The Expression and Role of Fibroblast Growth Factor Receptors in

Xenopus laevis Regenerating Hindlimbs.

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Limb regeneration is a complex phenomenon that occurs in amphibians such as, urodeles and anurans. This complicated process has been well characterized morphologically in the salamander and frog species. However, only recent studies have been aimed at the molecular and cellular level. In pursuing research to identify signals involved in the process of limb regeneration, I have used the *Xenopus laevis* system, since it is capable of regenerating limbs only during its tadpole stages and loses this ability as it approaches metamorphosis. This study provides a descriptive analysis of the expression of genes coding for Fibroblast Growth Factor Receptors (FGFRs) which have been recognized as key signals during both limb development and regeneration. In this study, we present the expression patterns of five FGFRs in pre-metamorphic (regenerating) and post-metamorphic (non-regenerating) hindlimb outgrowths resulting after amputation. Demonstrating differences in receptor expression, we devised functional studies using inhibitors and tissue transplantation to illustrate the importance of these signals during the limb regeneration process.
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Limb Regeneration: Models.

Some amphibians, in contrast to reptiles, birds and mammals have the ability to replace a severed limb. Spallanzani (1769) first reported that salamanders are capable of regeneration, the ability to restore an injured or amputated body part. Urodeles are amphibians that keep their tails; such as the newt Notophthalmus viridescens, and can regenerate an amputated limb during either their larval or adult life, whereas the anuran (tailless), such as the Xenopus laevis, can regenerate a limb only during its tadpole stages (Korneluk and Liversage, 1984). Although mammals have not been found to possess regenerative abilities, the process of limb regeneration in amphibians has been compared to tissue regeneration at the distal tips of the phalanges in both mice and humans and wound healing in mammals (Borgens, 1982; Ord & Stocken, 1984; Reginelli et al., 1995).

Limb regeneration and development demonstrate similar patterns.

The principles that regulate patterning of the regenerating limb may be similar to those involved in patterning during initial limb development (Cohn and Tickle, 1996). The first event in the regenerative process is the covering of the wound by specialized epithelial cells, which is a process known as wound healing. This process is extremely important to the manufacturing of the necessary signals that will be incorporated in the wound epithelium. It is well known that if this wound covering is prevented, either by covering the tissue with
grafted tissue or by the insertion of the amputated limb into the gut, no
regeneration occurs (Goss, 1956). Wound healing begins immediately after
amputation and discontinues once the wound epithelium has formed its covering.
Following wound epithelium formation, a blastema forms through the
dedifferentiation of muscle, cartilage and nerve cells, and mesodermal tissues
(original stump tissues) become undifferentiated mesenchymal cells (Driesch,
1902). This blastema then enlarges due to cellular proliferation and eventually
the cells redifferentiate into specific tissues that reconstitute the amputated part
(Butler, 1933).

The basic similarity between limb regeneration and limb pattern development
is based on the formation of this blastema. The blastema resembles embryonic
cells present during normal limb development and has the capacity to differentiate
into specialized cells. Muneoka and Bryant (1982) through transplantation
experiments with the regenerating axolotl *Amblystoma mexicanum* demonstrated
that the patterning mechanisms involved in the developing and regenerating limb
are similar. They were able to show that normal limbs formed from ipsilateral
grafts of limb bud/limb bud and limb bud/blastema, but supernumerary limbs
formed through contralateral grafts. This suggests that something common to
both developing and regenerating limbs provide for the pattern signals to the
limb. In the developing chick wing and the regenerating limb, epithelium tissue
proliferates to form an apical ectodermal ridge (AER; developing wings/limbs) or
a wound epithelium (also called epidermal cap in regenerating limbs) that caps
the wing/limb bud (Zwilling, 1955; Fallon and Lopez, 1994). It is this covering
that signal the underlying cells to proliferate in both systems. The developing limb bud consists of mesenchymal cells that undergo proliferation and differentiation enabling the patterning and establishment of an entire limb (Chevallier et al., 1977; Geduspan and Solursh, 1992).

The regenerating blastema consists of dedifferentiated tissue that redifferentiates into mesenchymal tissue. Spallanzani (1769), Morgan (1901) and many others have indicated that skeletal elements formed during limb regeneration are histologically similar to those present in developing limbs. On the cellular level, ultrastructural studies provide similarities between the regenerating blastema and the embryonic limb bud (Bryant et al., 1971; Bryant et al., 1977; Muneoka and Bryant, 1982). Their findings also suggest that muscle differentiation follows similar patterns between development and regeneration in limbs. However, differences exist at the molecular level. Tassava et al. (1993), Estrada et al. (1993), and Tassava and Acton (1989) have recognized certain antigens: such as WE3, WE4 and WE6 expresses in the wound epithelium and have been functionally studied and believed to be involved in secretion and cell adhesion processes. WE3 and WE4 have been implicated as actin-binding proteins and WE6 has been identified as keratin. These proteins, although present, most likely are not the signals for initiating limb regeneration; thus, the search for the molecular signals responsible for triggering dedifferentiation and regeneration are currently being investigated.
**FGFs and FGFRs likely play roles in the regeneration process.**

Fibroblast growth factors (FGFs) and FGF receptors (FGFRs) likely play roles in amphibian limb regeneration and pattern formation in development. Members of the FGF family have been found to be present in the newt wound epithelium (FGFR-1 and FGFR-2, Poulin, et al., 1993; Poulin, and Chiu, 1995) and mesenchyme (Hondermarck and Biolly, 1990; Boilly et al., 1991). These molecules also have been implicated in the function of the limb bud AER in vertebrate systems (Orr-Urtreger et al., 1991; Herbert et al., 1990). FGF-2 has been found to induce regeneration in the chick wing bud when applied locally to the amputation site (Taylor et al., 1994). Niswander et al. (1993) and Vogel et al. (1995) have demonstrated that in the absence of an AER (the ectodermal tissue which directs the growth of the normal limb/wing) in chick wing buds, FGF is capable of replacing AER function by inducing the process of growth and pattern formation. It is apparent that these signals have induction capability and may be the target signals that initiate the regeneration process.

**Characterization of FGF and FGFRs.**

The FGF family consists of at least ten related genes (FGF1-FGF10) that are classified on the basis of conserved coding sequences (Johnson and Williams, 1993; Emoto et al., 1997). FGF is a small polypeptide (13 Kda) that was first isolated by Gospodarowicz (1974) and was shown to be mitogenic in 3T3 cells. Since then, FGF protein-receptor interactions have been recognized in a number
of different mechanisms of action, such as, proliferation, differentiation, and mesoderm induction, which depend on the cell type and the receptors involved (Fernig and Gallagher, 1994). This multifunctional characteristic of FGF is what distinguishes it from the other growth factor members (Johnson and Williams, 1993).

Most FGFs are secreted and affect target cells by binding to one of five known FGF receptors (FGFR1, FGFR2, FGFR3, FGFR4a and FGFR-4b) and eventually activates transcription factors that provide a nuclear response. The actual number of FGFs and FGFRs is actually much greater, since there are alternative translation initiation sites for the FGF ligands and multiple alternative splicing forms of these receptors (Johnson et al., 1991).

A clarification of nomenclature for different FGF and FGFR became necessary when discoveries of multivariant genes were recognized (Table 1). Our discussion will focus primarily on the five receptors because they will be the focus of our investigation. FGFR-1 was first isolated from chicken embryos using radiolabeled FGF-1 and FGF-2 (Lee et al., 1989). Since then, it has also been isolated from human; h2, h3, or flg (Dionne et al., 1990), in the mouse; designated flg as well (Mansukhani et al., 1990), and Xenopus; xFGFR-1 (Musci et al., 1990). Following the discovery of these genes, FGFR-2 (Freisel and Dawid, 1991), FGFR-3 (Keegan et al., 1991), FGFR-4 (Partanen et al., 1991), and FGFR4b (Dr. I. Hongo, Japan-not published) have been cloned in these systems. There is approximately 50-75% sequence homology amongst these receptors. Moreover, the receptor sequence homologies between species are quite
high: 98% between human/mouse and 88% human/Xenopus (Freisel and Maciag, 1995, Patrie et al., 1995). FGFRs' structure can provide clues to its binding affinities for specific ligands as well as their mode of action. Each receptor, has an intracellular tyrosine kinase located at its C-terminal that binds substrates. Its extracytoplasmic region contains a 14 amino acid kinase insert domain that has different binding affinities for FGFs based on the immunoglobulin (Ig)-like domains (Johnson et al., 1991).

**Alternative Splicing and Ligand Specificity**

Structural variants of these receptors are produced through alternative splicing of their RNA transcripts and appear to occur in a cell and tissue specific manner (Table 1). The first loop may or may not be excised leaving two or three loop receptor variants proposing no alteration in binding, and at the present time has not been functionally characterized. The genes for FGFR-1, 2, and 3 contain three mutually exclusive and consecutive exons that encode the 3' half of the last Ig loop (Robbie, 1995). When a FGFR contains three Ig-like domains it is called an alpha (α) type; if it has only the second and third loop it is designated beta (β); and when it is missing a secretory signal it is called gamma (γ; Johnson and Williams, 1993). The Ig-like domain is the binding site for FGF ligands. Alternative splicing also results in structural differences in tyrosine kinase domains (Shi et al., 1993). FGFR-1, FGFR-2 and FGFR-4 have membrane bound localization; whereas, FGFR-3 has been found to be localized in the nucleus of breast epithelial cells (Johnston et al., 1995). Splice variants of FGFR-1 and
FGFR-2 also have been identified that give rise to secreted protein forms of the extracellular ligand binding domain (Freisel and Maciag, 1995).

FGFs are known to exert their effects through high affinity binding to receptors on the surface of cells (Dohrman et al, 1993). These proteins can bind to multiple receptors with different binding affinities, allowing for tissue specific regulation in response to FGF action. FGF binding is accomplished with the aid of proteoglycans, namely heparin, heparinase and heparin sulfate (HS) (Gao and Goldfarb, 1995; Itoh and Sokol, 1994; Kan et al., 1993). These molecules act to bind several FGF ligands, forming a web that cross-links and dimerizes receptors. The binding site for HS-FGF on FGFRs spans the second to approximately half the third loop and has no variant forms; whereas, loop III has three receptor forms: IIIa, IIIb, and IIIc (Spivak-Kroizman, 1994). A receptor that only contains IIIa, thus a IIIa variant, is a secretor variant, while those variants that differ in their IIIb and IIIc combinations provide an alteration in binding specificity for ligands. The activation of FGF on cells also can be accomplished by membrane bound low affinity glycosaminoglycan co-receptors (GAG) that are composed of a linear heteropolysaccaride bound to a proteoglycan with its sugars sulfated providing high charges (Jackson et al., 1991).

**FGFR Signaling.**

The signaling pathway and activation of these FGF receptors involves ligand-receptor binding (Patric et al., 1995). Binding of a ligand causes the receptors to dimerize, initiating a conformational change that results in the activation of
protein tyrosine kinase (PTK) The protein kinase domains phosphorylate each other to initiate downstream signaling (Burgess and Maciag, 1989). Every point in this system is crucial to the activation of transcription factors. If the ligand does not bind, most signals are discontinued. Dimerization has been recognized as important in catalytic events. The complexity of regulation of FGFRs comes from differences in their catalytic domains (Shi et al., 1993). There are two forms of FGFR, one with a full catalytic kinase domain (form 1), and the other with a truncated catalytic domain (form 2; Spivak-Kroizman, 1994). When 1-1 Forms dimerize catalytic domains are fully functional, 2-2 dimers are silent, and 1-2 pairs are relatively inactive, although some have slight action (Shi et al., 1993). Since we will be using the tyrosine kinase domain as a point of manipulation to functionally inhibit FGFR signaling, it is important to describe the structure and pathways of these PTK groups.

Protein kinases impart their activity by binding or orienting their substrate site (intracellular receptor protein region with a Src homology 2 domain (SH2) with a phosphate donor that is either ATP or GTP complexed with a cation (Mg\(^{++}\) or Mn\(^{++}\); Amaya et al., 1991). A phosphate is then transferred from the donor to the hydroxyl acceptor residue (serine, threonine, or in the case of FGFRs, tyrosine) of the substrate (Yaish et al., 1988). There are two general classes of PTK’s: the receptor tyrosine kinase that contains an extracellular ligand binding and intracellular catalytic domain with intrinsic kinase activity, and the receptor associated tyrosine kinase that transmit signals from the membrane receptor by interacting with cytoplasmic membrane proteins (Freisel and Maciag, 1995).
Three signaling processes are currently known to occur in PTK systems. The first pathway involves autophosphorylation triggering the Ras and the MAP kinase cascade eventually initiating transcription factors that regulate genes involved in the cell cycle (Haung et al., 1995; Johnson and Williams, 1993). This pathway has been mostly found to be characteristic in cell proliferation and tumor formation. A second PTK pathway involves the activation of a G-protein which stimulates phospholipase C (PLC) to splits phosphatidylinositol 4,5-biphosphate (PIP$_2$) into two second messengers: inositol 1,4,5-triphosphate (IP$_3$) and diacylglycerol (DAG; Shilling et al., 1994). This process eventually leads to the release of calcium and the activation of protein kinases on the receptor processes. Since this pathway involves the use of secondary messengers, it most likely involves differentiation (Fernig and Gallagher, 1994). The third pathway causes the phosphorylation of the stat1 transcription factors and allows protein translocation into the nucleus. This method of PTK signal transduction has not yet been characterized with function for FGF processes, but is probably also involved in the regulation of the cell cycle, cell growth and differentiation (McKeehan and Kan, 1994). A major phosphorylation site on FGFR-1 is Tyr766 at the carboxy terminus. This site has also been identified as the src homology (SH) 2 domain in PLC (Ryan and Gillespie, 1994). Another major autophosphorylation site has been localized at Tyr653 and is phosphorylated by intermolecular mechanisms, as opposed to the intramolecular activation of Tyr766 (Hou et al., 1993).
**FGFR Inhibitors.**

Recently, commercial companies (Calbiochem, La Jolla, Ca. and Sugen Inc., San Francisco, Ca) and researchers have developed kinase inhibitors to understand these signaling pathways and how they affect cell regulation. There are two groups of competitive inhibitors aimed at two areas in the signaling processes. One group of inhibitors acts on the catalytic domain by using a pseudosubstrate sequence to prevent substrate interactions and the other group acts on the regulatory domains by blocking the activity of ATP/GTPase either through binding to the regulatory domain or inhibiting cofactor binding (Levitzki, 1990). The difficulty with using these inhibitors to evaluate function is that they sometimes affect other processes through non-specific actions on a given system in vivo.

Tyrophostin-A23 (3,4-dihydroxybenzylidene malononitrile; figure 1A) is a potent and broad ranged PTK inhibitor that primarily focuses on epidermal growth factor; however, it has been found that Tyrophostin A-23 is unstable in solution and that its derivatives are potent src tyrosine kinase inhibitors (Ramdas et al., 1995). *In vitro* experimentation has shown these derivatives to be an effective inhibitor of FGF receptor activity at a Ki (concentration constant) of 20-35 μM, EGF receptor at a Ki of 45μM as well as others reviewed by Ramdas et al. (1995). These derivatives as well as Tyrophostin A-23 act by competitively inhibiting the substrate site of the tyrosine kinase receptor.

Oxindole-based compounds (also called Indolinones) are a new class of protein tyrosine kinase inhibitors that demonstrate the ability to inhibit the
tyrosine kinase action of FGFR-1, PDGF (Mohammadi et al., 1997) and FGFR-2 (McMahon, Sugen, Inc. San Francisco, Ca., 1997-unpublished). SU4984 (3-(4-(1-formylpiperazine-4-yl)-benzylidenzyl)-2-indolinone; figure 1B) blocked the tyrosine kinase activity of the FGFR in 3T3 NIH cells at a Ki of 20-40μM as well as platelet derived growth factor and the insulin receptor, but not the epidermal growth factor (Mohammadi et al., 1997). Oxindole based inhibitors, namely SU5402, SU4984, SU76516, SU76568 and SU76636 were given to us by Dr. McMahon from Sugen, Inc. in California. SU76516, SU76568 and SU76636 have not been characterized to date, but their inhibition is believed to be much more specific to FGFR-1 and FGFR-2 than SU4894 and SU5402 has been demonstrated strongly and specifically inhibit FGFRs at a concentration between 10-20μM (McMahon, unpublished and Mohammadi et al., 1997). Indolinones act to inhibit the kinase activity of FGFR-1, affecting the catalytic (competitive inhibitor of the ATP site) domain of the receptors.

**FGFRs involvement in various human diseases.**

FGFR have been identified in various human diseases involving limb and craniofacial development. A mutation in FGFR-1 can cause Pfeifer syndrome that is characterized by limb defects and abnormal skull and facial shape due to premature differentiation of cartilage and bone (Cross and Dexter, 1991). FGFR-2 has been identified as the gene responsible for Crouzon, Jackson-Weiss, and Apert syndromes (Su, 1997; Bellus et al., 1995). All of these syndromes are caused by gene mutations altering normal development and causes limb and facial
defects (Park et al., 1995). Mutations in FGFR-3 can cause achondroplasia resulting in dwarfism which can be lethal (Bellus et al., 1995; Tavormina, 1995). In these cases, FGFR appears to be turned on, without the necessity of a signal or FGF ligand, preventing cartilage growth. However, when FGFR-3 gene knockout in mice was performed, and expansion of endochondrial growth occurs, suggesting that other mechanisms and genes are involved (Su, 1997).

**FGF and FGFR expression and action in limb development.**

In the developing chicken limb bud, FGF signals from the AER are necessary for normal outgrowth and patterning of the limb (Niswander et al., 1993). FGFs are believed to be the mitogenic signal present in the AER that induces cells to differentiate during limb development. When chick limb mesenchyme is placed in cell culture at clonal density with FGF-2, muscle differentiation increases and the terminal differentiation of myoblasts is inhibited (Goldfarb, 1991). As growth of the limb occurs *in vivo*, posterior cells are the first to leave the area under the AER (progress zone) and to differentiate. These cells migrate in a proximal to distal direction. When the AER is removed, cell growth is inhibited, however, when FGF-2 (also called bFGF) or FGF-4 is introduced to these limb bud cells, growth continues (Fallon et al., 1994; Niswander et al., 1993). When beads soaked in FGF 1, 2, and 4 were placed in the lateral plate mesoderm opposite somites 20-26 in chick embryos, they were stimulated to produce additional limbs (Cohn et al., 1995). FGF-8 is believed to be involved in mitogenesis, signaling for the proliferation of the underlying mesenchymal cells,
causing limb elongation (Crossley et al., 1996; MacArthur et al., 1995; Mahmood et al., 1995; Vogel et al., 1996).

The developing mouse has been shown through *in situ* hybridization studies to express FGFR-1 (flg) and FGFR-2 (bek) mRNA in the lateral mesoderm and the limb bud mesenchyme (Orr-Urtreger et al., 1991). bek appeared to be localized in the surface ectoderm and chondrocytes in the developing limb expressing strongest in the interdigital web, whereas flg seems to be distributed in the mesenchyme. FGFR-3 expression appears to be primarily found in the cartilage rudiments of developing bone, around the periosteum during endochondrial ossification and in resting cartilage (Peters et al., 1993). Northern blot expression studies demonstrated that FGFR-4 appears to be moderate in fetal human striated muscle, pancreas and adrenal gland (Partanen et al., 1991; Stark et al., 1991). Only FGFR-4 can be induced by heparin alone to dimerize and autophosphorylate; however, the role of heparin to induce physiological changes is still uncertain (Fernig and Gallagher, 1994). Newborn mice that were transgenic for FGFR-1 were born without lungs (Park et al., 1995; Herbert et al., 1990). These studies demonstrate that FGF and its receptors are expressed in a tissue specific manner and that it is crucial to understand how they relate to the developmental process.
**FGF and FGFR presence in limb regeneration.**

Related to the roles of FGFs in limb development, the presence of FGF and its receptors has been studied during limb regeneration processes in the axolotl *Amblystoma mexicanum* and in the newt, *Notophthalmus viridescens*, to ascertain FGF’s role. In the axolotl, FGF-1 has been found to be present in the wound epithelium and the mesenchyme (Boilly et al., 1991; Hondermarck and Boilly, 1990) and its receptor-ligand binding is heparinase sensitive (Forough et al., 1991; Itoh and Sikol, 1994). FGF receptors have been found to be spatially and temporally distributed in a specific manner in the regenerating newt limb (Poulin et al., 1993). During the blastema stages of regeneration, FGFR2 expression is localized in the wound epithelium basal layer and in the perichondrium (bone cell precursors). Alternative splicing of FGFR2 produces two different transcripts, KGFR and bek. These two isoforms delineate more specific expression patterns present during the stages of regeneration associated with growth and blastema cell proliferation. These transcripts differ in the second half of the third Ig-like loop domain, and their expression is present in different tissues at different times in the regeneration process. In the pre-blastema stage, the KGFR mRNA transcript is present only in the basal layer of the wound epithelium, whereas the bek expression is first presented in the perichondrium, then as regeneration progresses its presence is in the mesenchyme. In contrast, FGFR1 was expressed exclusively in the blastema mesenchymal cells (Poulin et al., 1993; Poulin and Chiu, 1995).
**Xenopus laevis offers a system to study limb regeneration.**

Although the newt has been an exceptional model system to study the limb regeneration, *Xenopus laevis* limb regeneration only exists during the early tadpole stages and loses this ability after metamorphosis. In 1962, Dent has described the process of *Xenopus* limb regeneration in detail, providing evidence that as *Xenopus* increases in age, it loses its ability for complete regeneration. The later the amputation takes place during its tadpole stages the more incomplete the regeneration. If the animal is amputated postmetamorphically (stage 59 and older), it only forms a cartilagenous (hypomorphic) spike; therefore, this system offers us a means to discriminate those signals that no longer are present when regenerative ability is lost.

**Limb patterning and regeneration in the Xenopus laevis hindlimb.**

Since staging the animals for limb regeneration is crucial to our experiments, it is necessary to briefly describe the developmental process of the limb in *Xenopus laevis* in accordance to Nieuwkoop and Faber (1976). In its early stages, specifically stages 48 through 50 (approximately 7.5-16 days after fertilization), the hindlimb becomes apparent as a semicircular bud with a conical definition on its distal side. At stages 51 through 53 (approximately 17-25 days post-fertilization), the hindlimb becomes more cone-like and paddle shaped with slight indications of a flat foot, increasing in length rather than width. It is at this developmental stage that the limb becomes pigmented, forming melanophores,
and develops a 4th and a 5th toe. This is the point at which amputation would provide complete limb regeneration. At stages 54 through 59 (26-45 days post-fertilization), the tadpole hindlimb grows to its full nature. *Xenopus laevis*, during its normal and final stages of limb development, grows 3 black clawed digits, and the fourth and fifth digits remain unclawed. The limb being extremely small and delicate is not the only indicator in the staging process. The tentacles are also helpful in aging the tadpoles, because they become longer at the end of stage 53 and decreased in length as they approached metamorphosis at the end of stage 59.

The anuran process of regeneration after a mid-shank amputation at stage 53 to 55 (Nieuwkoop and Faber, 1976) follows that of normal urodele (newt) regeneration: wound covering, dedifferentiation, cell proliferation, redifferentiation and maintenance of growth, and termination of growth (Butler, 1933). As mentioned above, the wound epithelium is considered to be the initiator of the regeneration process by providing the signal to cells underneath it to dedifferentiate and form a blastema (Stocum, 1984; Tsonis, 1996). We believe that FGF and FGFR are likely candidates for such signals.
Research Objectives.

This comparative study between regenerative (pre-metamorphic) and non-regenerative (post-metamorphic) stages that are present during *Xenopus laevis* hindlimb regeneration was designed to pinpoint possible factors that might initiate the limb regeneration process and lead us toward discovering the mechanisms involved in tissue regeneration in vertebrates. We therefore, examined the expression of five FGFRs during *Xenopus laevis*’ pre-metamorphic stages (tadpole stages); a developmental time in which limb regeneration is permissive, and post-metamorphic stages (froglet stage); a period in which this ability is lost, in order to correlate expression patterns with regenerative ability. Here, we conclusively show that expression does relate to the regulation of this process. We have further studied the role of these receptors by using specific inhibitors. Our study implicates FGFR-1 and FGFR-2 as the major players, which regulate the ability for limb regeneration.
Fibroblast Growth Factor Receptors Regulate the Ability For Hindlimb Regeneration in *Xenopus laevis.*

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ABSTRACT

During outgrowth of the developing limb, signals from the apical ectodermal ridge (AER), such as Fibroblast Growth Factors (FGF), are paramount for limb patterning. Similarly, FGF 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 fathom the role of FGFR signaling in limb regeneration, we have examined the expression patterns of xFGFR-1, xFGFR-2, xFGFR-3, xFGFR-4a and xFGFR-4b in *Xenopus laevis*. This amphibian model provides a system where both regenerating (pre-metamorphic; tadpole or larva stage) and non-regenerating (post-metamorphic; froglet stage) hindlimbs can be studied. In pre-metamorphic hindlimbs (stage 53) all the receptors were expressed in the wound epithelium and the underlying mesenchyme. In post-metamorphic limbs (stage 61), however, transcripts for xFGFR-1 and xFGFR-2 were absent from the wound epithelium. The expression results for xFGFR-1 and xFGFR-2 were corroborated at the protein level by employing specific antibodies. Thus, it appears that both FGFR-1 and FGFR-2 are the prime candidates involved in the outgrowth signaling during the regeneration process. FGFRs role in regeneration was further investigated by using specific inhibitors to FGFRs during pre-metamorphic regeneration. These compounds inhibited the normal limb outgrowth and resulted in the majority of the cases, to generate cones and spikes reminiscent of growth that is seen in amputated post-metamorphic limbs. Lastly, since the FGFR signals expression differences are derived from the wound
epithelium, we have preliminary results demonstrating the successful transplantation and conversion of post-metamorphic limbs to recapture their regenerative ability. These results are discussed in relation to future regenerative models and the possibilities of inducing regeneration in non-regenerative systems.
INTRODUCTION

Urodeles and anurans are two commonly used systems to study limb regeneration. Urodeles, such as the newt *Notophthalmus viridescens* can regenerate an amputated limb during either its larval or adult life (Tsonis, 1991), whereas the anuran *Xenopus laevis* can regenerate its limb only during the tadpole stage (Bossilico et al., 1992; Korneluk and Liversage, 1984). The first event in the regenerative process is the covering of the wound by specialized epithelial cells, then the muscle, cartilage, nerve cells and mesodermal tissues become undifferentiated mesenchymal cells forming a blastema (Driesch, 1902). This blastema then enlarges due to cellular proliferation and eventually redifferentiates into specific tissue cells that reconstitute the amputated part (Butler, 1933; Tsonis, 1996). *Xenopus laevis*’ intrinsic ability for limb regeneration during stage 52-55 (pre-metamorphic; stages delineated according to Nieuwkoop and Faber, 1976) discontinues at the onset of metamorphosis at stage 59 (post-metamorphic). At this stage of development, the limb is non-regenerative and grows a cartilagenous stump. Although the newt has been an excellent model to study limb regeneration, *Xenopus laevis* offers a system containing both regenerative and non-regenerative limb capacities, therefore, it can be used to comparatively reveal differences in signal presence or 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 ten related genes (FGF1-FGF10; reviewed in Johnson and Williams, 1993; Emoto et al., 1997) and are small polypeptides (≈13 Kda, Gospodarowicz, 1974). Most FGFs are secreted and affect target cells by binding to one of five known FGF receptors (xFGFR-1, xFGFR-2, xFGFR-3, xFGFR-4a and xFGFR-4b). Members of the FGF family and their receptors are present in the wound epithelium (Boilly, B. 1991; Poulin et al., 1993; Poulin and Chiu, 1995). Although their regulatory roles for limb outgrowth and patterning in regeneration are not fully understood, evidence has been presented from other systems and imply that FGFs are imperative signals for this process. In particular, during chick limb bud development, signals from the apical ectodermal ridge (AER; 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 (Niswander et al., 1993). Therefore, by analogy, these factors could be the important factors for the initiation of the limb regeneration process as well.

Several studies using the axolotl, *Ambystoma mexicanum*, and the newt, *Notophthalmus viridescens*, have shown the presence of FGFs and FGFRs in the wound epithelium during limb regeneration (Boilly et al., 1991; Poulin and Chiu, 1995). FGF-1 (aFGF) and FGF-2 (bFGF) were found to be present in the wound epithelium (epidermal cap) and the blastema in the regenerating limbs of the axolotl (Biolly et al., 1991). These FGF receptors have been found spatially and temporally distributed in a specific manner in the regenerating newt limb (Poulin
et al., 1993; Poulin and Chiu, 1995). During the blastema stages of regeneration, nvFGFR2 expression is localized in the wound epithelium basal layer and in the perichondrium (bone cell precursors). In contrast, nvFGFR1 was expressed exclusively in the blastema mesenchymal cells (Poulin and Chiu, 1995). This expression is consistent with the idea that FGF might be in the wound epithelium and involved in the signaling for limb outgrowth during regeneration.

The primary objective of this research project was to determine the expression patterns of FGFRs during limb regeneration in Xenopus laevis, which loses the ability to regenerate its limbs after metamorphosis. Therefore, this system can reveal differences in growth factor presence and action. For this, we have selected five FGFRs cloned from Xenopus laevis and we have examined their expression via in situ hybridization and by immunofluorescence. Having correlated expression of two receptors (xFGFR-1 and xFGFR-2) with the ability for limb regeneration, we examined the input of their function by using specific inhibitors. In addition, we will also present data demonstrating induction of limb regeneration in stage 61 (post-metamorphic and non-permissive) amputated froglet host limbs when the wound epithelium from stage 53-55 (pre-metamorphic and permissive) is transplanted.
MATERIALS AND METHODS

Reagents

Unless otherwise specified, all materials were analytical reagents of the highest grade commercially available from Acros Organics (Princeton, NJ), Aldrich Chemical Company (Milwaukee, WI), Atlanta Biochemicals (Norcross, GA), Boehringer Mannheim (Indianapolis, IN), Fisher Scientific (Pittsburgh, PA), Miles, Inc. (Indianapolis, IN), Oxford Labware (St. Louis, MO), Sigma Chemical Company (St. Louis, MO), and Vector Laboratories (Burlingham, CA), and were used without further purification.

Animals

Laboratory bred tadpoles were purchased from Xenopus One Inc., WI and kept at room temperature in dechlorinated tapwater (One drop Stress-Coat Aquarious Water Conditioner, 1ml/lgal mixed with food, Aquarious Pharmaceuticals, Inc. PA) maintained under oxygenation. The animals were put on a twelve hour light cycle and were fed two to three times daily. The feeding and cleaning procedure were as follows. 1. Tanks were cleaned every other day by brush and fresh tap water. While the animals were netted, the tank was filled with de-chlorinated tap water to give the animals an inch of swimming room. 2. Two teaspoons of powdered food (provided by Xenopus-One, MI) were added to a 1 L blender filled with 250ml tap water with the inclusion of a drop of dechlorinator. The mixture was then blended to a uniform color or until all
granules dissipated. 3. This mixture was then added to each tank until the tank was barely cloudy.

Tadpoles were staged prior to amputation according to Nieuwkoop and Faber (1976) for each experiment. A total of 100 tadpoles were used in the expression studies and 140 for inhibitor studies. Tadpoles were amputated at stages 53 (pre-metamorphic) at which regeneration is possible and stage 61 (post-metamorphic), when amputation produces a cartilagenous spike. At the correct stages, tadpoles were anaesthetized using 1% 3-aminobenzoic acid ethyl ester (Sigma Chemical Company) or by submerging animals in chilled water (3-6°C) prior to dissection. Due to the delicate nature and small size of the limbs, a surgical scalpel and diamond sharp forceps (Roboz Surgical Instruments Company, Inc., 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 post-metamorphic limb. Tissues for expression studies were then collected and fixed at 3 days, 10 days and 15 days post-amputation. Animals that were used for inhibitor studies were sacrificed after a period of 23 days.
Probes

xFGFR-1
Received from: Robert Freisel, Red Cross Laboratories, Maryland
Vector: pBluescript SK DH5alpha
Antibiotic Resistance: Ampicillin
Cloned in BamH1 site
Antisense Promotor: T3  Digested With: Xho1
Sense Promotor:  T7  Digested With: Xbal
Genbank Accession number:  M55163

xFGFR-2
Received from: Robert Freisel, Red Cross Laboratories, Maryland
Vector: pBluescript SK DH5alpha
Antibiotic Resistance: Ampicillin
Cloned in BamH1 site
Antisense Promotor:  T7  Digested With: NotI or Xba
Sense Promotor:  T3  Digested With: Kpn1 or Xho1
Genbank Accession number:  MM62322

xFGFR-3
Received from: Dr. Ikuko Hongo Dr. H. Okamoto, Ibaraki, Japan
Vector: pBluescript II SK- DH5alpha
Antibiotic Resistance: Ampicillin
Cloned in EcoRV
Antisense Promotor:  T3  Digested With: AccI
Sense Promotor:  T7  Digested With: NotI
Genbank Accession number:  AB007035

xFGFR-4a
Received from: Dr. Ikuko Hongo Dr. H. Okamoto, Ibaraki, Japan
Vector: pBluescript II SK- DH5alpha
Antibiotic Resistance: Ampicillin
Cloned in EcoRV
Antisense Promotor:  T3  Digested With: Hind III
Sense Promotor:  T7  Digested With: Xbal
Genbank Accession number:  AB007036

xFGFR-4b
Received from: Dr. Ikuko Hongo & Dr. H. Okamoto, Ibaraki, Japan
Vector: pBluescript II SK- DH5alpha
Antibiotic Resistance: Ampicillin
Cloned in EcoRV
Antisense Promotor:  T7  Digested With: BamHI
Sense Promotor:  T3  Digested With: HindIII
Genbank Accession number:  AB007036
**In situ hybridization**

200 limbs that were used for expression studies were processed for paraffin embedding using *in situ* hybridization precautions because it is extremely important to keep all samples sterile of contamination, since any RNase will confound these experiments. All tools and glassware were either autoclaved or baked and all solutions were either autoclaved or filtered. Tissues were first rinsed in a 1:1 1x phosphate-buffered-saline, pH 7.2 (PBS)/diethyl pyrocarbonate (DEPC Acros, Princeton) then fixed in 4% paraformaldehyde in PBS (4°C) overnight. Tissues were dehydrated through an alcohol series (30, 50, 70, 80, 95, 100%), cleared in Hemo-D (Fisher Scientific, Pittsburgh), then coated and embedded in molten paraplast (Oxford Labware, St. Louis). Six-microns-thick sections were cut using a Spencer model “820” microtome (Fisher Scientific), allowed to spread in pre-warmed DEPC, and mounted on vectabond (following company’s protocol; Vector laboratories, Burlingham) pretreated and cleaned slides. These sections were melted on a slide warmer at 45°C for 30 minutes and kept at 4°C until ready for use.

The sections were processed for *in situ* hybridization using protocols provided by the Boehringer Mannheim (Indianapolis) Digoxygenin (DIG)-labeled RNA method with slight modifications according to Furlow et al. (1997). Plasmids containing FGFR genes were digested with the appropriate restriction enzymes. Riboprobes (both antisense and sense- see above) were made using either T7, Sp6 or T3 RNA polymerase and labeled using the DIG RNA kit from Boehringer-Mannheim. Slides were placed on a slide warmer (45°C) for 20 minutes, then
dewaxed in Hemo-D (Fisher-Scientific) until paraffin was removed. All solutions were made in DEPC water unless otherwise stated. Slides were rehydrated in an ethanol series then post-fixed in 4% paraformaldehyde (room temperature, pH 7.2) and digested with 250 μg/ml pepsin on a slide warmer (37°C) for 7 to 12 minutes. The slides were then fixed again in 4% paraformaldehyde for 10 minutes and washed in 1x PBS/DEPC. Sections were then acetylated in 0.25% acetic anhydride in trichloroethanolamine (pH 8.0) for 10 minutes and washed again in 1x PBS/DEPC. Tissues were rehydrated in an ethanol series and allowed to dry on a slide warmer at 45°C. Antisense and sense DIG labeled probes were diluted to 1000-1500 ng/ml with hybridization solution (50% Formamide, 1mM EDTA, 10mM Tris/HCl pH 7.5, 600 mM NaCl, 0.25%SDS, 10% PEG 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 in separate moist chambers (sense and antisense groups) and kept 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 SSC. First, slides were washed at 5xSSC briefly, then treated in RNase solution (Fisher-Scientific) for 30 minutes and washed in 2xSSC for 1 hour and 0.1xSSC for an additional hour. The slides were briefly rinsed in buffer 1 (0.1M Tris/HCl pH 7.6, 0.15 M NaCl) for 5 minutes. The slides were blocked in buffer 2 (0.1M Tris/HCl pH 7.6, 0.15 M NaCl made with 10% heat inactivated horse serum) 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 not allowed to dry throughout the entire immunological detection procedure. Slides were then washed 4x at 5 minutes in buffer 1 and incubated 10 minutes in buffer 3 (100mM Tris/HCl pH 9.5, 100mM NaCl, 50mM MgCl₂) and developed in NBT/BCIP (nitroblue tetrazolium/bromochloroindoylphosphate from Boehringer-Mannheim) for 2-18 hours.

**Immunofluorescence Antibody Staining**

6 μm-thick sections were cut and deparaffinized in Hemo-D for 10 minutes or until paraffin was removed. Tissues were rehydrated by ethanol series. During the embedding procedure, limbs were fixed in 4% paraformaldehyde. This presented an autofluorescence problem that was overcome by treating the sections with 0.1% sodium borohydride in PBS (pH 8.0) for 3 cycles at 10 minutes. Saponin (Sigma) was used to add permeability to cell membranes, which enabled antibodies to bind to the intracellular receptor complex. A mixture of 0.1% saponin/10% goat serum/PBS pH 7.2 was made to dilute both primary and secondary antibodies. Antibodies were purchased from Santa Cruz Biotechnologies. 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 described by company) 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 described by company) was used at a 1:30 dilution. These antibodies have been shown to cross-react with amphibian systems, specifically in the newt (Notrophthalmus viridescens) and in the frog (Rana pipins) by McDevitt et al. (1997). Anti-rabbit IgG conjugated with FITC (Fluorescein isothiocyanate; Vector Laboratories) was used at a 1:300 dilution for immunodetection. Negative controls for these antibodies involved applying the secondary antibody to tissues without the primary antibody. In addition, we performed positive tests for these antibodies on newt tissues (regenerating eye and limb) and mouse testes.

**Inhibitors**

Six inhibitors, specific to FGFR-1 and FGFR-2, were used to characterize the importance of these receptors in the process of limb regeneration. Tyrophostin A-23 was purchased from Calbiochem (La Jolla, Ca) and 5 synthetic oxindole-based inhibitors, namely SU5402, SU4984, SU76516, SU76636 and SU76568 were provided by Dr. McMahon (from Sugen, Inc., San Francisco, Ca). All inhibitors were made soluble in 100% dimethylsulfoxide (DMSO; 200μl), then mixed with water/food at approximately 250 ml (final concentration=27 μM) per 30 animals. This concentration was determined from previous in vivo in-lab experiments and in vitro studies with 3T3 cells (Mohammadi et al., 1997). The pre-metamorphic (stage 53) animals were immersed in inhibitor solution post-amputation and kept in this solution for 23 days after amputation. Since DMSO has been demonstrated to not affect the limb
regeneration process at these concentrations, DMSO-treated controls were not used (Tsonis et al., 1994). This treatment was found to be the least toxic on the animals.

At the end of the experiment, control (untreated premetamorphic hindlimbs) and treated limbs were fixed in Bouin’s fixative (at least 24h). These limbs were then rinsed in 70% alcohol and decalcified in 5% trichloroacetic acid (TCA) in 70% alcohol for 48 hours. The limbs then underwent dehydration by ethanol series, were cleared and infiltrated with a 1:1 solution of 100% xylene:100% ETOH, embedded in paraplast and sectioned six-microns-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 Chemicals). These limbs were then stored in 100% methyl benzoate (Sigma Diagnostics, St. Louis). Treated limbs that grew spikes, or did not match the control group morphologically, were embedded and sectioned using a microtome and stained with Harris hematoxylin modified solution and 1% eosin Y in 95% ethanol (Sigma Diagnostics, St. Louis).

**Victoria Blue Staining**

Bouin’s fixative was removed from regenerated limbs with 2% ammonium hydroxide solution for 48 hours with frequent changes. The limbs were immersed in 10% hydrogen peroxide for depigmentation (time varies), dehydrated 1 hour in 50% and 1 hour in 70% ethanol and stained with 1% Victoria Blue B dissolved in 70% alcohol for 2 hours. The limbs were then de-stained via a graded ethanol
series up to 95% ethanol until the stain was at a desired level. For storage, the limbs were placed in methyl benzoate and pictures were taken by a CCD videocamera viewed with an Olympus stereoscopic microscope.

**Hematoxylin and Eosin Y Staining**

Tissue sections were placed in 100% xylene until all wax was removed. Tissues were hydrated with a graded ethanol series (100, 95, 75, 55, 35%) to tap water. Once hydrated to tap water, tissues were placed in hematoxylin until sections began to appear blue (approximately 2 minutes). Tissues were immediately rinsed in tap water to remove excess dye, then transferred back to ethanol for dehydration up to 95% ethanol and placed in Eosin Y (diluted to 1% in 95% ethanol) for a short time until desired pink color stain was obtained. Tissues were washed in absolute ethanol, further dehydrated in 100% xylene and a cover slide was mounted with 100% permount (Fisher Scientific, Pittsburgh).

**Transplantation Experiment**

30 tadpoles at stage 53 were amputated following surgical guidelines outlined above and allowed to regenerate to an early cone (7 days). Wound epithelium was carefully isolated from the regenerates and cultured for two days using amphibian tissue media: 30% L-15, 30% Eagles medium, 9% fetal calf serum, 5% conditional medium, 1% fungizone, 1% penicillin/streptomycin, 11.2 μg/ml tetracycline, 15.0 μg/ml gentamycin, 10.0 μg/ml insulin, made in sterile de-
ionized water. In order to assure that the isolated wound epithelium was uncontaminated and that no mesenchymal cells were present, wound epithelium was washed with serum free media (Sigma) two times and immersed in 10 μl Dil (Indocarbocyanines; Molecular Probes, Indianapolis) in 1ml of serum free media and allowed to incubate overnight. Dil is a cationic fluorescent dye that accumulates on hyperpolarized membranes and translocates into the membrane of the cultured tissue (Molecular Probes, cat., 1997). Three days prior to transplantation, 20 post-metamorphic (stage 67-tailless) frogs were amputated at the ankle of their hindlimbs as previously explained. Pre-metamorphic wound epithelium was rinsed with serum free media two times and transplanted under the first few cell layers of the healed wounds of 10 post-metamorphic frogs. The remaining 10 frogs were used as control animals, in which, they were punctured and treated as the experimental limbs, but no wound epithelium was transplanted. Animals were allowed to grow and were observed for limb regeneration. Some limbs were taken 2 weeks after implantation, imbedded in O.C.T. Compound (Miles, Inc, IN) and processed for frozen sectioning using a cryostat. This was to insure that the transplanted tissue had been incorporated and reorganized in the host limb. These tissues were observed under fluorescence (rhodamine filter) and bright-field microscopy. Intact limbs were viewed and captured by a CCD videocamera and Sony printer.
Pictures

All black and white photos 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 CCD videocamera (DEI-470, Optronics Engineering, MI) 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

Characterization of the stages of limb regeneration in *Xenopus laevis*.

The normal regenerative process in *Xenopus laevis* tadpole limbs amputated at pre-metamorphic stage 53 (Nieuwkoop and Faber, 1976) as depicted through histological sections. Regenerating limbs were taken at 3, 9, 12, 15, 18, 21 and 23 days post-amputation. The following will be a description through observation of histological and morphological structures of the normal limb regeneration process in *Xenopus laevis*:

*Epithelial Wound Healing/Dedifferentiation* - 3 days post-amputation. There is no basal lamina formed and the wound epithelium is approximately two cell layers thick as seen through electron microcopy (Dent, 1962).

*Early Cone* - (see fig. 2A) 5 to 10 days post-amputation. A blastema has been formed without apparent differentiation.

*Late Cone* - 11 to 15 days post-amputation. Cartilage and muscle differentiation is easily identifiable through light microscopy.

*Early Palette* - (See fig. 2B) 16 to 17 days post-amputation. The apical end of the limb bud loses its conical shape and begins to flatten out. Muscles and cartilage become more prominent.

*Late Palette* - (see fig. 2C) 18 to 19 days post-amputation. The flattened end of the limb becomes more widespread and the wound epithelium thickens.

*Early Toe* - 20-27 days post-amputation. Toes begin to sprout from the palette.
Late Toe- (see fig. 2D) 25 days post-amputation. Toes go through their final stages of regeneration.

When *Xenopus laevis* was amputated at **post-metamorphic** stage 61 (Nieuwkoop and Faber, 1976); inspection at 15 days post-amputation indicates that normal limb regeneration does not take place. Instead, there is the formation of a cone (as shown in expression studies; fig. 3-7), then a spike containing cartilagenous elements (not shown), which is identical to spikes presented from inhibitor experiments (fig. 8D, 10C and D). The post-metamorphic re-growth is also covered by a wound epithelium. However, as it will become apparent from our data, this is molecularly different from the wound epithelium during pre-metamorphic regeneration.

**In situ hybridizations with xFGFRs in pre-metamorphic and post-metamorphic hindlimb blastemas.**

Sense and Antisense DIG labeled probes to xFGFR-1, 2, 3, 4a and 4b were used for **in situ** hybridization studies with *Xenopus laevis* regenerating and non-regenerating hindlimbs. Since the expression patterns at 3 days and 10 days were the same as those shown at 15 days post-amputation, we present results from the 15 day pre-metamorphic and post-metamorphic hindlimb blastemas.

At 15 days post-amputation, regenerative blastemas from stage 53 tadpoles (Nieuwkoop and Faber, 1976) are characterized as a late cone with a wound epithelium that has a basal layer separating it from the underlying mesenchymal
cells. xFGFR-1 and xFGFR-2 expression was high in the wound epithelium and mesenchyme and present in the surrounding cells of the perichondrium (fig. 3A and 4A, respectively). In post-metamorphic hindlimbs (stage 61), xFGFR-1 and xFGFR-2 expression was absent from the wound epithelium, but exhibited some expression to its underlying mesenchyme (Fig. 3B and 4B, respectively).

xFGFR-3 expression was seen in the wound epithelium, the mesenchymal cells and in the cartilage condensations (Fig. 5A). Signal in post-metamorphic limbs was present in both basal cell layer of the wound covering and the underlying mesenchyme (Fig. 5B); however, when compared to the pre-metamorphic tissues, expression was less prominent. xFGFR-4a was expressed in the wound epithelium and mesenchyme in both the pre- and post-metamorphic hindlimbs (Fig. 6A/B) and FGFR-4b exhibited moderate signal in the wound epithelium, mesenchyme and in the perichondrium (Fig. 6C) of the pre-metamorphic limb. Expression was seen in the wound epithelium basal cell layer and mesenchyme of the post-metamorphic limb (Fig. 6D). Pre-metamorphic and post-metamorphic hindlimb tissues presented with sense probes were used as negative controls and were absent of expression (fig. 3B/D, 4B/D, 5B/D, FGFR4a and FGFR4b-not shown).

**Immunofluorescence with FGFR-1 and FGFR-2 presence in pre-metamorphic and post-metamorphic hindlimb blastemas.**

Although *in situ* hybridization results are conclusive to determine presence of mRNA transcripts, immunohistochemistry is the more convincing technique to
provide evidence that genes are truly being translated into proteins. We used two primary rabbit polyclonal antibodies; one for FGFR-1 (flg) and another for FGFR-2 (bek). Antibodies for FGFR-3, 4a and 4b were not available for the amphibian system. Figure 7 depicts immunofluorescence staining of flg and bek antibodies on paraffin sections of 15d regenerates from pre-metamorphic regenerates and post-metamorphic non-regenerates as well as their respective negative controls. flg (Fig. 7A) and bek (Fig. 7C) epitopes were present in both the wound epithelium, its basal layer and underlying mesenchymal cells in the pre-metamorphic regenerating limb; however, antibodies (flg and bek) did not detect protein presence in post-metamorphic stumps (Fig. 7 B/D, respectively) and the negative controls show low background (7E/F).

**Effect of FGFR Inhibitors on Xenopus laevis limb regeneration.**

*In situ* hybridization and immunofluorescence expression studies have indicated that FGFR-1 and FGFR-2 are present in *Xenopus laevis*’ pre-metamorphic regenerative limbs (stage 53), but absent in post-metamorphic froglet non-permissive limb stumps (stage 61). This demonstrates that FGFR-1 and FGFR-2 might be important signaling molecules from the wound epithelium correlated with the limb regeneration ability. In order to connect FGFR expression with a function during limb regeneration, we have used Tyrophostin A-23 and 4 Oxindole based compound inhibitors (SU5402, SU4984, SU76568, SU76516 and SU76636) to halt the FGFR signal transduction pathway.
Tyrophostin-A23 is a broad range tyrosine kinase inhibitor, demonstrating its ability to inhibit growth factor signal transduction in vitro (Yaish et al., 1988) and its derivatives that are produced due to its instability in solution, have been shown to inhibit FGF receptor signaling in vitro (Ramdas et al., 1995). Oxindole-based inhibitors have been shown to affect both FGFR-1 and FGFR-2.

After amputation, animals were separated into groups of 30 per tank and treated with each inhibitor solution for 23 days, at which point we terminated this experiment. We then compared the treatment effects on limb regeneration. These results are presented in Table 2. Controls (untreated limbs) showed normal regeneration and by 23 days they were at the toe stage (fig. 8A). However, in the majority of the treated animals, regeneration was inhibited. Inhibition of the regeneration was characterized by the presence of only a cone or spikes, which are reminiscent of the outgrowth after amputation of post-metamorphic limbs. Tyrophostin A-23 was capable of effecting the regeneration process in 74% of limbs tested (Table 2; percentages represent the total # of limbs affected/total limbs for each group). SU76568 had the most profound inhibiting effect on regenerating limbs with a inhibition rate of 96%, followed by SU5402 at an inhibition of 90%, while SU76516 showed inhibition in 89% of the limbs, SU76636 in 82% and SU4984 in 68%. In the control group, we observed that only 13% of the limbs were delayed, without apparent abnormalities.

The effects of inhibition on regeneration can be seen histologically in figures 8, 9 and 10. In figure 8, we compare representative regenerates (external view) for different groups, taken at 23 days. In the control (untreated) regenerate, toes
have re-grown (figure 8A). In figure 8B, we can observe a regenerate that has been arrested at early cone stage due to treatment with SU4984. Figure 8C shows a SU76516-treated regenerate at a late cone stage. A cartilagenous spike reminiscent of post-metamorphic limbs developed after Tyrophostin A-23 treatment can be seen in Figure 8D.

In figure 9, we can observe abnormal limbs that regenerated to the toe stage, stained with Victoria Blue B which stains cartilagenous elements. In Figure 9A, we can observe the normal limb regenerate at 23d post-amputation. SU4984 caused a duplication at the level of the autopodium resulting in two sets of two toes (Fig. 9B). SU76516 inhibited the normal formation in the tibia/fibula area with a fusion of these bones (Fig. 9C). An example of a hypomorphic regenerate induced by SU76516 is shown in Figure 9D.

Further inspection of inhibited regenerates or spikes through sectioning and staining, demonstrated that the process of regeneration in the affected limbs was not only simply delayed, but was altered considerably (Fig. 10). Figure 10A 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 10B, we can observe a 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, since this section shows a well-differentiated muscle (from stump), directly underneath the cone. In figures 10C and 10D, we show the characteristic histology of spikes with growth elements covered by a thin layer of epithelium.
Tyrophostin A-23 treated limbs grew spikes (Table 2) which seemed to grow almost immediately after treatment, producing a fairly large spike by 23 days (10C); whereas, other spikes, such as the one depicted in figure 10D produced by treatment with SU4984 were shorter in length.

**Transplantation of Xenopus laevis regenerating hindlimb wound epithelium to non-regenerating post-metamorphic hindlimbs.**

Our studies so far have indicated the importance of FGFR-1 and FGFR-2 in regulating limb regeneration. FGFR-1 and FGFR-2 were expressed in the wound epithelium during limb regeneration, but were absent from the wound epithelium in post-metamorphic, non-regenerating *Xenopus laevis* hindlimbs. In addition, inhibitors affected limb regeneration. We have, therefore, deduced that the wound epithelium must contain signals that are necessary for the onset of the limb regeneration process. It is tempting to suggest that exogenous expression of FGFR-1 and FGFR-2 in the wound epithelium of post-metamorphic amputated hindlimbs should induce and restore limb regeneration in the *Xenopus* system. If this were true, it would solidify the importance of FGFRs in the limb regeneration process. Such a hypothesis can be pursued by creating transgenic *Xenopus* that would be capable of expressing these receptors in the wound epithelium of post-metamorphic limbs. Such experiments are feasible, but quite laborious, and therefore, beyond the scope of the present study. We, however, pursued some experiments which the wound epithelium from pre-metamorphic blastemas were transplanted onto stumps of amputated post-metamorphic hindlimbs. We were
interested in seeing if such a wound epithelium would provide the necessary signals for the induction of limb regeneration. Indeed, we observed induction of regeneration in a stage 67 froglet hindlimb after amputation and transplantation in two out of ten frogs. Figure 11A shows a regenerate 25 days after amputation; note the presence of toes connected by a web. Figure 11B depicts a section through a 14d stump of a stage 67 amputated hindlimb, transplanted with stage 53 wound epithelium. The transplanted wound epithelium was treated with 1% DiI, in order to visualize successful graft transplantation. Figure 11C is the same section stained with hematoxylin and eosin y viewed through a bright-field microscope.
Figure 1:

Molecular Structure of FGFR Inhibitors

(A) Tyrophostin A-23; the structure contains benzylidenemalosine that incorporates its phenyl moieties of tyrosine and compete for the tyrosine kinase substrate site of protein tyrosine kinases.

(B) SU4984; the structure contains oxindole residues that contact the tyrosine kinase at the site in which adenine of adenosine triphosphate binds, acting as a competitive inhibitor.
Figure 1: Molecular Structure of FGFR Inhibitors

A  Tyrophostin A-23

3,4-ditrihydroxy-cis-cinnammonitrile

B  SU4984 (example of Oxindole Based Compounds)

3-(4-(1-formylpiperazin-4-yl)-benzylidenyl)-2-indolinone
Figure 2:

Characterization of the stages of Limb Regeneration in *Xenopus laevis*.

Bright-field photographs taken with a stereoscopic microscope; 9x magnification. Stage 53 *Xenopus laevis* regenerating hindlimbs at 7d (A), 14d (B), 18d (C) and 24d (D) post-amputation: (A) represents an early cone (B) represents a late cone/early palette, (C) represents a late palette, (D) represents a late finger regenerate.
Figure 3:

*In situ* hybridizations with xFGFR-1 in pre-metamorphic and post-metamorphic hindlimb blastemas. Paraffin sections through a *Xenopus laevis* regenerating hindlimb blastema (stage 53, 15 days post-amputation; (A) Antisense and (C) Sense). xFGFR-1 shows expression in the wound epithelium (we) and the mesenchyme (m). Paraffin sections through a *Xenopus laevis* non-regenerating hindlimb blastema (Stage 61, 15 days post-amputation; (B) Antisense and (D) Sense). xFGFR-1 highly expresses only in the mesenchymal tissue (m) but is absent from the wound epithelium (we). Pictures were taken with a bright-field microscope (magnification at 400x) by CCD videocamera.
Figure 4:

*In situ* hybridizations with xFGFR-2 in pre-metamorphic and post-
metamorphic hindlimb blastemas. Paraffin sections through a *Xenopus*
*laevis* regenerating hindlimb blastema (stage 53, 15 days post-amputation;
(A) Antisense and (C) Sense). xFGFR-2 is expressed in the wound epithelium
(we) and mesenchymal tissue (m), and perichondrium (p). Paraffin sections
through a *Xenopus laevis* non-regenerating hindlimb blastema (Stage 61, 15
days post-amputation; (B) Antisense and (D) Sense). xFGFR-2 is absent
from the wound epithelium but is present in the mesenchyme (m). Pictures
were with a bright-field microscope (magnification at 400x) by CCD
videocamera.
Figure 5:

In situ hybridizations with xFGFR-3 in pre-metamorphic and post-metamorphic hindlimb blastemas. Paraffin sections through Xenopus laevis regenerating hindlimb blastema (stage 53, 15 days post-amputation; (A) Antisense and (C) Sense; although cartilage cannot be seen, no presence was detected in sense tissue). xFGFR-3 shows expression in the wound epithelium (we), mesenchyme and in the cartilage condensations (c). Paraffin sections through a Xenopus laevis non-regenerating hindlimb blasrema (Stage 61, 15 days post-amputation; (B) Antisense and (D) Sense). xFGFR-3 shows expression in the mesenchyme and in the basal layer of the wound epithelium (arrow). Pictures with a bright-field microscope (magnification at 400x) by CCD videocamera.
Figure 6:

*In situ* hybridizations with xFGFR-4a and xFGFR-4b in pre-metamorphic and post-metamorphic hindlimb blastemas. Paraffin sections through a *Xenopus laevis* regenerating hindlimb blastema (stage 53, 15 days post-amputation); xFGFR-4a shows high expression in the wound epithelium (we), mesenchyme (m) and in the cartilage condensations (c). (B) Paraffin sections of a *Xenopus laevis* non-regenerating hindlimb blastema (Stage 61, 15 days post-amputation); xFGFR-4a shows high expression in the wound epithelium as well as its underlying mesenchyme (m). (C) Paraffin sections through a *Xenopus laevis* regenerating hindlimb blastema (stage 53, 15 days post-amputation); xFGFR-4b shows expression in the wound epithelium (we), mesenchyme (m) and around the perichondrium (p). (D) Paraffin sections of a *Xenopus laevis* non-regenerating hindlimb (Stage 61, 15 days post-amputation); XFGFR-4b expression is present in the basal cell layer of the wound epithelium and in the mesenchymal tissues. Pictures were with a bright-field microscope (magnification at 400x) by CCD videocamera.
Figure 7:

Immunofluorescence: FGFR-1 and FGFR-2 presence in pre-metamorphic and post-metamorphic hindlimb blastemas. (A) Paraffin sections through a Xenopus laevis regenerating hindlimb blastema (stage 53, 15 days post-amputation); FGFR-1 (flg) shows high expression in the wound epithelium (we), mesenchyme (m) and in the cartilage condensations (c). (B) Paraffin sections of a Xenopus laevis non-regenerating hindlimb (Stage 61, 15 days post-amputation); FGFR-1 (flg) is absent from the wound epithelium (we) as well as its underlying mesenchyme. (C) Paraffin sections through a Xenopus laevis regenerating hindlimb blastema (stage 53, 15 days post-amputation); FGFR-2 (bek) shows expression in the wound epithelium (we) only. (D) Paraffin sections of a Xenopus laevis non-regenerating hindlimb (Stage 61, 15 days post-amputation); FGFR-2 (bek) is absent from these tissues. (E,F) Negative controls depicting paraffin sections through 15-day Xenopus laevis hindlimb blastemas (stage 53 and stage 61, respectively). Pictures were taken with a fluorescent microscope at a magnification of 200x.
Figure 8:

Effects of Tyrophostin A-23 and Oxindole inhibitors on *Xenopus laevis* regeneration. Bright-field photographs taken of stage 53 *Xenopus laevis* regenerating hindlimbs at 23d post-amputation; all pictures were taken with a stereoscopic microscope; 9x magnification. (A) represents a normal regenerated limb at 23 days post-amputation. (B) represents an early cone when treated with SU4984 for 23 days post-amputation. (C) represents a late cone when treated with SU4984 for 23 days post-amputation. (D) represents a spike when treated with Tyrophostin A-23 for 23 days post-amputation.
Figure 9:

Victoria Blue B cartilage staining of control and inhibited limbs.

23 day post-amputated *Xenopus laevis* regenerated limbs were stained with Victoria Blue B, pictures taken by a CCD videocamera under stereoscopic microscope at a magnification of 6x. (A) represents a normal regenerated hindlimb (t; tibia, f; fibula). (B) represents a limb affected by SU4984; a duplication (d) has occurred at the distal end of tibiafibularis region, producing mirror image skeletal elements; note the tibiafibularis is fused (fu). (C) represents a limb affected by SU76516; the limb has been fused (fu) at the tibiafibularis region. (D) represents a limb affected by SU76636; the limb has toe deformations (lack of growth) and abnormalities (a).
Figure 10:

Effects of Tyrophostin A-23 and Oxindole-based inhibitors on *Xenopus laevis* regeneration (sections). Hematoxylin and Eosin Y stained paraffin sections. (A) represents a normal 7d regenerating early cone (400x); wound epithelium (we) is a few layers thick, cartilage condensations (c) are forming behind the mesenchymal tissues (m). (B) represents a SU4984-treated 23d regenerating early cone demonstrating tissue disorganization (200x). (C) represents a Tyrophostin A-23-treated 23d regenerative spike formed with little evidence of muscle and skeletal elements (100x). (D) represents a SU4894-treated 23d regenerative spike that is abnormal and with a smaller form (100x). Pictures were taken under a bright-field microscope by a CCD videocamera.
Figure 11:

Transplantation of *Xenopus laevis* regenerating hindlimb wound epithelium to non-regenerating post-metamorphic amputated limbs offers patterned re-growth: (A) represents re-growth in a stage 67 amputated hindlimb transplanted with stage 53 regenerative wound epithelium tissue after 25 days taken by stereoscopic microscope (9x); re-growth is incomplete (3 digits) and a web has formed. (B) represents a 14d stage 67 amputated hindlimb transplanted with stage 53 regenerative wound epithelium tissue conjugated with Dil dye taken by fluorescent microscope with a rhodamine filter (200x); the wound epithelium tissue has migrated and reorganized the host tissue, (C) bright-field representation of (B) stained with hematoxylin and eosin Y. Fluorescence and bright-field pictures were taken by CCD videocamera.
### Table 1: Alternative Splicing in FGFR and Ligand Specificity

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|                 | 90.32% | 68.29% | 82.14%  | 89.29%  | 96.30%  | 74.07%         | 12.50%   |

*Percentages=Total # of Early/Late/Spikes/Total Limbs
DISCUSSION

We have demonstrated that there are five FGF receptors that are present in the wound epithelium and underlying mesenchyme during *Xenopus laevis* limb regeneration (figures 3-7). xFGFR-1 and xFGFR-2 expression is absent from the wound epithelium in post-metamorphic hindlimb outgrowth when *Xenopus laevis* loses its ability for normal limb regeneration (Figures 3B, 4B and 7B and D). 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’s (bek) expression surrounded 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 (Orr-Urtreger et al., 1991; Stark et al., 1991). In the regenerating newt limb, nxFGFR-1 was highly expressed in the mesenchyme but not in the wound epithelium, and nxFGFR-2, as well as its spliced variants bek and KFGR, were expressed in the wound epithelium and mesenchyme (Poulin et al., 1993; Poulin and Chiu, 1995). 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 limb regeneration. However, the most
convincing evidence that FGFRs are the regeneration signals is offered when pre-metamorphic stage 53 tadpoles amputated limbs were treated with FGFR inhibitors. After *Xenopus laevis* loses its ability to regenerate due to changes occurring through metamorphosis, amputation thereafter results in a disorganized cone or a cartilagenous spike. Our results demonstrate that morphologically similar spikes are generated when FGFR inhibitors were used during the normal process of regeneration (Figures 8D, 10C-D). Since our expression analysis showed that xFGFR-1 and xFGFR-2 were only present in the wound epithelium of the pre-metamorphic regenerating limbs, we can deduce that the wound epithelium does contain the regenerative signals linked to the FGF signaling pathway. Our preliminary transplantation studies support this and might provide a meaningful extension in inducing regeneration in vertebrate models.

Our expression analysis with xFGFR-3, xFGFR-4a and xFGFR4b suggests that these receptors are important in the maintenance of pre-metamorphic limbs. xFGFR-3 expression in regenerating limbs seems to correlate with the developing limb in its expression in cartilage formation. Since xFGFR-4a and 4b have not been detected for presence in developing or regenerating limbs, our data might suggest that they play roles in post-metamorphic spike formation.

Identifying the signal for regeneration is of paramount importance to the field. For example, are these signals common in limb, eye, liver and tail regeneration? Can the regenerative abilities in newts be unified under a molecular mechanism? After lens removal in the newt, a regenerating lens appears to grow only from the dorsal iris by dedifferentiation of pigmented epithelial cells (McDevitt, 1997).
Interestingly, FGF and their receptors are expressed and control lens differentiation (Robinson et al., 1995). Furthermore, FGFR-1 expression is confined in the dorsal iris, and inhibition of its function with an FGFR-1 specific inhibitor results in the arrest of lens regeneration (Del Rio-Tsonis et al., 1997 unpublished). In this sense FGFR-1 seems as important for lens regeneration as for limb regeneration. Comparative research, analogous to the one outlined in this paper, can be extended to other regenerative systems, for example in mice, which are capable of regenerating only their digit tips once amputated (Borgens, 1982; Mahmood et al., 1995; Reginelli, et al., 1995).

We have investigated signals that permit limb regeneration and have used the Xenopus model because comparisons can easily be made between pre-metamorphic tadpoles and post-metamorphic 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 system where genetic manipulation is easier and can establish new models for regeneration research.
CONCLUSIONS

1. Through expression studies, we demonstrate that FGFR-1 and FGFR-2 show patterns correlated with the induction ability of *Xenopus* to regenerate its limbs.

2. Furthermore, by inhibiting FGFRs, we demonstrate that limb regeneration is inhibited or shows characteristics as seen in post-metamorphic amputated limbs.

3. These experiments point to a crucial role of wound epithelium signals in regulating limb regeneration.

4. The wound epithelium is capable of inducing limb regeneration in post-metamorphic limbs upon transplantation.

5. Extension of these studies in other animals or in the employment of transgenic technologies could lead to new genetic models for limb regeneration.
ABBREVIATIONS

a abnormal
AER apical ectodermal ridge
ATP adenosine triphosphase
BCIP bromochloroindoylphosphate
c cartilage condensations
DAG diacylglycerol
DEPC diethyl pyrocarbonate
DIG digoxigenin
DMSO dimethyl sulfoxide
ETOH ethyl alcohol
f fibula
FGF fibroblast growth factors
FGFR fibroblast growth factor receptors
FITC fluorescein isothiocyanate
GAG glycosaminoglycan
GTP guanosine triphosphate
HS heparin sulfate
IP₃ inositol 1,4,5-triphosphate
m mesenchyme
mRNA messenger ribonucleic acid
NBT nitroblue tetrazolium
p periosteum
PBS phosphate-buffered-saline
PEG polyethylene glycol
PIP₂ phosphatidylinositol 4,5,-bisphosphate
PLC phospholipase C
PTK protein tyrosine kinase
Tyr tyrosine
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