Fibroblast growth factor receptors regulate the ability for hindlimb regeneration in *Xenopus laevis*

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During outgrowth of the developing limb, signals from the apical ectodermal ridge, such as fibroblast growth factors, are paramount for limb patterning. Similarly, fibroblast growth factor molecules and their receptors are synthesized in the wound epithelium of the regenerating limb blastema, implicating an analogous function to limb development. To address this issue further and to understand the role of fibroblast growth factor receptor signaling in limb regeneration, we have examined the expression patterns of x-fibroblast growth factor receptors-1, -2, -3, -4a, and -4b in *Xenopus laevis*. This amphibian model provides a system in which both regenerating (premetamorphic; tadpole or larva stage) and nonregenerating (postmetamorphic; froglet stage) hindlimbs can be studied. In premetamorphic hindlimbs (stage 53), all of the receptors were expressed in the wound epithelium and the underlying mesenchyme. In postmetamorphic limbs (stage 61), however, transcripts for x-fibroblast growth factor receptors-1 and -2 were absent from the wound epithelium. The expression results for x-fibroblast growth factor receptors-1 and -2 were corroborated at the protein level by employing specific antibodies. Thus, it appears that expression of both fibroblast growth factor receptors-1 and -2 is associated with the ability for limb regeneration. The role of these receptors in regeneration was further investigated by using specific inhibitors to fibroblast growth factor receptors during premetamorphic hindlimb regeneration. These compounds inhibited the normal limb outgrowth and resulted, in the majority of the cases, in outgrowths of cones or spikes reminiscent of growth that is seen in amputated postmetamorphic limbs. Thus, fibroblast growth factor receptors-1 and -2 expression and function should be regarded as paramount for the ability of limb regeneration in *Xenopus*.
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Urodeles and anurans are two common types of amphibians that are used to study limb regeneration. Urodeles, such as the newt *Notophthalmus viridescens* can regenerate an amputated limb during either its larval or adult life, whereas the anuran *Xenopus laevis* can regenerate its hindlimbs during only the tadpole stage.[1,2] The first event in the regenerative process is the covering of the wound by specialized epithelial cells. Subsequently, muscle, cartilage, and mesodermal tissues become undifferentiated mesenchymal cells forming a blastema. The blastema then enlarges because of cellular proliferation and eventually redifferentiates into the tissues that reconstitute the amputated part.[2] *X. laevis*'s ability for limb regeneration during stage 52–55 (premetamorphic; stages delineated according to Nieuwkoop and Faber[3]) discontinues at the onset of metamorphosis at stage 59 (postmetamorphic). At this stage of development, the hindlimb is nonregenerative and grows a cartilaginous stump. Although the newt has been an excellent animal model to study limb regeneration, *X.*

AER	Apical ectodermal ridge
DEPC	Diethylpyrocarbonate
DMSO	Dimethylsulfoxide
EGF	Epidermal growth factor
FGFs	Fibroblast growth factors
nvFGFR	Notophthalmus viridescens FGFR
PBS	Phosphate-buffered saline solution
PDGF	Platelet-derived growth factor
SSC	Sodium citrate/sodium chloride solution

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laevis offers an animal species that contains both regenerative and nonregenerative limb capacities; therefore, it can be used to reveal comparatively differences in specific gene expression correlated with the ability for limb regeneration.

Fibroblast growth factors (FGFs) and FGF receptors (FGFRs) could provide signals that might play a crucial role in limb regeneration and pattern formation. The FGF family consists of at least 10 related genes (FGF1–FGF10; reviewed in Johnson and Williams[4] and Emoto et al.[5]). Their gene products are small polypeptides of approximately 13 kDa.[6] Most FGFs are secreted and affect target cells by binding to one of five known FGF receptors (FGFR-1, FGFR-2, FGFR-3, FGFR-4a, and FGFR-4b).[7–9] Although their regulatory roles for limb outgrowth and patterning in regeneration are not fully understood, evidence has been presented from other systems, implying that FGFs are imperative signals for this process. In particular, during chick limb bud development, signals from the apical ectodermal ridge (AER), which is considered the equivalent of the wound epithelium during newt limb regeneration, are responsible for supporting limb outgrowth. Removal of the AER inhibits limb development, but when FGF-soaked beads are added into AER-less limb buds, outgrowth is restored.[10,11] Also FGFs have been shown to induce limb development when applied to the flank of chick embryos.[12–14] Therefore, by analogy, these factors could also be important for the initiation of the limb regeneration process as well.

Several studies using the axolotl, *Amblystoma mexicanum*, and the newt, *N. viridescens*, have shown the presence of FGFs and FGFRs in the wound epithelium during limb regeneration. FGF-1 (acidic FGF) and FGF-2 (basic FGF) were found to be present in the wound epithelium and the blastema in the regenerating limbs of the axolotl.[15] FGF receptors have been found spatially and temporally distributed in a specific manner in the regenerating newt limb.[16,17] During the blastema stages of regeneration, notophthalmus viridescens (nvFGFR-2) expression is localized in the wound epithelium basal layer and in the perichondrium. In contrast, nvFGFR-1 was expressed exclusively in the blastema mesenchymal cells.[17] Such expression patterns are consistent with a role of FGF as a molecule from the wound epithelium signaling for limb outgrowth during regeneration.

The primary objective of this study was to determine the expression patterns of FGFRs during hindlimb regeneration in *X. laevis,* which loses the ability to regenerate its limbs after metamorphosis. Therefore, this system should reveal differences in this signaling pathway. For this, we have selected five FGFRs cloned from *X. laevis*, and we have examined their expression via in situ hybridization and by immunofluorescence. Having associated expression of two receptors (xFGFR-1 and xFGFR-2) with the ability for hindlimb regeneration, we also show that if these receptors are blocked by specific inhibitors, limb regeneration is impaired.

MATERIALS AND METHODS

Laboratory-bred *Xenopus* tadpoles were purchased from Xenopus One, Inc. (Ann Arbor, MI), and kept at room temperature in dechlorinated tap water maintained under oxygenation. The animals were put on a 12-hour light cycle and were fed two to three times daily. Two teaspoons of powdered food (Xenopus One) were added to a 1-L blender filled with 250-ml tap water. The mixture was then blended to a uniform color or until all granules dissipated.

Tadpoles were staged prior to amputation according to Nieuwkoop and Faber[3] for each experiment. Tadpoles were amputated at stages 53 (premetamorphic), when regeneration is possible, and stage 61 (postmetamorphic), when amputation produces a cartilaginous spike. At the correct stages, tadpoles were anaesthetized using 1% 3-aminobenzoic acid ethyl ester (Sigma Chemical Co., St. Louis, MO) or by submerging animals in chilled water (3 to 6 °C) prior to dissection. Because of the delicate nature and small size of the limbs, a surgical scalpel and diamond sharp forceps (Roboz Surgical Instruments Company, Inc., Rockville, MD) were used to remove the presumptive ankle region of the hindlimb in the early tadpoles and to remove tissue below the knee in the postmetamorphic limb. Tissues for expression studies were then collected and fixed at 3-, 10-, and 15-days postamputation. Animals that were used for inhibitor studies were collected after a period of 23 days.

Hybridization probes and in situ hybridization

The following probes were obtained for these studies. FGFR-1—Vector: pBluescript SK DH5alpha, cloned in BamH1 site. Antisense promoter: T3 (Xho1 digest). Sense promoter: T7 (Xba1 digest). Genbank Accession number: M55163. xFGFR-2—Vector: pBluescript SK DH5alpha, cloned in BamH1 site. Antisense Promoter: T7 (Not1 or Xba1 digest). Sense promoter: T3 (Kpn1 or Xho1 digest). Genbank Accession number: MM62322. Both xFGFR-1 and -2 were obtained from Dr. Robert Friesel, American Red Cross, Rockville,

MD. xFGFR-3—Vector: pBluescript II SK-DH5alpha, cloned in EcoRV. Antisense promoter: T3 (AccI digest). Sense Promoter: T7 (NotI digest). Genbank Accession number: AB007035. xFGFR-4a—Vector: pBluescript II SKDH5alpha, cloned in EcoRV. Antisense Promoter: T3 (Hind III digest). Sense promoter: T7 (XbaI digest). Genbank Accession number: AB007036. xFGFR-4b—Vector: pBluescript II SKDH5alpha, cloned in EcoRV. Antisense Promoter: T7 (BamHI digest). Sense promoter: T3 (Hind III digest. Genbank Accession number: AB007036. XFGFR-3, 04a, and -4b were all obtained from Drs. Ikuko Hongo and H. Okamoto, Ibaraki, Japan.

Limbs that were used for expression studies were processed for paraffin embedding. Sections that were 6-μm thick were cut, and these sections were melted on a slide warmer at 45 °C for 30 minutes and were kept at 4 °C until ready for use.

Plasmids containing FGFR genes were digested with the appropriate restriction enzymes. Riboprobes (both antisense and sense [see above]) were made using either T7 or T3 RNA polymerase and were labeled using the DIG RNA kit (Boehringer-Mannheim, Indianapolis, IN). Slides were deparaffinized, rehydrated in an ethanol series, and then postfixed in 4% paraformaldehyde (room temperature, pH 7.2) for 20 minutes. The sections were then treated with 250 mg/ml pepsin (Sigma, St. Louis, MO) on a slide warmer (37 °C) for 7 to 12 minutes. The slides were fixed again in 4% paraformaldehyde for 10 minutes and were washed in 1x phosphate-buffered saline (PBS)/diethylpyrocarbonate (DEPC). Sections were then acetylated in 0.25% acetic anhydride in trichloroethanolamine (both from Fisher Scientific, Pittsburgh, PA) (pH 8.0) for 10 minutes and washed again in 1x PBS/DEPC. Tissues were rehydrated through an ethanol series and allowed to dry on a slide warmer at 45 °C. Antisense and sense DIG labeled probes were diluted to 1,000–1,500 ng/ml with hybridization solution (50% Formamide, 1 mmol ethylenediamine tetraacetic acid, 10 mmol Tris/HCl, pH 7.5, 600 mmol NaCl, 0.25% sodium dodecyl sulfate, 10% polyethylene-glycol 6000, 1x Denhardt's solution, and 200 μg/ml yeast tRNA) and heat denatured at 85 °C for 5 minutes. Probes were incubated with sections at 60 °C overnight (16 hours). The next day, all washes took place at 55 °C in a water bath, with the exception of 5x sodium citrate/sodium chloride solution (SSC). First, slides were washed briefly at 5x SSC, then were treated in RNase solution (Fisher-Scientific, Pittsburgh, PA) for 30 minutes, and were washed in 2x SSC for 1 hour and 0.1x SSC for an additional hour. The slides were briefly rinsed in buffer 1 (0.1 mol Tris/HCl pH 7.6, 0.15 mol NaCl) for 5 minutes. The slides were blocked in buffer 2 (0.1 mol Tris/HCl pH 7.6, 0.15 mol NaCl made with 10% heat inactivated horse serum (Sigma)) for 1 hour. They were then incubated for 2 hours with anti-DIG antibody conjugated to alkaline phosphatase (Boehringer-Mannheim) prepared in buffer 2, which was modified to 1% heat-inactivated horse serum in a humidified chamber of buffer 1. Slides were then washed four times at 5 minutes in buffer 1 and incubated 10 minutes in buffer 3 (100 mmol Tris/HCl pH 9.5, 100 mmol NaCl, 50 mmol $MgCl_2$) and developed in nitroblue tetrazolium/bromochloroindoylphosphate (Boehringer-Mannheim) for 2 to 18 hours.

Immunohistochemical staining

Tissues were fixed with 4% paraformaldehyde (PFA) and processed for paraffin embedding. Paraffin sections that were 6-μm thick were cut and deparaffinized in Hemo-D (a cleaning agent for histology; Fisher Scientific) for 10 minutes or until paraffin was removed. Tissues were rehydrated by ethanol series and were then treated with 0.1% sodium borohydride in PBS (pH 8.0) for three cycles at 10 minutes. A mixture of 0.1% saponin (Sigma)/10% goat serum (Sigma)/PBS pH 7.2 was made to dilute both primary and secondary antibodies. Antibodies were purchased from Santa Cruz Biotechnologies, Santa Cruz, CA. Rabbit polyclonal antibodies for FGFR-1 (flg; epitope corresponding to amino acids 808–822 at the carboxy terminal with no cross-reactivity to bek, FGFR-3, or FGFR-4 as the company described) was used at a 1:30 dilution. FGFR-2 (bek; epitope corresponding to amino acids 789–802 at the carboxy terminus with no cross-reactivity to flg, FGFR-3, or FGFR-4 as the company described) was used at a 1:30 dilution. These antibodies have been shown to cross-react with amphibian systems, specifically in the newt (*N. viridescens*) and in the frog (*Rana pipiens*).[18] Antirabbit IgG conjugated with fluorescein isothiocyanate (Vector Laboratories, Burlingame, CA) was used at a 1:300 dilution for immunodetection.

FGFR inhibitor studies

Two specific FGFR inhibitors (SU5402 and SU4984; SUGEN, Inc., Redwood City, CA) were used for our studies. They are 3-substituted indolin-2-one that have been shown specifically to inhibit FGFR-1 autophosphorylation, with SU5402 being more potent in this function. SU5402 was shown to lack inhibitory activity when tested on other protein kinases, such

Table 1. Expression of FGFRs in 15-day blastema of pre-metamorphic and postmetamorphic limbs

	Stage 53		Stage 61	
Receptor	**Wound epithelium**	**Mesenchyme**	**Wound epithelium**	**Mesenchyme**
xFGFR-1	+	+	–	+
xFGFR-2	+	+	–	+
xFGFR-3	+	+	+	+
xFGFR-4a	+	+	+	+
xFGFR-4b	+	+	+	+

+ and – indicate presence or absence, respectively.

as platelet-derived growth factor (PDGF) receptor, epidermal growth factor (EGF) receptor, or insulinlike growth factor receptor. SU4984, however, was found to inhibit PDGF receptor as well.[19] In addition to these two inhibitors, we also used the commercially available Tyrophostin A-23 (Calbiochem, La Jolla, CA), which has also been shown to inhibit FGFR-1 and EGF receptor.[20] All inhibitors were made soluble in 100% dimethylsulfoxide (DMSO; 200 ml) and were then mixed with water/food at approximately 250 ml (final concentration was 27 mmol) per 30 animals. This concentration has been shown to be effective in in vitro studies.[19] The premetamorphic (stage 53) animals were immersed in inhibitor solution postamputation and were kept in this solution for 23 days after amputation.

At the end of the experiment, control (DMSO-treated animals) and treated limbs were fixed in Bouin's fixative. These limbs were then rinsed in 70% alcohol and decalcified in 5% trichloroacetic acid in 70% alcohol for 48 hours. The limbs then underwent dehydration by ethanol series, were cleared and infiltrated with xylene, embedded in paraplast, and sectioned 6-μm thick onto pretreated slides. Those limbs that underwent regeneration to the finger stage were whole-mount stained for cartilage with a 1% Victoria Blue B in 70% ethanol (Aldrich Chemical Co., Milwaukee, WI). These limbs were then stored in 100% methyl benzoate (Sigma Diagnostics, St. Louis, MO). Sections were stained with Harris hematoxylin modified solution and 1% eosin Y in 95% ethanol (Sigma Diagnostics).

Photography

All black and white photographs were taken with an Olympus camera connected to either an Olympus BH-2 microscope or an Olympus SZ-PT stereoscopic microscope. All color pictures were taken with a Sony CCD videocamera connected to either an Olympus BH-2 microscope or an Olympus SZ-P2 stereoscopic microscope, viewed with a Sony monitor and printed with a Sony mavigraph videoprinter.

RESULTS

The normal process of limb regeneration after amputation of premetamorphic *X. laevis* hindlimbs has been reported before.[21,22] Later here, we provide a brief description of the stages to familiarize the reader with this system.

Wound healing/dedifferentiation was 3-days postamputation. There was no basal lamina formed, and the wound epithelium was approximately two cell layers thick as seen through electron microcopy.[22] Early cone was 5- to 10-days postamputation. A blastema was been formed without apparent differentiation. Late cone was 11- to 15-days postamputation. Cartilage and muscle differentiation was easily identifiable through light microscopy. Early palette was 16- to 17-days postamputation. The apical end of the limb bud lost its conical shape and began to flatten. Muscles and cartilage became more prominent. Late palette was 18-to 19-days postamputation. The flattened end of the limb became more widespread, and the wound epithelium thickened. Early toe was 20- to 25-days postamputation. Toes began to sprout from the palette. Late toe was 25-days postamputation. Toes were in their final stages of regeneration.

When *X. laevis* was amputated at postmetamorphic stage 61,[3] normal hindlimb regeneration did not take place. Instead, there was formation of a cone or of a spike containing cartilaginous elements.

In situ hybridization with xFGFRs in premetamorphic and postmetamorphic hindlimb blastema

A summary of the expression experiments is presented in Table 1. We observed two different patterns:

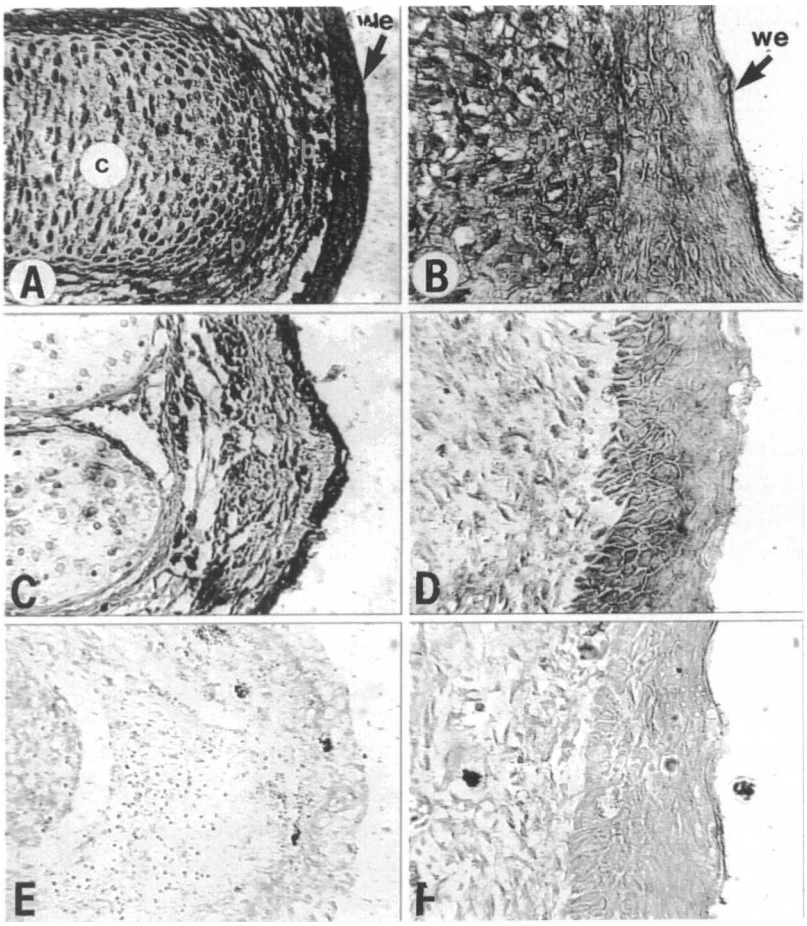

Figure 1 Expression patterns of FGFRs in *Xenopus* hindlimb regeneration. **A,** Expression of xFGFR-1 in a 15-day blastema after amputation of a stage 53 hindlimb. Note the high expression (depicted as blue color) in the wound epithelium (we), the blastema (b), perichondrium (p), and cartilage (c). **B,** Expression of xFGFR-1 in a 15-day regenerate after amputation of a stage 61 hindlimb. There is some expression in the mesenchyme (m), but the wound epithelium is negative. **C,** Expression of xFGFR-4b in a 15-day blastema after amputation of a stage 53 hindlimb. Similar to xFGFR-1, note the expression in the wound epithelium and the underneath mesenchymal cells of the blastema. **D,** Expression of xFGFR-4b in a 15-day regenerate after amputation of a stage 61 hindlimb. Note the expression in the blastema and in the basal layer of the wound epithelium. **E** and **F,** Hybridization with a sense probe (xFGFR-1) to show the background in a 15-day regenerate after amputation of a stage 53 and stage 61 hindlimb, respectively.

xFGFR-3, xFGFR-4a, and xFGFR-4b were expressed in the wound epithelium and the mesenchyme of the premetamorphic and postmetamorphic amputated hindlimbs. However, xFGFR-1 and xFGFR-2 differed in that they were absent from the wound epithelium of amputated hindlimbs of stage 61 animals. These patterns were very similar when blastemata were collected at different time intervals, ranging from 3- to 15-days postamputation. Figure 1 presents examples of these characteristic expression patterns. Figure 1, *A,* shows expression of xFGFR-1 in a 15-day blastema after amputation of a stage 53 hindlimb. Note expression (depicted by the blue color) in the wound epithelium, the underlying mesenchyme, and the cells of the perichondrium and cartilage. Expression of xFGFR-1 in the stage 61 nonregenerating hindlimbs 15 days after amputation is shown in Figure 1, *B*. Although some expression is seen in the mesenchyme, the wound epithelium is negative. This pattern of expression was the same when xFGFR-2 was used (not shown). Figures 1, *C* and *D*, show expression of xFGFR-4b in the wound epithelium and the mesenchyme in both premetamorphic and postmetamorphic hindlimbs, respectively. These sections were taken 15-days postamputation. In Figure 1, *D,* note expression of xFGFR-4b in the basal layer of the wound epithelium. Such an expression was not seen with xFGFR-1 and xFGFR-2 (compare with Figure 1, *B*). Although the signal indicating expression in the wound epithelium of postmetamorphic amputated hindlimbs is not as strong as the one observed in the wound epithelium of the amputated premetamorphic limbs, it is, however, obvious. xFGFR-3 and xFGFR-4a exhibited similar patterns to xFGFR-4b (not shown).

Immunohistochemical localization of FGFR-1 and FGFR-2 gene products

Although in situ hybridization results are able to show the presence of mRNA transcripts, immunohistochemistry could provide corroborative evidence that the genes are being translated into proteins, which, in fact, are responsible for the specific action. To address this, we used two primary rabbit polyclonal

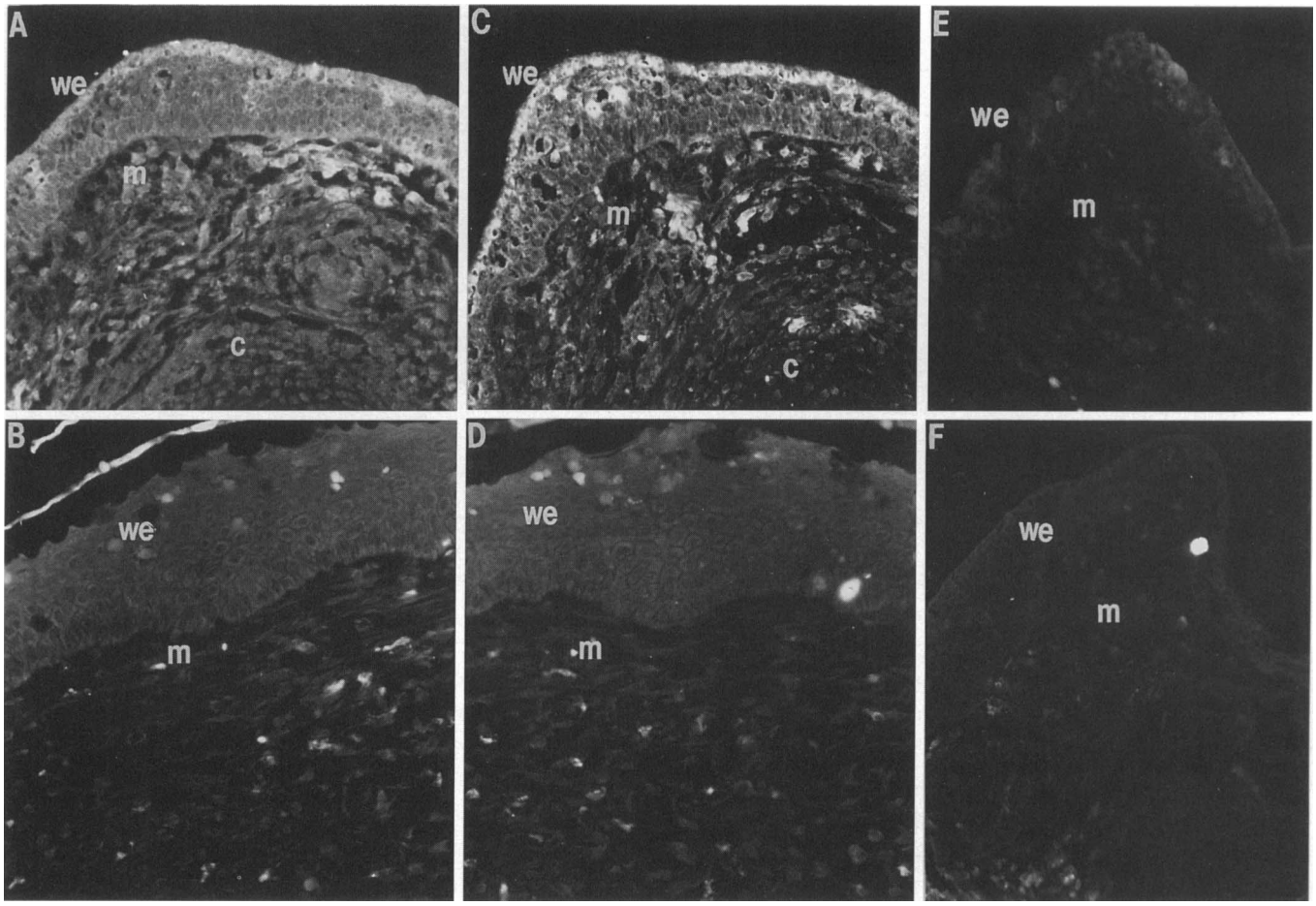

Figure 2 Immunohistological detection of FGFR-1 and FGFR-2 protein in premetamorphic and postmetamorphic hindlimb blastema. All panels are sections obtained from paraffin-embedded *X. laevis* tissue. **A,** Regenerating hindlimb blastema (stage 53, 15 days postamputation); FGFR-1 (flg) shows high expression in the wound epithelium (we), the mesenchyme (m), and the cartilage condensations (c). **B,** Nonregenerating hindlimb (stage 61, 15-days postamputation); FGFR-1 (flg) is absent from the wound epithelium (we), as well as its underlying mesenchyme. **C,** Regenerating hindlimb blastema (stage 53, 15-days postamputation); FGFR-2 (bek) shows expression in the wound epithelium (we) only. **D,** Nonregenerating hindlimb (stage 61, 15-days postamputation); FGFR-2 (bek) is absent from these tissues. **E** and **F,** Negative controls of 15-day hindlimb blastemas (stage 53 and stage 61, respectively). Pictures were taken with a fluorescent microscope at a magnification of x200.

antibodies: one for FGFR-1 (flg) and another for FGFR-2 (bek). Antibodies for FGFR-3, -4a, and -4b that cross-react with this amphibian system were not available to us. Figure 2 depicts immunofluorescence staining using flg and bek antibodies on paraffin sections of 15-day regenerates after amputation of premetamorphic and postmetamorphic hindlimbs. FGFR-1 (Figure 2, *A*) and FGFR-2 (Figure 2, *C*) were present in both the wound epithelium and underlying mesenchymal cells in the premetamorphic regenerating limb 15-days postamputation. However, these receptors were not detected in postmetamorphic stumps 15-days postamputation. In Figure 2, *E* and *F*, the negative controls are presented to show the background.

Effect of FGFR inhibitors on X. laevis limb regeneration

Our in situ hybridization and immunofluorescence protein expression studies have shown that FGFR-1 and FGFR-2 are present in the wound epithelium of premetamorphic regenerating hindlimbs but absent in the postmetamorphic. This suggests that FGFR-1 and FGFR-2 might be important signaling molecules from the wound epithelium associated with the ability for limb regeneration. In order to associate FGFR expression further with a function during limb regeneration, we have used specific inhibitors to determine whether these patterns of expression are also necessary for the process of limb regeneration.

Table 2. Effects of FGFR inhibitors on limb regeneration

	Receptor Inhibitor			
Limb regeneration growth stage	**SU5402**	**SU4894**	**Tyrophostin A-23**	**Control**
Spikes	18	6	14	0
Early cone	23	12	16	5
Late cone	15	10	10	9
Early palette	1	9	5	37
Late palette	2	1	5	32
Early finger	2	1	2	20
Late finger	1	2	2	9
Total	62	41	54	112
Percent of affected limbs:	90.32	68.29	74.07	12.50

After amputation, animals were treated with each inhibitor solution for 23 days, at which point we terminated this experiment. We then evaluated the effect of treatment on limb regeneration. These results are presented in Table 2. Controls (untreated limbs) showed a normal pattern of regeneration, and by day 23, most of the regenerating hindlimbs were at the late stages of regeneration. However, in the majority of the treated animals, regeneration was inhibited. Inhibition of the regeneration was characterized by the growth of cones or spikes, which are reminiscent of the outgrowth after amputation of postmetamorphic limbs. SU5402 was the most potent, showing an effect in 90% of the cases. SU4984 affected 68% of the regenerating limbs, and Tyrophostin A-23 affected 74%. SU5402 is needed in lower concentration than SU4984 to inhibit autophosphorylation of FGFR-1.[19] Because we used the same concentration for both, this could explain why SU5402 showed a better effect on regeneration. In the control group, we observed that only 13% of the limbs were delayed, without apparent abnormalities. (Table 2). For each group, the affected limbs are defined as the sum of regenerates with spikes, early and late cones. Limbs at or past the palette stage were considered unaffected.

The effects of inhibition on regeneration can be seen histologically in Figures 3 and 4. In Figure 3, we compare representative regenerates (external view) from different groups, taken at 23 days after amputation. In the control (untreated) regenerate, toes have regrown (Figure 3, *A*). In Figure 3, *B*, we can observe a regenerate that has been arrested at early cone stage because of treatment with SU4984. Figure 3, *C*, shows a SU5402-treated regenerate at a late cone stage. A cartilaginous spike reminiscent of postmetamorphic limbs developed after Tyrophostin A-23 treatment can be seen in Figure 3, *D*.

Further inspection of inhibited regenerates or spikes through sectioning and staining showed that the process of regeneration in the affected limbs was not simply delayed but was altered considerably (Figure 4). Figure 4, *A*, shows a section through an untreated regenerate 7 days after amputation. This early cone is characterized by the presence of a wound epithelium and blastema formation. In Figure 4, *B*, we can observe an SU4984-treated regenerate 23 days after amputation. A small cone has been developed with considerable disorganization. This cone shows obvious abnormality in its polarity as well. In Figure 4, *C and D*, we show the characteristic histology of spikes with cartilage elements covered by a thin layer of epithelium induced by Tyrophostin A-23 and SU5402, respectively.

DISCUSSION

We have shown that there are five FGF receptors that are present in the wound epithelium and underlying mesenchyme during *X. laevis* limb regeneration. xFGFR-1 and xFGFR-2 expression is absent from the wound epithelium in postmetamorphic hindlimb outgrowths when *X. laevis* loses its ability for normal hindlimb regeneration. The expression patterns are consistent with what is known about FGFR presence in developing and regenerating limbs.

In developing mouse limb buds, FGFR-1 (flg) expression is distributed in the mesenchyme, and its expression is related to the onset of newly differentiated structures. FGFR-2 (bek) is expressed in the surface ectoderm, as well as the mesenchymal condensations, whereas FGFR-3 has been primarily found in the cartilage rudiments of the limb and FGFR-4 in striated muscle.[23,24] In the regenerating newt limb, nvFGFR-1 was highly expressed in the

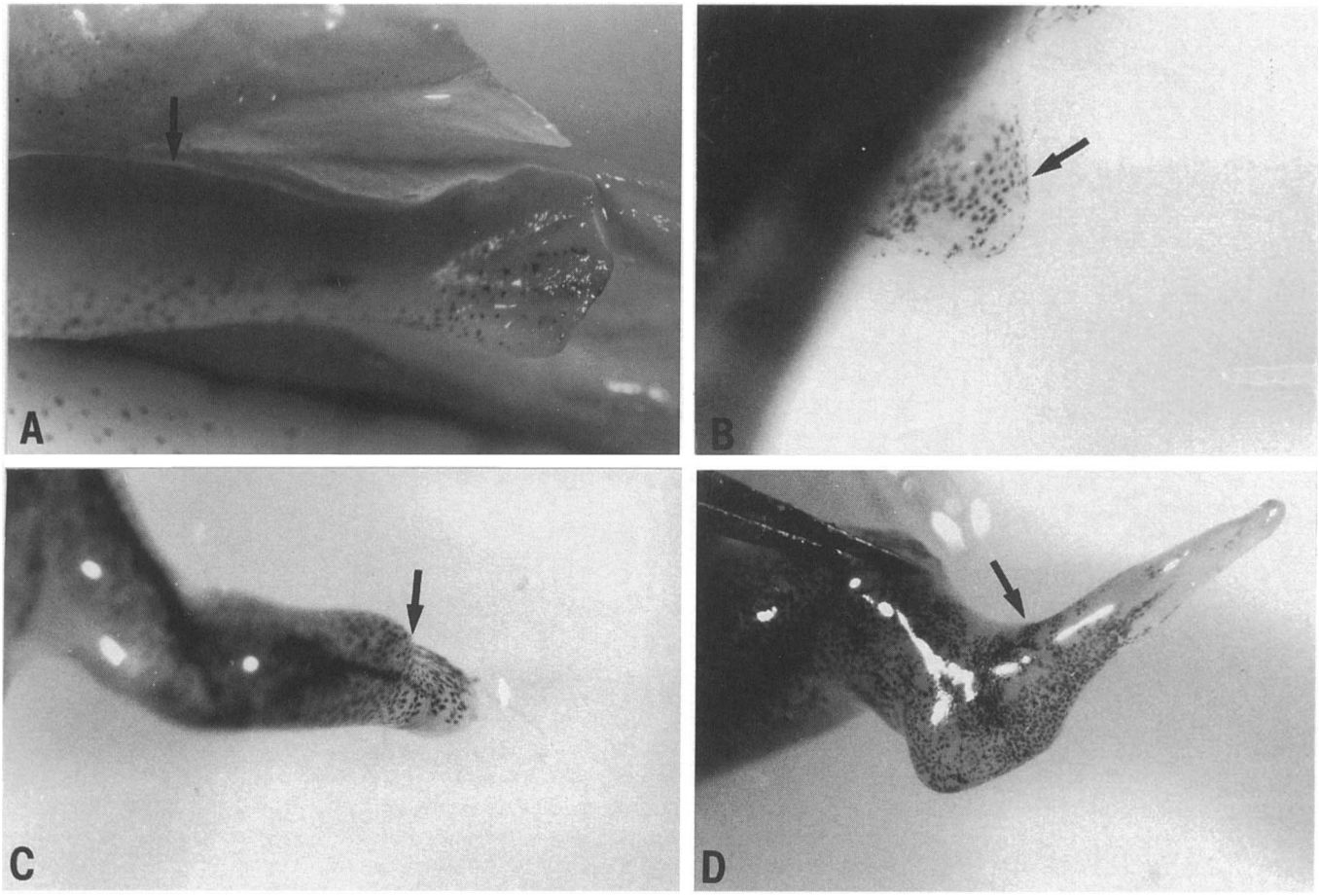

Figure 3 Effects of FGFR inhibitors on *X. laevis* hindlimb regeneration. Limbs from stage 53 animals were amputated and treated. All of these figures represent regenerates at 23-days postamputation. **A,** Untreated control with normal regeneration. **B,** Treatment with SU4984 has resulted in only a cone formation. **C,** A regenerate at late cone stage resulted after treatment with SU5402. **D,** A spike formation after treatment with Tyrophostin A-23. *Arrows* indicate the level of amputation.

mesenchyme but not in the wound epithelium, and nvFGFR-2, both its spliced variants bek and KFGR, were expressed in the wound epithelium and mesenchyme.[16,17] One difference between FGFR-1 expression between the newt and the frog is that FGFR-1 is expressed in the *Xenopus* wound epithelium. This is probably a species-specific difference between the two regenerating systems.

Clearly, the expression patterns for FGFR-1 and FGFR-2 presented in this study suggest a role of these receptors in hindlimb regeneration. However, the most convincing evidence that FGFRs are regeneration signals is offered when premetamorphic stage 53 tadpoles amputated hindlimbs were treated with FGFR inhibitors. After *X. laevis* loses its ability to regenerate because of changes occurring through metamorphosis, amputation thereafter results mostly in a disorganized cone or a cartilaginous spike. Our results show that morphologically similar spikes are generated when FGFR inhibitors were used during the normal process of regeneration (Figures 3 and 4). Because our expression analysis showed that xFGFR-1 and xFGFR-2 were only present in the wound epithelium of the premetamorphic regenerating limbs, we can deduce that the wound epithelium does contain the signals linked to the FGF signaling pathway associated with limb regeneration.

While this article was in preparation, two reports appeared that provide more evidence for the role of FGF signaling in limb development and regeneration. When FGFR-2 gene was mutated in mouse, the mutant mice did not form a limb bud. Further, FGF-8 was absent in the mutant presumptive limb ectoderm, and the mesenchyme-specific FGF-10 was downregulated.[25] In another study, expression of FGF-8 was found to be associated with limb regeneration in *Xenopus*, the same way as FGFR-1 and FGFR-2 were found to be in our study.[26]

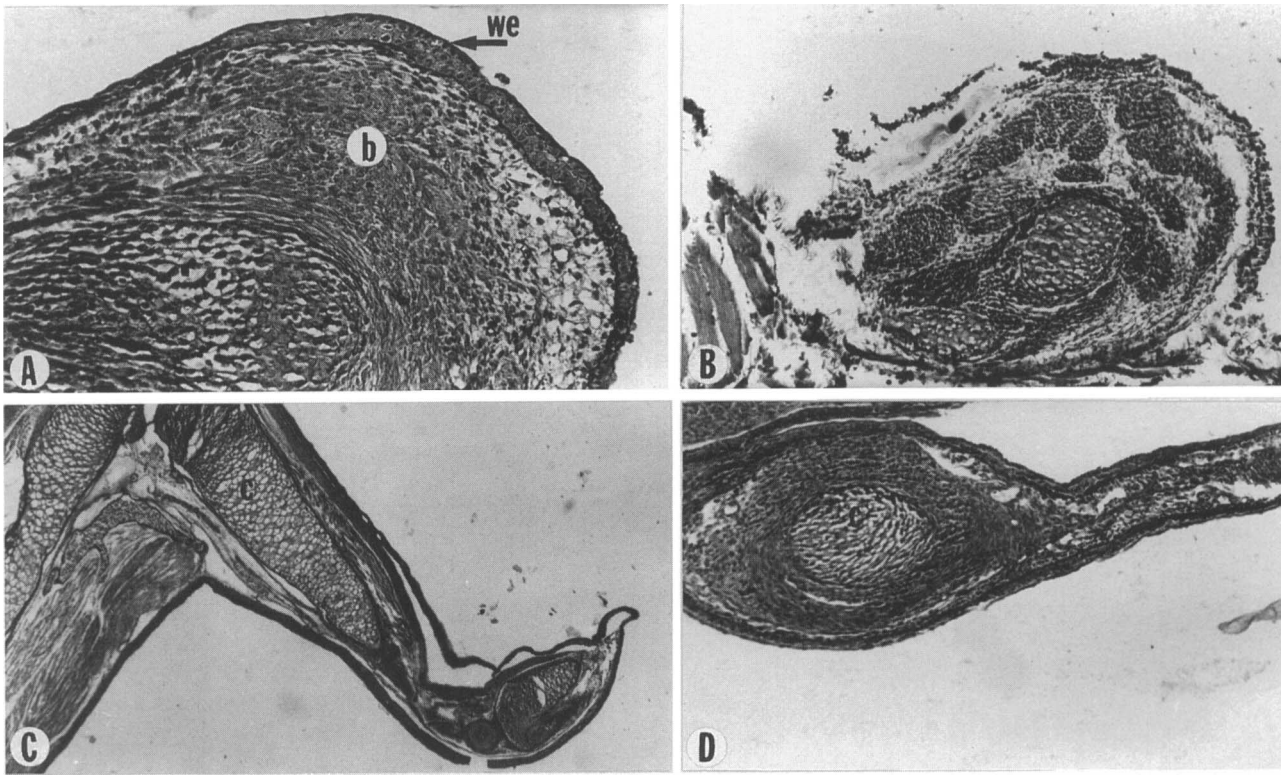

Figure 4 Histological examination of the effects of inhibitors on *X. laevis* hindlimb regeneration. Limbs from stage 53 animals were amputated and treated. After 23 days, representative samples of affected regenerates were embedded in paraffin, sectioned, and stained with hematoxylin and eosin to reveal their tissue organization. **A,** Untreated control regenerating early cone 7 days after amputation; note the well formed blastema (b) covered by the wound epithelium (we). **B,** Section through a regenerate treated with SU4984. Considerable tissue disorganization can be seen in the cone containing cartilaginous elements (c). **C,** A section through a regenerate (spike) after treatment with Tyrophostin A-23 with cartilage (c). **D,** A section through a spike formed after treatment with SU5402.

We have investigated signals that permit limb regeneration and have used the *Xenopus* model because comparisons can easily be made between premetamorphic tadpoles and postmetamorphic frogs. Our results provides evidence that FGFR-1 and FGFR-2 are crucial factors involved in the induction of limb regeneration. Extension of these studies to other models of regeneration and animals through transgenesis might lead to the development of an animal model in which genetic manipulation is easier and can establish new models for regeneration research.

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