

free diffusion of molecules between the epidermis and mesenchyme.

Fibroblast growth factors (FGFs) belong to a large family of proteins that affect cell proliferation, differentiation, survival and motility, and function as key signalling molecules in embryogenesis (Szebenyi & Fallon 1999). Several FGFs are expressed during early limb development and have been shown to play important roles in limb initiation and in the establishment of growth and patterning centres such as the apical ectodermal ridge (AER) and the zone of polarizing activity (ZPA), respectively (Martin 1998; Yonei-Tamura *et al.* 1999; Tickle & Munsterberg 2001). Transduction of FGF signals in early limb development appears to be mainly mediated by the high affinity transmembrane tyrosine kinase receptors FGFR1 and FGFR2 (Deng *et al.* 1997; Xu *et al.* 1999), and FGFR2 appears to be the key receptor for modulating limb bud initiation (Xu *et al.* 1998). One of the spliced forms of FGFR2, FGFR2-IIIb, has been shown to play an essential role in maintaining the AER, though it does not appear to be required for limb bud initiation (Arman *et al.* 1999; Revest *et al.* 2001).

In addition to its key roles in limb development, FGF signalling is also likely to play an important role in the process of limb regeneration in amphibians. It has been shown using either *Xenopus* larvae or mature newts and axolotls that FGF1, FGF2, FGF8 and FGF10, as well as FGFR1 and FGFR2, are expressed in regenerating limbs

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(Christen & Slack 1997; D'Jamoos *et al.* 1998; Cannata *et al.* 2001; Christensen *et al.* 2001, 2002; Dungan *et al.* 2002). FGF10 application can stimulate a regenerative response in *Xenopus* limbs at late larval stages when, normally, no regeneration occurs, whereas FGF1, FGF2 and FGF8 do not appear to have such inductive ability (Yokoyama *et al.* 2001). FGF1 and FGF2, however, have been suggested to play a role in the proliferation of blastemal cells. FGF1 is strongly expressed in the AEC and weakly in the mesenchyme, is able to stimulate blastema cell proliferation, and its inhibition using blocking antibodies reduces thymidine incorporation in the blastema (Boilly *et al.* 1991; Zenjari *et al.* 1996; Dungan *et al.* 2002). FGF2 has been detected in the AEC and in the nerves present in the blastema, and is mitogenic for blastemal cells in culture (Mescher & Loh 1981; Mullen *et al.* 1996; Corcoran & Ferretti 1999). FGFR1 is expressed in the mesenchyme of early- and medium-bud blastemas but is not detectable in the AEC (Poulin *et al.* 1993). Two FGFR-2 variants, *KGFR* and *bek*, have been detected during regeneration. At very early stages of regeneration *KGFR* is present in the basal layer of the AEC, while *bek* expression is restricted to the periosteum; in the blastema, both *KGFR* and *bek* variants are present in the mesenchyme (Poulin & Chiu 1995).

We have analysed the limb regeneration process in two salamandrids, *Triturus carnifex* and *Triturus vulgaris meridionalis*. The first species is characterized by adult animals 15 cm in length while specimens of *T. v. meridionalis* hardly reach 7 cm in length. Although we have found that both species eventually regenerate, the appearance of a blastema is much delayed in *T. carnifex* as compared with *T. v. meridionalis*. To understand which factors may be mainly responsible for these differences in blastema formation and in the progression of regeneration in these two species, we have monitored formation of the blastema, its innervation and proliferation, and expression of FGF1 and FGF2. The data obtained from this study suggest that the two species show strong differences in the onset of proliferation, and this appears to correlate with differences in formation of an AEC, innervation and FGF2 expression. Furthermore, we show that in blastemal cells FGF2 may act in an autocrine fashion.

2. MATERIAL AND METHODS

(a) Tissue preparation

Adult animals, collected near Pisa (Italy), were anaesthetized in 0.1% tricaine (3-aminobenzoic acid ethylester methane sulphate salt, Sigma), forelimbs were amputated proximally to the elbow and collected at different times after amputation. Experimental operated animals were maintained either at 18–20 °C or 25 °C as specified in § 3. Regenerated tissues were either fixed in 4% paraformaldehyde (PFA, Merck) in PBS (phosphate buffered saline, 100 mM of phosphate buffer, 120 mM of NaCl, pH 7.4) for paraffin embedding, or cryomounted for cryostat sectioning. Sections were stained with toluidine and methylene blue or DAPI, or processed for immunohistochemistry.

(b) DAPI staining and immunohistochemistry

Adult animals, left to regenerate at 18–20 °C, were injected with 250–350 µl (depending on the size of the animal) of 0.3%

colchicine (Sigma) 24 h and 48 h before collecting the blastema. At each stage of interest, at least three samples were obtained. Sections (7 µm) of regenerating limbs at different stages were equilibrated in PBS and nuclei stained for 10 min with 400 µg ml⁻¹ DAPI (Sigma: D-1388) diluted in PBS. At least 400 nuclei per specimen were counted.

All immunohistochemical studies were carried out on regenerating limbs from animals left to regenerate at 25 °C. Staining with the anti-keratin monoclonal antibody (mAb) LP1K (Lane *et al.* 1985) and the anti-neurofilament mAb RT97 (Hybridoma Bank, University of Iowa, USA) was carried out on cryostat sections fixed with acid alcohol as previously described (Ferretti *et al.* 1989).

The reactivities of the anti-FGF2 mAb (Transduction Laboratories, Mamhead, UK), anti-FGF1 goat polyclonal antibody (C-19, Santa Cruz Biotechnology), and of the anti-FGFR2 polyclonal antibody (C-17, Santa Cruz Biotechnology) were assayed on 7 µm paraffin sections and detected as previously described (Zhang *et al.* 2000, 2002). Staining of cultured blastemal cells for FGF2 was carried out after fixing the cells for 10 min in 4% PFA. Controls where the primary antibody was omitted were always negative.

(c) Cell culture

BIH1 cells, long-term cultures derived from limb blastemas, were cultured as previously described (Ferretti & Brookes 1988), apart from the fact that 0.1% bovine skin gelatine (Sigma, Poole, UK), instead of collagen, was used to coat the dishes (Corcoran & Ferretti 1999). Cells were serum-deprived for 3 days before assessing changes in FGF2 expression either by immunocytochemistry or by RT-PCR. In some experiments 10 ng ml⁻¹ FGF2 was added to cells that had been maintained in the absence of serum for 3 days.

(d) RT-PCR

Total RNA was isolated using TRI REAGENT (Sigma) according to the manufacturer's instruction and reverse transcription carried out as previously described (Zhang *et al.* 2000).

MCM3 amplification was performed using the primers 5'-AATCGTTGGCACCAGAGTA-3' (sense) and 5'-GTCAAACTCATCAATGCA-3' (antisense) as described (Albani *et al.* 1998). The primers used for the normalization were SAT2F (5'-GCCTAGCTGATGAAGGGTGAT-3') and SAT2R (5'-GTCACAGATCTGGGTTTAATCCA-3') specific for a 200 bp fragment of the *N. viridescens* satellite 2 (Epstein *et al.* 1986).

3. RESULTS

(a) Time-course of regeneration

in *T. v. meridionalis* and *T. carnifex*

Gross morphological analysis of regenerating limbs of *T. v. meridionalis* and *T. carnifex* maintained at 20 °C has shown that whereas the time-course of the initial stages of regeneration in *T. v. meridionalis* is comparable to those reported in other related species (Iten & Bryant 1973), in *T. carnifex* there is no evidence of regeneration for several days (figure 1), though complete regeneration eventually also occurs in this species. Initial indication of a regenerative process in *T. carnifex* maintained at 18–20 °C is apparent approximately four weeks after amputation, when an early bud can be observed. By this time, regenerating limbs of *T. v. meridionalis* have reached the late bud stage.

Figure 1. Cartoon shows the approximate times required to reach the initial stages of limb regeneration, as defined by Iten & Bryant (1973), in *Triturus vulgaris meridionalis* and *Triturus carnifex* maintained at 20 °C.

Figure 2. Limb stumps from *Triturus carnifex* and *Triturus vulgaris meridionalis* 24 h after amputation. Sections are stained with toluidine and methylene blue. (a) *T. carnifex*: epidermal cells from the stump skin (s) have migrated to form a wound epidermis (we) that covers the cut surface; the gap in the wound epidermis (*) is an artefact due to a tear caused by the underlying bone (b). (b) *T. v. meridionalis*: wound healing is complete. Scale bar, 400 µm.

Regeneration progresses more rapidly in both species when the regenerating animals are maintained at 25 °C, but regeneration in *T. carnifex* always lags significantly behind. Unless otherwise specified the experiments presented here were carried out on animals maintained at 25 °C.

(b) Wound healing and blastema growth

To assess whether the different rates of regeneration observed in *T. v. meridionalis* and *T. carnifex* could be at least partly due to differences in the wound-healing process, we examined limbs from the two species 1 day after amputation. As shown in figure 2, it is clear that by 24 h after surgery wound healing is complete in both species.

To establish whether the delay in the appearance of a bud is due to defective accumulation of blastemal cells at the early stages of regeneration, we carried out morphological and immunocytochemical analysis to assess whether, in *T. carnifex*, dedifferentiation is delayed, and formation of the AEC, which is required for blastema outgrowth, is impaired. We stained cryostat sections of regenerates from the two species with the anti-keratin antibody LP1K, which we have previously shown to be a good marker of blastemal cells and of the AEC (Ferretti *et al.* 1989; Corcoran & Ferretti 1997).

As in other urodeles (Ferretti *et al.* 1989), expression of LP1K in mesenchymal cells is induced early in response to amputation (3–5 days) both in *T. carnifex* (figure 3a) and *T. v. meridionalis* (not shown), indicating that the early process of blastemal cell accumulation begins at a similar time. At early bud and blastema stages most blastemal cells are labelled both in *T. carnifex* and *T. v. meridionalis* (figure 3b,c).

Since adequate innervation is required for blastema growth at the early stages of regeneration, we have investigated whether there are obvious differences in the innervation of the blastema in the two species by staining limb regenerates at different times after amputation with the neuronal marker RT97 (figure 3d–f). RT97 reactivity shows that whereas nerve fibres are easily observed in the wound epidermis of *T. v. meridionalis* soon after amputation (figure 3d), they are not detected in *T. carnifex* (figure 3e), suggesting that an insufficient nerve supply may affect the onset of proliferation. Indeed, proliferating cell nuclear antigen (PCNA) analysis of regenerating limbs 4 days after amputation shows greater reactivity in *T. v. meridionalis* than in *T. carnifex* (data not shown). By the early-bud stage, fibres entering the wound epidermis can also be observed in *T. carnifex* (figure 3f).

Figure 3. Immunoreactivity of LP1K (green, *a–c*) and RT97 (red, *d–f*) in *Triturus carnifex* (*a,b,e,f*) and *Triturus vulgaris meridionalis* (*c,d*) at different stages of regeneration. (*a*) Oblique section of the tip of *T. carnifex*'s stump 4 days after amputation; note some induction of LP1K reactivity in the mesenchyme. (*b*) Longitudinal section of *T. carnifex* early bud; note that the majority of blastemal cells and a few cells in the wound epidermis (we) are LP1K-positive. The asterisk indicates an artefactual tearing of the wound epidermis from the underlying mesenchyme. (*c*) Longitudinal section of *T. v. meridionalis* mid-bud; LP1K reactivity is observed in the majority of blastemal cells and the wound epidermis. (*d*) Longitudinal section of *T. v. meridionalis* stump tip 4 days after amputation; note RT97-positive large nerve fibres (arrowhead) in the mesenchyme and fine nerve fibres (arrows) entering the wound epidermis (we). The insert top right shows a higher magnification of the wound epidermis. (*e*) Longitudinal section of *T. carnifex*'s stump tip 4 days after amputation. No RT97-positive fibres are observed in the wound epidermis (we) at this stage, though nerve fibres (arrowhead) are detected in the mesenchyme. (*f*) Longitudinal section of *T. carnifex* early bud. By this stage, RT97-positive fibres entering the wound epidermis are present. Scale bars, 50 µm (*c–f*); 200 µm (*a,b*).

Figure 4. Percentage of mitotic figures present in the blastema mesenchyme at different stages of regeneration in animals maintained at 18–20 °C for (*a*) *Triturus carnifex* and (*b*) *Triturus vulgaris meridionalis*. Several representative sections from at least three regenerates obtained from different animals were analysed for each time-point and a minimum of 400 nuclei per specimen counted. Error bars represent the standard deviation.

To quantify and compare accurately the proliferative activity of blastemal cells in regenerating limbs from the two species, we counted the number of mitotic cells, identified by DAPI staining, in limbs at different stages of regeneration (figure 4). In these experiments the animals were left to regenerate at 18–20 °C. By 12 days after amputation, the mitotic index in *T. v. meridionalis* regenerating limbs was twice that of 16 day *T. carnifex* limbs. In both species high mitotic activity was observed at the same regeneration stage, medium bud, which in the experiment shown here occurred at 16 days in *T. v. meridionalis* and at 43 days in *T. carnifex*, and was comparable in extent. This analysis shows that there is indeed a delay in the

onset of significant mitotic activity in *T. carnifex* as compared with *T. v. meridionalis*. Nonetheless, a clear correlation between mitotic index and the progression of regeneration is observed in both species, suggesting that the differences in regenerative capability are not related to intrinsic differences in blastemal cells.

These experiments indicate that delayed regeneration in *T. carnifex* is more probably owing to the lack of mitogenic stimuli than to impaired blastemal cell accumulation. We therefore monitored the expression of FGFs in the two species, as members of this family of growth factors appear to be important both for limb development and for regeneration.

Figure 5. FGF and FGFR2 immunoreactivity in *Triturus carnifex* and *Triturus vulgaris meridionalis* assessed by immunocytochemistry (positive staining is brown). (a) FGF1 reactivity in early bud regenerate of *T. carnifex*. (b) FGF1 reactivity in late bud regenerate of *T. carnifex*. Expression of FGF1 is largely localized to the epidermis of the regenerating limbs. (c) FGF2 reactivity in 4 day regenerate of *T. v. meridionalis*. (d) FGF2 reactivity in 7 day regenerate of *T. v. meridionalis*. (e) FGF2 reactivity in 7 day regenerate of *T. carnifex*. (f) FGF2 reactivity in 12 day regenerate of *T. carnifex*. (g) FGFR2 reactivity in 4 day regenerate of *T. carnifex*. (h) FGFR2 reactivity in 12 day regenerate of *T. carnifex*. (i) FGFR2 reactivity in 4 day regenerate of *T. v. meridionalis*. Scale bars, 100 µm.

(c) *FGF and FGFR-2 expression in regenerating blastemas*

FGF1 and FGF2 have been proposed as key factors in controlling blastema growth. We therefore investigated whether there was a correlation between their expression and the progression of regeneration in *T. v. meridionalis* and *T. carnifex*.

We examined FGF1 expression in *T. carnifex* between 4 days and six weeks after amputation, and found that FGF1 protein was detectable in the epidermis at all the stages of regeneration examined (figure 5*a,b*), suggesting that this growth factor does not play a primary role in initiating blastemal cell proliferation. We therefore focused our study on FGF2 expression.

Four days after amputation, the wound epidermis at the tip of the regenerating limb of *T. v. meridionalis* has already thickened to form an AEC and FGF2 expression is clearly observed within this structure (figure 5*c*). Expression of FGF2 in the AEC is still detectable at 7 days (figure 5*d*). In *T. carnifex*, by contrast, there is no epidermal thickening even 7 days after limb amputation and the wound epidermis covering the stump is FGF2-negative (figure 5*e*). FGF2 reactivity is observed transiently in the wound epidermis of *T. carnifex*, but at increased times after amputation (*ca.* 12 days, rather than 4 days as in *T. v. meridionalis*), and coincides with the appearance of the AEC (figure 5*f*). By the medium-late bud stage the wound epidermis is FGF2-negative both in *T. v. meridionalis* and in *T. carnifex*, though some reactivity is observed in the regenerating cartilage (not shown). These results show that maximal expression of FGF2 in the wound epidermis coincides with the onset of blastemal cell proliferation and the initial phase of blastema growth. FGF2 expression in regenerating limbs is not restricted to the wound epidermis, and some FGF2-positive cells can be observed at the tip of the stump.

Since FGFR2 has been shown to be the main FGFR in the wound epidermis, we also compared the expression of this receptor during limb regeneration in the two species. Unlike FGF2, in the wound epidermis FGFR2 can be detected in both species, from at least 4 days after amputation until the late-bud stage (figure 5*g-i*). Staining is also observed in several tissues of the stump and in blastemal cells. The staining intensity in the wound epidermis is always significantly higher in *T. v. meridionalis* than in *T. cristatus*, but in both species, higher levels of FGFR2 expression are observed in the epidermis at the onset of blastemal cell division and in the early growth phase. As observed in other species, FGFR2 is also expressed in the mesenchyme. These results support the view that FGF signalling is important for blastema growth and may account for differences in the regenerative capability between the two species.

(d) *FGF signalling in blastemal cells in vitro*

To gain further insight into the importance of FGF signalling in blastemal cell growth, we have examined FGF2 expression in cultured blastemal cells, the BIH1 cell line, under different culture conditions. As previously reported, cells maintained in serum-free medium do not proliferate, but this effect can be reversed by the addition of TPA or FGF2 (Corcoran & Ferretti 1999). Blastemal cells grown in the presence of serum that does not contain FGF2

appear to produce FGF2 as indicated by immunohistochemistry (figure 6*a*). By contrast, FGF2 is undetectable in cells cultured without serum (figure 6*b*). Addition of FGF2 to the medium, however, appears sufficient to stimulate FGF2 mRNA (not shown) and protein expression in these cells (figure 6*c*), suggesting an autocrine mechanism of this cytokine.

To better understand the involvement of FGF2 in the control of proliferation of blastemal cells, we investigated its effect on the expression of members of the minichromosome maintenance (MCM) protein family that are present in proliferating cells (Treisman *et al.* 1995). These highly conserved factors are essential for DNA replication licensing activity and for ensuring that the process takes place only once for each cell cycle (Tada & Blow 1998). We have found that specific primers chosen in the conserved region of the *MCM3* isolated from *T. carnifex* ovary (*TcMCM3*; Albani *et al.* 1998) amplify a transcript highly homologous (95%) to *TcMCM3* in blastemal extracts (data not shown); the same primers detect *MCM3* RNA in BIH1 cells grown either in the presence of serum or in serum-free medium containing FGF2, but not in serum-free medium alone (figure 7). These results support the view that FGF2 plays a key role in blastemal cell growth by activating the molecular pathways involved in the cell cycle control.

4. DISCUSSION

The work presented here has identified differences in the regenerative capability between two newt species, *T. v. meridionalis* and *T. carnifex*. We have shown that the regeneration time is longer in *T. carnifex* as compared with *T. v. meridionalis*, and in particular that there is a striking difference in the time required for blastema formation. It does not seem likely that this is mainly due to differences in size between these two species. The onset of limb blastema formation in other species of comparable or even larger size than *T. carnifex*, such as *Pleurodeles waltl*, is very similar to that of *T. v. meridionalis* and *Notophthalmus viridescens*, though the overall regenerative process may take slightly longer as a larger structure has to be replaced.

(a) *AEC formation and wound epidermis innervation are delayed in slow regenerating urodeles*

The initiation of blastema formation depends on the accumulation of progenitor cells at the tip of the stump beneath the wound epidermis and their ability to proliferate. It is evident that dedifferentiation does occur in *T. carnifex*, since reactivity of the cell dedifferentiation marker, LP1K, and morphological analysis, indicate the presence of blastemal cells at the tip of the stump only 4 days after amputation. The analysis of the temporal distribution of the mitosis under the AEC suggests that in *T. carnifex* there is an extended dedifferentiation phase and delayed onset of proliferation.

It is well established that for the process of dedifferentiation neither formation of the AEC nor an adequate nerve supply are required. By contrast, both the AEC and innervation are essential for cell proliferation to begin, and be maintained, during the early stages of regeneration (Singer & Salpeter 1961; Thornton 1968; Singer 1974; Dinsmore & Mescher 1998).

Figure 6. Expression of FGF2 in cultured blastemal cells by immunohistochemistry. (a) FGF2 immunoreactivity in cells cultured in the presence of serum. (b) FGF2 immunoreactivity in cells cultured without serum. (c) FGF2 immunoreactivity in cells cultured without serum but with 10 ng ml^{-1} FGF2. Scale bars, $50 \mu\text{m}$.

Figure 7. Expression of *MCM3* in cultured blastemal cells evaluated by RT-PCR: (a) cells grown in medium containing serum; (b) cells grown in serum-free medium and FGF2; (c) cells grown in serum-free medium containing 10 ng ml^{-1} FGF2. The *Notophthalmus viridescens* satellite 2 was used for normalization.

As described several decades ago, after limb amputation the wound epidermis is rapidly invaded by a large number of nerve fibres (Singer 1949). We have found that in *T. carnifex* innervation of the wound epidermis is rather slow as compared with *T. v. meridionalis*. Although we have not carried out a quantitative study on the density of innervation in the limbs of *T. carnifex* and *T. v. meridionalis*, it was evident that comparable RT97 reactivity was observed in the limb stumps of the two species. It is conceivable that the slow fibre outgrowth observed in *T. carnifex* is due to a less vigorous regenerative capability of the nervous system itself in this species. Analysis of regeneration in *T. carnifex* after spinal cord transection, where an initial loss of epithelial organization and migration of the ependymal cells of the cord stump is followed by re-epithelialization, has indicated that re-epithelialization occurs at approximately eight and a half weeks from injury in this species, as compared with approximately four weeks in the axolotl (Margotta *et al.* 1991; O'Hara *et al.* 1992). It is very likely that differences in innervation, possibly owing to slow nerve regrowth, may account at least in part for the different regenerative abilities.

Formation of the AEC in *T. carnifex* is also significantly delayed as compared with *T. v. meridionalis*. In *T. carnifex* the wound epidermis starts to thicken at least one week later than in *T. v. meridionalis* and does not contain a significant amount of FGF2 over this period. The mechanisms controlling formation of the wound epidermis during regeneration are not fully understood. It is likely that both signals from the mesenchyme and from the nerve are required for its induction. The initial phase of

dedifferentiation and blastemal cell accumulation does not seem to be impaired in *T. carnifex* and regeneration can proceed once the nerve has regrown. Therefore, it seems unlikely that an intrinsic defect in mesenchymal signalling to the wound epidermis is the primary cause for delayed AEC formation in this species. It has been suggested that the nerve influences formation of the AEC, since no AEC formation and blastema growth is observed when regenerating limbs are transplanted without spinal cord grafts that can innervate them (Thornton 1956). Dense innervation of the wound epidermis itself, however, does not seem an absolute requirement for AEC formation and progression of regeneration, as long as there is a sufficient density of innervation in the underlying blastema (Sidman & Singer 1960; Thornton 1960). A further indication that innervation can affect the AEC comes from work showing that expression of certain AEC proteins, such as 9G1 and the transcription factor *Dlx-3* are downregulated following limb denervation (Onda & Tassava 1991; Mullen *et al.* 1996). Therefore, it appears probable that the delay in AEC formation observed in *T. carnifex* results from the limited nervous supply observed at the early stages of regeneration in this species. As the early stages of limb regeneration depend on the presence of both an adequate density of innervation and of the AEC, we conclude that the delayed onset of regeneration in *T. carnifex* results from a slow development of these elements in response to amputation in this species.

(b) *FGF2 expression is required for blastemal cell proliferation*

Our analysis of FGF2 expression in *T. v. meridionalis* and *T. carnifex* limb regenerates has shown that FGF2 is transiently expressed at the early stages of regeneration and is no longer detectable by the medium-bud stage in either species. By contrast, expression of FGFR2, that is required for the initiation of limb development (Xu *et al.* 1998), is detectable in both species throughout regeneration, though the intensity of staining appears to be higher during the most active proliferative phase. These results suggest that FGF2 is a limiting factor for the progression of regeneration. It is most unlikely, however, that FGF2 is the main trigger for regeneration. The initial stages of regeneration, wound healing and dedifferentiation are apparently unaffected by the lack of FGF2. Furthermore, in *Xenopus* larvae FGF2 alone, unlike FGF10, is unable to stimulate limb regeneration (Yokoyama *et al.* 2001).

The fact that expression of the FGF2 protein in regenerating limbs coincides with AEC formation and the onset

of blastemal cell proliferation both in *T. v. meridionalis* and *T. carnifex*, suggests that this factor is required for early blastema growth. This is consistent with the proposed role for FGF2 in limb regeneration both in adult urodeles and *Xenopus* larvae, though in the former model FGF2 may be released by the nerves, whereas in the latter it seems to be produced mainly by the mesenchyme (Mullen *et al.* 1996; Cannata *et al.* 2001). Early limb-bud development and regeneration are nerve-independent, and FGF2 produced in the mesenchyme of larval limb buds appears to be important for its growth (Cannata *et al.* 2001). The loss of FGF2 in the mesenchyme of developing *Xenopus* limbs coincides with the loss of regenerative capability and the switch to nerve dependency. Both in late *Xenopus* larvae and adult urodeles, some effects of denervation can be reversed by FGF2 and it has therefore been proposed that FGF2 is one of the components of the 'nerve-dependent' mitogenic stimuli for blastemal cell proliferation. Our *in vitro* results are also consistent with previous studies reporting that cell proliferation and enhancement of the *MCM3* gene expression are strictly correlated (Yamamoto *et al.* 2001).

Though it is not yet clear whether the main source of FGF2 to the blastema is the nerve itself, or the AEC upon nerve stimulation, the results presented here suggest that delayed regeneration in *T. carnifex* is, at least in part, due to delayed deployment of FGF to the regenerate. This possibility is supported by previous *in vitro* studies (Corcoran & Ferretti 1999) and those presented here. FGF2 not only induces proliferation of cultured blastemal cells, but can also upregulate itself, suggesting that a finely tuned FGF2 loop is important for regeneration.

The other FGF we have examined in this study, FGF1, is expressed in the wound epidermis throughout regeneration. These results indicate that FGF1 cannot compensate for the early lack of FGF2, or other factors that may control blastemal cell entry into the cell cycle, in *T. carnifex*. On the basis of much work showing the mitogenic effects of FGF1 on blastemal cells (Boilly *et al.* 1991; Zenjari *et al.* 1996; Dungan *et al.* 2002), however, it is tempting to speculate that FGF1 may play a role in maintaining blastemal cells cycling after the initial priming to enter the cell cycle. Though at present the FGFs most involved in blastema growth seem to be FGF1 and FGF2, several other FGFs are involved in limb development and regeneration. Therefore, it will be important to systematically examine them all in a comparative fashion, taking advantage of systems with different regenerative capabilities to further assess their roles in regeneration.

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