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**Migration and *In Vivo* Transfection of Cells in the Regenerating
Caudal Fin of Zebrafish**

By

© **Christopher W. Brown**

A Thesis Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
of the Degree of

Master of Science

Department of Anatomy & Neurobiology
University of Ottawa
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Abstract

Regeneration of complex body structures involves a cascade of events including cell dedifferentiation, migration, proliferation, differentiation and patterning of the regenerated tissues. Understanding of the cellular and molecular mechanisms that govern these events will provide a better knowledge of the regeneration process. Limb regeneration has been most intensively studied in the urodele amphibians, however, the refinement of the *danio rerio*, zebrafish, as a genetic system permits the study of the genetic control of development and regeneration on a much larger scale. In addition, the epimorphic regeneration process of the zebrafish fin is very similar to the regeneration process of the tetrapod limbs, with the advantage that the fin presents a relatively simple anatomy, and complete restoration of the regenerate within a few weeks.

Two specific aims have been studied. On a cellular level, in order to specify the origin of cells that contribute to the regenerate, we used the lipophilic carbocyanine dye, Di-I, to label and subsequently to characterize the epidermal and mesenchymal cell contributions from the stump tissue of the caudal fin to the regenerate. This study is the first reported use of Di-I as a method to follow cell migration in the regenerating fin. We show that, after amputation, epidermal cells in the caudal fin migrate from all levels to the epidermal cap, and present characteristic migration patterns that suggest a role other than only in wound healing, but also likely in initiating the regeneration process. In addition, induction of various types of injuries within the caudal fin provided insight into the origin of the signal that initiates the migration of the epidermal cells to the region forming the epidermal cap.

On a molecular level, we investigated a method to introduce genes of interest into the regenerating caudal fin. By injecting Green Fluorescent Protein (GFP) reporter constructs into the blastema of regenerating caudal fins, we successfully transfected blastemal cells. Using this approach, we try to misexpress the *sonic hedgehog* gene in the regenerating fin in order to further investigate the function of this gene in the formation and patterning of the dermal bone of the fin.

Résumé

La régénération de structures corporelles complexes implique une seule cascade d'évènements tels que la dédifférenciation, migration, prolifération, différenciation cellulaire et l'établissement du patron des tissus régénérés. La compréhension des mécanismes cellulaires et moléculaires qui gouvernent ces évènements contribuera à une meilleure connaissance du processus de régénération. La régénération des membres a été intensément étudiée chez les urodèles, cependant, le raffinement du *danio rerio* en tant que système génétique permet d'étudier le contrôle génétique du développement et de la régénération à une plus grande échelle. De plus, le processus de régénération épimorphique de la nageoire du *danio* est très semblable à celui des membres de tétrapodes, avec l'avantage que la nageoire présente une anatomie relativement simple et une restauration complète du régénérat en quelques semaines.

Deux objectifs spécifiques ont été étudiés. Au niveau cellulaire, afin de déterminer l'origine des cellules qui participent à la formation du régénérat, nous avons utilisé le colorant lipophile carbocyanine, Di-I, pour marquer puis déterminer la contribution des tissus du moignon de la nageoire caudale au régénérat. Cette étude est la première à rapporter l'utilisation du Di-I comme méthode pour suivre la migration cellulaire dans la nageoire en cours de régénération. Nous montrons qu'après amputation, les cellules épidermiques de la nageoire caudale migrent tout le long de la nageoire vers la coiffe épidermique, et présentent des caractéristiques de migration qui suggèrent un rôle non seulement dans la cicatrisation mais aussi probablement dans l'initiation du processus de régénération. De plus, l'induction de divers types de blessures dans la nageoire caudale a apporté des informations quant à l'origine du signal qui initie la migration des cellules épidermiques vers la région formant la coiffe épidermique.

Au niveau moléculaire, nous avons cherché une méthode permettant d'introduire des gènes dans la nageoire caudale en cours de régénération. En injectant des constructions contenant le gène codant pour la "Green Fluorescent Protein" (GFP) dans le blastème de nageoires caudales en cours de régénération, nous avons transfecté avec succès des cellules du blastème. En utilisant cette approche, nous avons essayé de faire exprimer de façon ectopique le gène *sonic hedgehog* dans la nageoire en cours de régénération afin de poursuivre l'analyse de la fonction de ce gène dans la formation et l'établissement du patron des os dermiques de la nageoire.

List of Abbreviations

AER	Apical Ectodermal Ridge
BCC	Basal Cell Carcinoma
BMP	Bone Morphogenetic Proteins
bp	Base Pairs
BrdU	Bromo-2'-deoxy-uridine
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
CMV	Cytomegalovirus
CS	Calf Serum
d	Day(s)
Di-I	1,1'-dioctadecyl-3,3,3',3'-tetramethyl indocarbocyanine perchlorate
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DPI	Decapentaplegic
ECM	Extracellular Matrix
EtOH	Ethanol
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
FITC	Fluorescein Isothiocyanate
GFP	Green Fluorescent Protein
h	Hour(s)
HH	Hedgehog Protein
kDa	Kilo Dalton
M	Molar
MMP	Metalloproteinase
mRNA	Messenger Ribonucleic Acid
MCS	Multi-Cloning Site
MSX	Vertebrate <i>muscle segment homeobox</i> Protein
µg	Microgram
µm	Micrometer
ng	Nanogram
nl	Nanoliter
NaN ₃	Sodium Azide
PBS	Phosphate Buffer Saline
PFA	Paraformaldehyde
psi	Pounds per square inch
PTC	Patched Protein
PDGF	Platelet Derived Growth Factor
PZ	Progress Zone
RA	Retinoic Acid
RAR	Retinoic Acid Receptor
RNA	Ribonucleic Acid
TGF-β	Transforming Growth Factor beta

SHH	Sonic Hedgehog Protein
SP	Substance P
w	Weight
v	Volume
ZF4	Zebrafish Fibroblast cell line
ZPA	Zone of Polarizing Activity

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Migration and *In Vivo* Transfection of Cells in the Regenerating Caudal Fin of Zebrafish

Introduction

The Relevance for Regeneration Studies

Understanding the complete mechanism(s) of regeneration could present a cure for many diseases or injuries that cause tissue damage or organ failure. Although modern cellular and molecular biology have uncovered many fundamental mechanisms involved in the development of multicellular organisms (Saltzman, 1998), the challenge in understanding the formation of any organ is to integrate the multiple developmental processes that are occurring simultaneously at many levels of organization (Browder *et al.*, 1991).

In vertebrates, the mechanisms of organogenesis are studied in the urodele amphibian and the teleost fish, primarily because of their regenerative abilities during the adult phase. Tissue and organ culture techniques, transplantation and ablation experiments as well as molecular studies are identifying the tissue repair mechanisms which lead to the reactivation of embryonic programs that generate growth, pattern formation, and morphogenesis (Brockes, 1997). However the entire regeneration process is far from being completely understood. Specifically, the fundamental questions concerning the origin and developmental potentials of the

cells that produce the regenerate, and the roles of the adjacent tissues in organizing the regenerate (Morgan, 1901) remain unanswered and are the topic of focus herein.

The Study of Regeneration in Urodele Amphibians

The ability to regenerate complex structures is widespread in metazoan phylogeny, but among vertebrates the urodele amphibians are exceptional (Brockes, 1997). The remarkable regenerative ability of the urodele limb makes it an excellent system for the study of regeneration, the favorite subject of investigators being newts (*Notophthalmus*, *Pleurodeles* and *Cynops*) and axolotls (*Ambystoma*) (Géraudie and Ferretti, 1998). The urodeles regenerate their limbs by the proliferation of new tissue from the wound surface which progressively replaces the missing parts, a process called epimorphic regeneration (Browder *et al.*, 1991; Corcoran and Ferretti, 1997). Following limb amputation, the stump is covered by surrounding epithelial cells that form a wound epidermis. Blastemal cells accumulate underneath the wound epidermis by the dedifferentiation of cells from the stump tissue, proliferate and redifferentiate into new limb structures (Browder *et al.*, 1991; Brockes, 1997; Géraudie and Ferretti, 1998). These cellular contributions made by the adult tissues to the regenerating limb are regulated by changes at the molecular level, specifically those governing enzymes, extra cellular matrix components, adhesion proteins, growth factors and gene transcription (Reviewed in Géraudie and Ferretti, 1998).

Although the wound epidermis and the blastema are different tissues with distinct functions, what distinguishes them from the normal wound healing process is their unique interaction which is essential for the initiation and progression of regeneration (Géraudie and Ferretti, 1998; Tsonis, 1996). Before the introduction of teleosts as a model for regenerative studies, it is necessary to discuss the details of the unique epidermal-mesenchymal interaction in urodeles.

a. Wound Epidermis

The early steps of the regeneration process resemble those found in normal wound healing. Within minutes after limb amputation, white blood cells, erythrocytes, neutrophils and fibrinogen are released from the injured blood capillaries and a fibrin clot covers the wound surface (Tsonis, 1996). The clot consists of platelets embedded in a mesh of cross-linked fibrin fibers derived by thrombin cleavage of fibrinogen, and serves as a temporary shield protecting the wound tissue. Importantly the clot also serves as a reservoir of cytokines and growth factors that are released as activated platelets degranulate (Martin, 1997). During the vertebrate wound healing process, growth factors are thought to initiate the wound closure (Boilly *et al.*, 1991; Boilly, 1992; Poulin *et al.*, 1993; Poulin and Chiu, 1995; Tsonis, 1996; Martin, 1997) by providing chemotactic cues to recruit circulating inflammatory cells, and by initiating tissue movements or re-epithelialization and connective tissue contraction (Martin, 1997; Bromberek *et al.*, 1998). The formation of the clot itself also provides a matrix over and through which cells can migrate during the repair process (Martin, 1997).

As early as 1 hour after limb amputation, the epidermal cells surrounding the wound lose their hemidesmosomes, change shape and detach from the basal lamina (Norman and Schmidt, 1967; Repesh and Oberpriller, 1978, 1980). Within 2 hours, the flattened epidermal cells adhere to and migrate along the fibrin clot over the amputated surface (Tsonis, 1996). Although the exact mechanism of epidermal cell migration is unknown, it is thought to mimic lamellipodial action where the cell extends pseudopodia, forms nascent attachments, assembles and contracts the cytoskeleton, and finally, as it translocates forward, disengages distal adhesions (Martin, 1997; Tsonis, 1996).

At least two factors are thought to trigger the epidermal cells to move: substratum contact mediated by integrin adhesion receptors, and chemokinetic factors that appear to be required for continued crawling (Matsumoto *et al.*, 1995).

Integrins, a family of more than 20 heterodimeric proteins, do not simply bind cells to extra cellular matrix (ECM), but also act as potent signaling molecules (Dike and Inber, 1996; Matsumoto *et al.*, 1995; Plopper *et al.*, 1995). The molecular mechanism by which integrins act as binding proteins and signaling proteins is known as tensegrity. Tensegrity predicts that cells are hard-wired to respond immediately to mechanical stresses transmitted over cell surface receptors that physically couple the cytoskeleton to ECM or to other cells (Ingber, 1997). In the case of integrins, their initial heterodimeric binding induces their condensation into focal contacts, forming anchors to the extracellular matrix and signal-transducing complexes on the cytoplasmic surface. The cytoplasmic complex in turn transduces

environmental signals into biochemical responses, effecting changes in cell motility, ion transport and gene transcription (Matsumoto *et al.*, 1995; Kanazashi *et al.*, 1997; Rudzki and Jothy, 1997; Lelièvre *et al.*, 1996; Roskelley *et al.*, 1995) through force-dependent changes in scaffold geometry, or through other molecular mechanisms (Ingber, 1997; Giancotti, 1997; Fuchs *et al.*, 1997; Sjaastad and Nelson, 1997; Akiyama, 1996; Bissell *et al.*, 1996).

Growth factors, as previously mentioned, such as fibroblast growth factors (FGF's), isoforms of platelet derived growth factor (PDGF), or transforming growth factor (TGF- β) to name but a few (Martin, 1997), are also able to activate the cellular machinery that mobilizes the cells. For example, based on the results from tissue culture experiments, it is likely that the growth factor receptors that bind their respective ligands as well as the integrin receptors that bind specific extracellular adhesion molecules, are able to regulate cell locomotion through the GTP-binding protein Rac (Ridley *et al.*, 1992; Ridley, 1994). Furthermore, there is an interaction between both growth factor receptors and integrins that occurs through their common intracellular signaling pathways. In this way, each receptor type can either amplify or attenuate the other's signal and downstream response (Matsumoto *et al.*, 1995).

Approximately 12 hours after the limb is amputated, the surrounding epidermal cells have migrated from the edges of the wound to cover the open surface and thereby form the wound epidermis, now referred to as the epidermal cap (Browder *et al.*, 1991; Brockes, 1997). During closure of the wound surface, there is no

mitosis or DNA synthesis (Tsonis, 1996), further supporting the role of migration during epidermal cap formation. Variation in rates of migration have been correlated to the matrix coating density for hepatocytes plated on various ECM's such as laminin, fibronectin and collagen (Mooney *et al.*, 1995), thereby suggesting that the rapidity with which the wound heals could be in relation to the ECM components surrounding the wound.

The fact that the regenerative process is inhibited if the epidermal cap is removed, supports the role of the epidermal cap in initiating and maintaining the cues required for regeneration (Thornton, 1957; Thornton and Thornton, 1965). Although the signaling mechanism is not yet understood, it could be the combination of changes in the ECM (Greenberg and Hay, 1982), cytokines (Géraudie and Ferretti, 1998), growth factors (Reviewed in Martin, 1997) and tensegrity (Ingber, 1997) which initiates a second messenger signaling cascade within the epidermal cap, that starts the process of recruitment and proliferation of progenitor cells from the mature tissue of the stump.

The initiation of cytolysis of the stump tissues by the epidermal cap is thought to favor the release of blastemal progenitor cells (Singer and Salpeter, 1961; Thornton, 1968; Stocum, 1985; Schmidt, 1968). Significant changes in the connective tissues at the level of amputation are apparent within 24 to 48 hours. The first event identified to occur, is the degradation of certain components of the extracellular matrix (ECM) by enzymes of the matrix metalloproteinases (MMP's), none of which are detected in normal limbs but they are up-regulated as early as 2

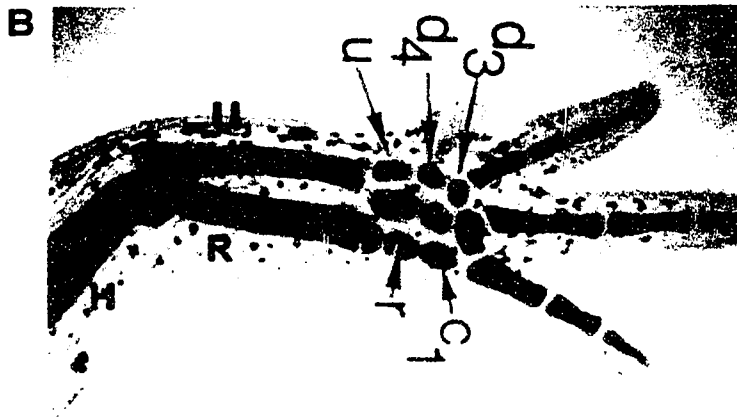
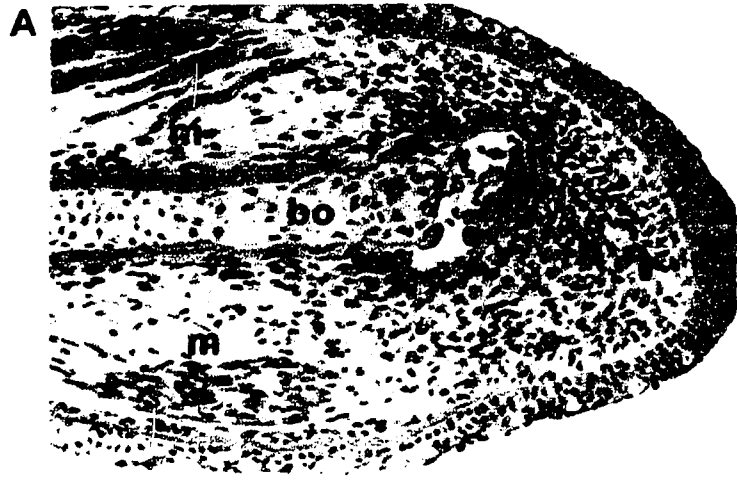
days after amputation (Yang and Bryant, 1994; Miyazaki *et al.*, 1996). MMP's degrade ECM components such as collagen (Grillo *et al.*, 1968; Dresden and Gross, 1970) and laminin (Géraudie and Ferretti, 1998). Laminin is of particular interest because of its presence in the basement membrane, which is an intermediate layer of extracellular proteins situated in between the epithelial and mesenchymal cells (Timpl *et al.*, 1983; Timpl and Brown, 1996). Laminin, a component of the basement membrane, plays a role in maintenance of tissue integrity (Timpl, 1989). At this point in normal wound repair in humans, basal lamina is synthesized, MMP expression is quickly shut off, and epidermal hemidesmosomes adhere to the basal lamina (Compton *et al.*, 1989). In contrast, during limb regeneration laminin is degraded (Gulati *et al.*, 1983; Géraudie and Ferretti, 1998). This is of great functional importance because epithelial-mesenchymal interactions are indispensable for the progression of regeneration and are impaired by the presence of a basement membrane (Salpeter and Singer, 1960; Tsonis, 1996). For example, the proliferation of the blastema is initiated by members of the FGF family which are synthesized by the epidermal cap but target the receptors (FGFR's) in the mesenchyme (Poulin *et al.*, 1993; Poulin and Chiu, 1995). This mitogenic ability (Mescher and Gospodarowicz, 1979; Mescher, 1983) is possible due to the degradation and delay in formation of a basement membrane that allows for a unique epidermal-mesenchymal interaction (Géraudie and Ferretti, 1998).

Another important contribution of the epidermal cap to the regeneration process, is that of positional identity. The faithful regeneration of distal structures

upon amputation implies that the cells along the limb know their position, or that they acquire the knowledge of their position upon amputation. Since only structures distal to the amputation plane can be regenerated normally, this means that the amputation plane determines the proximal boundary. On the other hand, evidence indicates that the epidermal cap imposes the distal boundary. The manner in which the cells determine their positional information between both boundaries creates the specific pattern unique to every tissue (Tsonis, 1996). For example, if the limb is amputated at the level of the hand, it will only regenerate the hand (Figure 1) and if it is amputated at the level of the shoulder, it will regenerate the whole limb. An experiment which better illustrates positional identity consists in grafting a distal blastema, formed from an amputated hand, into a proximal shoulder stump. This results in the regeneration of the normal limb in which the hand is derived from the blastema and the intermediate structure between the shoulder and the hand originates from the stump (Pescitelli and Stocum, 1980). How the cells can specify their location on a proximo-distal axis at the molecular level remains unclear. Retinoids and their receptors have been proposed to play a role in the determination of positional identity due to their ability to respecify axial identity. Specifically, 9-*cis* retinoic acid, a derivative of vitamin A, is one of the signaling factors known to affect normal regeneration (Viviano *et al.*, 1995). Recent work has provided evidence that the wound epidermis is a source of 9-*cis* retinoic acid (Viviano and Brockes, 1996), and it has been shown, that under certain conditions, retinoic acid has the ability to respecify proximo-distal and dorso-ventral patterning during

Figure 1: Regeneration of forelimbs of *Ambystoma maculatum* larvae.

Forelimbs amputated at the mid-humerus level and analyzed in longitudinal sections (A) and whole mount skeletal preparations stained with methylene blue (B). (A) Early bud blastema stage. ec, epidermal cap; b, blastema; m, mesenchyme; bo, bone. (B) Four-finger stage, all carpals have differentiated and the first three digits are already fully shaped. r, radial carpal; u, ulnar carpal; C1, central carpal; d3 and d4, carpals articulating with digits 3 and 4 (figure obtained from Géraudie and Ferretti, 1998).



amphibian limb regeneration (Kim and Stocum, 1986; Ludolph *et al.*, 1990). This vitamin A metabolite is acting through nuclear receptors belonging to the steroid/thyroid receptor superfamily which activate the transcription of target genes by binding either as homodimers or as heterodimers to specific DNA response elements (Means and Gudas, 1995). In urodeles, five different Retinoic Acid Receptors (RARs) have been identified, all of which are expressed in both the epidermis and the blastema (Ragsdale *et al.*, 1989, 1992a), RAR- δ being expressed at very high level in the blastema of the newt limb (Hill *et al.*, 1993; Giguere *et al.*, 1989; Ragsdale *et al.*, 1989, 1992b). Although the biological significance of the receptors distribution and function has yet to be elucidated, it is plausible that the 9-*cis* retinoic acid in the wound epidermis acts through the RAR to influence the behavior of the underlying mesenchymal cells, and subsequently confers the positional identity of the cells within the regenerate.

The information so far available about the epidermal cap, reveals that some of the dramatic changes induced by amputations are equivalent to those induced by skin wounding, such as epidermal cell migration. On the other hand other processes, such as MMP's activity and a subsequent prolonged delay in basement membrane formation, are intimately linked to the process of limb regeneration (Salpeter and Singer, 1960; Tsonis, 1996; Géraudie and Ferretti, 1998). Three roles are now attributed to the wound epidermis that are critical for the regeneration process: induction of dedifferentiation by hydrolysis of the mesenchyme, initiating dedifferentiated cell cycling and providing positional values for the start of

differentiation and pattern formation (Tsonis, 1996). These roles must be kept in mind when describing the activities of the blastema during regeneration.

b. Blastema

The blastema develops steadily through stages defined as early, medium and late bud (Singer, 1952). During the early bud stages (3 to 4 days after amputation) (Figure 1) the blastemal cells, a mound of undifferentiated mesenchymal progenitor cells, proliferate and accumulate under the epidermal cap (Géraudie and Ferretti, 1998). All stump tissues, except for the epidermis, are source of progenitor cells for the formation of the blastema (Wallace, 1981; Liversage, 1991; Ferretti and Brockes, 1991). How blastemal cells originate from the stump tissues, and the molecular mechanism governing this process, is still unclear. Two possible origins of blastemal cells are, as previously mentioned, the dedifferentiation of mature tissues in the stump that would regress to an undifferentiated state and reenter the mitotic cycle (Liversage, 1991), or the activation of populations of normally quiescent stem cells in the different stump tissues (Cameron *et al.*, 1986). The two possibilities are not mutually exclusive, and different tissues may contribute cells to the blastema through different mechanisms.

The division of the blastemal cells is at least under control of growth factors originating from the epidermal cap (Tassava and Mescher, 1975), the peripheral nervous system (Singer, 1952, 1974; Thornton, 1970), and possibly the blastemal cells themselves (Géraudie and Ferretti, 1998). In the adult newt, the blastema

proliferates for about 2 to 3 weeks post-amputation (middle bud stage) in order to achieve a critical mass before cell differentiation and restoration of the missing part. The period of proliferation varies for different species, depending on the size and the age of the animal (Tsonis, 1996).

Among the molecules that appear to play a key role in controlling the growth and patterning in the blastema are, the previously mentioned FGF's (Boilly *et al.*, 1991; Boilly, 1992; Hondermarck and Boilly, 1992) and their receptors (Poulin *et al.*, 1993; Poulin and Chiu, 1995), as well as several homeodomain proteins (Savard *et al.*, 1988; Tabin, 1989; Savard and Tremblay, 1995; Crews *et al.*, 1995; Simon *et al.*, 1995), retinoids (Viviano and Brockes, 1996; Stocum, 1991; Géraudie and Ferretti, 1997), bone morphogenetic proteins (BMP's) (Géraudie and Ferretti, 1998), and signaling proteins (Imokawa and Yoshizato, 1997; Endo *et al.*, 1997). Although the functional significance of some of these proteins is minimal or unknown, analysis of the gene expression patterns as well as understanding the regulation of both the genes and their products in comparison with their roles during embryonic development, could determine how the molecular signals govern limb regeneration. The emerging picture is that these genes play very similar roles in both embryonic limb development and limb bud regeneration, however slight differences could be attributed to types of progenitor cells and the presence of positional cues in the limb stump (Géraudie and Ferretti, 1998).

As in the formation of the epidermal cap, many of the ECM and membrane proteins with adhesion properties are expressed in a coordinated spatiotemporal

fashion during blastema formation. This is not surprising because changes in cell interaction and cell migration are key factors in blastema formation (Géraudie and Ferretti, 1998). The upregulation of hyaluronate, a major hydrophilic polyanionic glycosaminoglycan, helps to provide an environment suitable for cell migration (Prehm, 1984; Toole, 1991) and could facilitate the migration of blastema progenitor cells from the stump by promoting expansion of the distal portion of the stump (Tsonis, 1996; Géraudie and Ferretti, 1998). Other ECM components are involved in the stabilization of tissues by the interaction of the matrix proteins with adhesion molecules found on the cell surface. As previously mentioned, this type of interaction can act as a signal that activates cellular mechanisms such as cell motility, ion transport and gene transcription (Matsumoto *et al.*, 1995; Kanazashi *et al.*, 1997; Rudski and Jothy, 1997; Lelièvre *et al.*, 1996; Roskelley *et al.*, 1995), thereby contributing to the morphogenesis of the regenerate.

Cell differentiation begins during the late bud stage and proceeds in a proximal to distal direction. The first tissue to differentiate in the blastema is cartilage that appears initially at the ends of the persisting bone, which is completed by progressive addition to its distal end. Next, the more distal skeletal elements are added, just as they are in normal embryonic limb outgrowth. When the cartilaginous reconstruction is complete, the regenerated skeleton is transformed into bone. Muscle is formed by both *de novo* appearance around the cartilage and terminal addition to persisting muscles, and the vasculature and axons infiltrate the blastema from the stump and reconstruct their original patterns. Complete morphological and

functional reconstruction of the missing part is achieved between 6 and 10 weeks post-amputation, depending on the species, the age of the animal and the temperature at which the animal is kept (Géraudie and Ferretti, 1998).

c. Innervation

The nerves play a significant role in controlling the regeneration of the limb (Browder *et al.*, 1991). Once formed, the epidermal cap becomes well innervated (Tsonis, 1996) and has been shown to contain neuropeptides (Vethamany-Globus, 1987; Globus and Alles, 1990). However, the precise role of the neuropeptides found in the epidermal cap is unknown and it is also unknown if their expression reflects a general response to injury or is specific to limb regeneration (Géraudie and Ferretti, 1998).

Early regeneration blastema require the presence of both the epidermal cap and an adequate level of nervous supply for their outgrowth (Todd, 1823; Singer, 1974; Tassava and Mescher, 1975; Carlone and Mescher, 1985; Sicard, 1985; Brockes, 1987; Fekete *et al.*, 1987; Singer and Géraudie, 1991). The nervous system is thought to control cell growth, in the blastema, during the G1 phase of the cell cycle (Goldhamer and Tassava, 1987). It has been postulated that this cell cycle control is exercised either directly or indirectly by neurotrophic factors secreted by the nerves (Singer, 1974; Singer *et al.* 1976; Jabaily and Singer, 1977; Brockes, 1987; Ferretti and Brockes, 1991; Dinsmore and Mescher, 1998). One such secreted neuropeptide is substance P (SP), an undecapeptide that functions as a neurotransmitter in the

central and peripheral nervous system and appears to have a mitogenic effect on the blastemal cells in the newt limbs (Globus *et al.*, 1983).

Innervation is not required during the initial formation of blastemal cells and their release from stump tissues (Tassava and Olsen, 1985), since a limb that is denervated prior to amputation will undergo normal wound healing and accumulate some blastemal cells. However, the blastemal cells do not proliferate and regeneration is inhibited (Singer, 1952, 1974; Thornton, 1970). On the other hand, if the limb is denervated after a blastema has formed, regeneration proceeds but the regenerated limb is smaller in size. Therefore regeneration depends on the presence of the nerve only during the phase of rapid proliferation of blastemal cells (Singer and Craven, 1948).

The continued proliferation of the blastemal cells is due to the interaction between the nervous tissue and blastema which initiates the release of FGF-1, and possibly other factors, from the blastemal cells. These factors in turn stimulate the blastema to proliferate (Zenjari *et al.*, 1996, 1997).

Teleosts as a Model to Study Regeneration

The regenerative capability of teleost fish fins has been studied since the end of the eighteenth century (Morgan, 1900; Nusbaum and Sidoriak, 1900) and has been documented in species such as *Fundulus heteroclitus* (Goss, 1969), *Salaria pavo* (Bernhard and Wagner, 1992), *Tilapia melanopleura*, *Cyprinus carpio*, *Carassius auratus* (Becerra *et al.*, 1996; Santamaría and Becerra, 1991; Santamaría *et al.*,

1992, 1996; Mari-Beffa *et al.*, 1996a) and the infamous zebrafish, *Danio rerio* (Géraudie *et al.*, 1994, 1995; White *et al.*, 1994; Akimenko *et al.*, 1995; Ferretti and Géraudie, 1995).

Several features make the zebrafish a highly suitable experimental model, in particular its optical properties allow embryologists to view every stage of development. The embryo's clearness is advantageous during early development since every cell can be readily visualized (Kimmel *et al.*, 1995) and this also allows the observation of dynamic processes such as movements during gastrulation or in the later stages of development (Warga and Kimmel, 1990). Additionally, individual cells or groups of cells are easily labeled with fluorescent dyes and followed as development proceeds, allowing lineage analysis of different cell types (Kimmel *et al.*, 1994; Lee *et al.*, 1994a; Raible *et al.*, 1992). Furthermore, transplantation techniques have been established for a wide variety of tissues, allowing one to combine mutant and wild type tissues to address such issues as when a particular gene product is required during the development of a certain tissue (Shih and Fraser, 1996; Schilling *et al.*, 1996; Weinstein *et al.*, 1996; Halpern *et al.*, 1995; Eisen, 1992).

On a molecular level, the refinement of the zebrafish as a genetic system permits the study of the genetic control of development. Since this species allows for large-scale genetic screens in a vertebrate, a large number of mutations and a rapidly growing number of genes have been described in this species (Mullins and Nüsslein-Volhard, 1993; Driever *et al.*, 1994). The latter offers a useful tool to study

fin regeneration and the expression of a number of genes in regenerating zebrafish fins has recently been reported (White *et al.*, 1994; Jones *et al.*, 1995; Akimenko *et al.*, 1995; Laforest *et al.*, 1998). Resembling amphibian limb regeneration, the genes that are expressed in the regenerating zebrafish fin recapitulate expression patterns found during the initial development of the bony rays in the fins of the larvae (Akimenko *et al.*, 1995; Laforest *et al.*, 1998). Examples include the homeobox genes of the *msx* family (Akimenko *et al.*, 1995) and genes encoding the signaling molecules such as sonic hedgehog (SHH), BMP's or the SHH receptor gene, *patched1* (*ptc1*) (Laforest *et al.*, 1998). Although a number of these genes are also involved in embryonic fin bud development, their expression patterns are not the same as what is observed during larval fin development and fin regeneration (Laforest *et al.*, 1998).

Most teleost fish fins undergo a similar three stage regeneration process: the wound healing, blastema formation, and the fin ray formation/distal outgrowth (Bernhard and Wagner, 1992) - stages that are reminiscent of its counterpart in the limb (Géraudie and Singer, 1992). Although teleost fins and amphibian limbs are very different anatomically, zebrafish is thought to be an example where certain patterning demands may have converged with the regulation of highly differentiated limbs in tetrapods (Coates, 1994), suggesting that the epimorphic regeneration process (i.e. wound healing, blastema formation, blastema proliferation and differentiation) between certain teleosts and urodeles are similar because their mechanisms are conserved (Tsonis, 1996). Discussing the details of fin regeneration

will outline the similar regenerative characteristics common among certain teleosts fish and urodele amphibians, but before this is done, a short description of fin development is necessary.

a. Fin Development

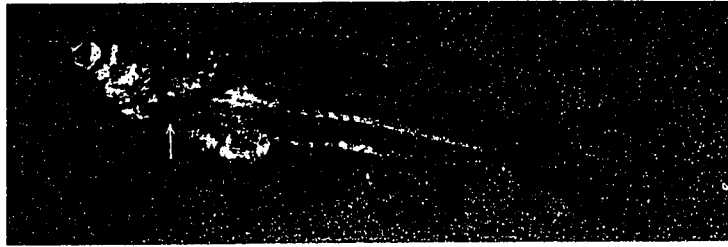
The zebrafish fins are classified into two groups, the paired fins which comprise of the pectoral and pelvic fins, and the unpaired fins which include the caudal, anal and dorsal fins (Figure 2A). During embryonic development the pectoral fins develop independently while a single sagittal fold, termed the median fin fold, is developing around the posterior part of the embryo and will give rise to the unpaired fins. It is thought that the mesenchymal cells of the paired fins originate from the lateral plate mesoderm and ventral edges of the somites (Harrison, 1895; Bouvet, 1974a, 1974b; Géraudie and François, 1973), whereas the mesenchyme of the unpaired fins are thought to derive from neural crest cells (Smith *et al.*, 1994). However, despite their different mesenchymal origins, both the paired and unpaired fins undergo similar fin ray development/regeneration processes.

Morphological analysis, experimental manipulations, and molecular studies have suggested that the development of the pectoral fin bud is under control of similar organizing centers as identified in the limb buds (Figure 3). In the tetrapod limb bud, the Apical Ectodermal Ridge (AER) provides growth factors required for the proximo-distal outgrowth of the bud (Niswander and Martin, 1992; Suzuki *et al.* 1992; Riley *et al.*, 1993) from the progress zone (PZ), a region of mesenchymal proliferation located at the distal end of the bud underlying the (AER) (Géraudie

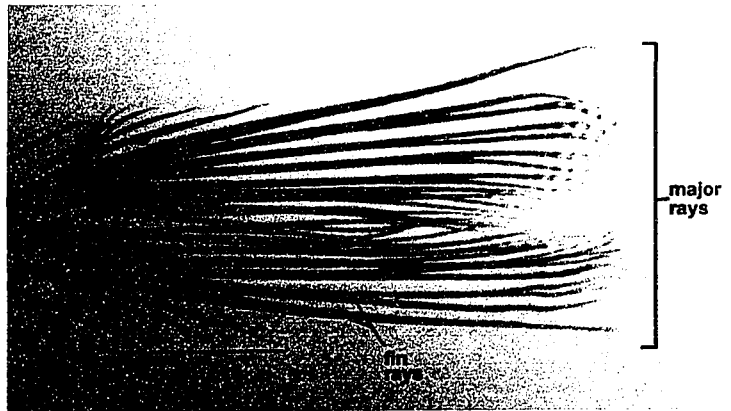
Figure 2: Dermal skeleton of the zebrafish fin.

(A) The fins of the zebrafish are classified into two groups, the paired fins which comprise of the pectoral and pelvic fins, and the unpaired fins which include the caudal, anal and dorsal fin. (B) The caudal fin of the zebrafish is composed of 5 minor rays located on the lateral sides of the fin, and a series of 18 major rays separated by soft tissue. The rays originate at the base of the fin in the subepidermal location, and consist of dermal bone. (C) The rays, called lepidotrichia, are composed of two parallel and symmetrical segmented elements defined as the hemirays. Caudally, each lepidotrichium forms a few dichotomous branches along the proximo-distal axis of the fin. Each lepidotrichia ends with a row of long rigid elastoidin rods called actinotrichia. The segmented structure of the lepidotrichia gives the flexibility to the fin ray.

A. Adult Zebrafish



B. Caudal Fin Skeleton



C. Fin Ray

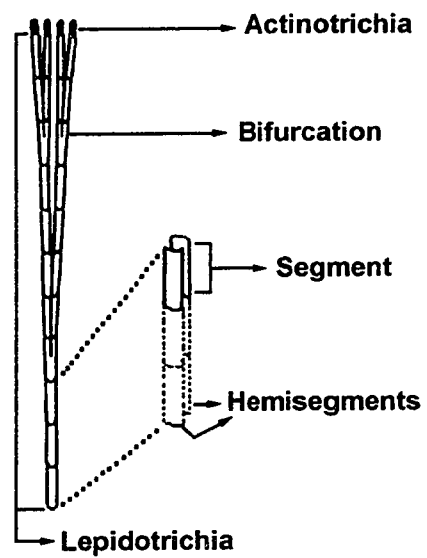
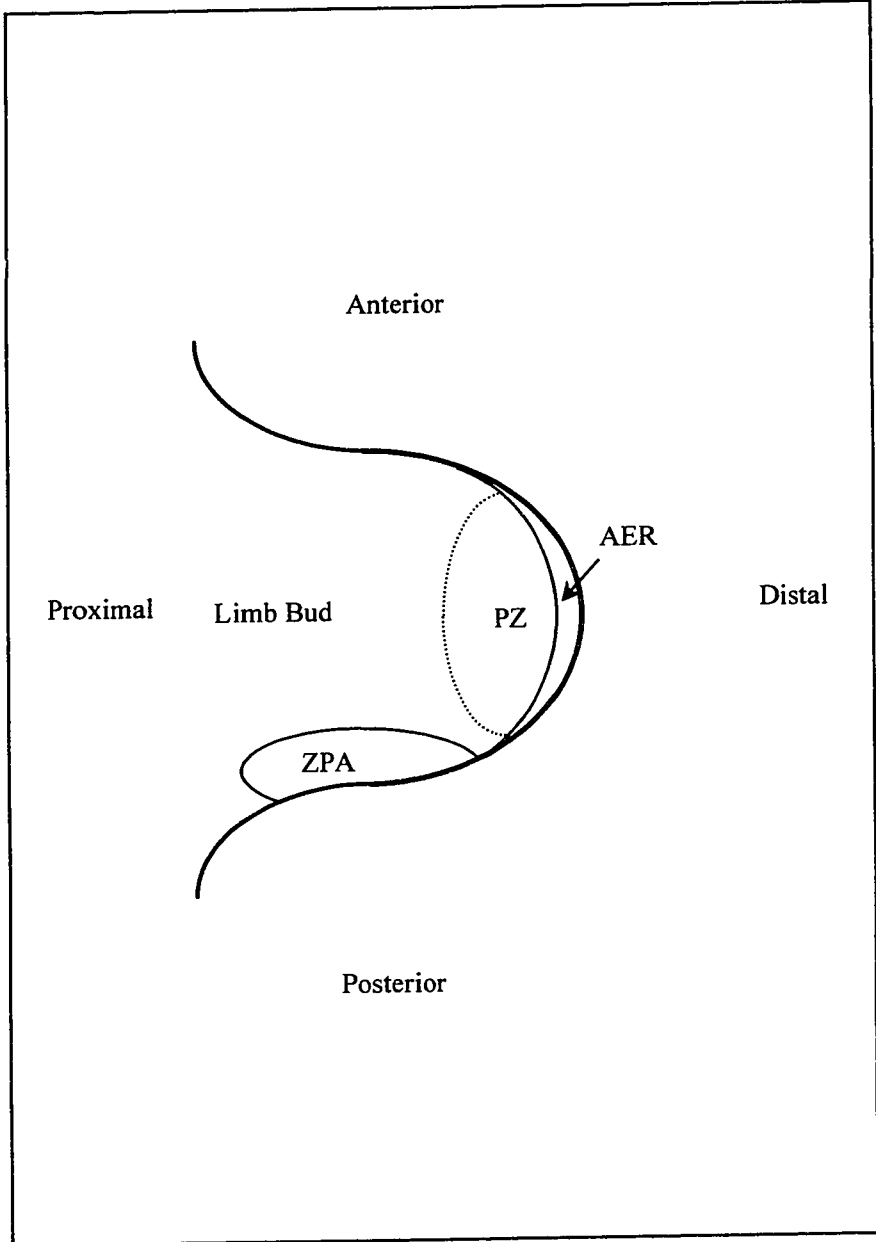


Figure 3: Limb bud schematic showing the signaling centers.

The limb bud is thought to possess three organizing centers. The Apical Ectodermal Ridge (AER) is a thickened epidermal layer which supports the proliferation of undifferentiated mesenchymal cells in the Progress Zone (PZ), allowing the limb to grow proximo-distally. The Zone of Polarizing Activity (ZPA), polarizes the anterior-posterior axis of the limb bud, and is dependent on the presence of the AER. It is thought that similar signaling centers are present in the pectoral fins.



and François, 1973; Wood, 1982; Géraudie, 1980). The Zone of Polarizing Activity, located in the posterior margin of the limb bud, is responsible for the antero-posterior patterning of the bud. It has also been demonstrated that cues from both the ZPA and the AER of the chick wing bud are required for each others maintenance, as well as the subsequent growth of the bud (Vogel and Tickle, 1993; Riddle *et al.*, 1993).

During teleost embryonic development, mesenchymal cells gather to form the pectoral fin primordia, which are visible as placode-like accumulations of cells underneath the epithelium that covers the yolk. The fin buds are first visible as two mounds on the dorsal surface of the yolk on each side of the trunk starting during the 2nd day of development. As the buds develop, a thickened epidermal cap termed the pseudo apical ectodermal ridge becomes prominent at their tips. It is likely that the ridge plays a morphogenic role during the outgrowth of the bud, similar to that of the limb bud of tetrapods. A most significant connection between the zebrafish fin bud and the tetrapod limb bud is the posterior mesenchymal domain of the bud, corresponding to the zone of polarizing activity (ZPA) which is thought to play a role in polarizing the posterior mesenchyme and thereby patterning the antero-posterior axis of the limb bud (Saunders and Gasseling, 1968).

Gene expression analysis in zebrafish suggest that a similar ZPA signaling center exists in the pectoral fin bud. The distal epithelial fold capping the bud that developed from the pseudo apical ectodermal ridge eventually expands into the blade of the fin proper during the larval stages.

During larval development, the unpaired fins arise from the finfold starting around the fourth week of life, and is concomitant to the formation of the fin ray structures. This 2nd phase of development occurs in a sequential order, starting with the caudal fin, followed successively by the anal, the dorsal fins, the pectoral and finally the pelvic fins.

The caudal fin of the adult zebrafish is composed of 5 minor rays located on the lateral sides of the fin, and a series of 18 major fin rays separated by soft tissue each of which can be considered an independent developing unit (Figure 2B) (Géraudie and Singer, 1992). The rays originate at the base of the fin in the subepidermal location, grow distally by progressive addition of segments that once formed cannot elongate but increase in diameter and bifurcate along the proximo-distal axis of the fin.

The skeleton of the rays consists of dermal bones (Moss, 1963), which unlike the endoskeleton that undergoes endochondral ossification, are formed from the mineralization of a proteoglycan matrix synthesized by scleroblasts. The dermal rays are called lepidotrichia, and are composed of two parallel and symmetrical segmented elements defined as the hemirays (Figure 2C). Within the hemiray (intra-ray) there are fibroblasts, melanophores, capillaries and nerve bundles, whereas the inter-ray tissue is composed of a thin layer of connective tissue that also contains capillaries and is covered by the epidermis. Caudally, each lepidotrichium ends in a double palisade of long rigid elastoidin rods called actinotrichia that line the internal

surface of the last segment of each lepidotrichia (Figure 2C) (Becerra *et al.*, 1983; Chandross and Bear, 1979).

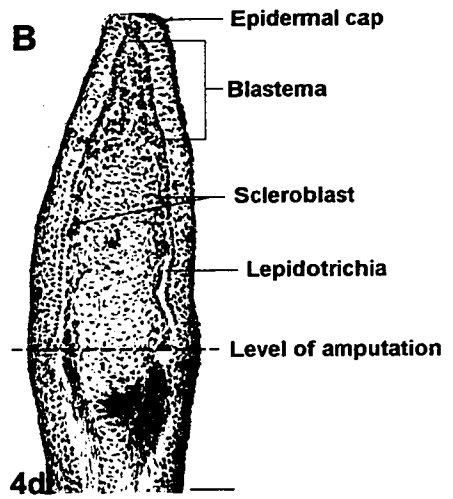
b. Wound Epidermis

Like the limb regenerate, a fin regenerate comprises of a wound epidermis that covers the stump within 6 hours in the zebrafish (Poleo *et al.*, submitted) and between 12 to 24 hours in other teleosts (Géraudie and Singer, 1992; Becerra *et al.*, 1996; Bullock, 1978). Once fully formed, the wound epidermis is termed the epidermal cap and covers the underlying mesenchymal cells termed the blastema (Figure 4).

Blood lost after amputation is minimal and healing begins immediately after injury (Becerra *et al.*, 1996). Blood from the injured capillaries accumulate at the wound, forming a temporary closure. It is thought that in fish, as in higher vertebrates, a change in cell-cell interaction and cell-ECM interactions initiates the epidermal cells, surrounding the cut edge, to undergo a dedifferentiation process. This transient epidermal cell state is thought to be due, in part, to the regeneration-associated modifications of glycosidic residues. Specifically, it is suggested that mucous cells among the epidermis of the caudal fin increase the synthesis and secretion of glycoproteins that play specific roles in initiating dedifferentiation. Effects of this dedifferentiation process causes the epidermal cells to loose their firm anchorage on the basement membrane, take on a flatten appearance and results in a separation of the epidermis from the underlying tissues (Mittal and Munshi, 1974). The change in glycoprotein residues is also thought to initiate the mobility of

Figure 4: Proximo-distal sections of zebrafish regenerating caudal fins.

Hematoxylin stained proximo-distal sections of regenerating fins. (A) 1 day after amputation the epidermal cap is formed and the underlying cells will give rise to the blastema. (B) 4 days after amputation, the blastema is proliferating and gives rise to the underlying tissues, such as the scleroblasts that are thought to secrete the lepidotrichial matrix which forms the new dermal skeleton. ec, epidermal cap; b, blastema. Scale bars: 30 μ m.

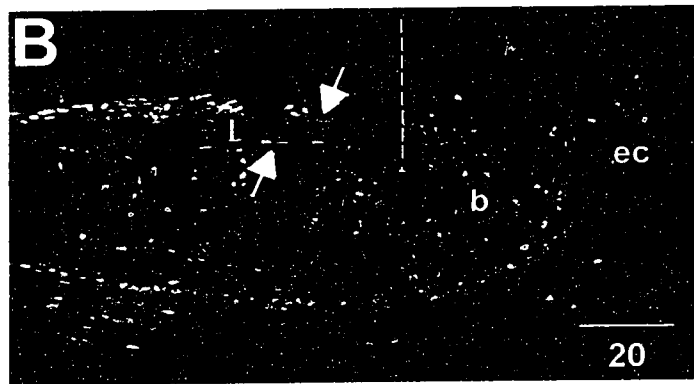
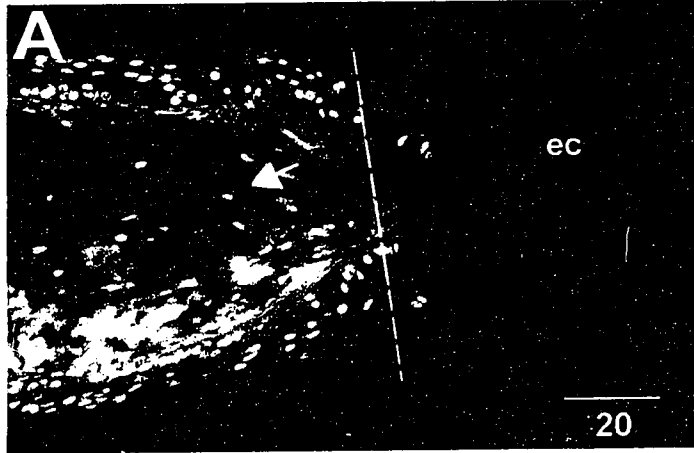


the epidermal cells (Marí-Beffa *et al.*, 1996a) thereby permitting them to migrate over the wound. This migration is thought to occur, as in higher vertebrates, by lamellipodial movement of individual epithelial cells. It is the *Malpighian*, or filament-containing cells of the fish epidermis that are thought to migrate rapidly towards the wound site (Bullock, 1978), and the fact that no mitosis is observed in the cells of the wound epithelium at this stage (Bernhard and Wagner, 1992; Santamaría *et al.*, 1996; Becerra *et al.*, 1996), supports the role of migration during epidermal wound formation. In the zebrafish regenerating caudal fins, recent cell proliferation analysis using BrdU, a thymidine analogue which is incorporated into DNA during S phase of the cell cycle and subsequently detected by immunocytochemical methods, has confirmed the lack of proliferation in the epidermal cells which form the wound epidermis (Figure 5) (Poleo *et al.*, submitted); thereby suggesting that epidermal cells migrate to form the wound epidermis.

The lack in formation of a basement membrane between epidermis and the mesenchyme allows for the epidermal cap to lay in direct contact with the underlying mesenchyme. This close contact is thought to play a role in patterning and positional identity similar to that of the pseudoapical ectodermal ridge in developing fin buds (Géraudie and François, 1973; Wood, 1982; Géraudie, 1980). As in urodele amphibians, a decrease in contact between the blastema and the epidermal cap attenuates the regenerative process (Marí-Beffa, 1996b). This lack of regeneration is due to the inhibition of blastema proliferation, suggesting that the

Figure 5: Proximo-distal sections of regenerated caudal fins showing the pattern of cell proliferation by incorporation of BrdU.

Both panels are from a single pulse of BrdU injection, 7 hours prior to fin collection. (A) 24h after amputation, the epidermal cap is covering the stump. It does not contain any labeled cells. The epidermis below the plane of amputation is strongly labeled as are some cells in the mesenchyme (arrow) which are involved in the formation of the blastema. (B) The blastema of a regenerating fin, 2 days after amputation, showing a high percentage of proliferating cells. Some of the cells located a few μm below the plane of amputation in the mesenchyme are labeled as well as some cells surrounding the lepidotrichia (arrow indicates scleroblast). The lateral epidermis presents a high rate of cell proliferation, however, the epidermal cap rarely contained labeled cells. Dashed line indicates level of amputation. Asterisk denotes erythrocytes. b, blastema; ec, epidermal cap; l, lepidotrichia. Scale bars: $20\mu\text{m}$ (Poleo *et al.*, submitted).



state of mesenchymal cell differentiation and or proliferation is controlled by the epidermis in the regenerating fin (Becerra *et al.*, 1996; Santoz Ruiz, 1996).

Although more is known about the underlying mechanisms and key factors involved in amphibian regeneration, the role of the epidermal cap in inducing mesenchymal dedifferentiation, and providing positional values for the start of differentiation and pattern formation during fin regeneration (discussed in the following section) is highly reminiscent of the role of the epidermal cap during limb regeneration, supporting the idea that perhaps the regeneration mechanism is not confined to a precise tissue and was preserved during evolution (Tsonis, 1996).

c. Blastema Formation

Growth of the blastema in zebrafish is apparent 48 hours after amputation (Figure 5B) (Poleo *et al.*, submitted; Géraudie *et al.*, 1995), but can be as late as 4 or 5 days post amputation depending on the fish species (Bernhard and Wagner, 1992).

Like in the limb regenerate, the blastema forms underneath the epidermal cap. However, unlike the tetrapod limb, the fin regenerate consists of two types of blastema arranged in a succession of adjacent blastemata at the tip of the amputated fin, the confluent and synchronous development of which gives rise to the fin regenerate. The loose mesenchymatous blastema, located between the rays, is thought to give rise to the inter-ray tissues. On the other hand, a dense mesenchymatous blastema, located at the tip of the severed ray, gives rise to the fin ray and intra-ray tissue (Géraudie and Singer, 1992) and is the blastema examined herein.

It is thought that the blastema, in fish as in urodeles, is formed from preexisting mesenchymal cells that dedifferentiate, proliferate and then differentiate into all cell types necessary to reestablish the amputated tissues (Becerra *et al.*, 1996; Géraudie and Ferretti, 1998). Although the idea that stem cells participate in blastema formation is still considered (Géraudie and Singer, 1992), studies have yet to confirm their presence and contribution to the regenerate in teleosts.

As some of the blastemal cells differentiate, their shape as well as their external membrane characteristics change according to the position that they occupy within the regenerating fin (Mari-Beffa *et al.*, 1996a). These changes are thought to depend on the presence of cues from the epidermal cap (Mari-Beffa, 1996b; Becerra *et al.*, 1996), cell-cell interaction and cell-ECM interactions (Géraudie and Singer, 1992; Becerra *et al.*, 1996; Mari-Beffa *et al.*, 1996a). It has also been demonstrated that teleost fins fail to regenerate if deprived of their nerve supply (Goss and Stagg, 1957), implying the trophic influence of nerves as another factor in controlling teleost fin regeneration (Becerra *et al.*, 1996).

As blastemal cells leave their distal proliferative region, they progressively differentiate to give rise to all cell types involved in the regeneration of connective and skeletal tissues (Becerra *et al.*, 1996; Géraudie and Singer, 1992). Cells lining the lateral sides of the blastema differentiate into scleroblasts or lepidotrichial forming cells which start to synthesize and secrete the lepidotrichial matrix in the epidermal/blastemal interface which gives rise to the dermal bone, whereas other blastemal cells differentiate into fibroblasts that restore the intra-ray connective

tissue (Santamaría and Becerra, 1991). The relatively rapid dedifferentiation, followed by a continuous cycle of proliferation and redifferentiation of the blastema, occurs until the amputated fin is restored to its original form, generally within a few weeks (Santamaría *et al.*, 1992).

While many histological and morphological data document fin regeneration, little is known about the underlying molecular mechanisms. Using key genes known to play a fundamental role during fin and limb development, and studying their expression patterns during fin regeneration, may provide some insight into the mechanism of fin regeneration. For example, the homeobox gene *msxB* is expressed in the AER and the progress-zone (PZ) of the developing fin buds of zebrafish embryos (Akimenko *et al.*, 1995), and during caudal fin regeneration its expression is reactivated in the blastema (Akimenko *et al.*, 1995). Other genes, such as *shh* (discussed herein), *ptc-1* encoding for the *shh* receptor (Laforest *et al.*, 1998), and members of the *fgf* family are also expressed during zebrafish fin development and are subsequently re-expressed during fin regeneration (Poleo and Akimenko, in preparation). Further study of these genes and others during fin bud development and fin regeneration could be advantageous in identifying similarities and differences in their functions, thereby leading to a better understanding of the molecular mechanism underlying the regenerative process.

Outlining the above regenerative process has made obvious the similarities between the process of urodele amphibian limb and teleost fish fin regeneration,

demonstrating the essential role and interaction of both the epidermal cap and the blastema that is required for the regenerative process.

The Sonic hedgehog Signaling Pathway

Embryologists have long performed experimental manipulations that reveal the striking abilities of certain structures in vertebrate embryos to impose pattern upon surrounding tissues. Recent work aimed at identifying signaling molecules that direct patterning has implicated secreted proteins encoded by a small number of gene families, such as that of *hedgehog* (Ekker *et al.*, 1995a). Hedgehog was identified in a saturation screen for mutants that affect larval cuticular patterning in *Drosophila* (Nüsslein-Volhard and Wieschaus, 1980). Subsequent studies have shown that members of the Hedgehog (HH) family of secreted proteins control a number of important inductive interactions in the development of both vertebrates and *Drosophila*, the best characterized HH family member being *Drosophila* HH and the vertebrate sonic hedgehog (SHH) proteins.

The *shh* gene has been cloned in mouse (Echelard *et al.*, 1993; Chang *et al.*, 1994), rat (Roelink *et al.*, 1994), *Xenopus* (Ekker *et al.*, 1995b), chicken (Riddle *et al.*, 1993; Johnson *et al.*, 1994), zebrafish (Krauss *et al.*, 1993; Roelink *et al.*, 1994) and human (Marigo *et al.*, 1995). In terms of the protein's function, both the vertebrate and *Drosophila* HH proteins undergo cleavage of a short signal peptide at the amino terminus followed by the autoproteolysis at an invariant histidine residue. The autoproteolysis ability relies mainly upon residues within the carboxy terminus,

and cleaves the protein into a large carboxy terminus and smaller cholesterol modified amino terminus fragment (Lee *et al.*, 1994b). The amino terminal is found to be highly cell associated and possesses the active signaling capability of the protein (Lee *et al.*, 1994b; Martí *et al.*, 1995). Between the related HH proteins, the carboxy terminal ends are phylogenetically divergent, whereas the amino terminus is highly conserved (Johnson and Tabin, 1995).

Specifically, the *shh* gene encodes a 430 amino acid (46kDa) protein which first undergoes cleavage of a signal sequence comprising of the first 27 amino acids. The autoproteolytic cleavage then releases a 27kDa carboxy terminus peptide and a 19kDa signaling amino terminal peptide (Johnson and Tabin, 1995). The binding of the amino terminal fragment to the cell is mediated by patched (PTC), a twelve pass transmembrane protein (Stone *et al.*, 1996), that acts as a receptor soaking up the HH signal (Ingham *et al.*, 1991; Sampedro and Guerrero, 1991; Nusse, 1996; Chen and Struhl, 1996; Marigo *et al.*, 1996a; Ingham, 1998). Vertebrate homologues of the *Drosophila ptc* gene have been isolated in mouse, chicken, zebrafish and human (Goodrich *et al.*, 1996; Marigo *et al.*, 1996b; Concordet *et al.*, 1996; Hahn *et al.*, 1996; Johnson *et al.*, 1996) and are highly expressed in cells which receive the SHH signal and are close to *Shh*-expressing cells (Goodrich *et al.*, 1996; Marigo, 1996a; Marigo and Tabin, 1996). It is thought that binding of the HH amino terminus to the PTC receptor relieves the PTC-dependent inhibition of Smoothed (Smo) (Marigo *et al.*, 1996b; Nusse, 1996) a protein with 7 transmembrane domains that belongs to the family of G-protein-coupled receptors (Heuvel and Ingham, 1996; Alcedo *et al.*,

1996). The signaling cascade that proceeds from the binding of HH is thought to downregulate cAMP dependent protein kinase A (PKA) and eventually mediates transcriptional activation of downstream targets through a yet unknown pathway (Figure 6) (Lepage *et al.*, 1995; Jiang and Struhl, 1995).

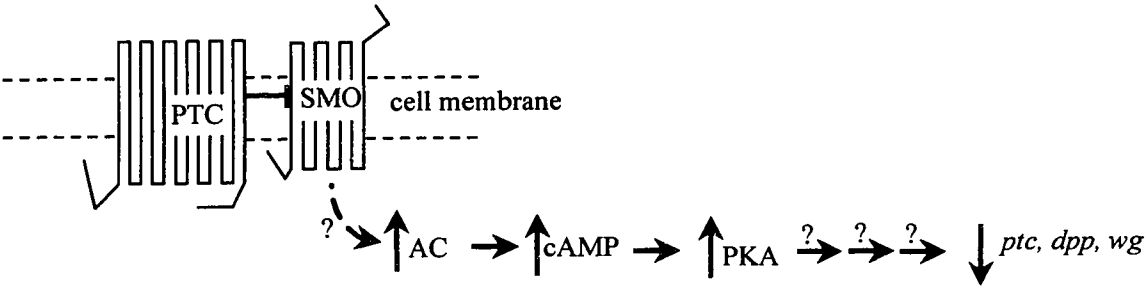
In terms of downstream targets, the *Drosophila* HH and the vertebrate SHH proteins appear to regulate the expression of *decapentaplegic* (*dpp*) and the vertebrate related bone morphogenetic protein *bmp2* and *bmp4* genes, respectively (Basler and Struhl, 1994; Bitgood and McMahon, 1995; Laufer *et al.*, 1994; Roberts *et al.*, 1995). For example, *bmp2*, whose domain of expression overlaps that of *shh* and *ptc* in the posterior margin of the chick limb bud, is up-regulated after *shh* ectopic expression in the anterior mesenchymal cells of the limb bud (Laufer *et al.*, 1994). In many other systems, members of the *bmp* family and *shh* are either co-expressed or expressed in close domains. For example, during tooth development, *shh* and *bmp2* transcripts are both located in the enamel knot which is proposed to function as an organizing center, while *bmp4* is first found in the adjacent dental mesenchyme at a later stage it is also located in the enamel knot (Vaahtokari *et al.*, 1996). These observations suggest that *bmp2* expression is somehow mediated by *shh* as a result of a conserved signaling mechanism.

Research in both developmental biology and in genetic analysis of human disease syndromes has facilitated remarkable progress in the knowledge of the HH signaling pathway. For example, nevoid basal cell carcinomas (BCCs), the most prevalent cancer amongst Caucasians, and medulloblastomas are associated with

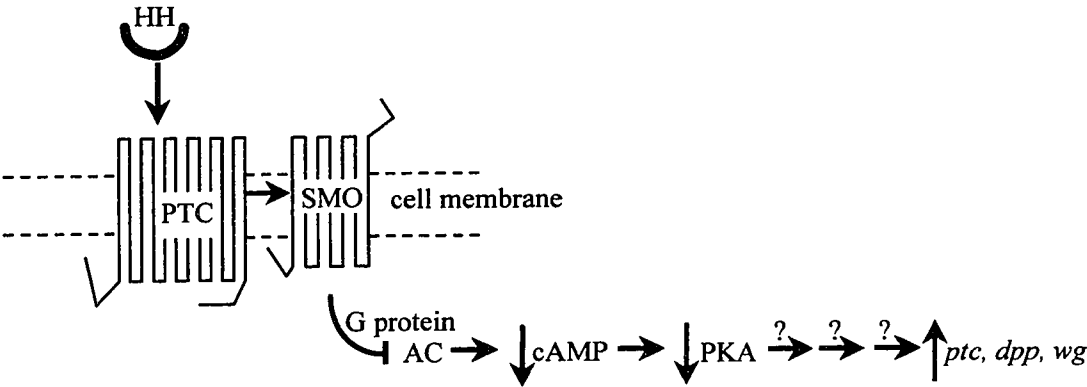
Figure 6: Transduction of HH signaling in *Drosophila*.

In target cells, HH signaling is mediated by two transmembrane proteins, patched (PTC), a twelve-transmembrane protein that has structural similarities to channel and transporter proteins, and smoothed (SMO), a seven-transmembrane protein similar to G protein-coupled receptors. According to one model (Nusse, 1996; Hammerschmidt *et al.*, 1997), SMO is a constitutive activator of HH target genes, but its activity is normally repressed by PTC. Although the regulative mechanism of SMO by PTC is unknown it is thought that there is a direct interaction between both PTC and SMO. (A) In the absence of HH adenylylase is constitutively active, producing cAMP and thereby activating PKA which leads to the repression of transcription of the HH downstream targets by a yet unknown mediator(s). (B) The repression of PTC on SMO is relieved by HH binding possibly to PTC itself, thereby activating the smo bound G-protein which inhibits the function of adenylylase, leading to decreased levels of cAMP and a decrease in PKA activity. Decreased PKA levels inhibit the repression of the HH downstream targets by some unknown mediator(s), leading to the transcription of HH downstream target genes.

A. In the absence of HH



B. In the presence of HH



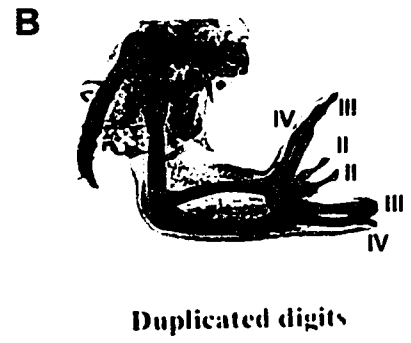
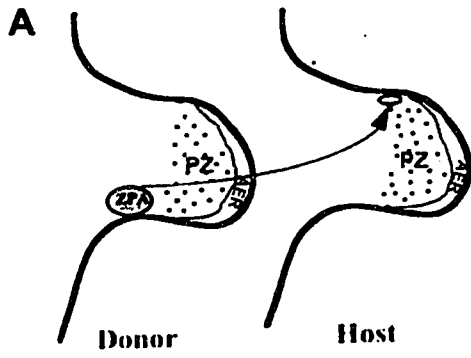
mutations in the HH receptor PTC (Hahn *et al.*, 1996; Stone *et al.*, 1996; Oro *et al.*, 1997; Ingham, 1998). Holoprosencephaly, a human disorder involving abnormal development of the forebrain and the midface, is associated with a translocation or a partial deletion of the *shh* gene (Belloni *et al.*, 1996; Roessler *et al.*, 1996). The continuing analysis of the HH pathway in model systems should yield further insight into the molecular basis of BCC, other cancers and disorders that in turn should lead to the development of new therapies for these conditions (Ingham, 1998; Hammerschmidt *et al.*, 1997). In addition, all these efforts are likely to enhance our understanding of the regulation and evolution of patterning mechanisms during animal development and regeneration.

Sonic hedgehog Expression Patterns during Fin Development and Regeneration

In chick it, has been demonstrated that transplanting the zone of polarizing activity (ZPA) from one limb bud to the anterior margin of another bud results in a mirror image duplication of the normal pattern of digits (Figure 7) (Saunders and Gasseling, 1968). The ZPA has been hypothesized to function by releasing a signal, termed a morphogen, which forms a gradient across the early embryonic bud and whose concentration determines the fate of the structural elements along the antero-posterior axis. Although SHH is found to be highly cell associated, its pattern of

Figure 7: A model for anterior posterior patterning.

This panel shows the result of transplanting the Zone of Polarizing Activity (ZPA) from one stage 20 chick limb bud to the anterior margin of another bud. The mature limb, which normally contains 3 digits (II, III and IV) now contains six digits (IV, III, II, II, III, and IV) in a mirror-image duplication of the normal pattern. The grey oval region on the posterior limb is the ZPA. The overlying distal ectoderm is the Apical Ectodermal Ridge (AER), and the dotted center region is the Progress Zone (PZ).



expression colocalizes with ZPA activity (Riddle *et al.*, 1993), implicating it as a candidate for the ZPA morphogen. The same type of mirror image digit duplication is obtained when a source of retinoic acid is placed in the anterior mesenchyme of a limb bud (Tickle *et al.*, 1982; Summerbell, 1983). Correspondingly, ectopic *shh* expression is detected in the anterior mesenchyme 24 hours after retinoic acid bead implantation (Riddle *et al.*, 1993). When fibroblast cells expressing SHH are implanted into the anterior mesenchyme of the limb bud, a mirror image duplication of the digits is also observed, thereby supporting the hypothesis that *shh* could act as a morphogen which specifies anterior-posterior axis in limb buds (Riddle *et al.*, 1993).

In the developing zebrafish embryo, *shh* is expressed in several key organizing centers such as in the notochord, the ventral structures of the neural tube and brain (Ekker *et al.*, 1995a), and in the posterior margin of the developing pectoral fin bud (Krauss *et al.*, 1993). The expression of *shh* in the posterior margin of the pectoral fin bud is found in a subset of cells whose position is analogous to the zone of polarizing activity (ZPA) of tetrapod limbs. Administration of retinoic acid (RA) to zebrafish embryos results in the anterior duplication of the *shh* expression patterns thereby mimicking duplication of the zone of polarizing activity (Akimenko and Ekker, 1995). Like in tetrapods, this suggests a role for *shh* in patterning the antero-posterior axis, and supports the hypothesis of a conserved limb/fin bud developmental mechanism between tetrapods and teleost fish.

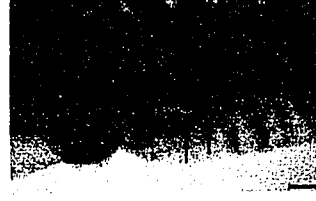
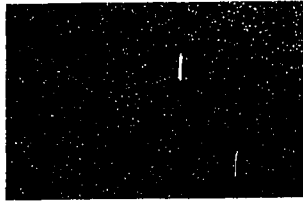
Shh expression in pectoral fin buds of zebrafish larvae diminishes after 80 hours and is no longer detectable at 4 days post-fertilization. At four weeks post-fertilization, *shh* is re-expressed in the developing larval fins in a sequential manner that correlates with the order of formation of the lepidotrichia (Figure 8A-E). *Shh* is expressed in one cluster of cells, on each side of the developing fin, distally located in the larval fin ray (Figure 8E) in a subset of cells of the basal layer of the epidermis. This expression is no longer detectable in the adult fins. This pattern of *shh* expression is indicative of a role for this gene in formation of the dermal bone of the fins, a pattern that is clearly distinct from that observed in pectoral fin buds (Laforest *et al.*, 1998). During regeneration of the caudal fin (Figure 8F-I), *shh* expression starts at 40 hours after amputation and remains present throughout the regeneration process. At 2 days, the expression of *shh* is confined within 100 to 250 cells in the basal layer of the epithelial compartment located at the level where the scleroblast cells of the blastema start to secrete the lepidotrichial matrix in the subepithelial space (Figure 8F), supporting the hypothesis that *shh* may be involved in dermal bone formation as during larval development.

In the fin regenerate, *bmp2* and *ptc1* are expressed in overlapping domains with *shh* expressing cells, as well as in adjacent mesenchymal cells. This pattern of expression allows on to propose that *bmp2* and *ptc1* may be part of the *shh* signaling pathway during fin regeneration as they are in other systems (Laforest *et al.*, 1998). As the fin regenerates, in a proximo-distal fashion, the domain of *shh* expression, as well as those of *bmp2* and *ptc1*, move distally in a way that they always remain

Figure 8: *Shh* expression in the developing fins of zebrafish larvae and during regeneration in the zebrafish caudal fin using whole mount *in situ* hybridization. (A) Caudal fins of 5.5mm larvae. (B) Anal fin of 6.5mm larvae. (C) Pelvic, (D) pectoral and (E) dorsal fin of a 7.2mm larvae. Note that fins in (A-I) are hybridized with the *shh* probe. During larval fin development (A-E) *shh* is expressed in each developing ray. (A) *Shh* expression in the rays of the developing caudal fin, precedes the formation of the lepidotrichia. (B) *Shh* is also strongly expressed in the analia-genetalia region. *Shh* expression subsequently appears in the distal part of the developing (B) anal, (D) pectoral then (C) pelvic fin rays always preceding the formation of the lepidotrichia. (E) The waving of the dorsal fin allows to show that there are two subsets of cells expressing *shh* in each fin ray (indicated by the two small arrows in one of the fin rays), one subset of cells on each side of the fin.

Induction of *shh* during regeneration of the caudal fin. *Shh* expression was determined at (F) 2 days; (G, I) 4 days and (H) 12 days after amputation. Two days after amputation, *shh* is strongly expressed just distal to the stump bone (F). Starting at three days the expression is moving distally from the level of amputation as the regenerate elongates. *Shh* is confined to one or two subset of cells in individual rays on each side of the fin (G) and like larval fin development it precedes the formation of the lepidotrichia. Two subsets of cells expressing *shh* are observed prior or during formation of a bifurcation (two fin rays on the right in (G)). *Shh* is expressed in only one subset of cells as soon as a bifurcation is formed (arrow in (H)) or in the lateral most fin rays of a caudal fin which never undergoes bifurcation (fin ray marked with an asterisk in (G)). (I) the distal part of the fin is slightly tilted to show subsets of *shh* expressing cells on each side of one fin ray, as during larval fin development in (E).

Note for (A-D) arrows indicate *shh* expression, anterior is to the left; h, cartilage condensation of hypural bones. For (F, G) open arrow indicates level of amputation; in (G) “p” refers to the proximo-distal plane of the caudal fin, whereas “t” refers to the transverse plane of the caudal fin. Scale bars A, 200µm; B, C, D, E, 160µm; F-I, 80µm (Laforest *et al.*, 1998).

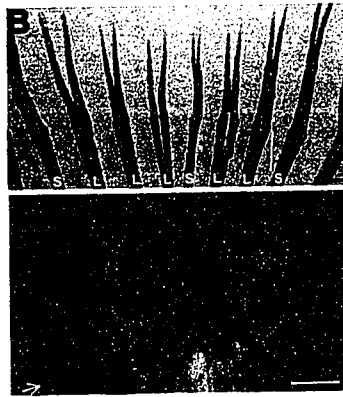
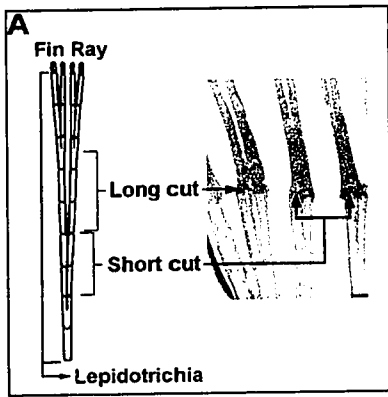


adjacent to the newest scleroblasts of the blastema. In any case, these patterns of *shh* expression resemble those observed during larval fin development, implying a recapitulation of developmental mechanisms during dermal bone regeneration (Laforest *et al.*, 1998).

During larval fin development and fin regeneration, when a unique group of *shh* expressing cells is present, it is always centered in the fin ray (Figure 8A-F), while when two groups of *shh* expressing cells are observed (Figure 8G, I), they are located on the lateral sides of the fin. Expression of *shh* in two groups of cells on one side of the major rays 2 to 17 is only observed whenever a bifurcation of the lepidotrichia is going to form. Once a bifurcation is made, only one group of cells is transiently present in each newly formed branch (Figure 8H). The most dorsal and ventral rays, 1 and 18, are the only major rays of the caudal fin that do not form any bifurcations and correspondingly only one group of cells expressing *shh* is detected on each side of these rays (Figure 8G, asterisk). These observations suggest a role for *shh* in the bifurcation of the dermal bone.

When adult fins are amputated immediately after the first branching point, termed a “short cut”, the once diverging dermal bone converges and fuses to form a single lepidotrichium which undergoes normal regeneration (Figure 9A, B) (Laforest *et al.*, 1998; Géraudie, unpublished observation). Correspondingly, cells in the basal layer of the epidermis express *shh* in a unique domain when the adjacent lepidotrichia are in the process of fusing (Figure 9C) compared to similar cells after an amputation that does not lead to the fusion of the lepidotrichia (Figure 8F, H).

Figure 9: Effect of amputation level on *shh* expression in regenerating caudal fins. (A) Schematized representation of the levels of amputation with respect to the first bifurcation of the caudal fin rays. The “short cut” was between 1-2 segments after the bifurcation. The “long cut” was made further than 2 segments distal to the bifurcation. The small insert shows the ray fusion induced by the “short cut”, but not by the “long cut”. (B) Representative fusion of the rays in a caudal fin that was amputated near the bifurcations. Due to the uneven level of the first bifurcations across the fin, some rays underwent a “short cut” (S) whereas some underwent a “long cut” (L). Only rays with short cuts have fused branches. (C) *Shh* expression in “short cut” fin rays at 4 days. The cells expressing *shh* form more diffuse patterns compared to fins that had a “long cut” (see Fig. 8G). Arrow in (C) indicates level of amputation. Scale bars: 80 μ m (Laforest *et al.*, 1998).



The time at which *shh* is expressed in a unique domain corresponds to the time during which fusion is taking place, again supporting the role of *shh* in dermal bone patterning (Laforest *et al.*, 1998).

The outlined parallels of the *shh* signaling pathway between tetrapods and teleost fish supports the hypothesis of a conserved involvement of the same molecules in limb/fin bud development. In addition, the above results imply a recapitulation of a developmental mechanism during regeneration in the zebrafish, suggesting a role for *shh* in dermal bone formation. This hypothesis is further tested in this study by misexpressing *shh* at specific locations in the regenerating caudal fin.

In Vivo Transfection as a Tool to Study Molecular Biology

The ability to engineer bacteria, yeast or mammalian cells to synthesize a desired protein or to generate a transgenic organism, such as a plant or animal, by introducing genetic material into the germ line or somatic cell is an example of how genetic engineering is providing new ways to study the functions of genes, RNA molecules and proteins and thereby revolutionizing all aspects of cell biology (Alberts *et al.*, 1994).

Transfection is the transfer of genetic material into a cell. Specifically, the gene or DNA sequence of interest is transferred into the nucleus of a cell. However, due in part to the size and charge of DNA and to the multitude of enzymatic and membrane barriers imposed by the cell, the spontaneous entry of intact DNA into

the cell and its subsequent expression in the nucleus has not been demonstrated as an efficient process (Felgner *et al.*, 1987). The use of a virus, a lipid complex, glycoprotein complex or calcium phosphate-coprecipitated DNA helps to overcome these obstacles and facilitates the entry of DNA into the cell (Mulligan, 1993; Benvenisty and Reshef, 1986; Wolff *et al.*, 1990; Wolff and Lederberg, 1994). With the retroviral vectors and adeno-associated virus vectors, the transferred DNA stably integrate into the chromosome of the target cell. However, the other transfer methods often result in the DNA entering the nucleus but not integrating into the chromosome, resulting in high but transient gene expression (Mulligan, 1993; Benvenisty and Reshef, 1986).

Although DNA transfection technology is very useful for molecular biological studies *in-vitro*, in some cases it may be incorrect to apply conclusions drawn from studies in cultured cells to the regulation of gene expression in whole animal (Benvenisty and Reshef, 1986). Therefore methods have been sought to introduce genes into animals *in vivo*.

The potentials of this *in vivo* transfection technology are extraordinary, as both a tool for molecular studies and ultimately for managing and correcting human diseases (Mulligan, 1993; Orkin and Motulsky, 1995; Strauss, 1998). Acquired maladies often have a genetic component that can theoretically be a target of *gene therapy* - a genetic correction strategy. For example, cancer in most cases is not inherited but results from genetic damage accumulated after birth (Blaese, 1995, 1997). In principle a normal gene could be delivered so that it physically takes the

place of a flawed version on a chromosome. However, in practice such targeted insertion of a gene into a chromosome is not yet achievable in people (Friedman, 1997).

Gene therapy is accomplished using two general techniques. Indirect or *ex vivo* gene therapy is achieved by removing target cells from the body, infecting them with viral vectors carrying the new genetic information, and then reimplanting them into the body. On the other hand a direct introduction of genes *in vivo*, without the use of viral vectors, is administered directly into the blood or tissues (Orkin and Motulsky, 1995). Direct *in vivo* gene transfer into postnatal animals has been achieved with the use of DNA encapsulated in liposomes, DNA entrapped in proteoliposomes containing viral envelope receptor proteins, calcium phosphate-coprecipitated DNA, and DNA coupled to a polylysine-glycoprotein carrier complex (Wolff *et al.*, 1990; Wolff and Lederberg, 1994). The injection of naked DNA directly into fertilized mammalian oocytes (Brinster, 1985) as well as in zebrafish embryos (Stuart *et al.*, 1988; Culp *et al.*, 1991) has also resulted in the production of transgenic animals. Although it was previously mentioned that the likelihood of spontaneous entry of DNA is limited due to obstacles imposed by the cell, post-natal tissues such as the mouse skeletal muscle has proven to be an exception, and has also resulted in successful expression of the injected gene (Wolff *et al.*, 1990; Tripathy *et al.*, 1996). The mechanism of entry of these polynucleotides into the muscle cell is unknown, however muscle may be particularly suited to take

up and express polynucleotides because of its structural features (Wolff *et al.*, 1990) or other characteristics which are further considered in the discussion section.

Although gene therapy has enormous biomedical and clinical potential, there are major difficulties concerning vector-host interaction and gene transfer efficiency, in addition to an inadequate understanding of the gene transfer mechanism, and a lack of suitable controls (Orkin and Motulsky, 1995). The refinement of these obstacles, is currently under investigation using various *in vivo* transfection analysis. With the use of various reporter constructs, *in vivo* transfection techniques can be studied to determine their gene transfer efficiency, compatibility with hosts, or shed insight into the gene transfer mechanism in addition to benefiting *in vivo* protein expression studies. For example, the injection of plasmid vectors containing the chloramphenicol acetyl transferase (CAT) or the β -galactosidase gene is used to detect transfected cells (Wolff *et al.*, 1990), and can be used to a certain extent to determine the transfection efficiency. In addition, fusing a reporter gene such as glutathione S-transferase (GST) with a gene of interest can also be used to detect protein expression (Peters *et al.*, 1995). Fusion proteins with a foreign reporter gene, such as the bacterial gene *lacZ*, has also contributed to studies on promoter function or lineage analysis (Lin *et al.*, 1994).

Typically, transgene expression is identified by *in situ* hybridization or by histochemistry in fixed tissues. However, the inability to easily detect transgene expression in living animals severely limits the utility of this technology, particularly in lineage analysis (Long *et al.*, 1997). A new technique utilizing the

green fluorescent protein (GFP) as a reporter gene overcomes this limitation and enables transgene expression to be observed in living organisms (Chalfie *et al.*, 1994).

An *in vivo* transfection technique is used herein as a potential tool to study zebrafish fin regeneration by misexpressing a gene of interest in the regenerating tissue, and could thereby also contribute to the understanding that is required for gene therapy to evolve into a successful clinical tool.

Objectives

Investigating the regeneration mechanism using the zebrafish fin as a model organism has several advantages over the predominant urodele amphibian limb regeneration studies (Browder *et al.*, 1991). First, the adult zebrafish can regenerate its entire fin structure in a few weeks (Santamaría *et al.*, 1992), in comparison to the 6 to 10 weeks required for amphibian limb regeneration (Géraudie and Ferretti, 1998). In addition, the fact that one amputated zebrafish caudal fin consists of 18 dense blastema in synchronous development (Géraudie and Singer, 1992), in comparison to only one blastema per limb in amphibians, can be advantageous for control purposes. Zebrafish therefore represent a valuable model for studying the events leading to the formation of a complex body structure on a much larger scale than it is possible in amphibians (Ferretti and Géraudie, 1995). Secondly, the process of recruitment of cells from the stump tissues, that allows the formation of a large blastema and its subsequent growth, is less complex in the fin than in the limb

due to less differentiated tissues and the lack of striated musculature (Géraudie and Singer, 1992). Therefore, in contrast to the tetrapod limb, the relatively simple anatomy of the teleost tail fin renders this organ an advantageous appendage in which to study the regeneration process (Becerra *et al.*, 1996). A third advantage in using zebrafish over amphibians, is that the abundant molecular analysis done on zebrafish embryonic development can be used to help study the regeneration process in adult zebrafish (Laforest *et al.*, 1998).

The refinement of the zebrafish as a genetic system allows for large-scale genetic screens in a vertebrate, and the identification of mutations that affect the development of this organism (Mullins and Nüsslein-Volhard, 1993; Driever *et al.*, 1994). These mutants will give the opportunity to isolate and characterize genes which could be involved in the regeneration process (Géraudie *et al.*, 1998). Additionally, the production of a genetic linkage map of the zebrafish genome (Postlethwait *et al.*, 1994), and a panel of zebrafish/mouse somatic cell hybrids will help to isolate and clone the mutated genes.

For the purpose of this study, the following two objectives are the point of focus:

a. Cell Migration during Zebrafish Caudal Fin Regeneration

During urodele limb and teleost fin regeneration, epidermal cells migrate to form the wound epidermis (Brockes, 1997; Tsonis, 1996; Bullock, 1978; Mari-Beffa *et al.*, 1996a), a tissue that is critical for subsequent outgrowth of the regenerate (Thornton, 1957; Thornton and Thornton, 1965; Becerra *et al.*, 1996; Santoz Ruiz,

1996; Mari-Beffa, 1996b). Recent analysis of proliferating cells during zebrafish caudal fin regeneration demonstrates that the cells of the wound epidermis do not proliferate, thereby suggesting that the epidermal cells form the wound epidermis by active migration (Poleo *et al.*, submitted). In addition, urodele mesenchymal tissues proximal to the level of ablation contribute to the formation of the blastema (Wallace, 1981; Liversage, 1991) as well as in teleosts (Ferretti and Brockes, 1991; Becerra *et al.*, 1996; Géraudie and Ferretti, 1998). Recent observations also suggest that mesenchymal tissue proximal to the level of ablation in the zebrafish regenerating caudal fin may contribute to the blastema (Poleo *et al.*, submitted).

In order to substantiate the implied similarities between the zebrafish fin regeneration events to that of other teleosts and urodeles, we investigated the migration of cells to the epidermal cap as well as the contribution of cells located in the mesenchyme to the formation of the regenerate in the zebrafish. This objective would also help in correlating gene expression patterns with the early events of caudal fin regeneration.

b. *In Vivo* Transfection in the Regenerating Caudal Fin of Zebrafish

As previously outlined, the expression patterns of *sonic hedgehog* (*shh*) during larval fin development and caudal fin regeneration suggest a role in dermal bone formation (Laforest *et al.*, 1998). We hypothesized that misexpressing *shh* within the regenerating caudal fin using a gene delivery technique could further substantiate the role of *shh* in formation of the bifurcation of the dermal bone. Therefore the second objective was to develop a gene delivery technique to use as a

tool to study the function of genes expressed during caudal fin regeneration, and more specifically to acquire insight into the function of *shh* during fin regeneration.

Material and Methods

Animals

Adult zebrafish, *Danio rerio*, are purchased from a local supplier. Fish are maintained in a 20 gallon aquarium at 28.5°C with a photo period of 14 hours of light and 10 hours of darkness, and fed regularly. Embryos are obtained by combining 3 - 4 male and female zebrafish in breeding cages the evening prior to the morning collection. The breeding cages are composed of two stacked plastic mouse cages where the floor of the top cage contains many holes. This allows the fertilized eggs, upon being laid, to fall between the space of the two cages. The next morning, the embryos are collected shortly after spawning which is induced by the onset of the photo period. Embryos are maintained at 28.5°C in 100mm petri dishes and staged according to hours post fertilization (p.f.) (Westerfield, 1995).

Fin Amputations

Adult zebrafish are anesthetized by immersion in water containing 0.17 mg/ml tricaine (3-aminobenzoic acid ethyl ester, A 5040, Sigma). The caudal fin is then amputated using a scalpel and the fish are returned to their tank. An amputation is defined as a cut orthogonal to the ray that removes a portion of the fin, whereas a gash is an orthogonal cut completely through a ray or through the inter-ray tissue which does not remove any portion of the fin. In contrast, a vertical laceration is a cut parallel to the ray, within the inter-ray tissue between successive rays without

the removal of any portion of the fin, and starts at the most distal portion of the fin and terminates at various levels along the fin.

At different times of regeneration, depending on the experiment, the fins are re-amputated at a level proximal to the level of the first amputation and fixed in phosphate buffer saline (PBS) (pH 7.0), containing 4% paraformaldehyde (PFA) (Westerfield, 1995).

Microinjections into Regenerating Caudal Fins and Embryos

The microinjection of Di-I or plasmid DNA was performed using borosilicate capillary pipettes with an inner diameter of 0.94 millimeters and an outer diameter of 1.2 millimeters (Sutter Instruments, BF120-94-10). Capillaries are pulled using a Sutter Instrument co. model p-87 Flaming Brown micropipette puller, and filled by capillary action. Using a nitrogen pressured injection apparatus (Pneumatic Picopump model PV830 World Precision Instruments), a controlled amount of Di-I or plasmid DNA solution is introduced into the tissue by varying the pressure and duration of the pressured nitrogen.

For injections in zebrafish embryos, the microcapillary perforates the chorion and a 30nl injection of a 50ng/ μ l plasmid DNA solution is made directly into each blastomere of a 1-4 cell stage embryos.

For injection into the caudal fin, adult zebrafish are anesthetized and injections of the Di-I solution are made into the various tissues of the caudal fin, as described in the following section. On the other hand the plasmid DNA injections (using only

DNA in supercoiled form) are made into the blastema of the regenerating caudal fin, using the same injection method for the DiI labeling in the caudal fin.

Lipophilic Carbocyanine Dye (Di-I) Labeling and Observation

Di-I (1,1'-dioctadecyl-3,3,3',3'-tetramethyl indocarbocyanine perchlorate; Molecular Probes) was chosen as a cell marker to observe cell migration during zebrafish caudal fin regeneration for the following reasons: first, the carbocyanine dye has been used successfully to observe cell movements *in vivo* (Psychoyos and Stern, 1996; Golden *et al.*, 1997). In addition, the lipophilic carbocyanine dye is completely insoluble in water but soluble in lipid and organic solvents and therefore only inserts into the membranes of the cells adjacent to the injection site, and is not transferred between cells (Stern, 1990). The dye's hydrophobic properties thereby inhibit the diffusion of the dye that could be misinterpreted as cell movement. Once applied to cells the dye diffuses laterally within the plasma membrane resulting in staining of the entire cell and inhibits fluorescence quenching. Also of great importance is the fact that the dye exhibits very low cell toxicity (Haugland, 1996), thereby ensuring the observation of the development/regeneration process and not an adverse effect of the dye.

A 0.25% w/v stock solution of Di-I in ethanol is further diluted tenfold in a 45°C 0.3M sucrose solution prior to microinjection (as per Selleck & Stern, 1991; Psychoyos and Stern, 1996). The ethanol is used as a solvent to dissolve the Di-I crystals (Haugland, 1996) and the sucrose solution is thought to act as an osmolarity regulating agent (Serbedzija *et al.*, 1990).

A 30 millisecond 12 psi injection delivered a small bolus of dye solution in the desired region, whereupon a small pink dot is visible, labeling ~20-100 cells. However in certain experiments the amount of Di-I injected is increased or decrease as noted.

Adult zebrafish are anesthetized as previously described, placed on an agarose surface to prevent damage to the microcapillary, and then injected with the Di-I solution in various tissues of the caudal fin depending on the experiment. The injections are done i) into the epidermal and mesenchymal tissues in between 2 rays, ii) in the epidermal layers surrounding the ray or iii) in the mesenchyme within a ray. The potential adverse effects of the injection procedure itself are examined by comparing fins injected with the previously described Di-I solution with uninjected, saline injected and ethanol diluted tenfold in 0.3M sucrose injected fins. No morphological differences were obvious, thereby suggesting that Di-I exhibits very low, if any, cell toxicity (Haugland, 1996; Serbedzija *et al.*, 1990) in the zebrafish caudal fin.

Unless otherwise noted the injection is done 24 hours prior to inducing the amputation, laceration or gash, in order to maximize the Di-I staining of cells that will migrate. The caudal fin is then injured at various levels in relation to the Di-I staining, and allowed to regenerate under normal conditions (previously mentioned in *Animals* section) with limited lighting due to the sensitivity of Di-I to light and primarily to limit fish activity which inhibits proper regeneration. The lighting was limited by covering the fish tank with a black garbage bag.

To observe the Di-I injected fish fin, the fish is anesthetized and then placed in a 60mm petri dish and the fin is covered with a cover slip to minimize fin movement, and to flatten the surface of the fin which helps in maintaining the same depth of field when a picture is taken. Using a microscope equipped with fluorescein optics and a mounted camera (Di-I has a distinctive orange/yellow fluorescence under a fluorescein or rhodamine filter), photographs are taken every 24 hours following amputation to observe and determine the rates and distances of the migrating Di-I labeled cells. The rate of cell migration is determined by measuring the distance of the Di-I labeled cell that has migrated furthest from the Di-I injection site, and dividing this distance by the amount of time that has elapsed since inducing the amputation.

In some cases the regenerating caudal fin injected with Di-I is completely removed and sectioned, as per the mentioned protocol, for further analysis. Visual inspection of regenerating caudal fins, on sections, is sufficient to attribute the location of a Di-I labeled cell to a specific tissue.

To ensure the accuracy of the results, a Di-I injection experiment is conducted in a minimum of three separate fish at the same time, and this experiment is later repeated in different adult zebrafish.

Vectors and Plasmid Constructs

a. Vectors

Two different vectors are used to engineer a construct for the *in vivo* transfection of *shh* in the regenerating caudal fin. The first vector is the commercially available pEGFPC1 C-terminal protein fusion vector (Clontech #6084-1). This vector encodes a red-shifted variant of wild type Green Fluorescent Protein (GFP) which has been optimized for brighter fluorescence and higher expression in mammalian cells. GFP expression is under control of the ubiquitous cytomegalovirus (CMV) enhancer/promoter region (Turner and Weintraub, 1994). Sequences flanking GFP have been converted to Kozak consensus translation initiation site to further increase the translation efficiency in eukaryotic cells. A multi-cloning site (MCS) is inserted between the EGFP coding sequence and the SV40 poly A. The SV40 polyadenylation signal downstream of the EGFP gene directs proper processing of the 3' end of the EGFP mRNA. The vector backbone also contains an SV40 origin for replication in mammalian cells expressing the SV40 T antigen. A neomycin-resistance cassette, allows stably transfected eukaryotic cells to be selected using G418. A bacterial promoter upstream of this cassette (P_{amp}) expresses kanamycin resistance in *E. coli* and an *f1* for single-stranded DNA production. The entire vector sequence of pEGFPC1 is 4.7 kb.

The pCMV5 construct originated in the department of Molecular Genetics and Biochemistry at UT Southwestern Medical Center in Dallas TX. It is one of a series

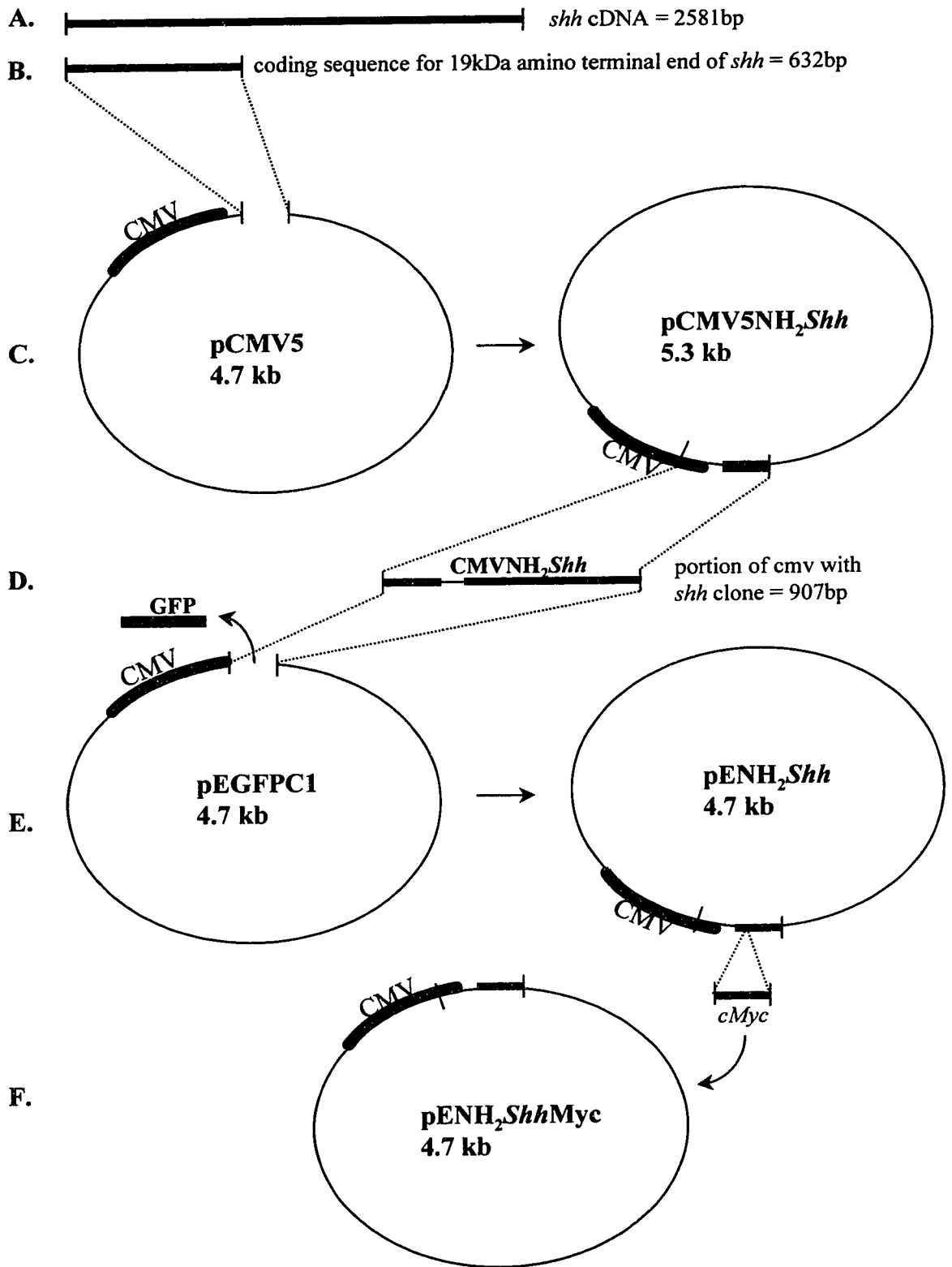
of 5 mammalian expression vectors which differ in polylinker sites and the presence/absence of a translational enhancer. Other than a different MCS, the pCMV5 has the same characteristics as the previously described pEGFPC1 vector, and the entire vector sequence is 5.7kb.

b. Plasmid Constructs

The zebrafish *Sonic hedgehog* (*shh*) cDNA clone was obtained from Vladimir Korzh (Roelink *et al.*, 1994). The amino terminus of the SHH protein contains the signaling capabilities of the full-length protein (Marti, 1995; Lee *et al.*, 1994b; Yang *et al.*, 1997). Therefore the 632bp cDNA region encoding the 19kDa N-terminal region of SHH, referred to as NH₂*Shh*, is used to generate two constructs that will ubiquitously express the amino terminus of the SHH protein in eukaryotic cells. To facilitate subcloning (Figure 10), the 632bp NH₂*Shh* fragment is originally cloned into the HindIII / SmaI site of the pCMV5 vector. The fragment is then excised at the SnaBI / SmaI sites, removing the original 632bp NH₂*Shh* sequence with an additional 275bp at the 5' end which codes for the 3' end of the ubiquitous CMV enhancer/promoter region, thereby producing the CMVNH₂*Shh*. This larger 907bp CMVNH₂*Shh* fragment is then subcloned into the SnaBI / SmaI site of the pEGFP vector, in which the corresponding 275bp of the CMV and the GFP with most of the MCS sequence had previously been removed, thereby producing the PENH₂*Shh*. The reason for using the backbone of the pEGFP vector to express SHH, is to minimize the differences observed between GFP expression and SHH expression to

Figure 10: Schematic of the pENH2*Shh* and the pENH2*ShhMyc* constructs.

(A) From the full length zebrafish *Sonic hedgehog* (*shh*) cDNA clone, (B) the 632bp cDNA region encoding the 19kDa N-terminal region of SHH, referred to as NH₂*Shh*, is used to generate two constructs that will ubiquitously express the amino terminus of the SHH protein in eukaryotic cells. (C) To facilitate subcloning, the 632bp NH₂*Shh* fragment is originally cloned into the HindIII / SmaI site of the pCMV5 vector. (D) The fragment is then excised at the SnaBI / SmaI sites, removing the original 632bp NH₂*Shh* sequence with an additional 275bp at the 5' end which codes for the 3' end of the ubiquitous CMV enhancer/promoter region, thereby producing the CMVNH₂*Shh*. (E) This larger 907bp CMVNH₂*Shh* fragment is then subcloned into the SnaBI / SmaI site of the pEGFP vector, in which the corresponding 275bp of the CMV and the GFP sequence had previously been removed, thereby producing the pENH₂*Shh*. (F) To detect the production of the 19kDa N-terminal region of SHH, 36bp encoding for the *c-Myc* is ligated into the CvnI site of the pENH₂*Shh* construct, which is located 96bp downstream of the NH₂*Shh* secretion signal (as per Bumcrot *et al.*, 1995), thereby generating the pENH₂*ShhMyc* construct. The *c-Myc* serves as an epitope tag allowing detection by the monoclonal antibody 9E10 (Evans *et al.*, 1985).



be due to backbone vector sequence. The final size of the pENH₂*Shh* construct is 4.7kb, identical to that of the pEGFPC1 vector.

To detect the production of the 19kDa N-terminal region of SHH, a *c-Myc* sequence is inserted into the pENH₂*Shh* construct. The *c-Myc* serves as an epitope tag allowing detection by the monoclonal antibody 9E10 (Evans *et al.*, 1985). Specifically, 36bp encoding for the *c-Myc* is generated by annealing two complementary oligonucleotide strands encoding for the 12 amino acid *c-Myc*. The *c-Myc* sequence is then ligated into the CvnI site of the pENH₂*Shh* construct, which is located 96bp downstream of the NH₂*Shh* secretion signal (as per Bumcrot *et al.*, 1995), thereby generating the pENH₂*ShhMyc* construct.

To ensure that the *c-Myc* sequence is in frame with the rest of the NH₂*Shh* sequence and that the NH₂*Shh* sequence was not altered during experimental manipulation, both the pENH₂*Shh* and the pENH₂*ShhMyc* constructs were sequenced.

c. Plasmid DNA Preparation

Plasmid DNA is prepared using the Qiagen midi plasmid purification protocol (Qiagen #12169) and is subsequently purified by equilibrium centrifugation in a cesium chloride-ethidium bromide continuous gradient, as described by Maniatis *et al.* (1989).

Cryostat Sectioning and Slide Mounting

Cryostat sectioning is used to obtain proximo-distal sections (Figure 4 and 5 along the proximo-distal plane of the as demonstrated in Figure 8G) of the regenerating caudal fins. Sectioning and mounting are performed as previously described by Westerfield (1995). After fixation, fins are embedded in a molten 50°C solution of 1.5% agar and 5% sucrose PBS. After the embedding solution hardens, a block containing the material to be sectioned is made with a scalpel. The block is then placed in a 30% sucrose PBS solution and stored at 4°C overnight (or until block has sunk in the sucrose solution). The block is mounted on the cryostat chuck by first preparing a raised platform by freezing a layer of OCT compound (Tissue Tek, Miles) with 2-methyl butane (27,034-2, Sigma) (-50°C) to the chuck. The agar block is then placed on the frozen layer of OCT compound, covered in OCT compound and frozen again with 2-methyl butane. The chuck and block are equilibrated to sectioning temperature at -25°C. Using the LKB cryostat, 16µm thick sections are cut and collected on glass slides. Slides used for observation under fluorescent microscope or immunohistochemistry are kept at -20°C before use.

If sections are to be stained with hematoxylin, a general purpose nuclear stain, they are placed in a filtered hematoxylin solution for 20 seconds, rinsed twice in distilled water and mounted as described below.

Slides are prepared for mounting by dehydrating for 5 minutes in a serial dilution of ethanol (20% EtOH; 40% EtOH; 60% EtOH; 80% EtOH; 90% EtOH;

2X 100% EtOH). Sections are cleared in two washes of xylene for 1 minute. Several drops of Cytoseal 60 mounting media (Stephens Scientific) are applied onto the tissue sections and a cover glass is laid over sections. Mounting media is allowed to dry overnight.

Transient Transfections in Cell Culture

Cells transiently expressing the 19kDa N-terminal region of SHH or the GFP, are generated using the following method. COS cells are transfected with 1 μ g of plasmid DNA (pENH₂*Shh*, pENH₂*ShhMyc* or pEGFPC1) per 60mm dish with 15 μ g/ml of lipofectamine reagent (Gibco BRL) in Dulbecco's Modified Eagle Medium (DMEM) (11995-065, Gibco BRL). After 6 hours, an equal volume of DMEM containing 20% fetal bovine serum (FBS) is added to the cells undergoing transfection, and the cells are left to incubate for 24 hours. Thirty hours after the start of transfection, the entire medium is replaced with fresh DMEM containing 10% FBS, and the cells are incubated for another 24h prior to immunocytochemistry. The transfection efficiency is determined after 24 hours to be 30% using the pEGFPC1 reporter construct.

To avoid losing cells during the washes incurred by the immunocytochemical detection process, cells are cultured in the following manner. Autoclaved cover slips are covered in a 1% gelatin (G8-500, Fisher Scientific) in 1X PBS solution, dried and placed into 60mm tissue culture petri dishes and rinsed with tissue culture media. A confluent culture of SHH expressing COS cells is then trypsinized (25300-

054, Gibco BRL) and plated in the petri dishes containing the gelatinized cover slips with DMEM with 10% FBS. Cells are cultured for 2 days or until cover slips are 90% confluent with adherent cells, then washed with 1X PBS.

Immunocytochemistry

The following two techniques are used to detect the production of the previously described pENH₂*Shh*Myc containing the 19kDa N-terminal region of SHH with a c-Myc epitope tag (*Plasmid Constructs* section).

a. In Cell Culture

When the transiently transfected cells, cultured on gelatin covered cover slips (as previously described in *Transient Transfection in Cell Culture* section), are 90% confluent, the cover slips are removed and fixed with 4% PFA for 0.5 hour. The cover slips are then placed in a 1X PBS solution containing 1M glycine (G 4392, Sigma) for 5 minutes. To avoid unspecific binding of antibodies, the cells are blocked with 5% Bovine Serum Albumin / Calf Serum (BSA/CS) and permeabilized in a 1X PBS solution containing 0.5% Triton X-100 (BP151-100, Fisher Scientific) and for 1 hour. The cover slips are then removed and incubated overnight at 4°C with a solution containing the mouse monoclonal 9E10 anti myc antibody diluted 1:500 in 3% BSA / 1X PBS. The following day, slides are rinsed 3 times with 1X PBS and then incubated for 60 minutes at room temperature with a solution containing the goat anti-mouse IgG antibody conjugated to fluorescein

isothiocyanate (FITC) (Jackson ImmunoResearch Laboratories, Inc.) in a 1:100 dilution with 3% BSA / 1X PBS. After 3 washes with a 3% BSA / 1X PBS solution, the sections are mounted using a solution of 50% glycerol, 50% PBS and immediately observed and photographed under the fluorescent light of a Zeiss microscope.

b. On Sections

The sections are permeabilized with 10 μ g/ml proteinase K for 10 minutes and then rinsed 3 times with PBS pH 7.5 before the sections are blocked, to avoid unspecific binding of antibodies, using a solution of 1X PBS, 0.1% BSA and 0.1% sodium azide pH 7.5 (PBS/BSA/NaN₃). The protein containing the Myc tag is immunocytochemically detected using a mouse monoclonal 9E10 anti Myc antibody diluted 1:500 in PBS/BSA/NaN₃. The slide is incubated overnight at 4°C in the diluted antibody solution, and then rinsed 3 times with PBS/BSA/NaN₃. The sections are then incubated for 60 minutes at room temperature with a goat anti-mouse IgG antibody conjugated to FITC (Jackson ImmunoResearch Laboratories, Inc.) in a 1:100 dilution in PBS/BSA/NaN₃. After 3 washes in PBS/BSA/NaN₃, the sections are mounted using a solution of 50% glycerol, 50% PBS and immediately observed and photographed under the fluorescent light of a Zeiss microscope.

Fluorescent Microscopy and Photographs

A Zeiss microscope equipped with epifluorescence optics (Fluorescein & Rhodamine optics set) and equipped with a mounted camera, is used to visualize and photograph the samples. Photographs of light samples are taken with Kodak Ektachrome Tungsten 160 ISA EPT 135-36 or 64 ISA EPT 135-36 color slide film. Photographs of fluorescent samples are taken using Kodak 400 ISA color slide film. Fluorescent whole mount caudal fin pictures require an average of 240 seconds of exposure, whereas fluorescent tissue sections or tissue culture samples only require an average of 60 seconds exposure.

Calculations

Statistical evaluation of the significance of the results, presented in the section discussing the green fluorescent protein expression in the regenerating caudal fin, is not possible using a standardized test format. Varying samples sizes eliminate the use of the Student's t-Test, and because sample sizes are not large enough, a population z-Test is not used (Weiss, 1995).

Results

I. Di-I Injections into Regenerating Caudal Fins

a. Migration of Cells within the Epidermal Layer after Amputation

To determine the basal level of cell migration, cells in non-regenerating caudal fins of adult zebrafish are labeled by injecting the lipophilic carbocyanine dye, Di-I, into the inter-ray tissue which is composed predominantly of epidermal cells (Figure 11). For these experiments, the tip of the microcapillary is introduced into the tissue pointing towards the distal end of the caudal fin (Figure 12A). A 30 millisecond, 12 psi injection delivers a bolus amount of dye at the injection site (Figure 12B, groups of cells identified as (b) and (c)). Increasing the pressure to 20 psi delivers a stream of dye that flows beyond the injection site and heads in the distal direction (Figure 12B, region delimited by square brackets). At 24 hour intervals, the labeled cells are observed to disperse at a rate of 80 μ m per day. This dispersion is most noticeable at three day intervals after the injection (Figure 12B-D, groups of cells identified (a), (b) & (c)). The fact that Di-I is hydrophobic and does not diffuse (Stern, 1990), suggests that the dispersion of the dye is a basal level of cell migration. All labeled cells, especially those that are labeled with lower amounts of dye, eventually lose their fluorescent stain. This is shown with the disappearance, 3 days after injection, of the labeled stream of cells in figure 12B, C (region delimited by square brackets). Although the lighting is minimized (as described in *Material and Methods* section) it is not eliminated. Therefore, one cannot rule out the possibility that the fading of

Figure 11: Transverse section of the caudal fin of an adult zebrafish.

Transverse cryosection through 3 fin rays. The inter-ray epidermal tissue (denoted by the large arrowheads) constitutes the major tissue in the inter-ray region, compared to the mesenchymal tissue (denoted by the arrows). The intra-ray mesenchyme is indicated by the asterisk. Open arrowhead indicates the hemiray portion of the lepidotrichia. Scale bar: 30 μ m.

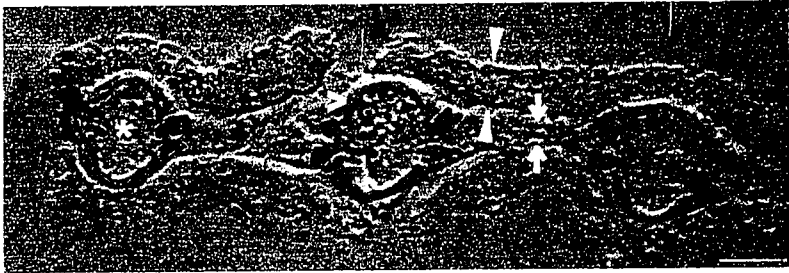
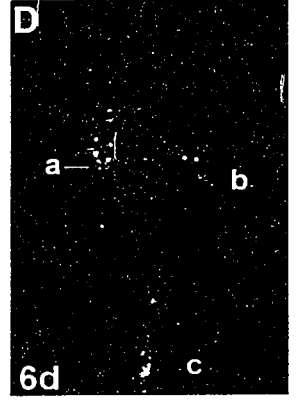
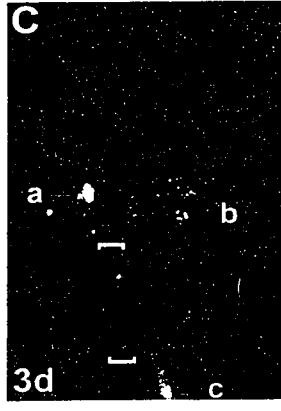
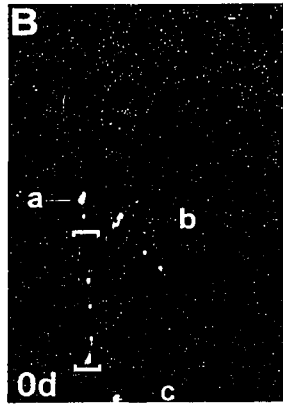
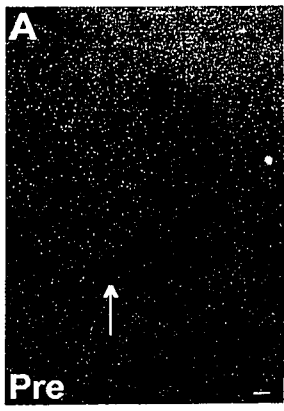


Figure 12: Basal level of cell migration in non-amputated caudal fin.

(A) Normal adult caudal fin is used for Di-I injections (arrow indicates direction of injection). (B) A 30 millisecond, 12psi injection delivers a bolus of dye at the injection site (groups of cells labeled (b) and (c)). Increasing the injection pressure to 20 psi, delivers a stream of dye ((a) and region delimited by brackets). (C) 3d after injection, the groups of cells labeled (a-c) occupy a dispersed pattern around the injection site, and the region delimited by the bracket is no longer a visible stream (compare with B). (D) 6d after injection, the groups of cells labeled (a-c) are not as intense as at 0d or 3d, and appear more dispersed around the region of injection. Scale bar: 80 μ m.

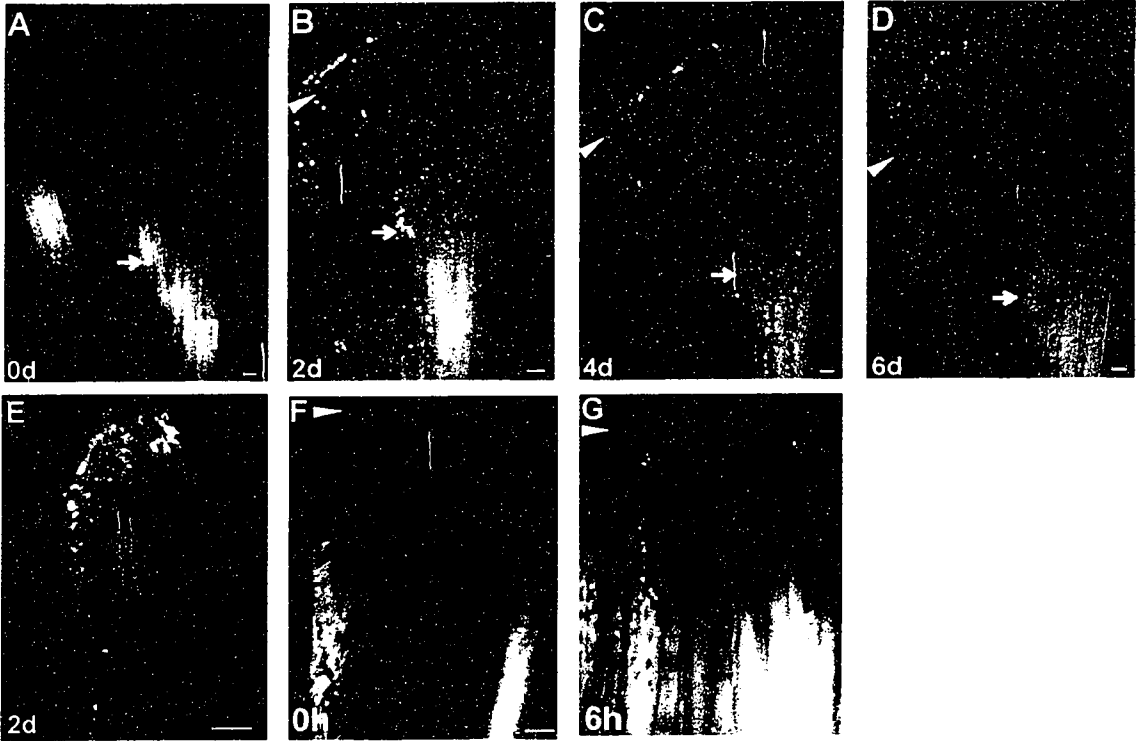


the dye could be due to the sensitivity of the dye to either the limited exposure to ambient light, or the fluorescent light when observing and photographing the fins. Both the basal level of migration and the fading of the dye were considered when observing Di-I labeled cells in amputated fins.

In order to examine the contribution of cells of the stump tissue to the regenerate, Di-I injected fins are amputated 24 hours after injection in the inter-ray tissue and observed at 24 hour intervals (Figure 13). In comparison to non-amputated controls (Figure 13A), the Di-I labeled cells in the amputated fins undergo an obvious dynamic response. First, there is a dramatic increase in the rate of migration of the labeled cells in the amputated fins (Figure 13A-D, compare the Di-I labeled cells relative to the arrow). In comparison to 80 μ m per day in the controls, migration rates as fast as 500 μ m per day, towards the level of amputation, until approximately 3 days are observed for labeled cells located at various distances from the plane of amputation (Figure 13A-D). Even the labeled group of cells located 1400 μ m from the level of amputation, migrate at a similar rate towards the level of amputation (Figure 13 A-C). Although the average migration rate of the quickest migrating cell is 500 μ m/day (or 21 μ m/h) towards the level of amputation during the first 3 days, a rate as fast as 33 μ m/h within the first 6 hours has been observed (Figure 13F, G), suggesting a non-linear migration rate of cells towards the level of amputation over time. In addition, there are distinctly different migration rates among cells, which suggests that there may be different cell types that migrate upon amputation of the caudal fin.

Figure 13: Cell migration in the regenerating caudal fin.

(A) Caudal fin injected with Di-I, at various levels on the proximo-distal axis, 24h before amputation. The arrow is a reference point and the bracket denotes group of labeled cells located at 1400 μ m from the future level of amputation. (B) 2d after amputation, labeled cells have migrated in a distal direction, at a rate of 500 μ m/day. Cells located closer to the level of amputation accumulate in the region forming the epidermal cap whereas cells labeled 1400 μ m from the level of amputation are still moving in a distal direction (as noted by comparing labeled cells to arrow in A and B). (C, D) 4d after amputation, the epidermal cap is highly labeled in the region where the labeled cells were located closer to the level of amputation. The cells that were at 1400 μ m from the level of amputation are also still migrating distally, however no labeled cells originating from this group of labeled cells is noticed in the epidermal cap. (E) Proximo-distal section of a 2d regenerated caudal fin injected with Di-I 24h prior to amputation. Labeled cells are observed in the region forming the epidermal cap and the lateral epidermis. (F, G) Cell migration is observed by injecting a caudal fin with Di-I 24h prior to amputation. (F) Amputation is induced at 350-500 μ m from the Di-I stained cells. (G) 6h after amputation, labeled cells have migrated distally towards the level of amputation. Large arrowhead indicates level of amputation. Scale bar: 80 μ m, E, 30 μ m.



Proximo-distal sections performed on fins 2 days after amputation, revealed that only labeled cells are found in the epidermal cap (Figure 13E), suggesting a migration of the cells along the lateral epidermis. This observation confirms the results of cell proliferation analysis using bromodeoxyuridine (BrdU) (Poleo *et al.*, submitted), which showed that 6 hours after amputation, a time during which epidermal cells contribute to the wound epidermis, there is no DNA synthesis and no proliferation/mitoses in the cells of the wound epidermis or in the lateral epidermis. Cell proliferation is only observed starting at 24h after amputation in cells of the lateral epidermis proximal to the plane of amputation, whereas the cells in the epidermal cap rarely divide (Figure 5) (Poleo *et al.*, submitted). The complementation of these results demonstrates that epidermal cells actively migrate to the region forming the epidermal cap, before the onset of proliferation.

The migration of epidermal cells from various distances towards the level of amputation is observed to occur over the course of several days (2-3d) despite the fact that the epidermal cap is formed within 24h. However, knowing that epidermal cells are critical to the regeneration process (Tsonis, 1996; Géraudie and Ferretti, 1998; Becerra *et al.*, 1996), we thought that the epidermal cells were continually migrating to the epidermal cap, even after it had been formed. To determine if this was the case, caudal fins are amputated and allowed to regenerate for 1, 2, 3 and 4 days. One day prior to observation, the fins are injected with similar amounts of dye within 200-600 μ m from the amputated region, a level from which cells have been observed to contribute to the epidermal cap. It is observed that cells located in the

lateral epidermis (sections not shown) contribute to the wound epidermis only within the first two days after amputation (Figure 14). Although the cells no longer contribute to the epidermal cap after 2 days, migration towards the level of amputation is still observed after this time point as previously demonstrated in figure 13C and D.

Once arrived at the epidermal cap, the labeled epithelial cells migrate in the epidermal cap towards the lateral fin rays (Figure 14B and D). It is important to remember that the apparent movement of cells towards the lateral sides of the fin is not due to proliferation, since the cells of the epidermal cap do not divide.

b. Observation of Labeled Mesenchymal Cells

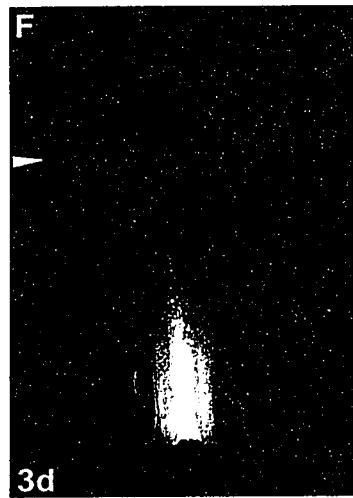
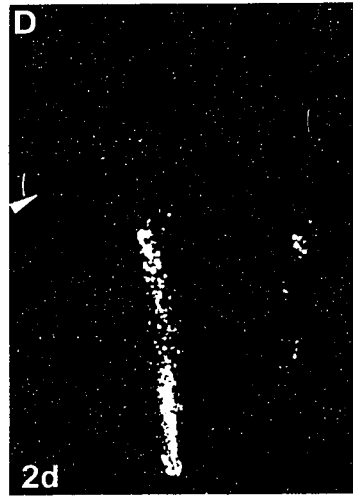
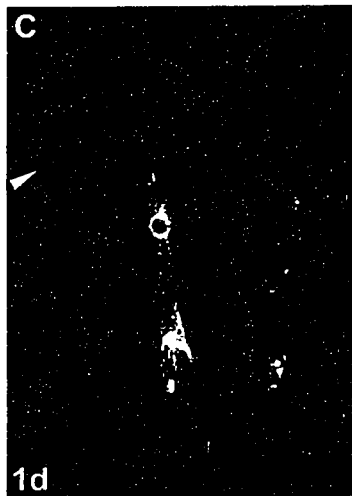
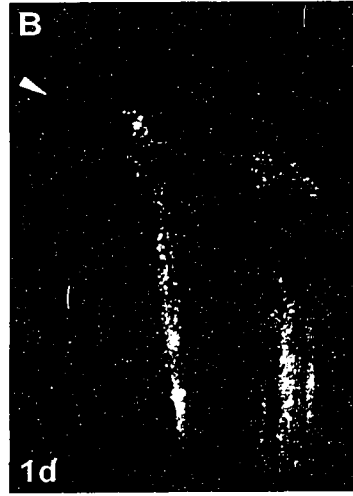
In urodele amphibians, all stump tissues, except the epidermis, are source of progenitor cells for the formation of the blastema (Wallace, 1981; Liversage, 1991; Ferretti and Brockes, 1991). Similarly, it is thought that in fish, the blastema is formed from the division of preexisting mesenchymal cells (Brockes, 1997; Géraudie and Ferretti, 1998). Cell proliferation analysis in regenerating caudal fins, using BrdU, has shown a high level of proliferation in mesenchymal cells at the level of amputation, suggesting that the cells in the mesenchyme might also contribute to the blastema in the regenerating caudal fin (Poleo *et al.*, submitted). To substantiate the possible contribution of these mesenchymal cells to the blastema, Di-I injections were performed within the intra-ray tissue 24 hours prior to amputation. When the Di-I is injected into the mesenchymal region within the intra-

Figure 14: Cell migration towards the epidermal cap.

Caudal fins are amputated and allowed to regenerate for 1, 2, and 3 days. One day prior to observation, the fins are injected with similar amounts of dye in the inter-ray tissue at 200-600 μ m from the amputated region. (A) 2 groups of cells labeled with Di-I in a non amputated fin. (C) and (E) are injected 1 and 2d after amputation, respectively. (B) 1d after amputation of fin shown in (A), the labeled cells have migrated to the epidermal cap. Note that the labeled cells within the epidermal cap are migrating towards the lateral side of the fin (towards the large arrowhead). (D) Same fin as in (C) but one day later. Labeled cells are also migrating to the epidermal cap during the 2nd day of regeneration, and as in (B) the cells in the epidermal cap are also migrating towards the lateral side of the fin (away from large arrowhead). (F) Same fin as in (E) but one day later. No labeled cells are contributing to the epidermal cap after the 2nd day of regeneration. Large arrowhead indicates level of amputation. Scale bar: 80 μ m.

Right after
Di-I injection

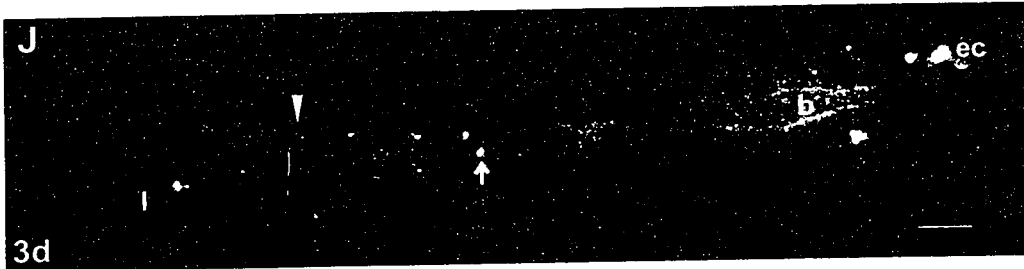
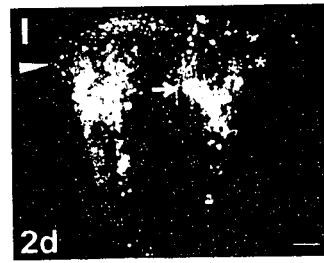
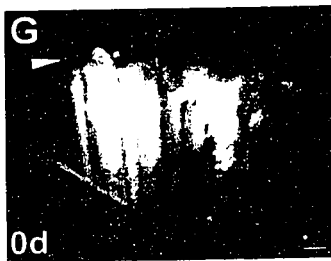
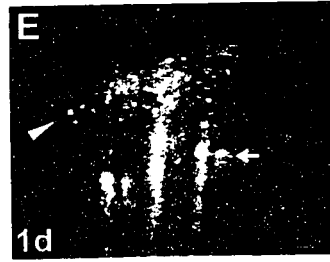
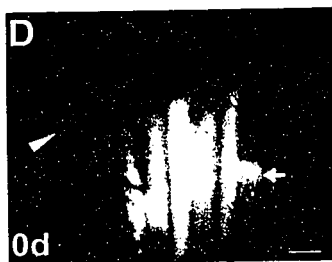
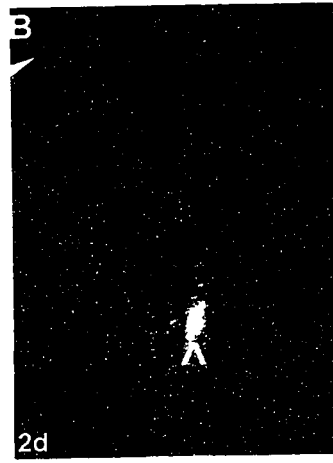
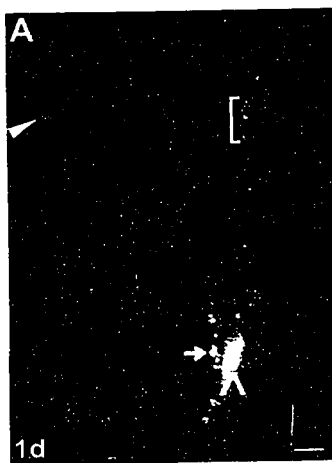
1 day after
Di-I injection



ray tissue at 800 μ m (Figure 15A-C) from the level of amputation, no migration of labeled mesenchymal cells towards the level of amputation is observed during the 6 days following amputation. Similarly, no obvious migration of labeled mesenchymal cells is observed when Di-I labeled cells are 250 μ m from the level of amputation (Figure 15D-F). To investigate the possibility that only the mesenchymal cells underlying the level of amputation give rise to the blastema in the regenerating tissue, the amputation was made within 80 μ m of the labeled mesenchymal cells (Figure 15G-I). However the observation of mesenchymal cell migration in whole mount is no longer possible when using Di-I at such a close distance to the level of amputation, since the method of Di-I injection labels both the inter-ray and intra-ray cells, and the epidermal cell migration thereby impedes the observation of the intra-ray mesenchymal cells. Proximo-distal cryosections eliminate this obstruction, however no labeled cells are found in a 3day regenerating blastema (Figure 15J). Because the blastema is a highly proliferative tissue it is possible that the mesenchymal labeled cells contributed to the blastema, but the fluorescence is diluted among the dividing cells thereby decreasing the intensity of the fluorescent labeling. However, labeled mesenchymal cells are located in the regenerate below the blastema (Figure 15J), suggesting that mesenchymal cells could have contributed to the early blastema formation. Therefore, further experiments should observe proximo-distal sections made at 1 or 2 days after amputation, in order to minimize the dispersion of the Di-I among the proliferating

Figure 15: Observation of labeled mesenchymal cells.

The mesenchymal tissues of the intra-ray are labeled with Di-I 24h prior to amputations. (A-C) Amputation is induced 800 μ m from the labeled cells. (A) 1d after amputation, the cells labeled in the adjacent inter-ray tissue (arrow) and epidermal tissue surrounding the ray (bracket) migrate in a distal direction, whereas the labeled intra-ray mesenchymal cells do not migrate (open arrowhead). (B) 2d and (C) 6d after amputation, the labeled epidermal cells have completely migrated to the epidermal cap, whereas the labeled intra-ray cells did not move. (D-F) When an amputation is induced 250 μ m from the labeled intra-ray cells, no cells from the intra-ray are observed to migrate 1d (E) and 2d (F) after amputation (arrow indicates labeled intra-ray cells). (G, H) When an amputation is made 80 μ m from the labeling site, no observation of migrating intra-ray cells on whole mount is possible using this experimental approach because the migration of epidermal cells to the epidermal cap impedes the observation of the intra-ray cells (asterisk in I), (arrow indicates level of labeled intra-ray cells). (J) Proximo-distal section performed on a 3d regenerated fin that had been amputated 80 μ m from labeled intra-ray cells, reveal no contribution of intra-ray cells to the blastema (arrow indicates labeled intra-ray cell in the regenerated tissue). Large arrowhead indicates the level of amputation. b, blastema; ec, epidermal cap; l, lepidotrichia. Scale bar: A-C and D-I 80 μ m; J, 30 μ m.



cells in the blastema.

With respect to the contribution of mesenchyme cells of the inter-ray tissue to the regenerate, labeled mesenchymal cells of the inter-ray tissue at a distance of 100 μm or more from the level of amputation, do not migrate during the regenerative process (not shown).

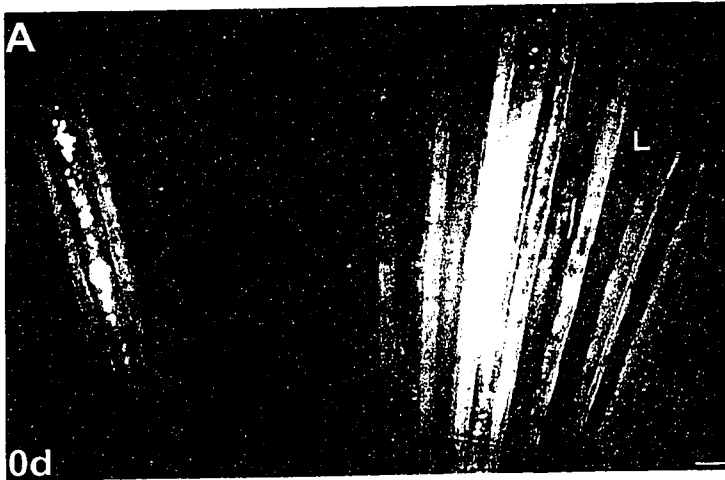
c. Investigating the Initiation of the Increase in Cell Migration

The dramatic increase in the rate of cell migration in the lateral epidermis is initiated by the amputation of the caudal fin. The amputation therefore triggers a signal(s) that is able to initiate cell migration within 6 hours of amputation, and can act over an extensive distance in the fin. To investigate the specific component of an amputation which initiates the increase in cell migration, Di-I labeled cells are observed after inducing various types of injuries on the caudal fin.

When a partial amputation of the fin is induced, removing only the 5 most lateral rays (Figure 16A), cells labeled in the lateral epidermis below the level of amputation and within 3-4 rays adjacent to the wound undergo an increase in migration rate (Figure 16C). In contrast, cells located at 6 or more rays from the partial amputation (Figure 16A) do not undergo an increase in cell migration after 2 days (Figure 16B). These results demonstrate that the initiation signal(s) for the increase in cell migration is induced by a partial amputation, and does not increase the rate of cell migration throughout the entire fin, but is specific to a region within at least 5 rays adjacent to the partial amputation.

Figure 16: Cell migration after partial amputation of the fin.

(A) 2 groups of cells in the inter-ray tissue are labeled on the ventral and dorsal lobes of a caudal fin, respectively. One day after Di-I injection, a partial amputation of the 5 lateral most fin rays (denoted by the “L”) is performed on one side of the fin. (B) and (C) Cell migration is observed 2 days after partial amputation was made. (B) Labeled cells in the inter-ray region of the lobe which has not been amputated do not show sign of migration. (C) Labeled cells in the inter-ray region close to the amputation are observed to have migrated distally and towards the wound. Scale: 80 μ m.



To narrow down the region of the wound which is responsible for initiating the increase in cell migration, 2 Di-I injections are made at the same level in the inter-ray tissue, one on the ventral and another on the dorsal lobe of the same caudal fin (Figure 17). At 24 hours after injection, a vertical laceration is induced in the ventral lobe (Figure 17A, B) and a partial amputation is induced in the dorsal lobe (Figure 17C) of the caudal fin. Although a vertical laceration initiates cell migration above basal levels (Figure 17D) 1d after injury, it does not initiate as rapid and as prolonged a migration as the amputation of a fin ray (Figure 17F). The migration of cells, underlying the amputation, continue to migrate to the epidermal cap at 2 days after amputation (Figure 17H). In comparison, the labeled cells below the vertical laceration are no longer observed to migrate at a rate above basal levels (Figure 17G). These results suggest that the initiation of cell migration is induced not simply by a wound, but a wound embodying the fin ray or the removal of tissue. However, the fact that a vertical laceration is healed within 24 hours (Figure 17B and E) must be taken into consideration as a factor which might affect the duration of cell migration, since a laceration does not undergo any subsequent tissue restoration, unlike an amputation. It is therefore possible that the cues required for tissue restoration could also initiate the intense cell migration.

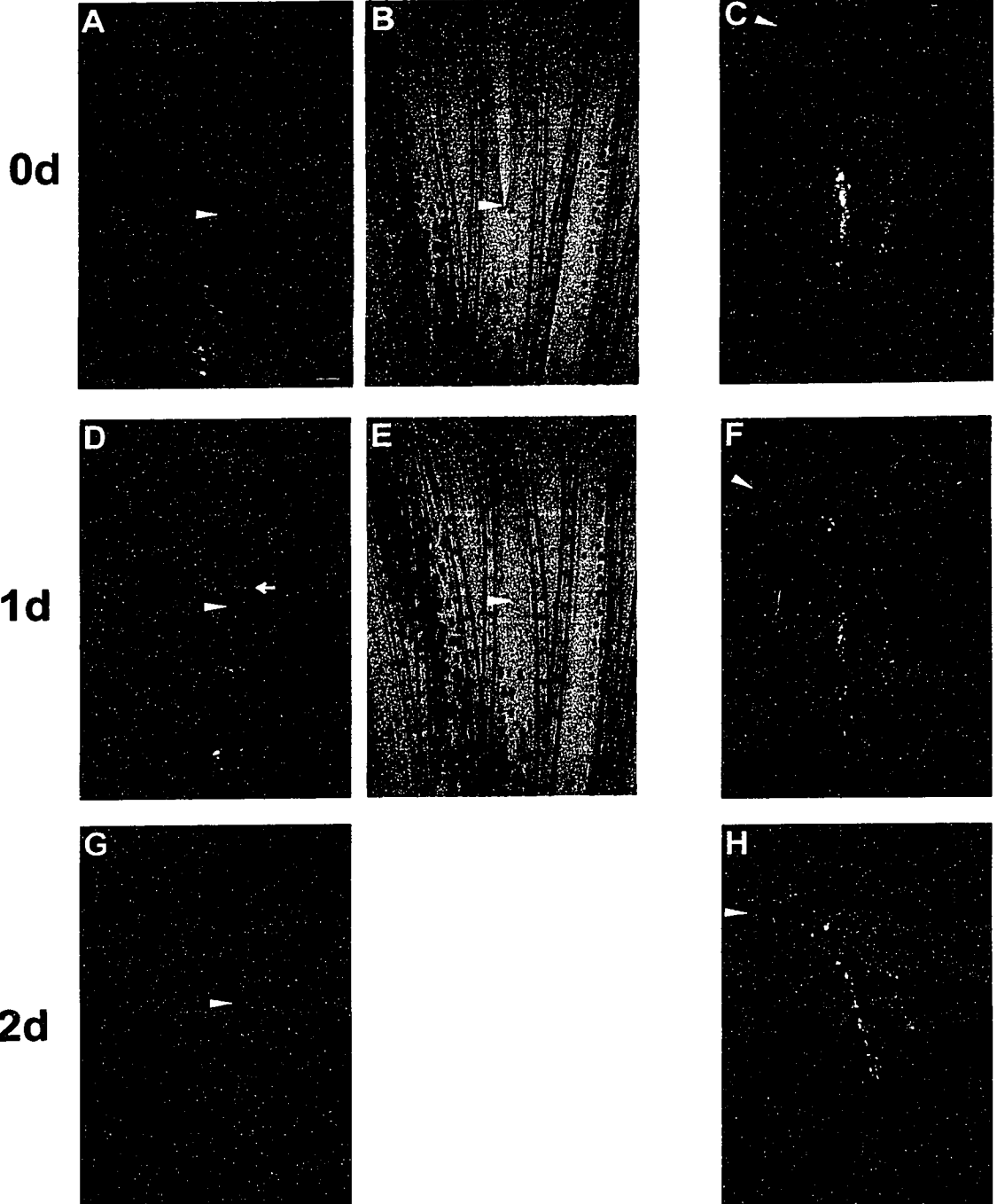
To investigate the role of the ablation of a fin ray in initiating the increase in intense cell migration, the effects of a gash (cut orthogonal to the ray) within one fin ray and within the inter-ray tissue are compared. Di-I is injected in several inter-ray regions at the same proximo-distal level. 24 hours after injection, gashes are

Figure 17: Wound encompassing the fin ray initiates intense cell migration.

Two groups of cells in the inter-ray tissue are labeled, at the same proximo-distal level, on the ventral and dorsal lobes of a caudal fin, respectively. (A, C, D, F, G, H) Photographed under fluorescent light. (B, E) Bright field image. (A, B) 1d after Di-I injection, a vertical laceration is induced in the ventral lobe (arrowhead indicates the proximal limit of the laceration), and (C) a partial amputation is induced in the dorsal lobe at approximately the same proximo-distal level from the labeled cells as the limit of the vertical laceration. (D) 1d after injury, a few labeled cells in the ventral lobe migrate to the region of the vertical laceration (arrow) which has completely healed at this time (E). (F) In comparison, many labeled cells underlying the partial amputation have migrated to the epidermal cap. (G) 2d after injury, labeled cells in the ventral lobe are no longer migrating above basal levels, (they are shown here using a rhodamine filter which is more sensitive to Di-I fluorescence). (H) Labeled cells underlying the partial amputation in the dorsal lobe are still migrating to the epidermal cap, 2d after injury. Large arrowhead indicates level of injury. Scale bar: 80 μ m.

Vertical Laceration

**Control
Amputation**



induced in individual fin rays (intra-ray) on the ventral side of the fin (Figure 18A, B) and another gash is induced in the inter-ray region on the dorsal side of the fin (Figure 18C, D). Both types of gashes are induced at the same distal level to the Di-I labeled cells in the fin. After 1 day of regeneration, there is an intense cell migration towards the gash induced in the ray (Figure 18E), unlike towards the gash induced in the inter-ray tissue (Figure 18G). Although this supports the correlation between a wounded ray and an increase in intense cell migration, the fact that a gash in the inter-ray tissue heals rapidly and does not undergo further tissue restoration, could slow down the migration of cells towards the inter-ray gash. In addition, once the cells have migrated to the region of the gash induced in the ray (Figure 18E), they do not remain in the wounded region but continue to migrate distally beyond the wound at a rate above basal levels as observed at 2 and 4 days (Figure 18I, J), a result that is not observed when a gash is induced in the inter-ray tissue (Figure 18K, L). These results suggest that the distal migrating epidermal cells do not necessarily play a role in tissue repair, since they continue to migrate beyond the distal wound, and may therefore be intrinsic to the regeneration process as considered in the *Discussion*.

In all the above results, the only direction in which the Di-I labeled cells migrate is towards the distal end of the fin. To determine if this is in fact the default direction, one gash is made, within a ray, distal to the Di-I labeled cells located in the adjacent inter-ray, and another gash is induced in the adjacent ray proximal to the Di-I labeled cells in the inter-ray tissue (Figure 19A, B). Results reveal that the

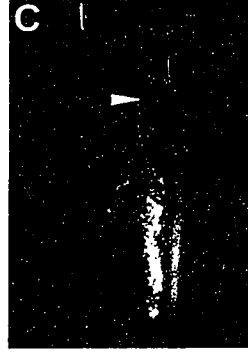
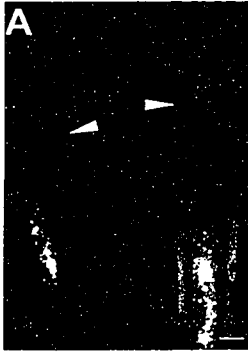
Figure 18: Wound embodying the fin ray initiates the intense cell migration.

Di-I is injected in several inter-ray regions at the same proximo-distal level in the caudal fin. (A, C, E, G, I, J, K, L) Fluorescent images and (B, D, F, H) bright field images. (A, B) 24h after injection, two gashes (denoted by large arrowheads) are induced within two separate fin rays at a level distal to the site of the Di-I labeled cells. (C, D) A gash is induced in an inter-ray region at the same distal level to the Di-I labeled cells. (E-H) 1d after injury, a number of labeled cells have migrated to the gash induced within the ray, as well as in the inter-ray tissue albeit not as obvious as the migration towards the gash induced within the ray. (I, J) 2d and 4d after injury, respectively, the labeled cells have migrated to and beyond the gash induced within the ray. In contrast, (K, L) 2d and 4d after injury, respectively, the labeled cells underlying the region of the gash in the inter-ray tissue are no longer migrating. Scale bar: 80 μ m.

Intra-Ray Gash

Inter-Ray Gash

0d



1d

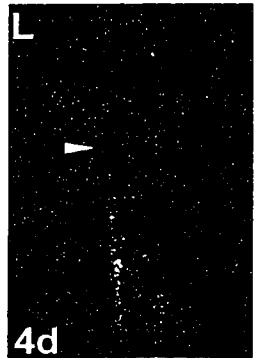
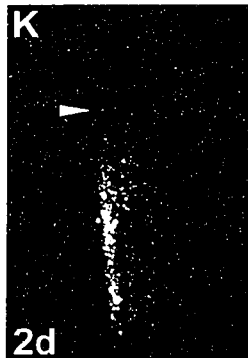
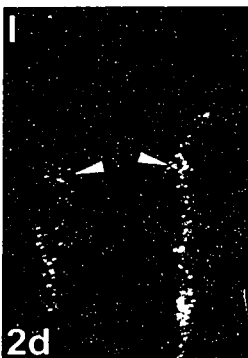
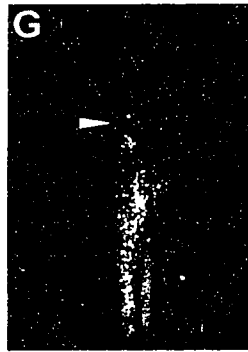
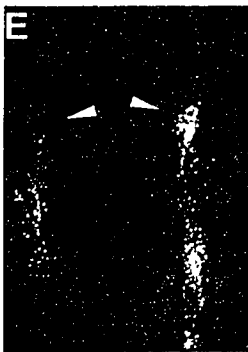
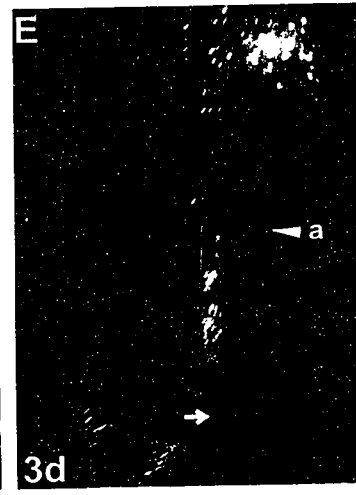
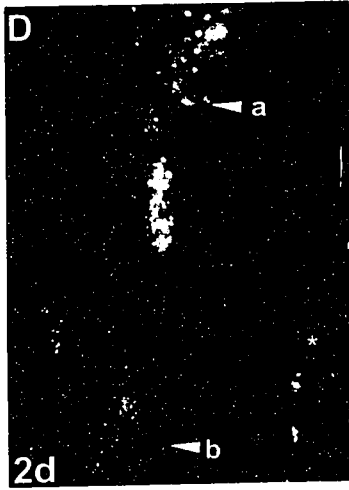


Figure 19: Cell migration is not only for wound healing.

(A) Light field image and (B, C, D, E) fluorescent images of the same caudal fin. 24h after injecting Di-I into the inter-ray tissue of the caudal fin, (A, B) a gash is induced within a ray distal to the labeled cells (arrowhead (a)), and another gash is induced in the adjacent ray proximal to the labeled cells (arrowhead (b)). Control injection is indicated by asterisk. (C) At one day after injury, the majority of the cells migrate in the distal direction towards the distal gash, whereas only a few cells (denoted by arrow) migrate towards the proximal gash. (D, E) At 2 and 4d, respectively, the cells which migrated toward the distal gash continue to migrate distally beyond the gash at a rate of $500\mu\text{m}/\text{day}$, whereas only a few cells have migrated towards the proximal gash (arrow in E) at a rate of $200\mu\text{m}/\text{day}$ for only a 24h period. Note that labeled cells to the left of arrowhead (b) arise from a more proximal injection and should be neglected. Scale bar: $80\mu\text{m}$.

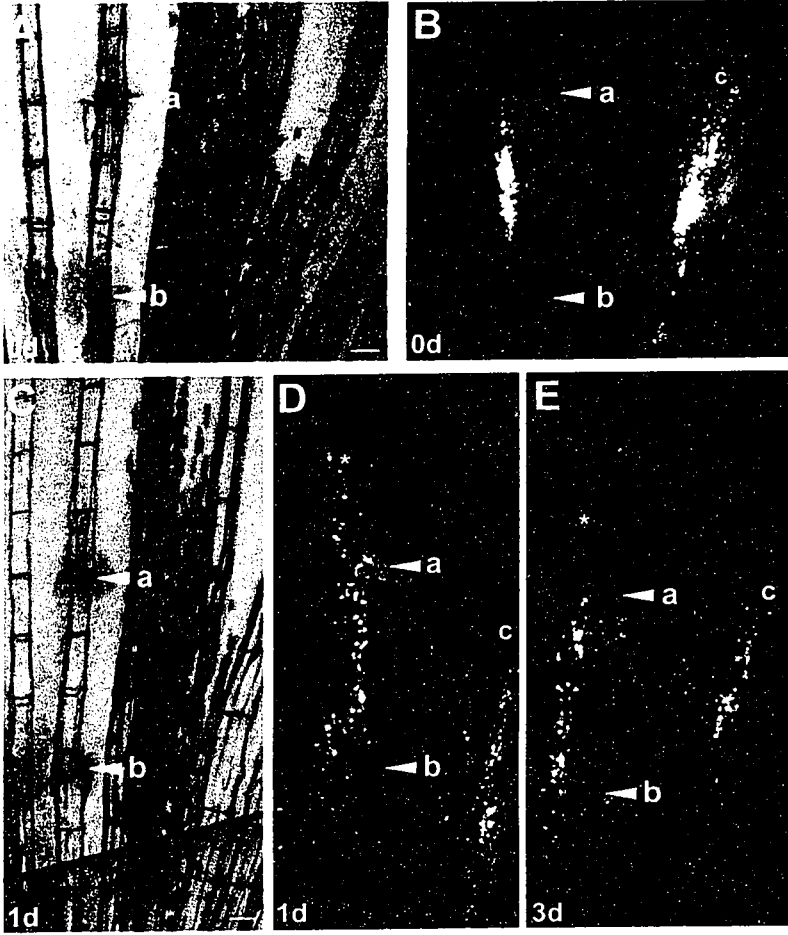


labeled cells start to migrate both towards the proximal (Figure 19C, arrow) and distal (Figure 19C, arrowhead (a)) gashes. However, only a few cells migrate in the proximal direction at a rate of 200 μ m/day, then stop after 24 hours and do not reach the distal wound (Figure 19 D, E). In contrast, many cells migrate towards the distal gash at a rate of 500 μ m/day and continue to migrate beyond the distal gash despite the absence of a more distal wound (Figure 19D, E). These results demonstrate that the majority of epidermal labeled cells migrate in the distal direction, and as previously observed, the migration of epidermal cells after injury are not only required for the wound healing process since they continue to migrate beyond the distal wound. Note that although the labeled cells are closer to the distal gash (100 μ m) than the proximal gash (250 μ m) in Figure 19B, the same experiment has been repeated at closer distances, and the results show that the distance of the gash from the labeled cells does not affect the number of migrating cells in the distal direction (not shown).

It has been shown that the nerve supply is critical for fin regeneration (Goss and Stagg, 1957; Becerra et al., 1996), and in the caudal fin, the nerve bundles are located within the intra-ray mesenchymal tissue (Becerra et al., 1983; Chandross and Bear, 1979). To investigate the possible role of the nerve bundles on initiating the intense increase in cell migration during regeneration, two gashes are induced within one ray proximally and distally, respectively, from a group of Di-I labeled cells located in the adjacent inter-ray (Figure 20A, B). The anterior gash at the base of the ray is to sever the nerve traversing through the rest of the ray, and the

Figure 20: Severed nerve in the fin ray does not impede migration of cells.

(A, C) Light field image and (B, D, E) fluorescent images of same caudal fin into which Di-I is injected in the inter-ray tissue. (A, B) 24h after injection, two gashes are induced within a ray, one above (arrowhead (a)) and one under (arrowhead (b)) the Di-I labeled cells. (C, D) 1 day after injury, the majority of the labeled cells migrate towards the distal gash and beyond (asterisk), whereas only a few labeled cells migrate towards the more proximal gash. These results are more pronounced at 3d after injury (E). Control injection is denoted as (c) in D and E. Scale bars: A, B, 80 μ m; C-E, 80 μ m.



posterior gash is to observe the response of the Di-I labeled cells. The migration of these cells was compared to the migration of control labeled cells located in the inter-ray tissue between 2 non-injured fin rays. Results demonstrate that the Di-I labeled cells distal to the severed nerve still migrate. Although some migrate towards the proximal gash at a rate of $200\mu\text{m}/\text{day}$ for only a 24 hour period (as previously shown in Figure 19), the majority migrates towards the distal gash at a rate of $500\mu\text{m}/\text{day}$ (Figure 20D) and even beyond the distal gash (Figure 20E), thereby demonstrating that severing the nerve within the ray did not inhibit the migration of the cells towards the injured regions. Although the nerve is severed, this does not eliminate the possibility that it can regenerate, and thereby initiate the intense cell migration. This possibility is further considered in the discussion.

II. In Vivo Transfection of the Regenerating Caudal Fin

The ability to introduce a gene of interest *in vivo* has been advantageous for studies in molecular biology, and in this study we are interested in producing or introducing SHH in the bifurcating region of the regenerating zebrafish fin. Of the methods available to introduce the SHH in zebrafish fins, bead implantation was ruled out because the bead does not remain in the tissue for a prolonged period due to the constant motion of the fin which dislodges the bead (further methods are described in *Expressing the Green Fluorescent Protein in the Regenerating Caudal Fin* section of the discussion). Of the remaining *in vivo* transfection techniques available, liposomal transfections did not show consistent results, and so the injection of naked plasmid DNA was chosen to try and express the gene of interest in the zebrafish regenerating caudal fin.

a. Green Fluorescent Protein Expression in Regenerating Caudal Fins

To develop a gene delivery technique as a tool to study the function of genes expressed during caudal fin regeneration, the pEGFPC1 expression vector containing the gene coding for the green fluorescent protein (GFP) is directly injected into both regenerating and non-regenerating caudal fins. Plasmid solutions at different concentrations ranging from 3 to 500ng in either a volume of 10 or 30nl, are injected into the interstitial space of the blastema of the regenerating caudal fin. In non-regenerating fins, which do not have a blastema, the solution is injected into

the tissue of the inter-ray region. Using fluorescent microscopy, GFP expression is detected *in vivo* as early as 12 hours after plasmid DNA injection into the blastema of 2 to 5 day regenerated caudal fins (not shown), whereas no fluorescence is detected in the non-regenerating fins. Examples of *in vivo* observations of transfected cells expressing GFP 24hr after injection of the pEGFPC1 plasmid are shown in figure 21A-C. These results demonstrate that unlike the unamputated fin, the tissue of the regenerating fin is susceptible to *in vivo* transfection of plasmid DNA. Cryosections of such fins reveal that the cells expressing GFP are located in the blastema (Figure 21D, E). In addition, the number of cells expressing GFP in the regenerating fins maximizes 24 hours after an injection into the blastema of a 3 day regenerated fin (Table 1, Figure 22).

When a blastema of a 3d regenerated fin is injected with pEGFPC1, the cells that express GFP closer to the level of amputation maintain their location and remain fluorescent for as long as one week (Figure 23). In comparison, the cells further distal from the level of amputation move distally and lose their fluorescence more rapidly (Figure 23). A possible reason for the loss of GFP expression in the more distal blastemal cells could be due to the proliferative nature of the blastemal cells, which when undergoing cell division dilutes the pEGFPC1 plasmid among daughter cells. However, dividing the plasmid among cells should increase the number of GFP expressing cells, a characteristic which is not observed, thereby suggesting that another mechanism is responsible for the more transient GFP expression in the distal blastemal cells.

Figure 21: The blastema of a regenerating caudal fin is susceptible to GFP plasmid DNA transfection.

(A-D) Observation of cells expressing GFP fluorescence 24 h after injection of the pEGFPC1 plasmid into the blastema of 3 day regenerating fins. (E) Bright field image of proximo-distal cryosection in (D). (A-C) Whole mount observation of the fin. The regenerating tissue is sensitive to varying concentrations of injected GFP plasmid DNA: (A) 100ng, (B) 50ng and 100ng ((a) and (b) respectively), (C) 50ng injections. (D, E) Proximo-distal cryosections, performed 24 hours after the blastema of a 3 day regenerated fin is injected with 100ng of GFP plasmid DNA, demonstrate that the cells located in the blastema express GFP. Large arrowhead indicates level of amputation. ec, epidermal cap; b, blastema. Scale bars: A-C, 80 μ m; D, E, 30 μ m.

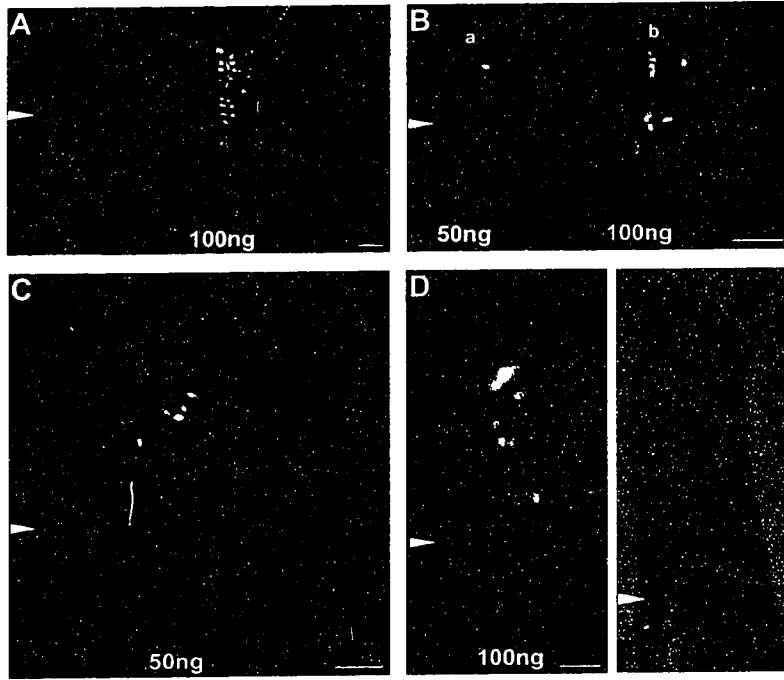


Table 1: Average number of fluorescent cells over time, in relation to amount of DNA injected into the blastema of a 3d regenerated caudal fin.
The number of fluorescent cells are counted at 0h, 24h, 48h and 72h after injection into the blastema of a 3d regenerated caudal fin.

Amount of DNA injected (ng)	n	Average number of fluorescent cells at the indicated time of observation				
		0h	24h	48h	72h	
3	15	0	6	6	6	
10	19	0	3	2	n/a	
20	8	0	10	8	8	
50	14	0	6	7	n/a	
70	2	0	4	n/a	n/a	
100	15	0	15	16	n/a	
120	9	0	4	3	3	
170	12	0	2	2	2	
200	12	0	13	n/a	n/a	
350	1	0	5	n/a	n/a	
500	12	0	6	n/a	n/a	

n=number of experiments

n/a=not available due to loss of GFP fluorescence

Figure 22: Average number of fluorescent cells over time, in relation to amount of GFP plasmid DNA injected into the blastema of 3d regenerated caudal fins.
n=number of experiments.

Average Number of Fluorescent Cells over Time, in Relation to Amount of GFP Plasmid DNA Injected into the Blastema of 3 Day Regenerating Caudal Fins.

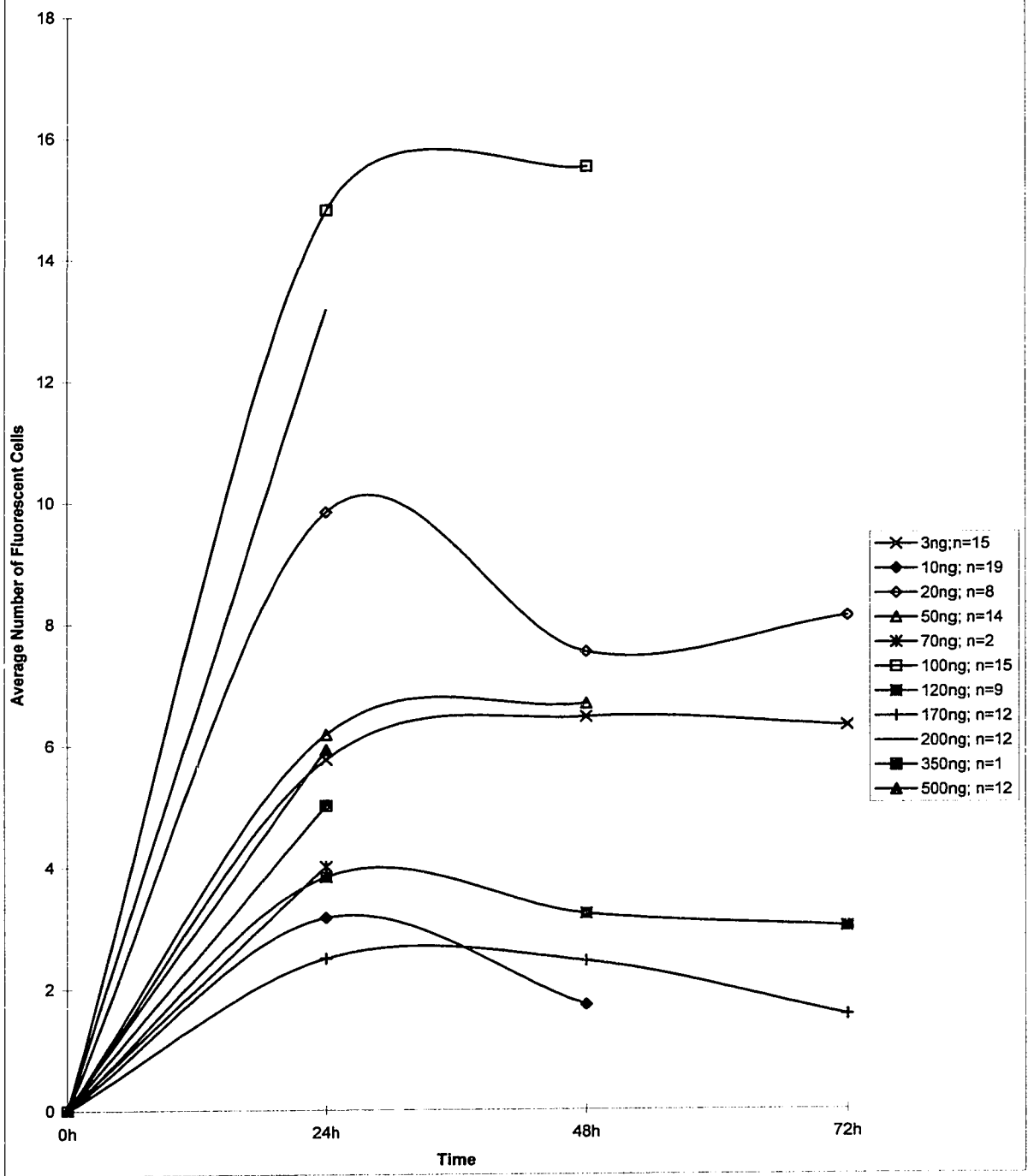
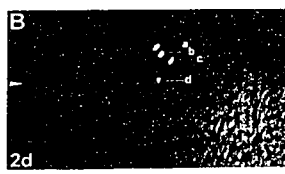
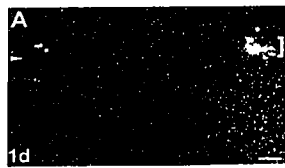


Figure 23: Cells expressing GFP in regenerating fin blastema are motile.

(A) The blastema of a 3 day regenerated fin is injected with 100ng of GFP plasmid DNA (bracket region in A) and observed after 24h. (B-D) Cells that express GFP closer to the level of amputation maintain their location (d) and remain fluorescent for as long as one week. In comparison, the cells further from the level of amputation move distally and lose their fluorescence more rapidly (a), (b) and (c). Large arrowhead indicates level of amputation. Scale bars: A, B, C, 80 μ m; D, 80 μ m.



Before the *in vivo* transfection method can be used as a tool to express proteins of interest into the regenerating caudal fin, it is necessary to determine the transfection efficiency as well as the optimum conditions under which transfection will occur in the regenerate. To determine these parameters, pEGFPC1 is used as the reporter construct and injected into the blastema of regenerating caudal fins in varying DNA plasmid concentrations and volumes (as previously mentioned). The data obtained has determined an optimum expression efficiency of 2 cells per nanogram of GFP plasmid DNA (Table 2). Furthermore, the maximum average number of blastema cells expressing GFP, which is 15 cells, is obtained with the injection of a volume of 30nl containing 100ng of plasmid DNA into the blastema of a 3 day regenerated fin (Table 3b, Figure 24). In comparison, the injection of 10nl containing 30ng of plasmid DNA into the blastema of a 3 day regenerated fin achieves a maximum average of 6 cells with the minimum volume in order to minimize the disturbance to the tissue (Table 3a, Figure 25). Although these results suggest an optimum range for GFP plasmid DNA transfection of the blastema in order to minimize or maximize the number of cells expressing GFP, more experiments are required to establish the significance of this data. However, these results are used herein as a guideline in the following set of experiments, in order to achieve maximum expression of SHH in the regenerating caudal fin.

Table 2: Average number of fluorescent cells per nanogram of injected GFP plasmid DNA.

The number of fluorescent cells are counted 24h after injection into the blastema of a 3d regenerated caudal fin. Dividing the number of fluorescent cells by the amount of DNA injected determined an optimum expression efficiency of 2 cells per nanogram of GFP plasmid DNA.

DNA injected (ng)	n	Average number of fluorescent cells	Average number of fluorescent cells/ng of DNA injected
0	0	0	0
3	15	6	2
10	19	3	0.3
20	8	10	0.58
50	14	6	0.1
70	2	4	0.06
100	15	15	0.15
120	9	4	0.03
170	12	2	0.01
200	12	13	0.066
350	1	5	0.01
500	12	6	0.01

n=number of experiments

Table 3: Average number of fluorescent cells 24h after injection the into the blastema of a 3d regenerating caudal fin versus the amount of GFP plasmid DNA injected in volumes of 10 nl and 30nl.

A. Injection Volume of 10nl

Amount of DNA injected (ng)	0	3	20	70	120	170
Average number of fluorescent cells	0	6	10	4	4	2
Standard Deviation	0	3	6	0	3	2
n	n/a	15	8	2	9	12

B. Injection Volume of 30nl

Amount of DNA injected (ng)	0	10	50	100	200	350	500
Average number of fluorescent cells	0	3	6	15	13	5	6
Standard Deviation	0	2	3	10	8	0	4
n	n/a	19	14	15	12	1	12

n=number of experiments

n/a=not applicable

Figure 24: Average number of fluorescent cells 24h after injection into the blastema of a 3d regenerating caudal fins versus the amount of GFP plasmid DNA injected in a volume of 30nl.

The general trend of the graph is obtained using a 5th order polynomial as a trendline.

Average Number of Fluorescent Cells 24h After Injection into the Blastema of 3d Regenerating Caudal Fins versus Amount of GFP Plasmid DNA Injected in a Volume of 30nl.

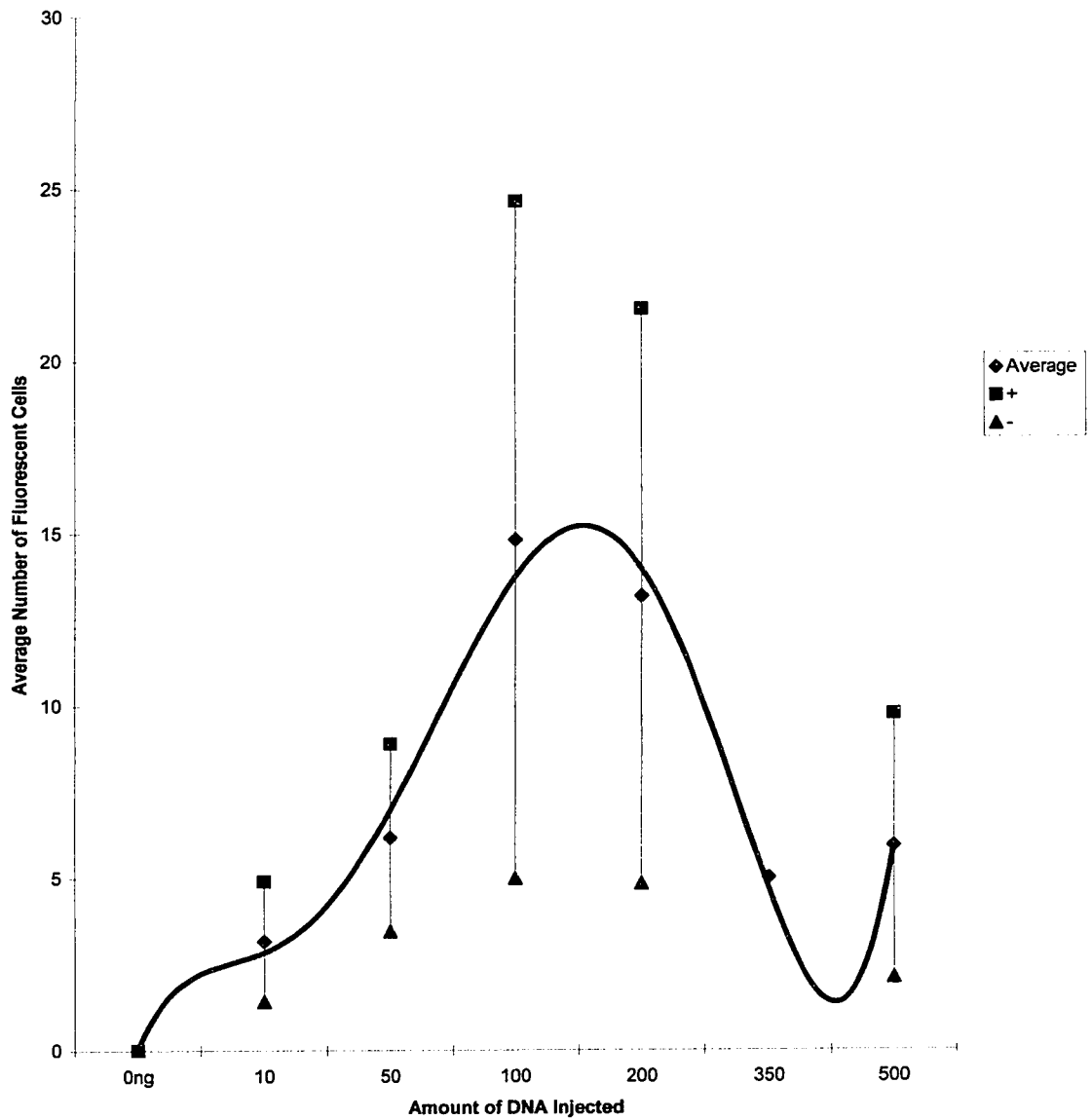
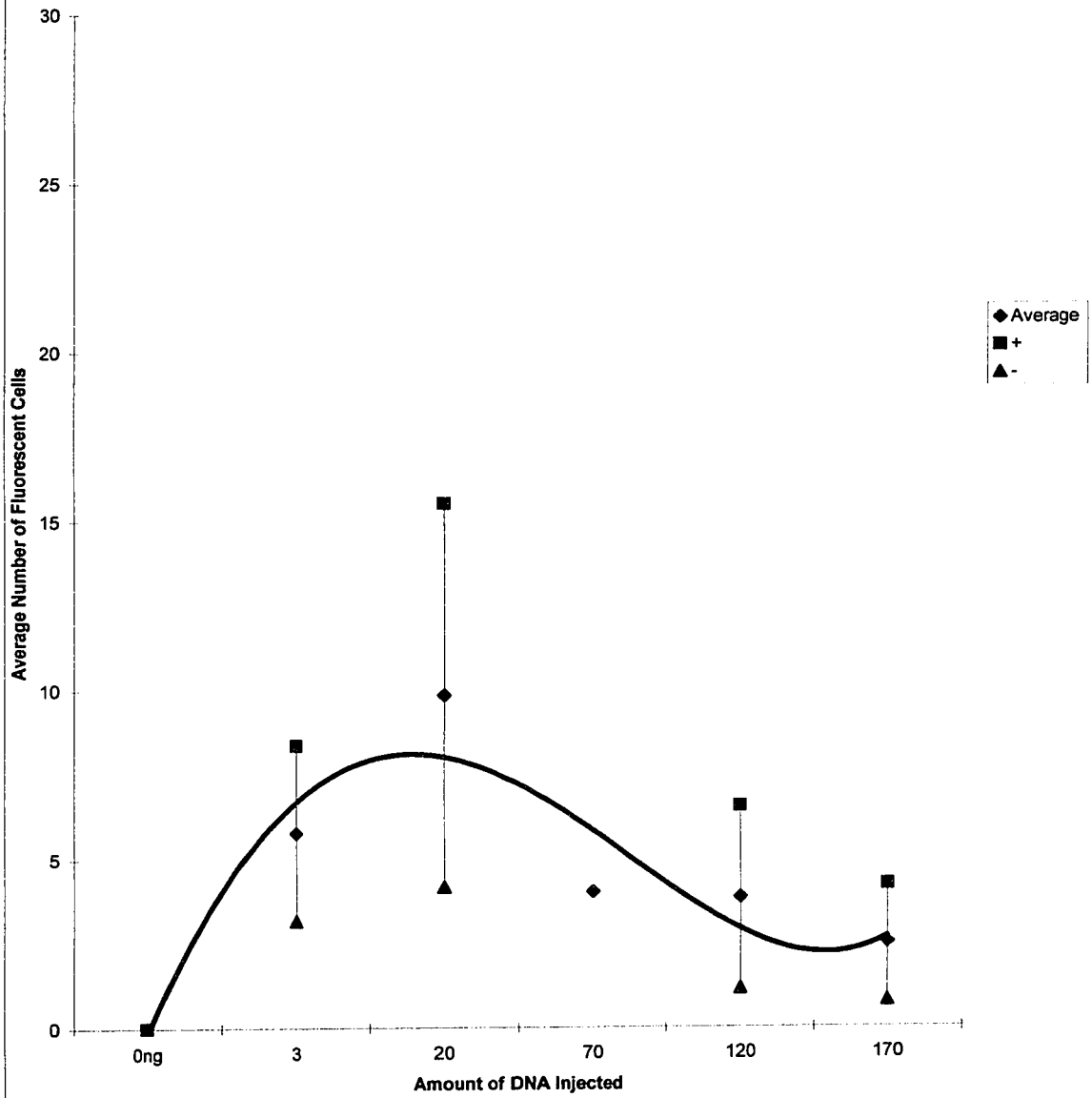


Figure 25: Average number of fluorescent cells 24h after injection into the blastema of 3d regenerating caudal fins versus amount of GFP plasmid DNA injected in a volume of 10nl.

The general trend of the graph is obtained using a 4th order polynomial as a trendline.

Average Number of Fluorescent Cells 24h After Injection into the Blastema of 3d Regenerating Caudal Fins versus Amount of GFP Plasmid DNA Injected in a Volume of 10nl.



b. In Vivo Transfection of Sonic hedgehog in Regenerating Caudal Fins

During larval fin development and fin regeneration, *shh* is expressed in one group of cells centered in the fin ray and this expression precedes the formation of the ray. However, when the bifurcation of the lepidotrichia is going to form, *shh* is expressed in two groups of cells on one side of the major rays 2 to 17. The most dorsal and ventral rays, 1 and 18, are the only major rays of the caudal fin that do not form any bifurcation and correspondingly only one group of cells expressing *shh* is detected on each side of these rays. These expression patterns suggest a role for *shh* in dermal bone formation and in formation of bifurcations. In addition, when adult fins are amputated immediately after the first branching point, termed a “short cut”, the once diverging dermal bone converges and fuses to form a single lepidotrichium which undergoes normal regeneration. Correspondingly, *shh* is expressed in a unique domain at the time during which fusion is taking place, and thereby supports the hypothesis that *shh* plays a role in dermal bone formation (Figure 9).

Since *shh* is expressed in a unique domain during the fusion of the lepidotrichia after a “short cut”, we hypothesized that if *shh* were misexpressed in a unique domain after a “long cut” it would result in the fusion of the bifurcating rays, and therefore confirm the role of *shh* in dermal bone patterning.

In order to misexpress *shh* in the regenerating fins, the coding sequence for the 19kDa amino terminal of SHH is inserted into an expression vector that is under the

control of the CMV promoter. Both the pENH₂*Shh* and pENH₂*ShhMyc* construct contain the coding sequence for 19kDa amino terminal of SHH, with the addition of a c-Myc epitope tag sequence inserted into the *shh* sequence of the pENH₂*ShhMyc* construct (as described in *Material and Methods* section).

The production of the SHH protein is verified by transfecting COS cells with the pENH₂*ShhMyc* construct and detecting the protein immunocytochemically using the 9E10 monoclonal antibody that detects the Myc epitope. Results confirm the transcription and translation of the protein (Figure 26A). To determine if the SHH protein is functional, 20ng of the pENH₂*Shh* or the pENH₂*ShhMyc* construct is microinjected into each blastomere of a 1-4 cell stage zebrafish embryos. Over 90% (n=65) of injected embryos develop a phenotype similar to those described by overexpressing SHH after a microinjection of *shh* mRNA (Figure 27), such as lack of ventricles in the brain (Figure 27C-F) and defects in the developing eyes which failed to develop lenses and epithelial pigmentation (Figure 26C-F) (Ekker *et al.*, 1995a). In comparison, the pEGFPC1 controls have a normal phenotype (Figure 27B).

Having confirmed the ability of the pENH₂*Shh* and the pENH₂*ShhMyc* constructs to produce a functional protein, they are then injected into caudal fins in the region of the “long cut”, just beyond the first branching point of the bifurcating lepidotrichia. Injections were made into caudal fins which had regenerated for 2 to 5 days, a period of time where *shh* is known to be expressed in the regenerating caudal fin thereby insuring that the components of the SHH signaling pathway are

Figure 26: The pENH2*Shh*Myc construct produces SHH *in vitro* and *in vivo*.

(A) Production of SHH is verified *in vitro* by transiently transfecting the pENH2*Shh*Myc construct into COS cells, and immunocytochemically detecting the Myc epitope. Staining is localized in the cytoplasm of the transfected cells. (B) 24h after injecting the pENH2*Shh*Myc construct into the blastema of a 3d regenerated fin, production of the amino terminal of SHH *in vivo* is immunocytochemically detected on proximo-distal cryosections (arrow in B). (C) Bright field image of (B) to determine the tissue in which the labeling is located. b, blastema; e, epidermis; ec, epidermal cap; l, lepidotrichia. Scale bar: B, C, 30 μ m.

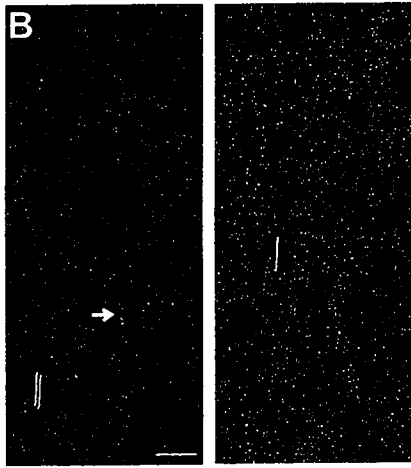


Figure 27: The pENH2Shh and pENH2ShhMyc constructs produce a functional SHH amino terminal protein in zebrafish embryos.

(A) Control wild-type 24h zebrafish embryo. (B-F) 20ng of plasmid vector DNA is microinjected into each blastomere of 1-4 cell stage zebrafish embryos. (B) Control embryo injected with pEGFPC1 does not show any abnormal phenotypes at 24h. (C-F) The pENH2*Shh* or the pENH2*ShhMyc* construct injected embryos are observed, at 24h, to develop phenotypes characteristic of *shh* mRNA injected embryos, such as lack of ventricles in the brain and defects in the developing eyes which failed to develop lenses and epithelial pigmentation (C-F) (Egger *et al.*, 1995a).



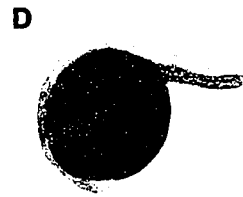
WT Control



pEGFPC1



pENH2Shh



pENH2ShhMyc



pENH2ShhMyc



pENH2ShhMyc

present. It is also during this time that the blastema is susceptible to transfection. The plasmid coding for the GFP is used as a control to ensure the tissue is susceptible to plasmid DNA uptake.

Observations are made every 24 hours following the injection for over a period of two weeks or until the fin is almost completely regenerated. Although 48 similar injections have been performed on 12 caudal fins with amounts of DNA ranging from 3ng to 6µg, no obvious morphological phenotypes in the lepidotrichia or other tissues of the regenerating fin have been observed as a result of these *shh* injections. The observation for possible morphological phenotypes included light microscopy of the whole mount fin as well as proximo-distal sections, however results were identical to that of controls.

The production of the Myc-tagged SHH protein in the regenerating caudal fin is verified by performing immunocytochemistry on cryosections 2 days after injection (Figure 26B, C). These results demonstrate that *in vivo* transfection in the regenerating caudal fin of the pENH₂*Shh*Myc constructs can produce the corresponding protein. However, detection of the exogenous protein was found in only 10% of the injections and is expressed in only a few cells, in comparison to the 100-250 cells which express endogenous SHH during regeneration. This suggests that the number of exogenous SHH producing cells may not have been sufficient in inducing a morphological phenotype in the regenerating fin.

Discussion

Amputation of either the fin of a teleost fish or the limb of a urodele amphibian, is followed by similar regenerative phases, the wound healing, blastema formation and proliferation and differentiation which allow the growth of tissue to restore the missing structure. These phases are interdependent, and each exhibits characteristic cell movements, cell proliferation, cell-cell interaction, cell-ECM interaction and gene expression patterns.

The purpose of this study is to determine and describe the migration of cells after the amputation of the zebrafish caudal fin, using Di-I as a cell marker. The second objective is to develop a gene delivery technique to be used as a tool to study the role of genes expressed during caudal fin regeneration.

Cell Migration during Zebrafish Caudal Fin Regeneration

Epidermal cell migration to the region forming the epidermal cap during regeneration has been well documented in the regenerating limb of the urodele amphibian (Tsonis, 1996), and has been observed in other teleosts (Bernhard and Wagner, 1992; Santamaria *et al.*, 1996; Becerra *et al.*, 1996). This study is the first reported use of Di-I as a method to label cells of regenerating fin. The results herein demonstrate that cells located within the epidermal layer of the regenerating caudal fin of zebrafish also contribute to the formation of the epidermal cap by migrating to this region, thereby suggesting a similar regeneration mechanism between zebrafish,

other teleosts and amphibians. In addition, this study reports the novel observation that the cells located within the epidermal tissue of the caudal fin, regardless of the distance from the level of amputation, are able to migrate towards the level of amputation.

While epidermal cells in various regenerating tissues have been shown to migrate by lamellipodial crawling (Ruth, 1911; Matsumoto, 1918; Martin, 1997; Tsonis, 1996), the exact mechanism of epidermal cell movement during zebrafish caudal fin regeneration is unknown. The observation that cells in the lateral epidermis contribute to the formation of the wound epidermis within 6 hours after amputation, a time where no proliferation above basal levels is detected in the surrounding tissues (Poleo *et al.*, submitted), is in support of an active migration process undertaken by the epidermal cells and not a passive movement imposed upon the epidermal cells by the expanding population of proliferating cells. This is further supported by Santos Ruiz and co-workers who, in goldfish, observed narrowing of the epidermis near the level of amputation which can be attributed to the loss of cells by migration towards the wound (Santos Ruiz *et al.*, 1996).

Although it is not known what type of epidermal cells undergo migration to form the wound epidermis in the zebrafish caudal fin, the fact that there are distinctly different migration rates among the migrating cells suggest that there may be different cell types which migrate upon amputation of the caudal fin. In addition, it has been observed by Bullock *et al.* (1978) that the *Malpighian* cells of the normal teleost epidermis, migrate to the region of injury. The *Malpighian* cells, or filament-

containing cells are mitotically active in the normal epidermis, however they do not undergo a mitotic burst during injury, further supporting an active migration towards the wound. The main characteristic of the *Malpighian* cell, is a dense cytoplasmic aggregation of filaments, which are continuous with desmosomes, thereby confirming that these cells are structurally suited to undergo active migration. Experiments to investigate the presence of such a cell type in the zebrafish could be done using antibodies directed against filaments, thereby identifying the location of cells with abnormally high amounts of filaments. These cells could then be further characterized based on histochemical techniques.

Even though part of the role of the migrating epidermal cells in forming the wound epidermis is to heal the wound, it is known that cues provided by the wound epidermis are critical to the regeneration process in both teleosts and amphibians (Tsonis, 1996; Brockes, 1997; Géraudie and Ferretti, 1998). The importance of the role of the epidermal cells in the regeneration process is further substantiated by their migration trend upon amputation. First, the majority of the epidermal cells in the regenerating caudal fin migrate at a 500 $\mu\text{m}/\text{day}$ rate towards the distal end, whereas a few cells migrate towards proximal gash at a rate of 200 $\mu\text{m}/\text{day}$ but only during the first 24 hours which demonstrates that the epidermal cells favor a distal migration and migrate at a quicker rate in this direction for a longer period of time than in any other direction. Second, when a gash is induced in the ray, the epidermal cells migrate distally towards the wounded region but then continue beyond the wound at a rate above basal levels. Both of these migration patterns show that the

epidermal cells migrate in a distinct manner upon injury which supports a role for them in the regeneration process other than only wound healing. Although the underlying molecular mechanism of how the wound epidermis contributes to regeneration is not yet known, the fact that the migration of cells to form the wound epidermis occurs before the onset of any cell proliferation (Poleo *et al.*, submitted) indicates a sequential order in which important regenerative cues might be acquired, suggesting a complementary role between both the wound epidermis and the onset of proliferation in early zebrafish fin regeneration. The lack of formation of a basement membrane between the wound epidermis and the underlying stump tissue allows a strong epithelial-mesenchymal interaction, and probably through the release of signaling molecules including fibroblast growth factors, results in the subsequent patterning and growth of the regenerate.

Even though migration of the epidermal cells to form the wound epidermis is observed to occur as early as 6h after amputation, the exact time at which migration is initiated after amputation is not accurately determined using the current Di-I labeling methods because different times of onset of migration are noticed with varying amounts of injected dye. The rationale behind this, is that the probability of labeling a cell that will migrate increases with the amount of Di-I injected. Therefore, in order to detect the first migrating cell, a high volume of Di-I would need to be injected. Unfortunately, a migrating cell is more difficult to detect in an environment that is saturated with Di-I, and so by injecting lower amounts of Di-I, the cells that are observed to migrate might not necessarily be the first migrating

cells. This problem can be avoided by increasing the sample size in which this experiment is conducted, thereby increasing the probability that with the same concentration of Di-I, the first migrating cell(s) will be labeled.

The epidermal cells only migrate towards the epidermal cap within the first 48 hours, yet after 24 hours the epidermal cap is developed enough to allow the initiation of blastema formation, supporting the thought that epidermal cells may provide more than simply proliferative cues and could play a role in controlling mesenchymal cell differentiation as has been suggested by some investigators (Becerra *et al.*, 1996).

Unlike epidermal cell migration, no migration of intra-ray mesenchymal cells of 3d blastema is observed in this study, yet several studies support the contribution of the underlying mesenchymal cells to the regenerating tissue (Brockes, 1997; Géraudie and Ferretti, 1998). Although cells in the intra-ray mesenchyme underlying the level of amputation are not observed to contribute to the formation of a 3d blastema, some Di-I labeled cells are located in the mesenchyme of the regenerate underlying the blastema, suggesting that mesenchymal cells contribute to the formation of the 1 or 2 day blastema. Subsequently, it could be argued that the observation at 3d is too late to observe the contribution of the Di-I labeled mesenchymal cells to the blastema. In addition, because the blastema is a highly proliferating tissue, the Di-I staining could be diluted to undetectable levels among the cells at 3d. It therefore remains to be determined, observing on sections at 1 or 2

days after amputation, if mesenchymal cells located adjacent to the wound contribute to the blastema in the regenerating caudal fin of the zebrafish.

A Wound in the Fin Ray Initiates an Intense Cell Migration

The dramatic increase in the rate of migration of cells labeled in the lateral epidermis is initiated by the amputation of the caudal fin. To investigate the origin of the signal(s) which initiates the intense cell migration, Di-I labeled cells are observed after inducing various types of injuries on the caudal fin. We have shown that a wound induced in the fin ray initiates a more prolonged and intense cell migration rate than any other wound, other than an amputation. In addition, the signal can act at great distance along the proximo-distal axis from the level of amputation, and yet it is limited laterally to within 3 adjacent rays.

The role of the nervous system in the initiation of cell migration was investigated because the nerve supply has been demonstrated to be critical for fin regeneration (Goss and Stagg, 1957; Becerra *et al.*, 1996), and the nerve bundles are located within the intra-ray of the caudal fin (Becerra *et al.*, 1983; Chandross and Bear, 1979). Although the nervous system within the ray could account for both the rapid dispersion of the signal that initiates the intense migration and the lateral confinement of the signal, severing the nerve did not prevent the intense migration signal. This suggests that the nerves within the ray do not play a role in signaling the intense cell migration. In the regenerating limb of urodeles, nerves have been suggested to play a role in initiating blastemal proliferation (Singer and Craven, 1948), and since the role of the nervous system in urodeles limb and teleost fin

regeneration share striking similarities (Géraudie and Singer, 1979), an equivalent role for the nerves within the ray of the caudal fin could explain why severing the nerve does not impede wound healing. However, it could also be argued that severing the nerve was not as effective as removing it from the caudal fin since in previous studies it has been demonstrated that there is a discharge of neurotrophic substances from the nerve endings during the early hours after nerve transection (Géraudie and Singer, 1979). To eliminate this possibility, the use of neurotoxins such as alpha-methyl-p-tyrosine which inhibits the production of the neurotransmitter catecholamine and has been shown to retard regeneration in limb (Taban *et al.*, 1976), or denervating the fin, could be used to ensure that neuronal transmission is inhibited in the regenerating caudal fin.

In amphibians, the substratum contact mediated by integrin adhesion receptors is thought to be the sensing mechanism which triggers the epidermal cells to move. As previously mentioned the integrins act as potent signaling molecules, physically coupling the ECM to the underlying cytoskeleton and transducing environmental signals into biochemical responses through force-dependent changes in scaffold geometry or other molecular mechanism (Ingber, 1997; Giancotti, 1997; Fuchs *et al.*, 1997; Sjaastad and Nelson, 1997; Akiyama, 1996; Bissell *et al.*, 1996). If such a mechanism is present in the caudal fin, then upon amputation the changes in ECM components (Géraudie and Ferretti, 1998) could initiate the signal for intense epidermal cell migration. In addition, it has been shown in cell culture, that the interaction between integrins and growth factor receptors, can regulate cell

locomotion (Matsumoto *et al.*, 1995). Although this could be the signaling mechanism, much is still to be learned about how cells convert the mechanical signals into a biochemical response, and if indeed such a signaling mechanism can transduce a signal as quickly and to such distances as what is observed after the amputation of the fin ray.

Expressing the Green Fluorescent Protein in the Regenerating Caudal Fin

There are many methods available to introduce a gene of interest in an organism, such as injecting viral vectors (retrovirus, adeno-virus) directly into the tissue (Riddle *et al.*, 1993), injecting a liposomal-DNA complex into the tissue (Nicolau *et al.*, 1983; Wolff *et al.*, 1990; Wolff and Lederberg, 1994), or the use of calcium phosphate-precipitated DNA which is injected into the tissue (Benvenisty and Reshef, 1986). It is also possible to introduce the protein of interest directly into the organism with the use of beads soaked in the protein (Yang *et al.*, 1997) or by implanting a pellet of cells stably expressing the protein of interest into the organism (Yang *et al.*, 1997) (all these methods are previously mentioned in the introduction in the *In Vivo Transfection as a Tool to Study Molecular Biology* section).

With regards to choosing a technique to express a protein of interest into the zebrafish regenerating caudal fin, the viral method was eliminated because there are no zebrafish specific retroviruses/adeno-viruses yet available, and we currently do not have the facilities for viral transfections. The method of implanting a bead into

the regenerating zebrafish fin was tried but the bead does not remain in the tissue for a prolonged period due to the constant motion of the fin which dislodges the bead. Of the remaining *in vivo* transfection techniques, the injection of naked plasmid DNA was chosen as method for expressing the gene of interest in the zebrafish regenerating caudal fin. The advantage of using naked plasmid DNA to transfect cells in the regenerating caudal fin, is that it transfects a very local group of cells and does not diffuse throughout the fin, the fish can be returned to normal conditions immediately, it is more effective than liposome-DNA complex (data not discussed), sterile conditions are not required, and is a relatively low cost method.

The transient transfection of the green fluorescent protein (GFP) in the cells of the regenerating caudal fin allows for real-time observation of transgene expression in living tissue. The GFP transfections are used to establish the optimum conditions under which transfection occurs in the regenerating caudal fin, so that a gene delivery technique can be developed to study the function of genes expressed during caudal fin regeneration. Establishing the optimum transfection conditions also resulted in some insights into the dynamic of fin regeneration.

Only the blastemal cells are identified as having the ability to be transfected by the injected plasmid DNA. The blastemal cells are particularly susceptible to transfection 3 days after amputation, a time when the blastema is well formed and is actively proliferating (Poleo *et al.*, submitted). Despite the electrical repulsion between cell surfaces and DNA, it is still unclear how the DNA enters or why some cells are more susceptible than others (Felgner, 1997). It is suggested that most non-

viral methods of gene transfer rely on normal mechanisms used by mammalian cells for the uptake and intracellular transport of macromolecules, such as the endocytic pathways (Mulligan, 1993). However, some tissues may simply favor the uptake of plasmid DNA because of their structures, such as the mouse skeletal muscle which may be particularly susceptible to take up and express DNA because of its unique multinucleation, sarcoplasmic reticulum and transverse tubule systems (Orkin and Motulsky, 1995). In this respect, it may be suggested that the undifferentiated and highly proliferating blastema provide the conditions whereby the blastema can take up DNA.

In addition to the blastemal cells being susceptible to *in vivo* transfection, the number of cells expressing GFP tends to maximize around 24 hours after injection and then decreases. This observation is thought to be due to the plasmid DNA not integrating into the chromosome, as is the case with most non viral transfer methods (Mulligan, 1993), and is thereby more susceptible to degradation. The end result is a transient gene expression, as is observed in all the *in vivo* blastemal cell transfections in the caudal fin.

In comparison to viral transfections that are approximately 100% efficient in tissue culture, the efficiency of naked plasmid DNA transfection *in vivo* is 10000 times less efficient and comparable to that of lipid encapsulated DNA transfections in tissue culture (Felgner, 1997). The optimal blastemal cell transfection efficiency was seen following an injection of 3ng of DNA in a volume of 10nl, resulting in 2 cells expressing a detectable level of GFP (Table 2). This expression efficiency is

sensitive to varying concentrations of plasmid DNA (Figure 24 and 25). The maximum average number of blastema cells expressing GFP is 15 cells which is achieved by injecting a volume of 30nl containing 100ng of pEGFPC1, however the efficiency of this injection is 4 times less than the optimum. Again, this suggests that the blastemal cells are sensitive to the amount of DNA injected, where amounts above 3ng results in a decrease in uptake efficiency. It is possible that high concentrations of foreign DNA in the cell or even in its environment might saturate the cells normal operating functions and or trigger a mechanism that inhibits the DNA uptake.

The injection of 10nl of a 3 μ g/ μ l GFP plasmid DNA solution into the blastema of the regenerating caudal fin, results in approximately 2 times more cells expressing GFP per nanogram than the 30nl injection of the same solution (Table 3, Figure 24 and 25). Although these results could be due to the expression efficiency being sensitive to varying amounts of plasmid DNA, in this case the volume injected must also be taken into consideration. These results suggest that the additional volume may also decrease the transfection efficiency. A plausible explanation for this could be that the additional volume disturbs the blastema and thereby leads to a decrease in the transfection efficiency. This is contrary to what has been suggested by Felgner *et al.* (1997), who state that inducing a small amount of tissue damage might play a role in increasing the transfection efficiency. Although these preliminary results suggest that the pEGFPC1 DNA concentration and perhaps the volume injected affect the transfection efficiency in the blastemal

cells of the regenerating caudal fin, whereby a 10nl injection containing 3ng of pEGFPC1 results in the optimum efficiency for the concentrations and volumes used in these experiments, further experiments need to be conducted in order to establish the significance of this data.

As demonstrated in this study, *in vivo* transfection of blastemal cells with GFP could be a useful tool to study the dynamics of fin regeneration. Specifically, the movement of the blastema cells can be visualized throughout the course of regeneration. Results demonstrate that the blastema cells that are closer to the level of amputation tend to maintain their location whereas the blastema cells further away from the level of amputation move distally (Figure 23). Although the mechanism of blastema cell movement is not known in the regenerating caudal fin, proliferative pressure is a possible cause of this movement, however the movement could also be due to active migration as is suggested in limb regeneration. In amphibian blastema the upregulation of hyaluronate, a glycosaminoglycan, is what helps provide an environment suitable for cell migration, and could facilitate the active migration of blastema progenitor cells from the stump (Tsonis, 1996; Géraudie and Ferretti, 1998). However, further ECM studies are required to determine if hyaluronate or similar matrix components are present in the regenerating caudal fin blastema.

In vivo transfection of blastemal cells with GFP is also useful to study blastemal cell differentiation. As regeneration progresses, the blastemal cells leave their distal proliferative region and progressively differentiate to give rise to all cell types

involved in the regeneration of connective and skeletal tissues (Becerra *et al.*, 1996; Géraudie and Ferretti, 1998). Correspondingly, the GFP expressing blastemal cells that are closer to the level of amputation tend to remain in their location and maintain their fluorescence for as long as a week, suggesting that the transfected blastemal cell may have differentiated (Figure 23). In contrast, the blastemal cells further away from the level of amputation move distally, and lose their fluorescence more rapidly. Although the more rapid loss of GFP fluorescence in the moving cells could be attributed to the proliferation of this tissue which dilutes the transfected plasmid DNA, the fact that the number of GFP expressing cells does not increase suggest that another mechanism may be responsible for the loss of GFP expression. Therefore the possibility that the proliferating blastemal cells are more susceptible to plasmid DNA degradation than a differentiating blastemal cell cannot be neglected. Further experiments involving blastemal cell counts and histological studies to determine the state of cell differentiation of the GFP expressing blastema are required to substantiate the explanation for the fact that the blastema cells located closer to the level of amputation express GFP for a longer period of time than the distal blastemal cells.

Although the potential for gene therapy is comparable to what aseptic techniques, antibiotics, vaccines and tissue transplantations have done to increase the quality and longevity of life, there are a few major obstacles to overcome. Specifically, there are many unanswered questions pertaining to the transfer of desired genes to appropriate cells and being able to obtain levels of expression for

effective treatment. But before these questions are answered, the efficacy of such a technique must be established (Orkin and Motulsky, 1995). The result of the pEGFPC1 transfection and expression of the GFP in the regenerating caudal fin of zebrafish have lead to insights regarding the efficiency of *in vivo* gene therapy. Specifically the concentration and perhaps the volume of the DNA solution plays a role in transfection efficiency. These factors must therefore be taken into consideration if one is interested in maximizing the production of a protein *in vivo*, since the amount of the protein being produced might have important biological implications.

Also to be considered, is the type of tissue in which the plasmid DNA is injected, since only the blastemal cells and not the epidermal cells of the regenerating fin produced GFP fluorescence. Similarly, Benvenisty and Reshef observed a 3 fold difference in plasmid DNA uptake within the same mouse and 8 fold difference between mice, when *in vivo* transfecting different regions of the liver using calcium phosphate precipitated DNA (Benvenisty and Reshef, 1986).

Another factor which might also influence the susceptibility for plasmid DNA uptake, is the state in which the cell is in, since the majority of cells expressing GFP are in the highly proliferative region. Similarly, Potter *et al.* have also encountered difficulties when trying to introduce DNA into terminally differentiated cells by electroporation (Potter *et al.*, 1984).

Altogether, four factors influence the transfection efficiency in the regenerating caudal fin of zebrafish using the naked plasmid DNA injection method, 1) the

concentration and 2) volume of the injected plasmid DNA, 3) the type of tissue in which it is injected and 4) the cell state. Note that all these factors may or may not be mutually exclusive, and could also vary with the size of the plasmid injected as well as the quality and form (linear, open circular or supercoiled) of DNA.

Misexpression of Sonic hedgehog in the Regenerating Caudal Fin

The expression pattern of *shh* in the developing larval fins and during caudal fin regeneration, is suggestive of a role for *shh* in dermal bone formation. In addition, the pattern of *shh* expression that precedes the bifurcation or the fusion of the rays, further substantiates the role of *shh* in dermal bone patterning (as previously discussed in *The Sonic Hedgehog Expression Patterns During Fin Development and Regeneration* section).

We hypothesized that the introduction or production of exogenous *shh* into the “long cut” region of a bifurcation would mimic the expression pattern of a “short cut” and thereby induce the fusion of the bifurcating rays. However, no detectable morphological effects were observed over the course of a week after the injections of either pENH₂*Shh* or the pENH₂*ShhMyc* constructs.

Other than causing the fusion of the rays, misexpression of *shh* could also have resulted in an increase in proliferation of cells as demonstrated by Wallace and Jensen (1997) who showed, using BrdU, that the amino terminus of SHH promotes the proliferation of mouse retinal precursor cells *in vitro*. A similar result was obtained by Bellusci *et al.* (1997) who, when overexpressing *shh* in mouse embryos,

observed an increase in proliferation of mesenchymal and epithelial cells during lung development. When Duprez *et al.* (1998) overexpressed SHH in the chick limb bud, they also observed an increase in proliferation of already committed myoblasts that resulted in an increase in myotube number *in vitro* and in hypertrophy *in vivo*. In addition, Duprez *et al.* (1998) noticed that SHH induced an accelerated process of differentiation of the muscle cells, leading to an enlarged and disorganized muscle in the forearm of the wing. In transgenic mice, overexpressing SHH in the epidermal cells of the skin, the development of basal cell carcinoma (BCC) like proliferations are observed throughout the skin surface (Oro *et al.*, 1997). However, when observing the whole mount as well as proximo-distal sections of regenerating fins misexpressing SHH using light microscopy, none of the above phenotypes are noticed. Future experiments should include more sensitive methods for observing the above outlined possible effects of *shh* misexpression in the regenerating fin, such as the use of BrdU to examine an increase in cell proliferation, or histological techniques such as hematoxylin to better determine the state of differentiation and organization of the cells surrounding the site of *shh* expression.

A possible explanation for the lack of morphological phenotypes in the fins misexpressing *shh* could be because of a low transfection efficiency that results in a low amount of SHH being produced. Using the GFP transfection results as an indicator, a maximum average of 15 cells are transfected in the blastema, a region whose cell count is in the 4th order of magnitude. It is therefore possible that the cells expressing SHH are not located in regions that are sensitive to SHH signaling

or not enough SHH is produced to effectuate a response. In light of what is observed with the GFP transfection experiments of the blastemal cells in the regenerating caudal fins, the cells expressing SHH might not remain in one local region for a long enough period of time to induce an effect because of the motility of the blastemal cells.

Suggestions for future studies include the use of retroviruses. As demonstrated by Riddle *et al.* (1993), it is possible to directly inject an avian specific retroviral vector, containing the *shh* gene, into the AER of developing chick limb buds and overexpress SHH. A similar experiment in zebrafish would include the use of a zebrafish viral vector containing *shh* and injecting it in the blastema or epidermis of the regenerating caudal fin, however a zebrafish specific retrovirus is not yet available. Failing that, cell pellets stably expressing SHH (as per Yang *et al.*, 1997) could also be implanted into the blastema of the regenerating caudal fin or in the blastema of the “long cut” region. However both the body temperature of the zebrafish (28.5°C) and the immune system in the adult fish, might present a problem in choosing the cell type to implant. It might therefore be advantageous to implant a zebrafish cell line (such as the zebrafish fibroblast cells (ZF4)) stably expressing SHH to maximize both the presence of the cell pellet and production of SHH.

Concluding Remarks

In light of the objectives, this study has demonstrated that the process of epidermal cell migration during caudal fin regeneration shares highly similar characteristics with that of other teleosts and urodele amphibians, suggesting a conserved and therefore an essential function during regeneration among these vertebrates. Analysis of epidermal cell migration using Di-I labeling also demonstrated some unique features which have not been reported in other studies, such as the rates of migration, distances of migration and direction of migration of the epidermal cells during zebrafish caudal fin regeneration.

With respect to developing a gene delivery technique to substantiate the role of *shh* during dermal bone regeneration, these results proved inconclusive although all indications demonstrate the expression of exogenous *shh* in the regenerating fin. However, the *in vivo* transfection of blastema cells with the GFP reporter construct proved to be a useful tool to further study the dynamics of fin regeneration, specifically blastema cell movement and differentiation.

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Publications and Abstracts:

(1) Full Papers:

Laforest, L., Brown, C., Poleo, G., Géraudie, J., Tada, M., Ekker, M., Akimenko, M.-A. (1998). Involvement of the sonic hedgehog patched1 and BMP2 genes in patterning of the zebrafish derma rays. *Development* in press.

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(2) Abstracts:

Laforest, L., Brown, C., Poleo, G., and Akimenko, M.A. Dual Effects of Retinoic Acid on *Sonic Hedgehog* signalling pathway in zebrafish. 6th International Limb Development and Regeneration Conference, Sun Valley, Idaho. May 17-21, 1998.

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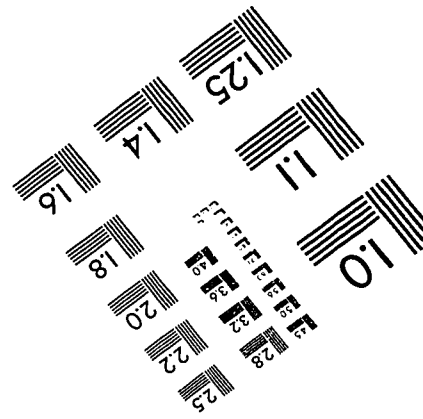
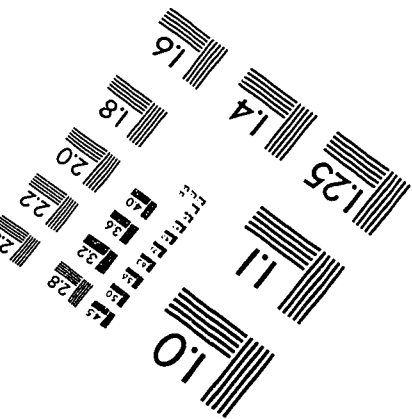
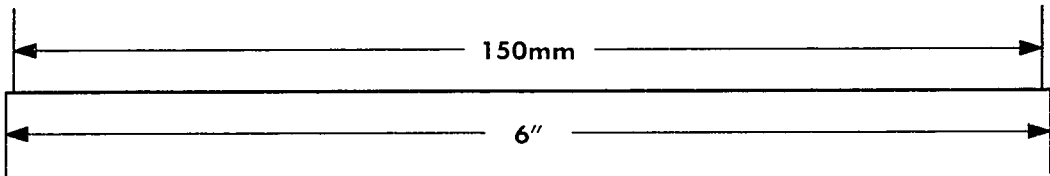
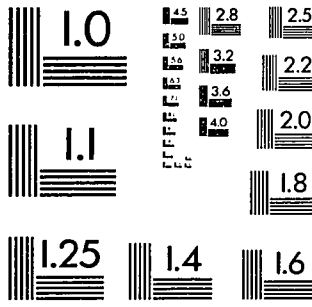
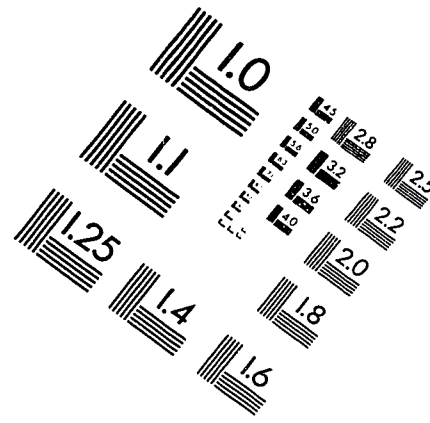
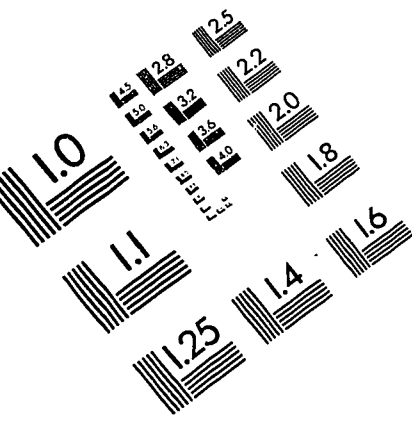
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IMAGE EVALUATION TEST TARGET (QA-3)



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