INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

Université d'Ottawa · University of Ottawa

Fibroblast Growth Factor 8 and Cell Proliferation in Zebrafish Fins

By

© Germán A. Poleo

Thesis submitted to the School of Graduate Studies and Research
University of Ottawa
in partial fulfilment of the requirements
for the M.Sc. degree in the

Department of Cellular and Molecular Medicine
Ottawa, Ontario May, 1998

Table of Contents

2.Chapter II: Characterization and expression of *Fgf8* in Developing and Regenerating Fins

List of Figure and Tables

Abstract

The fins of some teleost fishes, like many urodele amphibian limbs, have the ability to regenerate after injury. This regeneration process occurs in a sequential manner beginning with the closure of the wound by epithelial cells and followed by the establishment of a region of undifferentiated proliferating mesenchymal cells called the blastema. The cascade of events that results in the reestablishment of the structures lost to injury likely involves cell migration, proliferation and differentiation. To examine the pattern of cell proliferation taking place in the different tissues during the regeneration process of zebrafish (*Danio rerio*) caudal fins, I followed the pattern of incorporation of bromodeoxyuridine (BrdU) in the DNA of proliferating cells at various times after amputation. Before amputation, the fin mesenchyme in adult fish was seen as a compact tissue that did not proliferate. The epidermis, on the other hand, showed basal levels of cell division suggesting an ongoing process of growth and cell replacement. After fin amputation, epithelial cells migrated to cover the wound and to form the wound epidermis which did not show any signs of proliferation at any time of the regeneration process. However, the establishment of the wound epidermis was followed by an increase in the proliferating rate of the epidermal cells lateral to the wound epidermis. Starting 48 h after BrdU injections, labeled epidermal cells were found in the epidermal cap suggesting cell migration of proximal epidermal cells to this region. Mesenchymal cells from the most distal area of the stump started to incorporate BrdU 24 hours after amputation reaching a maximum rate of proliferation once the blastema was well consolidated.

Growing evidence suggests that key molecules involved in signaling pathways leading to limb bud development are also acting during fin bud development. Some of these signals are members of the fibroblast growth factor (*Fgf*) gene family which locally regulate growth and patterning in vertebrate embryos and during limb regeneration. I sequenced a cDNA clone encoding for the zebrafish FGF8 protein and analyzed its pattern of expression during zebrafish embryonic development and during fin regeneration. The zebrafish *Fgf8* cDNA sequence was 1958 base pairs long and contained an open reading frame which encodes a 233 amino acid protein. The predicted protein presented high sequence homology with FGF8 factors from other species. Genomic mapping analysis located the zebrafish *Fgf8* gene close to the centromere of chromosome XIII, in the vicinity of the *Pax2* gene. This mapping revealed a synthenic region between human and zebrafish genomes. During embryogenesis, *Fgf8* expression analyzed by whole mount *in situ* hybridization was observed in a number of signaling centres including the germ ring at gastrula stage, the tail bud, midbrain-hindbrain junction, developing somites and in the pseudo apical ectodermal ridge of the pectoral and pelvic fin bud. This pattern of expression, resembling that in chicken and mouse, suggests the conservation of the function of *Fgf8* during embryonic development in vertebrates. During fin regeneration, *Fgf8* expression was shown to be activated at the onset of blastema formation. Transcripts were found in the distal part of the blastema where high levels of BrdU incorporation were observed suggesting that FGF8 may be playing a role in maintaining blastema cells in an undifferentiated and proliferating state during fin regeneration.

proximité du gène Pax2. Cette cartographie a permis de révéler une région de synthénie entre les génomes humain et du danio. Au cours de l'embryogenèse, l'expression de Fgf8, analysée par la technique d'hybridation *in situ in toto*, est détectée dans plusieurs centres de signalisation dont la ceinture germinale au cours de la gastrulation, puis dans le bourgeon de la queue, la jonction entre le cerveau intermédiaire et le rhombencéphale, les somites et la crête apicale ectodermique des bourgeons des nageoires pectorales et des nageoires pelviennes au cours du développement larvaire. Ce profil d'expression, ressemblant à celui observé chez le poulet et la souris, suggère la conservation de la fonction de Fgf8 au cours du développement des vertébrés. Au cours de la régénération des nageoires, l'expression de Fgf8 est activée au moment de la formation du blastème. Les transcrits se situent dans la partie distale du blastème présentant un fort taux d'incorporation de BrdU; ceci suggèrant que FGF8 pourrait jouer un rôle dans le maintien des cellules du blastème dans un état non différencié et prolifératif au cours de la régénération.

Résumé

Les nageoires d'un certain nombre de poissons Téléostéens, comme les membres de plusieurs Urodèles, ont la capacité de régénérer après amputation. Le processus de régénération se passe de façon séquencielle débutant par la cicatrisation de la blessure par les cellules épithéliales, suivie par l'établissement d'une région composée de cellules mésenchymateuses non différenciées et hautement prolifératives, appelée le blastème. Les cellules du blastème se différencient ensuite, pour rétablir les structures manquantes de la nageoire. Afin d'examiner le profil de prolifération cellulaire dans les différents tissus au cours de la régénération de la nageoire caudale du Danio rerio, j'ai suivi le profil d'incorporation de la bromodeoxyuridine (BrdU) dans l'ADN des cellules en cours de prolifération à différents stades après amputation. Avant amputation, le mésenchyme des nageoires de poissons adultes est un tissu compact qui ne prolifère pas. En revanche, l'épiderme présente un niveau basal de divisions cellulaires qui suggère un processus constant de croissance et de remplacement cellulaire. Après amputation de la nageoire, les cellules épithéliales migrent pour recouvrir la blessure et former l'épiderme de cicatrisation qui ne présente pas de signe de prolifération à aucun moment de la régénération. Cependant, la formation de l'épiderme de cicatrisation est suivi par une augmentation du taux de prolifération des cellules de l'épiderme latérales à l'épiderme de cicatrisation. A partir de 48 h après injection de la BrdU, des cellules épidermales marquées à la BrdU sont observées dans la coiffe apicale épidermique suggèrant la migration de cellules épidermiques provenant de régions proximales vers cette région distale. Les cellules mésenchymateuses de la zone la plus distale du moignon commencent à incorporer la BrdU seulement 24 h après amputation, et atteignent un taux maximal de prolifération une fois que le blastème est bien établi.

Un nombre croissant de données suggère que les molécules clés impliquées dans les cascades de signalisation conduisant à la croissance du bourgeon de membre fonctionnent aussi lors de la croissance du bourgeon de la nageoire pectorale. Quelques uns de ces signaux sont des membres de la famille multigènique des "fibroblast growth factors" (Fgf) qui régulent localement la croissance et l'établissement du patron des embryons de vertébrés et des membres en cours de régénération. J'ai séquencé un clone d'ADNc codant pour la protéine FGF8 du danio et analysé le profil d'expression de ce gène au cours du développement embryonnaire du danio et de la régénération des nageoires. La séquence de l'ADNc de Fgf8 du danio est de 1958 paires de bases et contient une phase ouverte de lecture codant pour une protéine de 233 acides aminés. La protéine prédite présente un fort degré de similitude avec les facteurs FGF8 d'autres espèces de vertébrés. L'analyse de la cartographie génomique a permis de localiser le gène Fgf8 du danio près du centromère du chromosome XIII, à

Acknowledgments

I would like to thank my supervisors, Dr. Marie-Andrée Akimenko and Dr. Marc Ekker for allowing me to do my master studies in their laboratory. Thanks Marie-Andrée for being patient with my bad writing skills. Certainly my master will not only be in science but also in english as a second language.

I would like to thank all the people in the lab who made my life easier in Canada; Gary Hatch, Lucille Joly, Ted Zerucha, Genny Giroux, Nathalie Chartran; Cricho Brown, Jennifer Miles, Cristofre Martin and Cristof Nolte , you have been the best work team I ever had. I would like to give special thanks to Lynda Laforest for being a very good teacher and more importantly a very good friend.

I would like to thank Ms. Luisa Rebolledo at the Graduate Students' Association for her guidance and support.

I thank Gillian Lord and Shannon Goodwin at the Department of Cellular and Molecular Medicine for all their help.

I thank the Consejo Nacional de Investigaciones Científicas y Tecnológicas for their financial support during my MSc studies.

Lucia without your help and support I would have never made it. Emilia your beautiful smile encouraged me and gave me some peace of mind when the writing was hard.

ABBREVIATION LIST

AER: Apical ectodermal ridge

BrdU: 5-bromo-2-deoxyuridine

BSA: Bovine serum albumin

cDNA: Complementary deoxyribonucleic acid

DEPC: Diethyl pyrocarbonate

DNA: Deoxyribonucleic acid

DTT: Dithiothreitol

ECM: Extra cellular matrix protein.

ec: Epidermal cap

EDTA: Ethylenediamine tetra-acetate

EtOH: Ethanol

FITC: Fluorescein isothiocyanate

LFC: Lepidotrichia forming cells

LiCl: Lithium chloride

N-CAM: Cell adhesion molecules

PBS: Phosphate buffer saline

PCR: Polymerase chain reaction

PFA: Paraformaldhehyde

p.f: Post fertilization

PK: Protein kinase

PZ: Progress zone

RA: Retinoic acid

RNA: Ribonucleic acid

rpm: revolution per minute

SDS: Sodium dodecyl sulphate

WE: Wound epidermis

ZPA: Zone of polarizing activity

Gene and Protein Nomenclature

BMP: Bone morphogenic factor

engl: engrailed-1 gene

Fgf: Fibroblast growth factor gene

FGF: Fibroblast growth factor protein

FGFR: Fibroblast growth factor receptor protein

FGFR: Fibroblast growth factor receptor gene

Hox: Homeobox containing gene

msx: Muscle segment homeobox gene

Msx: Muscle segment homeobox protein

NvMsx: Newt muscle segment homeobox gene

Ptc: Patched, gene

RAR: Retinoic acid receptor

RXR: Retinoid X receptor

Shh: Sonic hedgehog, gene

SHH: Sonic hedgehog, protein

Wnt-7a: member of the *Wnt* family genes

WNT-7a: member of the Wnt protein

CHAPTER I: Cell Proliferation and Movement During Fin Regeneration in Zebrafish (*Danio rerio*)

I. INTRODUCTION

1. Limb and Fin Regeneration

Some animal species have the ability to regenerate a complete body part after natural damage or experimental amputation. This process is widespread in metazoa and occurs in a bi-directional fashion. When a planaria worm is transected, both sides can regenerate the missing part, the head regenerates a tail and the tail a new head. In adult vertebrates, the regenerating ability of a whole structure is restricted to a few species of fish and several urodele amphibians. In these cases, regeneration occurs in only one direction, along a proximo-distal axis. The events which take place during regeneration not only mimic the normal process of cell differentiation, but also patterning, since the regenerated structure is undistinguishable from the original.

Within vertebrates, the most intensively studied animals with regeneration capabilities have been urodele amphibians, such as newts and axolotls. Although the whole regeneration process is far from fully understood many clues have been found by developmental biologists using approaches such as transplantation, ablation, tissue and organ culture and most recently through molecular studies in amphibians.

1.1 Urodele amphibian limbs as a model in regeneration

The amazing capability that urodele amphibians have to regenerate different tissues of their bodies, such as the lens of the eye, limbs, tails, jaws and neuromast organs, has made them an excellent model to study such processes. The most intensively studied aquatic urodeles in terms of adult limb regeneration are newts (*Notophthalmus* and *Pleurodeles*) and the axolotl (*Ambystoma*) (Duellman and Trueb, 1986).

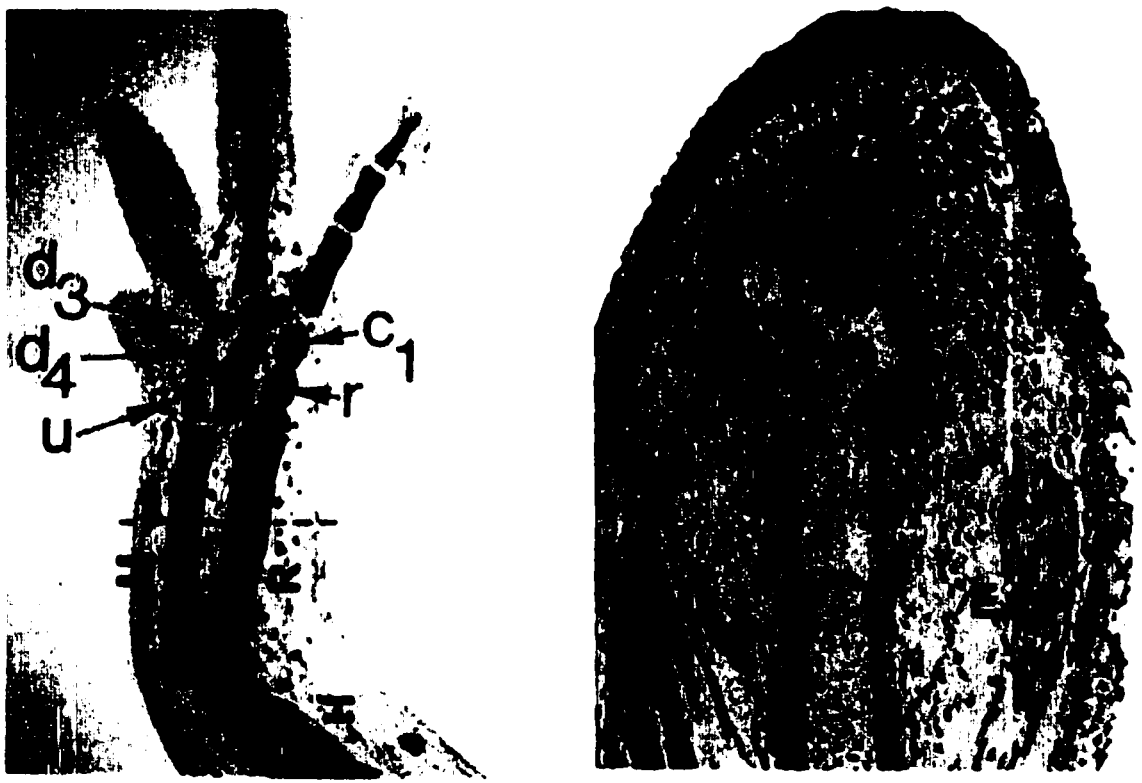
After limb amputation, regeneration occurs in an ordered process, beginning with the closure of the wound by migrating epidermal cells from the lateral side to the centre of

the stump (Hay and Fishman, 1961; Repesh and Oberpriller, 1980). Later, a blastema forms beneath the wound epidermis (Figure 1) from the different mesenchymal cell tissues (muscle, fibroblast, bone cartilage and nerves) that de-differentiate and re-enter the cell cycle (Wallace, 1981; Liversage, 1991; Ferretti and Brockes, 1991). Although, both processes, formation of the wound epidermis (WE) as well as the blastema are closely integrated, they present intrinsic characteristics.

a. Wound Epidermis

In newts, following amputation of the limb, the region of the wound is completely covered by migrating epithelial cells from the lateral surfaces of the stump within 12 hours, forming the wound epidermis (Reviewed in Brockes, 1997). Before the epidermal cells migrate to the surface of the wound, they change their shape and become detached from the basal epidermis. The cells use the substrate provided by the fibrin clot that is formed over the cut surface as a support in order to move. As this migration is occurring, an extracellular matrix is synthesized which is also used as a support for cell movement and as a signal which is able to activate gene expression (Gibbins, 1978; Greenberg and Hay, 1982; Tsonis and Eguchi, 1983). As these cells migrate neither mitosis, nor DNA synthesis occur in the epidermal cells covering the wound. This resembles normal wound healing after skin injury of other body parts, however, one important difference exists: the wound epidermis in regenerating limbs does not rapidly form a basement membrane, rather it maintains a direct contact with the mesenchyme (Salpeter and Singer, 1960). This direct contact exerts an influence in both directions from the mesenchyme to the epidermis and vice versa and likely confers upon the cells of the wound epidermis, their same new own characteristics which may differ from those of their progenitors, the lateral epidermal cells (Reviewed by Tsonis, 1996). When the basement membrane begins to be established it requires the synthesis and release of laminin and collagen type IV from the wound epidermis (Del Rio-Tsonis et al., 1992).

Figure 1. Forelimb of *Ambystoma maculatum* before amputation and during limb regeneration. **A**. Skeleton of the normal forelimb of *Ambystoma maculatum* showing the different skeletal structures and the level of amputation. H, humerus; U, ulna; R, radius; r, carpal radiale; u, carpal ulnare; cl, carpal centrale; d3 and d4, carpal articulating with digits 3 and 4; dashes indicate the level of amputation. **B**. Longitudinal section of a 4 weeks regenerating forelimb of *Ambystoma maculatum* amputated at the mid-humerus level. Within the first week after amputation, the blastema forms beneath the wound epidermis at the tip of the damaged stump tissue. Complete morphological and functional reconstruction of the missing part is reached between 6 and 10 weeks after amputation, depending on the species, the age of the animal, and the temperature at which the animal is kept (From Géraudie and Ferretti, 1998). b, blastema; we, wound epidermis; bo, bone.


d_3
d_4
u
c_1
r

Many different experiments have demonstrated the importance of wound epidermis during the regeneration process. If the wound epidermis is removed, the limb does not regenerate (Thornton, 1957) and epidermis grafts over the amputation result in the inhibition of regeneration (Mescher, 1976). This clearly implicates the wound epidermis in the initiation of regeneration. Different roles have been attributed to this tissue: The first role is to release several enzymes that induce histolysis of the mesenchyme tissue which may lead to de-differentiation (Schmidt, 1968); The second role is the induction of patterning and maintenance of cell proliferation (Tassava and Mescher, 1975; Tassava and Lloyd, 1977) by producing and releasing signaling molecules into the mesenchyme.

b. Blastema formation

Epithelial-mesenchymal interactions during the first days after limb amputation result in the subsequent formation of the blastema. It is well known that in urodele amphibians the blastema is formed from cells present in the mesenchyme without any participation of the epidermis, however, the origin of these cells is not well known. Two different hypotheses have been proposed to explain the origin of blastemal cells. One suggests that the cells come from a population of stem cells that are mobilized locally as a consequence of amputation. The other suggests that formation of the blastema results from de-differentiation of mature mesenchymal cells such as muscle, nerves, chondrocytes and fibroblasts, which regress to an undifferentiated stage and reenter the cell cycle. Experimental data provide stronger support to the second hypothesis. For instance, Multinucleated myotubes labeled with [^{3}H]thymidine and rhodamine-dextran (a cytoplasmic lineage tracer) implanted beneath the wound epidermis are first observed as proliferating mononucleated myogenic progenitor cells in the blastema and later as differentiated muscle cells (Lo et al., 1993). An interesting observation is that a few of the labeled myogenic progenitor cells are seen to differentiate into chondrocytes indicating that transdifferentiation can occasionally occur. Skeletal tissues and fibroblasts also contribute to the formation of the blastema (Muneoka et al., 1986).

Blastema formation is initiated by changes in the extracellular environment which take place in the connective tissue 24-48 hours after limb amputation (Géraudie and Ferretti, 1998). Mesenchymal cells that will eventually form the blastema are thought to be released through partial histolysis which alters cell-to-cell and cell-to-extracellular matrix components (ECM) interaction. Rearrangement of the ECM is regulated by different metalloproteinases, such as collagenase, gelatinase and stromelysin which have not been found to be expressed in non amputated limbs of newt and axolotl, but are up regulated 2 days after amputation (Yang and Bryant, 1994; Miyazaki et al., 1996). For example, the presence of collagenase is correlated with rapid degradation of collagen following amputation (Grillo et al., 1968; Dresden and Gross, 1970). Other ECM components shown to be modified during limb regeneration are laminin, fibronectin, tenascin and hyaluronate (Smith et al., 1975; Gulatí et al., 1983; Onda et al., 1991). Components of the ECM are involved in the stabilization of tissues by the interaction of the matrix proteins with adhesion molecules found on the cell surface. This type of interaction can act as a signal which activates cellular mechanisms such as ion transport and gene transcription (See Appendix)(Matsumoto et al., 1995; Leliévre et al., 1996; Kanazashi et al., 1997; Rudzki and Jothy, 1997). Transmembrane receptors such as integrins and cell adhesion molecules (*N*-CAM) involved in cell-cell and cell-ECM interactions have also been found to be differentially regulated following urodele limb amputation (Maier et al., 1986; Tsonis et al., 1997). Although the participation of the ECM during limb regeneration is evident, its precise role in the cascade of events preceding the release of mesenchymal cells from the stump in order to form the blastema has yet to be elucidated.

c. Innervation: a requisite for limb regeneration

Regeneration requires an adequate level of nervous supply. When the limb of an amphibian urodele is deprived of sensory and motor nerves prior to amputation, wound healing and blastema cell accumulation occur but regeneration is inhibited (Thornton, 1970). Conversely, if the limb is denervated after blastema formation, regeneration

proceeds but results in a limb of reduced size. This suggests that regeneration is dependent on nerves during the time of rapid proliferation of the blastema (Singer and Craven, 1948). Such control of the cell growth of the blastema by the nervous system is thought to take place through the release of neurotrophic factors such as: fibroblast frowth factors (FGF); glial growth factors (GGF); transferrin; neurotensin; nerve growth factors (NGF); and neuropeptides such as substance P (Singer et al., 1976; Jabaily and Singer, 1997; Brockes, 1987; Ferretti and Brockes, 1991; Dinsmore and Mescher, 1998).

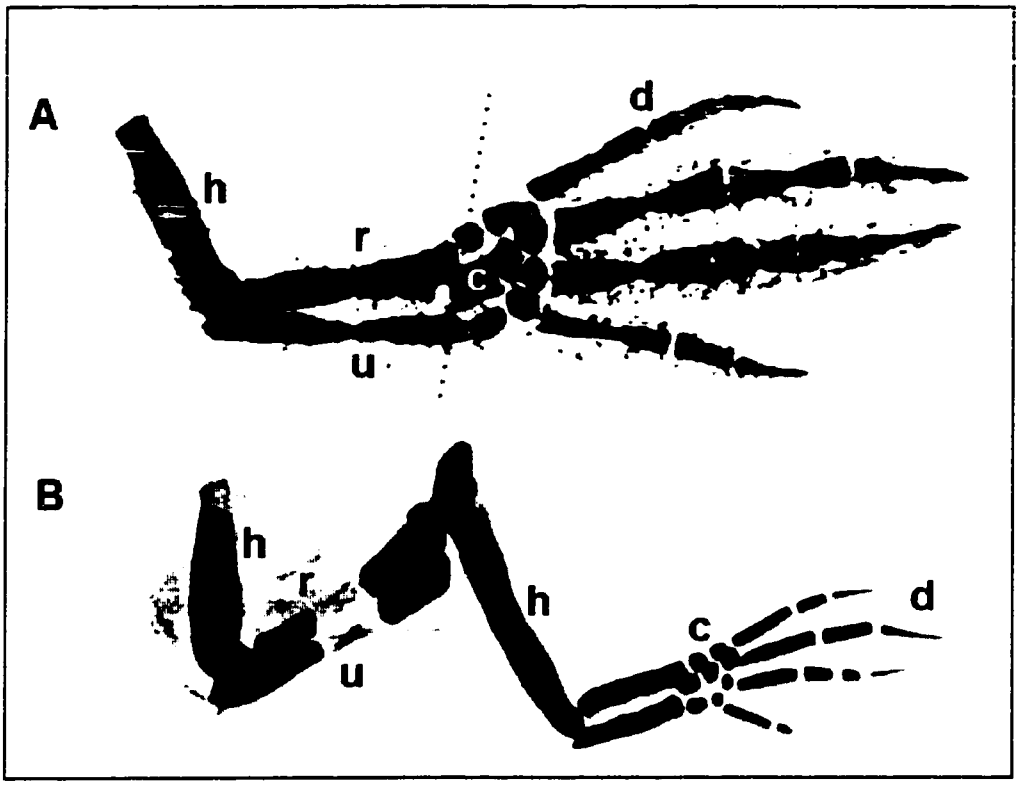
1.2 Positional Identity of the cells in the regenerate

One interesting characteristic of urodele limb regeneration is cell axial identity; blastema progenitor cells will only give rise to structures distal to their level of origin. For example, if the limb is amputated at the level of the hand, it will only regenerate the hand (Figure 2A) 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 a 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. 9-*cis* retinoic acid, a derivative of vitamin A is one of the signaling factors known to affect normal regeneration (Viviano et al., 1995).

Like other vitamin A derivatives or retinoids, 9-*cis* retinoic acid has a teratogenic effect when administered exogenously in embryos. The effects observed include induction of severe craniofacial and limb malformations, and affect patterning in systems undergoing regeneration (Reviewed by Brockes, 1990). This vitamin A metabolite acts through nuclear receptors belonging to the steroid/thyroid receptor superfamily which

Figure 2. Effects of retinoic acid on axolotl limb regeneration. **A.** Skeleton of a fully regenerated limb under normal conditions. Dashes mark the amputation plane **B.** Proximalization of limb structures by retinoic acid treatment during limb regeneration. Observe the formation of an extra humerus. h, humerus; r, radius; u, ulna; c, carp; d, digits. (from Tsonis, 1996).


A
h
r
c
d
u
B
h
r
u
h
c
d

activate transcription of target genes by binding either as a homodimer or as heterodimers to specific response elements (Means and Gudas, 1995). Two different families of receptors have been characterised, the Retinoic Acid Receptor (RARs) and the Retinoid X Receptors (RXRs). Each of these families present multiple isoforms which differ in binding affinity to different retinoids. In urodeles, five different RARs have been identified, all of which are expressed in both the mesenchymal blastema and epidermis (Ragsdale et al., 1989; Ragsdale et al., 1992). Although the biological significance of the receptors distribution and function has yet to be elucidated, it is thought that the synthesis of 9-*cis* retinoic acid is important in the maintenance of the wound epidermis and that it influences the behaviour of the underlying mesenchymal cells. Treatment with retinoic acid under specific conditions, of a urodele amphibian after limb amputation results in regeneration of skeletal structures with more proximal identity leading to a partial duplication of the proximo-distal axis (Figure 2B)(Niazi and Saxena, 1978; Thoms and Stocum, 1984). Pecorino and co-workers (1996b) have shown that a specific RAR (RARδ 2) is implicated in the positional identity of blastemal cells during limb regeneration. The group constructed a series of plasmids encoding chimeric proteins containing the DNA-binding domain sequences of various newt RAR isoforms (RAR: α1, α2, δ1a, δ1b and δ 2) fused to the ligand binding domain of the thyroid hormone. These were then transfected by particle bombardment into a blastema of a regenerating newt limb and the transfected blastema was grafted to a proximal stump. Using T3 (thyroid hormone) treatment to selectively activate a specific recombinant RAR, they were able to induce proximalization of the transfected cells only when activating the RAR δ2 isoform. This experiment clearly indicates the participation of RARδ2 in the specification of positional identity and supports the idea that different receptor isoforms are associated with different activities. For example, while RARδ1 has been shown to be responsible for the synthesis of RA-inducible WE3 antigen, a marker for secretory differentiation, which distinguishes

the wound epidermis from normal skin (Pecorino et al., 1994), RARα1 has been found to mediate growth inhibition of blastema cells (Schilthus et al., 1993).

1.3 Fibroblast growth factors in limb regeneration

Members of the fibroblast growth factor family such as FGF1 and FGF2 which are known to act as extracellular signaling molecules that induce growth and differentiation in cells of various origins (Boilly et al., 1991; Brockes, 1996) have also been found in the wound epidermis (Mullen et al., 1996; Zenjary et al., 1996a).

One possible role of FGF proteins is that they may be acting as signals which stimulate the proliferation of the underlying mesenchyme. The action of FGF proteins is mediated through the activation of a family of tyrosine kinase transmembrane receptors of which 4 have been identified to date (FGFR1 to 4)(for a review see Goldfarb, 1990). It has been suggested that the wound epidermis itself might be acting as a storage compartment for these factors as it contains large quantities of heparan sulphates which are known to bind FGFs with high affinity (Tsonis, 1996; Boilly et al., 1991). This binding may protect FGFs from proteolytic degradation (Goldfarb, 1990). This protection could have a significant biological impact by regulating the time lapse separating FGF synthesis and utilization as well as restricting diffusion into different tissues (Goldfarb, 1990). The influence of heparins on FGF activity can be seen when cell proliferation in the blastema of regenerating limbs decreases after exogenously application of sulphated polysaccharides (Zenjari et al., 1996). These polysaccharides seize the FGF proteins preventing them from binding to the receptors. As a consequence the mitotic activity decreases in blastemal cells.

Of the four *FGFR* genes (*FGFR1-FGFR4*) known, *FGFR1* and *FGFR2*, have been found to have a distinct spatiotemporal pattern of expression during regeneration in newts (Poulin et al., 1993; Poulin and Chiu, 1995). *FGFR2* is expressed in the basal layer of the wound epidermis during pre-blastema stage and later in mesenchymal cells. In contrast, *FGFR1* is expressed during early to mid-bud blastema but is absent from the wound

epidermis. The presence of both FGFs and FGFRs in the wound epidermis and in the blastema suggests their participation in epithelial-mesenchymal interactions which may be required for outgrowth and proliferation.

1.4 Gene expression during limb regeneration

Cell growth and differentiation have to occur in a cooperative orchestrated manner during limb regeneration in order to faithfully re-establish the missing structures. This process is guided by a complex network of signals. In the last decade, efforts to understand the mechanisms of limb regeneration have been focused in finding genes coding for such signals and in determining their interactions during initiation, growth and patterning of the regeneration process.

Hox genes have been identified as candidates for specifying positional information and cell differentiation of the vertebrate body and limb axes. These genes, first identified in *Drosophila*, share a highly conserved 180 bp nucleotide sequence called the homeobox and encoding for a DNA binding domain. The proteins encoded by the *Hox* genes act as transcription factors (McGinnis and Krumlauf, 1992). In *Drosophila*, they are responsible for the specification of cell and antero-posterior patterning, which results in the determination of each body segment (Lewis, 1978). In vertebrates, four *Hox* gene clusters have been characterized and have been designated *Hox* A, B, C and D. Each cluster contains up to 13 *Hox* genes which have been named based on their position within a given cluster. For example, members of cluster A are named *Hoxa1*, *Hoxa2*, *Hoxa3, 4, 5, 6, 7, 9, 10, 11* and *Hoxa13*. Co-linearity exists between the position along the rostro-caudal axis at which a particular *Hox* gene is expressed and the physical location of that *Hox* gene within the complex (Dolleé et al., 1989; Izpisua-Belmonte et al., 1991; Duboule et al., 1989). 3' genes in the cluster are expressed more rostrally and at earlier developmental stages than more 5' genes. Analysis of the expression patterns during limb development suggests that 5' *HoxA* genes are involved in patterning the proximo-distal axis and the 5' *HoxD* genes in the patterning of the antero-posterior limb axis. The

relationship between pattern of expression and function is further supported by experiments in which the *Hox* genes have been overexpressed in chicken or knocked out in mouse (Izpisúa-Belmonte and Duboule, 1992; Morgan et al., 1992; Dollé et al., 1993; Small and Potter, 1993; Yokouchi et al., 1995). When the most 5' gene of the *HoxD* cluster (*Hoxd13*), is mutated in mouse, homeotic transformation of the phalanges occurs. However, if either *Hoxd11* or *Hoxa11* are knocked out, digits, ulna or radius are affected (Davis and Capecchi, 1994; Davis et al., 1995). The resulting appendicular skeleton defects suggest that similar *Hox* genes function together to specify limb outgrowth and patterning along the proximo-distal axis.

Several *Hox* genes have been identified in newt and axolotl (Brown and Brockes, 1991; Simon and Tabin, 1993; Beauchemin et al., 1994; Gardiner et al., 1995; Nicolas et al., 1995). These *Hox* genes show a similar genomic organization and regulation during limb development to that of higher vertebrates. However, the expression pattern of some *Hox* genes in urodele amphibian limbs is not restricted to embryogenesis, as they are also expressed in the limb during adulthood (Belleville et al., 1992). It has therefore been proposed that their expression in urodele limbs can be related to the regeneration ability of these animals.

During limb regeneration, some of the *Hox* genes observed to be expressed, such as *Hoxa13* and *Hoxa9* in axolotl, present a distinct pattern of temporal and spatial colinearity from that observed during limb development (Gardiner et al., 1995). During axolotl limb development, *Hoxa9* and *Hoxa13* expression patterns are comparable to that in mouse and chick limb bud where *Hoxa9* is expressed early and is proximally restricted in the mesenchyme and *Hoxa13* is more distally expressed presumably specifying distal limb structures. In contrast, during regeneration, *Hoxa13* and *Hoxa9* are re-expressed 24 hours after amputation in the same population of stump cells, regardless of the level of amputation. It is only at later blastema stages that they show differential expression patterns along the proximo-distal axis (Gardiner et al., 1995). Although the same set of genes expressed during limb development is used to restore the missing part of an

amputated limb, the role they play in the process may be, at least at the early stages of regeneration, somewhat different.

Another homeobox gene expressed during limb regeneration in the newt is *NvMsx-1* (Crews et al., 1995) which is related to the *Msx-1* gene in mouse and chicken. During development of the mouse and chicken limbs, *Msx1* is expressed in a group of mesenchymal cells which form the progress zone underlying the apical ectodermal ridge (see Chapter 2). *Msx1* has been proposed to maintain the proliferation and undifferentiated state of these mesenchymal cells (Song et al., 1992). In the regenerating newt limb, *NvMsx1* transcripts are found in the distal mesenchymal cells of the blastema, underneath the apical epidermal cap. However, in newt, this gene does not seem to affect either proliferation or differentiation of myogenic cells in culture (Crews et al., 1995), suggesting a different role for the *Msx-1* orthologue in the urodele limb.

The segment polarity gene sonic hedgehog (*Shh*) is expressed in the posterior mesenchymal cells of the limb bud and is thought to participate in patterning of the antero-posterior axis of the limb bud (see Chapter 2). Recent studies show that *Shh* is re-expressed in a posterior domain of the regenerating blastema (Imokawa and Yoshizato, 1997). Axial reversal graft of the posterior blastema into the anterior part of the regenerating limb results in the development of a limb with supernumerary digits. These results resemble those obtained when *Shh* is ectopically expressed in the anterior part of the developing chick limb bud, and results in duplication of the distal skeletal structures in a mirror image (Tickle et al., 1975) suggesting that *Shh* may be involved in patterning the antero-posterior axis of the regenerate.

The molecular data currently available suggests that the same key molecules present during limb development are also found during limb regeneration and might participate in the same patterning mechanism. However, slight differences exist which, according to Géraudie and Ferretti (1998), "might be due to difference in the origin of the progenitor cells during development and regeneration and to the presence of positional cues in the stump of the adult newt limb".

1.5 Teleost fish fins as models to study regeneration.

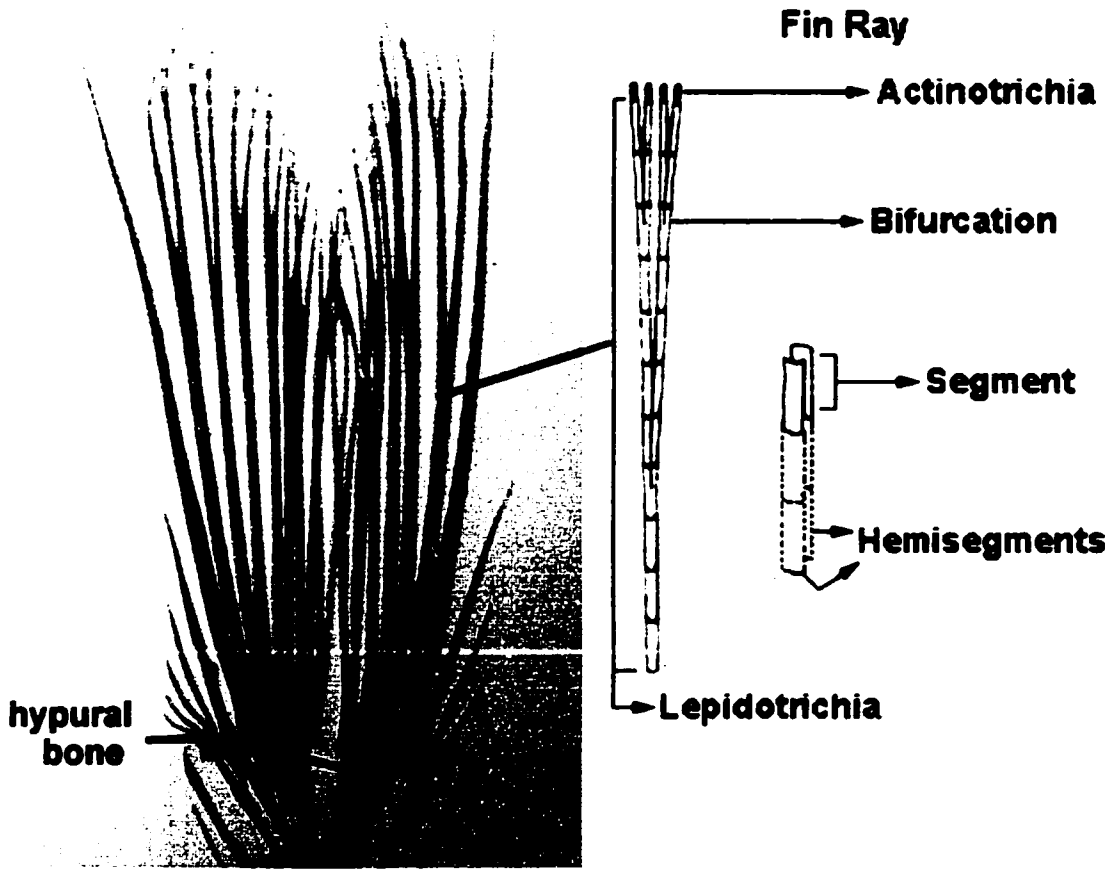
That some fish are able to regenerate their fins has been well documented since the last century but it has only been recently that research has started to give more detailed information on the process through histological and molecular studies. As I will show later, comparison of tissue structures and pattern of expression of different genes between fish and urodele during fin/limb regeneration suggests that the early steps of such processes are ruled by similar signaling networks. This, together with the relative simplicity of the structure of the fish fins, make the fin an ideal system for the studies of the regeneration process.

Many studies have shown the capability of some teleost fish to fully regenerate their fins (Géraudie and Singer, 1992; Wagner and Misof, 1992; Misof and Wagner, 1992; Becerra et al, 1996; Marí-Beffa et al., 1996). Unlike vertebrate limbs, fish fins present two different kinds of skeleton: endoskeleton and dermoskeleton. The first results from endochondral ossification of cartilage and the second from the mineralization of a proteoglycan matrix synthesized by scleroblast.

The dermoskeleton of teleost fins is formed by two different skeletal elements, the lepidotrichia and the actinotrichia (Figure 3). The lepidotrichia is composed of 2 series of parallel concave segments, or hemirays, facing each other. These hemirays occupy a subepidermal position and are attached to the endoskeleton by striated muscle (Géraudie et al., 1994). Each hemiray is the product of mineralization of a collagen basal lamella and sulphate glycosaminoglycans, synthesized and secreted by scleroblasts or lepidotrichia forming cells (LFC), cells of mesenchymal origin. The space between two hemirays (the intraray space) is filled with connective tissue and contains blood vessels, fibroblast cells and nerves.

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 branch depending on their position within the fin. Each hemiray distally

Figure 3. Skeleton of the caudal fin of adult zebrafish, revealed after alcian blue and alizarin red stainings. The exoskeleton of the caudal fin is composed of 18 major fin rays and of some lateral minor rays. The fin rays or lepidotrichia are of dermal origin since in contrast to endochondral bones, lepidotrichia ontogenesis occurs by direct mineralization of a collagenous matrix without passage through a cartilaginous precursor. Each lepidotrichia is composed of 2 parallel hemirays made of a series of concave hemisegments. The space between the hemisegments is filled by connective tissue which contains the blood vessels and the nerve bundles. The hemisegments are linked to each other with ligaments and the proximal end of the lepidotrichia are connected by striated muscles to the endoskeleton. All the lepidotrichia form branches along the proximo-distal axis except the most dorsal and ventral ones. The rays develop by addition of segments to the most distal part of the fin. The actinotrichia, the second component of the dermal skeleton of the fin rays, form an array of elastoidin fibrils lining the internal surface of the distal segment of each fin ray.


Fin Ray
Actinotrichia
Bifurcation
Segment
Hemisegments
Lepidotrichia
hypural bone

ends with a row of unmineralized elastoidin fibrils known as actinotrichia which give support to the edge of the tip of the fin (Figure 3)(Chandross and Bear, 1979; Géraudie, 1983). Actinotrichia appears before lepidotrichia, early in fin development and regeneration (Géraudie, 1992; Becerra et al., 1996), remaining always distally located in a constant polymerization-depolymerization balance (Marí-Beffa et al., 1989). The origin of the actinotrichia is still a subject of controversy. Some observations suggest that they could be synthesized by epidermal cells at the tip of the fin (Géraudie, 1980) showing some similarities with the nails in tetrapod limbs (Becerra et al., personal communication). However, only cells of mesenchymal origin surrounding the actinotrichia immunoreact with anti-actinotrichia antiserum suggesting that connective cells, and not epidermal cells, synthesize actinotrichia (Santos Ruiz et al., 1996).

Regeneration of the fin only occurs when the amputation is performed at the level of the dermoskeleton. If the amputation is made at the level of the endoskeleton or across of the articulation of the ray with the fin endoskeleton, regeneration does not occur (Géraudie et al., 1995). Each fin ray can be seen as an independent unit which presents intrinsic regeneration capabilities. However the amputation of the fin results in a synchronous regeneration of the rays at least during the early steps of fin regeneration and of the soft tissue between them.

Although the anatomy between amphibian limbs and fish fins is totally different, the process of regeneration shows similar features such as the formation of a special epidermal tissue at the tip of the amputated fin or wound epidermis and, later, the formation of an undifferentiated and proliferating tissue or blastema.

a. Wound Epidermis

As in urodele amphibians, the wound epidermis in fins plays an important role during the process of regeneration and has been suggested to control the degree of cell differentiation in the blastema (Becerra et al., 1996). Early after amputation, epidermal

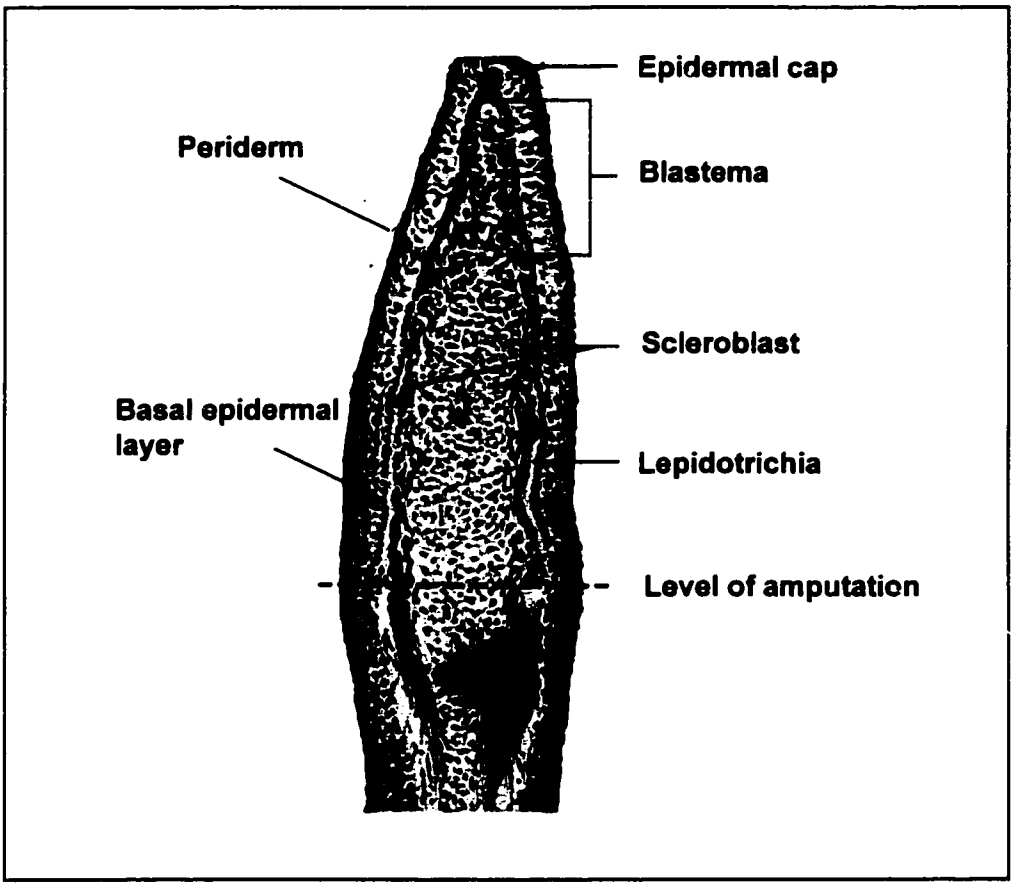
cells from the lateral side of the fin migrate to cover the wound making direct contact with the mesenchyme tissue before the basal lamina forms (Géraudic and Singer, 1992). Later, the wound epidermis forms (denominated epidermal cap at later stages of regeneration) as a stratified tissue (Figure 4) made of non dividing undifferentiated epidermal tissue (Géraudie et al., 1992; Santamaria et al., 1996a; Becerra et al., 1996; Bernhard and Wagner, 1992). This is a characteristic shared with the tip of the fin bud (pseudo apical ectodermal ridge) during ontogenesis (Géraudie, 1980). The lack of proliferation at the pseudo apical ridge has been related to the synthesis of actinotrichia in fin development (Géraudie, 1980).

b. Blastema

Once the wound is healed, a blastema forms beneath the wound epidermis (Figure 4). The time for blastema establishment depends on the fish species and size.

It is thought that the blastema, in fish as in urodeles, is formed from pre-existing mesenchymal cells that de-differentiate, proliferate and then differentiate giving rise to all types of cells required to re-establish the missing structures. This mechanism has not been as intensively studied in fins as in urodele limbs, but histological studies in regeneration of *Salaria pavo* and *Fundulus* fish show the blastema as a cytomorphological heterogeneous tissue in cellular composition (Misof and Wagner, 1992; Goss et al., 1969). Goss interpreted this as a possible cell lineage restriction suggesting that the cells of the blastema are not so fully dedifferentiated as their appearance might indicate keeping their identity during the de-differentiation, proliferation and differentiation process (Goss et al., 1969). Blastemal cells originating from scleroblasts will eventually form scleroblasts. As previously mentioned this process has been described in urodele amphibians, where de-differentiation of multinucleate myotubes yield mononucleate blastema cells, which then proliferate and primarily contribute to regenerate muscle tissues (Lo et al., 1993).

Figure 4. Proximo-distal section of a 3 days regenerated caudal fin of zebrafish, stained with hematoxylin. The epidermis is a stratified tissue whose basal layer is composed of cuboidal cells. The blastema, made of undifferentiated mesenchymal cells, is located in the most distal part of the mesenchymal compartment underneath the epidermal cap. The lepidotrichial matrix is secreted in the subepidermal space by the scleroblasts lining the lateral side of the mesenchymal compartment.
Dashes indicate the level of amputation.
m, mesenchyme; l, lepidotrichia; ec, epidermal cap; s, scleroblast; p, peridermis.


Epidermal cap
Periderm
Blastema
Scleroblast
Basal epidermal layer
Lepidotrichia
Level of amputation

Although de-differentiation is the most advocated mechanism in urodele amphibian blastema formation and can be probably extrapolated to fish, the idea that some stem cells participate in blastema formation in fish still persists (Géraudie and Singer, 1992). As the blastemal cells differentiate, their shape as well as their external membrane characteristics change according to the position at which they are within the fin (Marí-Beffa et al., 1996). These changes of the blastemal cells have been suggested to depend on nerve supply, cell-cell interactions and cell interactions with extracellular matrix components (Géraudie and Singer, 1979; Misof and Wagner, 1992; Marí Beffa et al., 1996; Becerra et al., 1996).

Positional identity in cells, in both urodele amphibians and fins specify their location on a proximo-distal axis, resulting in the normal regrowth of the structure distal to the level of amputation. In fish, even the pigment patterns are re-established after regeneration (Géraudie et al., 1995). As in amphibian limb regeneration, retinoic acid has the characteristic, under certain conditions of administration, to respecify proximo-distal and dorsal-ventral patterning. It increases the number of segments between the level of amputation and the first bifurcation and under different conditions, RA can also have teratogenic effects which results in the fusion of the fin rays (Géraudie et al., 1994; Géraudie et al., 1995).

How these effects occur in fish fins has yet to be determined. In zebrafish three subtypes of RAR retinoic acid receptors (α, β and γ) (White et al., 1994) and five RXRs (α, β, γ, δ, ε) (Jones et al., 1995) have been characterized, but only two, RARα and RARγ, have been reported to be expressed in the distal mesenchymal cells of the blastema of regenerating fins. Of the two, RARγ seems to be the predominant transcript in the fin regenerate. Its equivalent in newt, RARδ2 has been proposed to be involved in cell identity in the blastema of regenerating limbs (Pecorino et al., 1996). This suggests that RA might be playing a similar role in respecifying axial identity in regenerating fins.

The requisite for nerve supply is another characteristic that fin and limb regeneration share. The denervation of an amputated pectoral fin results in the lack of restoration of the missing part, and only wound healing takes place (Géraudie and Singer, 1979).

One feature that strongly differs between regeneration in amphibians and in fish is the speed at which it occurs. In fish, the process takes place much quicker with some fishes forming well developed fin components only a week after partial fin amputation. In contrast, the establishment of limb structures in urodeles can take several weeks. Thismight be due to the different anatomical complexity.

1.6 Regeneration in zebrafish (*Danio rerio*)

Most studies on fin regeneration have been done in teleosts such as *Salaria pavo* (Bernhard et al., 1992) *Tilapia melanopleura*, *Cyprinus carpio*, *Carassius auratus* (Becerra et al., 1996; Santamaria et al., 1996a; Marí-Beffa et al., 1996) and *Fundulus heteroclitus* (Géraudie and Singer, 1992). More recently, zebrafish has been included as an important model to analyze different developmental processes including fin regeneration. As many other teleost fishes, it has the capability to fully regenerate its fins after amputation.

Regeneration is now studied in zebrafish because zebrafish has become a very popular model to analyze vertebrate embryonic development. The increasing amount of data on gene expression during embryonic development makes the zebrafish an attractive model to study the molecular mechanism of fin regeneration.

The regeneration process in zebrafish is very similar to that described for other teleost fish, differing only in the timing of the different events such as wound healing, formation of the blastema and cell proliferation.

As previously mentioned, regeneration only takes place when the amputation is performed at the level of the dermoskeleton. After amputation, the wound is rapidly healed and growth of the regenerate is apparent 2 days after amputation. Formation of the rays can be seen a week after the injury and occurs in a proximo-distal manner. If the fin is amputated below the first bifurcation, replacement of the missing part occurs in an apparently faithful phenotype. However, a defect is observed in the pattern of the

branching forks, which form more distal than in the non amputated ray (Géraudie et al.,1995).

1.7 Gene expression during fin regeneration in zebrafish

The process of regeneration requires the activation of genes which are not normally expressed in non-injured adult fins. It is still not known which factors trigger the regeneration process, however the expression of some key genes known to play a fundamental role during fin/limb development has been investigated in regenerating fins. For example, members of the *HoxD* cluster (*Hoxd11*, *Hoxd 12*), are expressed in distal blastemal cells (Borday et al., 1998). Members of the zebrafish *Msx* homobox gene family, (*msxA*, *msxB*, *msxC and msx D*) are also expressed during regeneration (Akimenko et al., 1995). While *msx A* and *msxD* are re-expressed in the epidermal cap overlying the distal blastema, *msxB* and *msxC* are re-expressed in the distal blastema (Akimenko et al., 1995).

Other genes, such as *sonic hedgehog* gene (*shh*), its receptor *Patched-1* (*Ptc-1*), and a member of the *Fgf* gene family (*Fgf8*)(in this work, Chapter 2) reported to be expressed during zebrafish fin development are also re-expressed during fin regeneration.

However, despite some similarities in the patterns of expression of some genes, such as the *msx* genes, there are differences between the molecular mechanisms of embryonic fin development and regeneration. Some genes expressed during fin development are not re-activated during fin regeneration. For example, expression of the zebrafish *engrailed-1* gene (*eng1*), whose transcripts are found in the ventral ectoderm of the pectoral fin bud (Hatta et al., 1991), is undetectable in the regenerating fin (Akimenko and Géraudie, personal communication).

The products of the genes so far analyzed contribute either to patterning and growth or to differentiation of the regenerate, or to both processes. Nevertheless, it is clear that many transcription factors and signaling molecules are shared between the processes of fin development and regeneration, even though they may not exert identical functions.

1.8 Objectives

The regeneration process in fins implicates many different cellular changes during wound healing, formation of the blastema and growth of the regenerate to restore the missing structures. These different processes involve cell de-differentiation, proliferation, migration and differentiation. If we want to understand the molecular mechanisms of fin regeneration, it becomes essential to have a good analysis of the dynamic of cell proliferation during fin regeneration in the species analyzed, i.e. the zebrafish (*Danio rerio*).

In order to have a better understanding of such mechanisms in the zebrafish, I have examined and described the pattern of cell proliferation during early and late regeneration of the caudal fin. The cell proliferation pattern was followed using an analogue of thymidine, bromodeoxyuridine (BrdU), which, after injection in the fish, is rapidly uptaken by cells and incorporated into the nuclear DNA during S phase of the cell cycle. BrdU incorporation can be detected by immunocytochemistry using a mouse monoclonal antibody against BrdU. This method has been used intensively in cell and organ culture as well as in *in vivo* studies of cell proliferation.

The pattern of cell proliferation observed during caudal fin regeneration in zebrafish was compared with proliferation patterns described for other teleost fish (Becerra et al., 1996; Santos Ruiz et al., 1996).

II. MATERIAL AND METHODS

1. Animals

Adult fish purchased from a local supplier, were maintained in an aquaria at 28.5°C with a photoperiod of 14 hours light 10 hours darkness and fed daily (Westerfield, 1993).

2. Fin Amputation

Adult zebrafish were anaesthetized by immersion in water containing 0.17 mg/ml tricaine (ethyl-m-aminobenzoate, MS 222, Sigma). The fins were then amputated using a scalpel and the fish were returned to their tank. At different times of regeneration, depending on the experiment, the fins were cut off again and fixed in phosphate buffered saline solution (PBS) containing 4% paraformaldhehyde (PFA) (Westerfield, 1993).

Amputation of the caudal fins was always made proximal to the level of the first bifurcation of the rays.

3. Labeling of proliferating cells with BromodeoxyUridine (BrdU)

Labeling of proliferating cells with 5-Bromo-2-DeoxyUridine (BrdU, Sigma) was performed according to Santamaria *et al.*, 1996.

Adult fish were anesthetized by immersion in water containing tricaine.

Injections of 0.25mg/g wet weight BrdU in Hank's solution were made in the general cavity of the fish, with an insulin syringe, at various time points after caudal fin amputation (1, 2, 3 and 5 days regeneration). At specific time points after BrdU injections, the regenerated part of the fins were dissected out and fixed in 4% PFA for cryostat sectioning.

In each experiment, 6 different fish were injected with BrdU, and their caudal fins were used to perform immunocytochemical detection assays.

4. Cryostat Sectioning and slide mounting

Sectioning and mounting were performed as previously described in Westerfield et al; 1995.

After fixation, fins were embedded in a molten 50°C solution of 1.5% agar and 5% sucrose PBS. After hardening of the solution, a block containing the material to be sectioned was cut using a scalpel. These blocks were placed in a 30% sucrose PBS

solution and stored at 4°C overnight. The blocks were mounted on the cryostat chuck in a layer of OCT compound (Tissue Tek, Miles) and then frozen with 2-methyl butane (-50°C). 16 μm thick sections were made using a LKB cryostat and collected in glass slides. Slides used in immunocytochemistry were kept at -20°C before use.

5. Immunocytochemical detection

The sections were permeabilized with 10μg/ml of proteinase K for 10 min and then rinsed 3 times with PBS pH 7.5. Nuclear DNA was denatured with 2N HCL for 45 min at 37°C and then neutralized with 0.1M disodium tetraborate (Borax) for 10 min. Three washes in PBS pH 7.5 were done before the sections were blocked using a solution of PBS containing 3% bovine serum albumin (BSA) and 0.1% sodium azide.

The DNA that incorporated BrdU was immunocytochemically detected using a mouse monoclonal antibody against BrdU (Sigma) as a primary antibody, diluted 1:500 in 1X PBS, 0.1% BSA and 0.1% Sodium azide pH 7.5. Slides were incubated overnight at room temperature and rinsed 3 times with PBS/BSA. The sections were then incubated with a 1:100 dilution of goat anti mouse IgG antibody conjugated to fluorescein isothiocyanate (FITC) (Jackson ImmunoResearch Laboratories, Inc.) for 60 minutes at room temperature. After several washes in PBS, the sections were mounted using a solution of 50% Glycerol 50% PBS, observed and photographed under a fluorescent light of a Zeiss microscope.

III. RESULTS

1. Pattern of cell proliferation during initiation of fin regeneration

To determine the pattern of cell proliferation in regenerating fins, I analyzed the incorporation of the thymidine analogue, BrdU, into nuclear DNA during the S phase of the cell cycle in the mesenchyme and epidermal tissue of regenerating fins.

Two different types of controls were performed: one where incubation with the primary antibody was omitted (Figure 5A) and a second where sections of fins from fish not injected with BrdU were immunocytochemically treated (not shown). No specific antibody reaction was observed.

To determine the optimal time at which the highest number and intensity of labeled cells is observed, I first injected fish in the general cavity, with a single dose of BrdU (2,5 mg/g wet weight) and collected the fins at several time points afterwards (1/2, 1, 4, 7, 24, 48 and 72 hours). This first experiment was done on fish with an already well developed 5 day fin regenerate. I found that BrdU is incorporated into the DNA of lateral epidermal cells as fast as half an hour after its injection, indicating the high proliferation rate of this tissue and the short span of time in which BrdU is metabolized. Blastemal cells showed signs of labeling one hour after injection. However, the maximum number of cells labeled is seen in lateral epidermis and blastemal cells seven hours after BrdU injections. After this time, labeling gradually decreases in the epidermis and in the blastema until it is almost completely diluted in both tissues at 72 hours after BrdU injection (see Section 3. Proliferation and migration).

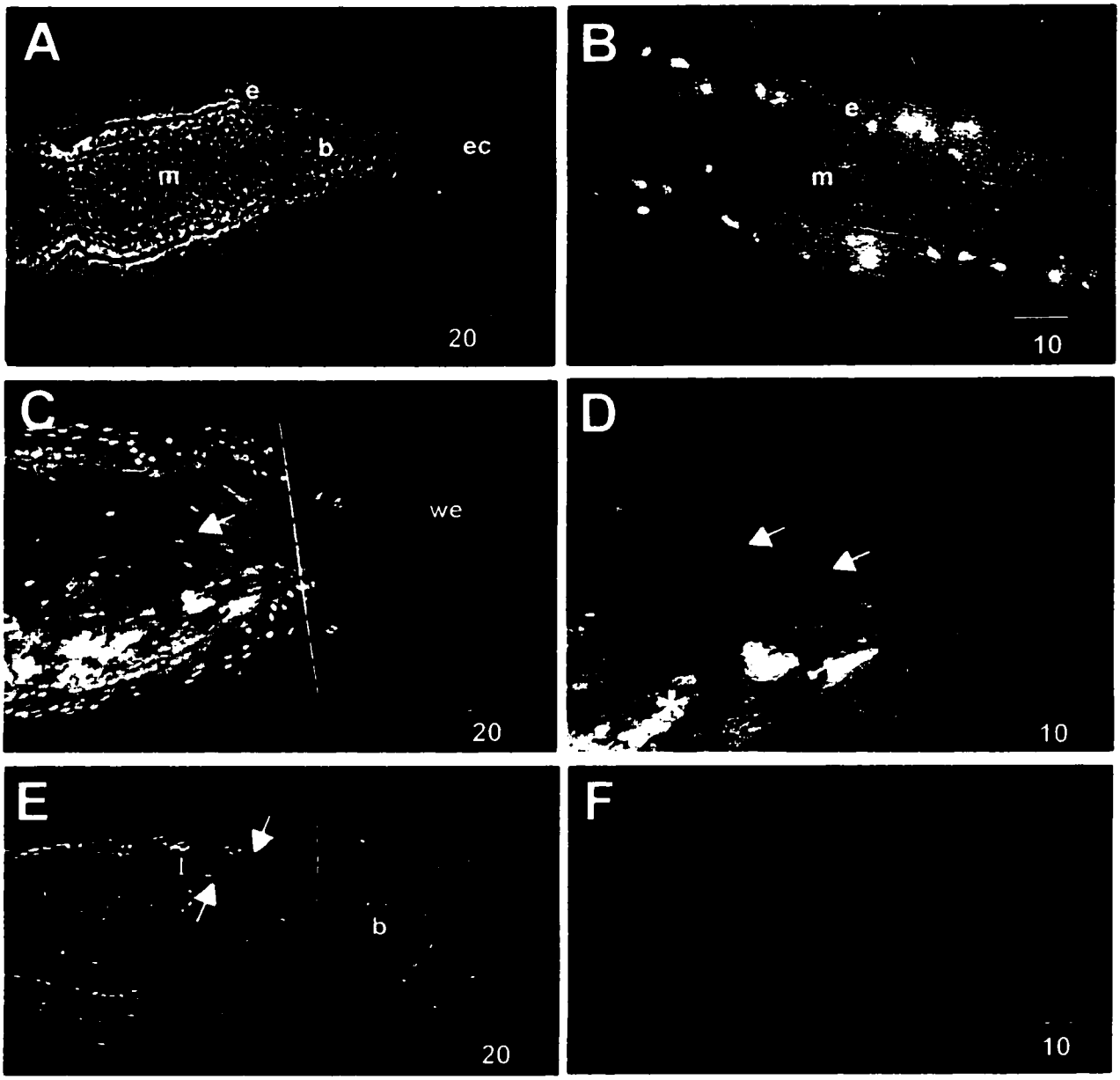
This work only shows qualitative results. Quantitative observations will eventually be necessary to establish the exact proliferative rate of the different cell types depending on their location within the regenerate.

Unless otherwise stated, 7 hours after BrdU injection was taken as the standard time to perform the rest of the experiments as it was the time at which the maximum of cells were labeled.

In order to observe the cell proliferation pattern during the first 72 hours after fin amputation, fins of adult fish were amputated and allowed to regenerate for 6, 24, 48 and 72 hours. BrdU was injected 7 hours prior to the collection and fixation of the fins.

In zebrafish, epidermal cells cover the stump as early as six hours after amputation. At that time, the cells of the wound epidermis did not show incorporation of BrdU (not shown). However, the lateral epidermal cells showed basal levels of BrdU incorporation

Figure 5. Proximo-distal sections of regenerated caudal fins showing the pattern of cell proliferation after incorporation of BrdU. BrdU was injected 7 hours prior to fin collection. **A.** Control. Fins incubated only with the secondary antibody showing no labeled cells. **B.** Non amputated fin showing the basal level of cell proliferation in the epidermis and no proliferation in the mesenchyme. **C.** Twenty four hours after amputation, the epidermal cap is covering the stump. It does not contain any labeled cells. The epidermis below the level of amputation is strongly labeled as are some cells in the mesenchyme (arrow) which are involved in the formation of the blastema. **D.** Enlargement of **C** showing labeled cells in the mesenchyme (arrows). **E.** The blastema of a regenerating fin, 2 days after amputation, showing a high level of cell proliferation. Some of the cells located a few micrometers beneath the plane of amputation in the mesenchyme are labeled as well as some cells surrounding the lepidotrichia (scleroblasts). The lateral epidermis presents a high rate of cell proliferation, while, the apical epidermis rarely contains labeled cells. **F.** 3 days after amputation, the blastema is strongly labeled. In contrast , cells proximal to the blastema region do not show signs of proliferation.
The asterisk in C indicates an artefactual signal attributable to erythrocytes whose fluorescence is distinguishable from the fluorescence of cells labelled with FITC antibody; b, blastema; m, mesenchyme; we, wound epidermis; ec, epidermal cap. Dashes in C and E indicate the plane of amputation. Scale bars in A,C,E: 20 μm; B,D,F: 10 μm.


A
e
b
ec
m
20
B
e
m
10
C
we
20
D
10
E
l
b
20
F
10

similar to those observed in the epidermis of non-amputated fins (Figure 5B). Twenty four hours after amputation, the wound epidermis forms a thicker tissue that rarely shows divisions. In contrast, the epidermis below the level of amputation is highly labelled (Figure 5C). The blastema and the basal layer of the epidermis are yet to be formed, but some fibroblast-like cells show signs of BrdU incorporation (Figure 5C-D, arrows). These cells may participate in the formation of the blastema.

At two days of regeneration, the blastema is composed of a group of packed cells distal to the level of amputation that present a high degree of incorporation of BrdU. It was interesting to find that a number of cells 75 μm under the level of amputation in the connective tissue, also showed signs of BrdU incorporation (Figure 5E), suggesting that these cells could eventually form part of the blastema. Similarly some of the cells surrounding the stump lepidotrichia, probably scleroblasts, also showed BrdU incorporation (Figure 5E, arrows). At this time, the basal epidermal layer is established and is composed of highly ordered cuboidal cells which are not labeled. At three days post amputation, the epidermal cap still does not show signs of proliferation and both the lateral epidermis and the blastema show high incorporation of BrdU (Figure 5F). However, the newly formed mesenchyme, proximal to the blastemal region, does not show signs of BrdU incorporation suggesting that these cells do not proliferate and are probably engaged in the process of cell differentiation (Figure 5F).

2. Pattern of proliferation in 3 days regenerated caudal fins (Transverse sections)

The caudal fin is composed of 19 rays separated from each other by soft tissue. In order to compare the proliferation pattern of interray and intraray tissues along the proximo-distal axis of the regenerate, a series of transverse sections were carried out on 3 day regenerated fins.

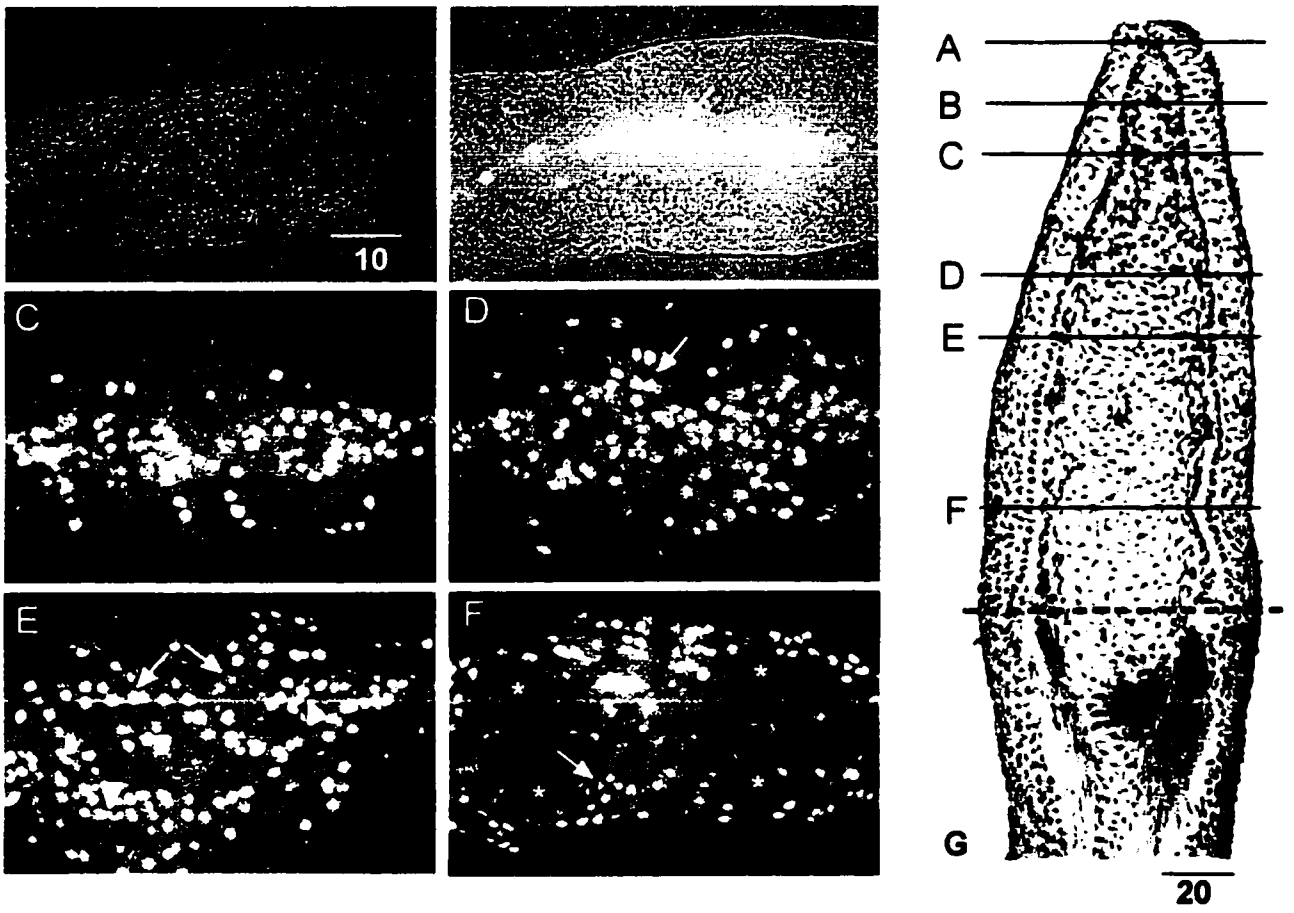
After three days of regeneration, the epidermal cap and the blastema are well established and the developing lepidotrichia are already visible as shown on the proximo-distal section of the caudal fin (Figure 6G).

The most distal transverse section, which only contains cells of the epidermal cap, did not show signs of BrdU incorporation (Figure 6A). At a more proximal level (20μm from section A), labeled nuclei can be seen in 30% of the blastemal cells while epidermal cells remain unlabeled (Figure 6B). The lack of labeling in some of the mesenchymal cells may be attributed to the dilution of the labeling due to the high proliferation rate in the blastema. Figure 6C shows two adjacent rays, 30μm proximal to section A. The mesenchyme is strongly labeled within each ray (asterisk) and to a lesser extent in the region between the rays (interray). At this level, some of the proliferating cells in the epidermis are distributed close to the basal epidermal layer of each fin ray. However, the basal layer of the epidermis remains unlabeled. It is at this level that the lepidotrichial matrix is starting to be deposited in the epidermal/blastema interface.

More proximal sections (62 μm below section A) show that a few cells in the basal layer of the epidermis incorporated BrdU (Figure 6D, arrow). The blastema is still strongly labeled in both the interray and intraray tissue. More proximally (87 μm below section A), the number of labeled basal epidermal cells increases (Figure 6E). Many cells, close to the forming hemi-ray, are labeled (Figure 6E, arrows). Closer to the plane of amputation, the number of labeled cells decreases progressively in the whole mesenchymal compartment (interray and intraray) (Figure 6F), indicating a non dividing tissue characteristic of differentiation. However, the epidermis still shows a strong degree of proliferation in the medial layers.

No incorporation of BrdU was observed in any of the sections of the outer layer of the epidermis (peridermis), suggesting that the cells of this epidermal layer do not divide.

Figure 6. Pattern of cell proliferation in transversal sections of a regenerating caudal fin 3 days after amputation. BrdU was injected 7 hours prior to fin collection. **A.** Section at the tip of the regenerated fin showing that cells of the wound epidermis did not incorporate BrdU. **B.** Section at a more proximal level (20 μm from section A) where some labeled cells can be seen in the blastema (arrow). Epidermal cells at this level rarely divided. **C.** 30 μm below section A, two adjacent fin rays are shown. The mesenchymal tissue, in the intra-ray (asterisk) as well as in the interray (star) shows a strong incorporation of BrdU. The epidermal basal cell layer is well established but does not show any sign of cell proliferation even though some epidermal cells, close to the basal layer, are labeled. **D** and **E.** 62 μm and 87 μm below section A, respectively. **D.** At the level of the newly forming lepidotrichia, the basal epidermal cells (arrows) incorporated BrdU. Arrowhead in E indicates the scleroblasts lining the newly forming hemisegment of the lepidotrichia. **F.** The new lepidotrichia have thickened (asterisk), the epidermis is still strongly labeled. A few mesenchymal cells have incorporated BrdU. The labeled cells are concentrated in the interray connective tissue. **G.** Proximo-distal section of a 3 days regenerated caudal fin stained with hematoxylin. Horizontal lines indicate the levels of transversal sections shown in panels **A** to **F**. Scale bars in A-F: 10 μm; G: 20 μm.


10
C
D
E
F
A
B
C
D
E
F
G
20

3. Proliferation and migration

Cells in the different tissues of regenerating fins can move as a result of "active" or "passive" migration. While in active migration the cells are responsible for their own movement, in passive migration the increase in cell number due to cell divison forces the cells to move and the tissue to grow. The movement of proliferating cells, whether passive or active, can be followed if labeled dividing cells are observed at different times of regeneration.

To observe the path followed by cells of the different tissues (epithelial and mesenchymal compartments), the regenerating cells were labeled by injecting BrdU into the general cavity of fishes five days after caudal fin amputation. The regenerated fins were cut again and fixed at different time points (7, 24, 48, 72 hours) after BrdU injection (Figure 7).

Seven hours after BrdU injection, five day regenerated fins show similar BrdU incorporation patterns to those observed in 3 day regenerated fins (see Figure 5F). However, at 5 days, the different tissues and fin components are more established giving a better overview of the proliferation process. The epidermal cap did not contain any labeled cells (Figure 7A). Some cells close to the peridermal layer showed labeling (Figure 7A, arrowhead), however, the medial and the basal epidermal layer did not. In contrast, in more proximal levels, labeling of the intermediate cell layers of the epidermis and basal epidermal layers were observed (Figure 7A, small arrow).

At the level where the lepidotrichia is forming, the number of epidermal cells that incorporated BrdU is considerably higher, especially towards the intermediate epidermal layers, decreasing as the plane of amputation is approached. The basal epidermal layer contains labeled cells immediately proximal to the level where the new hemirays are being synthesized (small arrow). At the distal part of the blastema, cells are strongly labeled (Figure 7A) on a proximo-distal length of about 50μm. Their number and intensity decrease towards the proximal part due to BrdU dilution resulting from cell

division, suggesting a high proliferation rate for this tissue. Surrounding the newly forming lepidotrichia, sclerobasts show labeled nuclei, up to 100 μm (arrows) into the proximal region of the regenerating fin. This indicates that the signal or signals that maintain this specialized cell dividing capacity is still present far from the apical cap and blastema.

In fish injected 24 hours prior to fixation (Figure 7B), the intensity of the signal in the blastema is lower but more widely dispersed than in the mesenchyme of fish injected 7 hours prior to fixation (Figure 7A). However, the pattern of labeled cells in the epidermis is similar to that in Figure 7A. In fish injected 2 days prior to fin fixation (Figure 7C), only a few labeled cells can be seen in the blastema. The intensity of the labeling in mesenchymal cells is even lower to that observed in figure 7B due to the dilution of BrdU among the cells. The epidermal cap, located where cells are not thought to proliferate, show labeled cells. This suggests that cells previously labeled in more proximal regions migrated towards the tip of the fin epidermis during its growth. The different intensities found in the labeled cells of the epidermal and mesenchymal compartments suggest distinct rates of proliferation.

Cells of the basal epidermal layer, 72 hours after BrdU injection (Figure 7C, arrow), are still strongly labeled indicating a low proliferation rate. In fish injected 3 days prior fin fixation (Figure 7D), most of the cells have lost their labelling. Only some cells close to the most outer layer of the epidermis (periderm) and in the proximal mesenchyme are still labeled.

These experiments allow us to somewhat follow the path of proliferating cells during the regeneration process and show that different types of cells present different proliferation rates.

Figure 7. Proximo-distal sections of 5 days regenerated fin showing cell proliferation after a single injection of BrdU at different times prior to fin collection, (**A**) 7 h., (**B**) 24 h., (**C**) 48 h. and (**D**) 72 h. **A.** Seven hours after BrdU injection the epidermal cap does not have any labeled cells, however 10 μm proximal, some cells close to the peridermis are labeled. More proximally, at the level of the forming lepidotrichia, the number of cells in the intermediate epidermis having incorporated BrdU considerably increases. The number of labelled cells decreases as the level of amputation is reached. The basal epidermal layer presents signs of proliferation at the level where the lepidotrichia matrix is released (small arrows). The blastema is strongly labeled (Asterisk) but labelling of the mesenchymal cells decreases proximally. Surrounding the lepidotrichia, for at least 100 μ m below the blastema, scleroblasts are labeled (Arrows). **B.** 24 hours after BrdU injection, the intensity of the labelling in the blastemal cells has decreased. Signs of BrdU incorporation is spread throughout the mesenchyme suggesting cell movement. The pattern of labeled cells in the epidermis is similar to that in A. **C.** 48 hours after BrdU injection, the overall intensity of cell labelling has decreased indicating BrdU dilution due to multiple cell division. Many cells in the epidermal cap are now labeled. The distal blastema does not show labeled cells while a number of more proximal mesenchymal cells present a weak signal. These cells are probably derived from the successive divisions of strongly labeled blastemal cells similar to those shown in A. **D.** At 72 hours most of the cells have lost their labelling, only some cells close to the peridermis show sign of BrdU incorporation (Arrows) as well as cells in the proximal mesenchyme (small Arrow).
l, lepidotrichia; m, mesenchyme; ec, epidermal cap; Asterisk in A, blastema. Dashes in A correspond to the level of amputation. Scale bars A-D: 10 μm.


A
l
m
ec
B
C
D

IV. DISCUSSION

The regeneration process in fish and amphibians can be divided into different phases corresponding to wound healing, blastema formation and growth of the tissue to restore the missing structure. In each phase, the tissues involved exhibit their own characteristic of cell movement, proliferation, cell-cell interaction and cell-ECM interaction.

The purpose of this study was to determine and describe the proliferation pattern during regeneration of the amputated zebrafish caudal fin, at different times following fin removal by labeling dividing cells with the thymidine analogue BrdU.

Both mesenchymal and epidermal cells were found to have distinct proliferating dynamics.

Non-amputated zebrafish fins showed incorporation of BrdU in the intermediate cell layers of the epidermis which were attributed to a basal level of proliferation probably required for the replacement of cells lost to "wear and tear" and/or for the constant generation of cells needed for ongoing fin growth. Such a basal level of proliferation was not observed in the teleost fish *Carassius auratus* (goldfish) when BrdU incorporation was analyzed 12 hours after injection (Santos Ruiz et al., 1996). Such a difference can be explained through the distinct proliferation rate of the epidermis for each fish which may also account for the considerably slower rate of regeneration in gold fish caudal fins compared to zebrafish. Another interpretation is that such a difference may be due to the different technical approaches used. In the studies of cell proliferation in goldfish caudal fin, BrdU labeled cells were detected using the peroxidase-antiperoxidase complex (PAP) which may be less sensitive than the fluorescein conjugated (FITC) used in our study (Dr. Jean-Yves Sire, Universite Paris VI, France, personal communication).

1. Cell proliferation in the epidermal layers of the fin regenerate

The regeneration process begins with the re-ephitelialization of the wound by epidermal cells from the lateral side of the stump. In zebrafish, wound healing occurs

very rapidly; 6 hours after fin amputation, the wound was completely covered by epithelial cells. At this time, the lateral epidermis shows basal levels of BrdU incorporation. This result is consistent with the idea that cell movement occurs by active migration and not by the pressure exerted by cell proliferation. This is further supported by Santoz Ruiz and co-workers who, in gold fish, observed narrowing of the epidermis near the level of amputation which can be attributed to the loss of cell layers by migration to the wound.

Twenty four hours after wound healing, the epidermis that first covers the injury becomes thicker, forming the epidermal cap and shows no sign of proliferation. At this time, the blastema and the epidermal basal layer are not yet formed, and the number of labeled cells in the lateral epidermis has considerably increased whereas in gold fish the first labeled cells appeared in the lateral epidermis 36 hours after amputation. The same high proliferation rate of the epidermis was observed at all the subsequent times of regeneration analyzed. The increase in cell proliferation is probably to compensate the loss of cell layers that occur during wound healing and later to provide cells required for the growth of the tissue. What signals or factors trigger such an increase in the proliferation rate of the epidermal cells is not known.

The epidermal cap did not show signs of BrdU incorporation during the whole process of regeneration in any of the fins observed. This lack of proliferation is also exhibited by other teleost fish such as gold fish (Santos Ruiz et al., 1996) and may be correlated to the low mitotic activity in the pseudo-apical ectodermal ridge of the bud during fin ontogenesis (Géraudie, 1980). Géraudie suggests that the absence of cell proliferation in the wound epidermis in fishes is due to their involvement in the synthesis of the actinotrichia since the actinotrichia differentiate directly under the wound epidermis of the regenerate (Géraudie and Singer, 1992). However, the absence of proliferation in this region is probably not a direct consequence of actinotrichia production. The reasons for this are: first, because DNA synthesis arrest is not a prerequisite for collagen synthesis to occur (Schiltz, 1979); and secondly, because of the observation that cells from the

connective tissue and not the basal epidermis are probably the ones that synthesize actinotrichia (Santamaría et al., 1996). Although the epidermis does not directly synthesize the actinotrichia matrix, its participation through epidermal-mesenchymal interaction cannot be ruled out.

Regardless of the reason for such non proliferating behaviour displayed by the epidermal cap, studies in urodele amphibians have demonstrated that without the wound epidermis, the regeneration process does not take place. This suggests a strong ephitelial-mesenchymal interaction that results in the patterning and growth of the limb, probably through the release of signalling molecules including retinoic acid, and fibroblast growth factors, which are found in the wound epidermis. A similar interaction may be taking place during fin regeneration since it is only after the wound epidermis is well established (24 hours after amputation) that the cells from the underlying mesenchyme (pre-blastema cells) start to incorporate BrdU. In gold fish, the first labeling observed in the blastema appeared between day 3 and 4 after amputation.

Analysis of BrdU labeled cells 2 days after injection showed that the movement of epidermal cells did not only take place during wound healing but continued toward the tip during the whole process of regeneration (Figure 7C). Labeled cells were not observed in the epidermal cap prior to 2 days. A likely explanation for this is that the labeled cells at the tip of the fin, seen 48 hours after BrdU injections, are descendants of more proximal cells that have divided (showing less intense BrdU labelling) and then migrated to this position. To further test this hypothesis, Christopher Brown in our lab, labeled epidermal cells with a lipophilic dye which labels cell membrane (DiI) at different distances from the level of amputation and followed their movement. He found that injections up to 900 μm from the level of amputation, gave rise to stained epidermal cap within 24 hours. These experiments confirmed the idea that the cells of the epidermis are originating from the lateral side of the fin and can migrate long distance before reaching the tip of the fin in a considerably short time.

Very little, if anything, is known regarding the underlying molecular mechanisms which lead to cell proliferation and movement during the process of regeneration. The following propositions could be formulated:

a) Lateral epidermal cell proliferation could be triggered by biochemical signals originating in the wound epidermis, as it is only after the wound epidermis is formed that epidermal proliferation rate increases. It is well known in amphibian urodele limbs that the wound epidermis is indispensable in order for the regeneration process to take place. However the underlying molecular mechanism is not yet known. Retinoic acid and some members of the FGF family are good candidates as signals present in the wound epidermis that can influence cell behaviour in the blastema as well as in the lateral epidermis of amphibians. Topical application of retinoic acid in mouse skin induces regeneration-like effects, skin irritation, increases epidermal DNA synthesis and hyperplasia (Lutzow-Holm et al., 1995). In human skin, FGF proteins together with epidermal growth factor (EGF) and insulin-like growth factors (IGF) are important mitogens for wound healing and are capable of stimulating epithelialization (Bhora et al., 1995). In rats, FGFR-1 has been found to be mainly expressed in newly forming epidermis during wound healing after burn (Takenaka et al., 1997) suggesting that FGF may affect the proliferation and differentiation of epidermal keratinocytes as well as angiogenesis.

b) Signals could be triggered as a result of a mechanical force induced by the change of tissue tension generated by the injury and tissue elongation. Ingber (1997) suggests that many signals can be activated by mechanical stresses resulting from the interaction of the ECM with a receptor complex associated with the cytoskeleton. However, much is still to be learned of how cells convert the mechanical signals into chemical responses (See Appendix).

It has been recently reported that cyclic strain of human keratinocytes, in vitro, show signs of cell proliferation, DNA synthesis, elongation, and protein synthesis (Takei et al.,

1997). The growth of keratinocytes is attributed to the previously mentioned mechanism, which implicates a network of growth factors, cytoskeleton, and the protein kinase family.

To study the participation of the cytoskeleton in epidermal cell migration and proliferation it will be interesting to see what happens when the microfilament structure is damaged. This can be done using cytchalasin B or D which is a fungal metabolite that binds to actin and disrupts its structure. I would expect a decrease in cell proliferation as well as migration since it is well known that epidermal cell movement during wound healing is dependent on actin (Donaldson and Dunlap, 1981; Pascolini et al., 1984)

c) Conversely, these epidermal cells could be responding to neurotrophic signals released by nerves. Denervation of the regenerating pectoral fin in the fish *Fundulus* results in a rapid decline of protein and DNA synthesis that results in no replacement of the missing part (Géraudie and Singer, 1979). However, wound healing occurs suggesting that denervation does not interfere with cell migration. Labeling epidermal cells within a ray using DiI and denervation of the same ray will give us more information regarding the relationship between neurotrophic factors and cell migration. It might be also interesting to follow the pattern of cell proliferation after denervation during caudal fin regeneration.

d) Finally these mechanisms could arise as a result of a combination of the above possibilities.

2. Cell proliferation in the mesenchymal compartment of the fin regenerate

In zebrafish, 48 hours after amputation, the blastema is present and shows a high rate of proliferation above the level of amputation (Figure 5E), whereas in gold fish blastemal cells are first labeled between day 3 and 4 after amputation.

At a distance up to 50μm proximal to the level of amputation, in the mesenchyme tissue, some fibroblast and scleroblast cells surrounding the lepidotrichia stump are labeled. This indicates that the signals involved in the activation of the cell cycle are

present relatively far from the level of amputation. Whether or not these cells are contributing to the formation of the blastema is still not known. Further analysis of the movement and proliferation of the cells located beneath the level of amputation, presently under investigation, will give some insight into the role of these cells.

As soon as the cells underlying the epidermal cap, composed of a morphological mass of undifferentiated and proliferating cells (Becerra et al., 1996), escape the influence of factors that induce proliferation, differentiation occurs (28 and 48 hours after BrdU injections, Figure 7B,C). The blastema gives rise to all mesenchymal cell types involved in tissue growth. While some cells become scleroblasts or lepidiotrichia forming cells and line the lateral border of the regenerate, others become fibroblasts (Misof et al., 1992; Becerra et al., 1996; Marí-Beffa et al., 1996).

It is still unclear which factors are responsible for keeping the blastemal cells undifferentiated and proliferating. However, genes such as two members of the *msx* homeobox gene family, *msxB* and *msxC*, and the *fibroblast growth factor 8* (*Fgf8*)(in this work, Chapter two), known to be involved in cell proliferation, have been found to be expressed in the distal part of the blastema, suggesting their participation in blastema maintenance.

Scleroblast was another cell type which presented specific proliferating characteristics. The pattern of BrdU incorporation that these cells showed surrounding the lepidotrichia of a 5 day regenerated fin (Figure 7A), suggests that scleroblast differentiation might be influenced by the degree of maturity of the lepidotrichial matrix; the most distal lepidotrichial matrix in the regenerate being the less mature (see Figure 8). Five days into the regeneration process, the hemisegments that form the lepidotrichia are still constituted of a collagen basal lamella as can be determined by alcian blue and alizarin red staining which stains collagen and calcified tissue, respectively (Figure 8). The rays increase in width primarily by deposition of a combination of ECM collagen with sulphated glicosaminoglicans, changing the proportions as more matrix is deposited and then

Figure 8. Five day regenerated fin stained with alcian blue and alizarine red. The large arrow indicates the wave of lepidotrichial matrix maturation that extends from the proximal to the distal portion of the regenerated fin. The small arrows show the first and second segmentation in each fin ray regenerate. The actinotrichia form an array of organized fibrils at the tip of each fin ray (Arrowhead).
Dashes indicate the level of amputation.

mineralize (Santamaria et al., 1992). This change in ECM components might be influencing differentiation of the scleroblast. ECM-dependent cell differentiation has been extensively studied in other models (Hay, 1989).

The extracellular matrix influences the cell differentiation processes which occur during chondrogenesis. Chen et al., (1995) suggests that program of chondrocyte differentiation is regulated, at least in part, by the surrounding extracellular environment. This process presents different switches during differentiation that drive the transitions from the proliferating state to maturation and from maturation to a hypertrophy state. Both of these transitions can be reverted if the cells are released from their surrounding environment. During each transition, the chondrocytes synthesize new matrix molecules, thus modifying the pre-existing microenvironment as differentiation progresses. For example, during maturation the chondrocytes secrete a cartilage matrix protein (CMP) which is a marker for postmitotic chondrocytes. Eventually, the cells undergo hypertrophy and synthesize type X collagen. In culture, this process occurs in a wave that begins in the centre of a chondrocyte colony and spreads to its periphery. A similar mechanism could be taking place during the formation of the dermal bone.

On the basis of their glycosylation pattern, scleroblasts were shown to exhibit different lectin affinities, which depended on their state of differentiation along the proximo-distal axis of the growing regenerate (Marí-Beffa et al., 1996). A set of morphological and chemical changes was observed during lepidotrichial matrix maturation, from the immature to the more mature region. Loss of affinity for lectins, high interaction of collagen-sulphated glycosaminoglycans and masking of collagen fibrils, are all related to the precalcificative maturation of the lepidotrichial matrix (Marí-Beffa et al., 1996).

It will be interesting to see the response of scleroblast to different glycosaminoglicans, However, to do so, tissue culture systems of fins scleroblast will be necessary.

V. CONCLUDING REMARKS AND SPECULATIONS

The present analysis discribes the pattern of cell proliferation during fin regeneration which together with cell lineage tracing carried out by Chritopher Brown in our laboratory, brings new iformation on the path followed by the cells during regeneration of the caudal fin in zebrafish.

From our observations the following can be stated:

- The pattern of proliferation of zebrafish caudal fin resembles, in several aspects, that reported for the *Carassius auratus* (gold fish), probably only differing in the proliferating rate of the different cells.
- Epidermal and mesenchymal cells exhibit distinct rates and patterns of proliferation.
- Cell proliferation in the blastema, as evidenced by BrdU incorporation, can be correlated to the expression of *msxB, msxC*, and *Fgf8* (see Chapter 2), genes that have been implicated in cell proliferation and differentiation in other tissues organism.

The immunohistochemical technique used here is currently being applied in our laboratory together with whole mount in situ hybridization (double labelling), which will allow us to establish a direct correlation between cell proliferation and gene expression.

It is probable that in the years to come we will see that many of the data collected from urodele amphibian research, such as for example: increase in the level of certain metalloproteinases, important changes in the composition of the ECM components, mechanisms involved in cell positional identity and expression of different patterning genes would be most likely applicable to fin regeneration. Giving more support to the idea that the regeneration process between fish and amphibians is similar in nature and that this mechanism has been conserved through evolution.

In summary, after fin amputation, lateral epidermal cells quickly migrate to cover the wound. Later on, 24 after amputation, the wound epidermis is consolidated as a non proliferating tissue. At this time the lateral epidermis begins to proliferate to compensate

for cell lost by the migration and to supply cell for the normal growth of the tissue. Cells in the mesenchyme reenter the cell cycle and form the blastema probably by response to signals from the wound epidermis. Once the blastema cells escape the influence of such signals they differentiate into all the different cells necessary to restore the lost tissue.

CHAPTER II: Characterization and expression of *Fgf8* in Developing and Regenerating Fins

I. INTRODUCTION

Teleost fish have five different fins that differ in function and morphology. These fins can be classified into two different groups: paired and unpaired fins. The unpaired fins, caudal, dorsal and anal, develop from a unique median epidermal fin border running dorsally and ventrally along the trunk and around the tip of the tail of the embryos.

Of the paired fins, the pectoral fins develop first during embryonic development as two bilateral buds over the yolk sac and the pelvic fins form later during larval development on both sides of the median fin fold anterior to the anus (Figure 9). Each fin, unpaired or paired, develops in a sequential order characteristic of each teleost species.

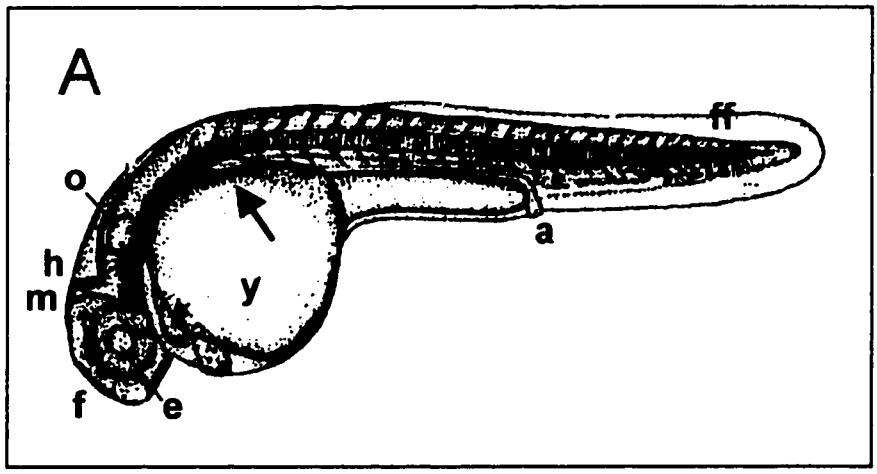
As paired fins in fish are phylogenetically related to limbs in tetrapods, I will briefly describe how limb buds in tetrapods develop.

1. Limb bud development in tetrapod species.

All tetrapods organize their limbs by following similar developmental principles. The limb bud emerges as the result of a thickening of the somatic layer of the lateral plate mesoderm. Outgrowth and patterning of the limb bud are regulated by three major signaling centres, the apical ectodermal ridge (AER), the zone of polarizing activity (ZPA) and the dorsal and ventral ectoderm that cover the limb bud (Figure 10). Signals from the three zones interact and influence the growth and differentiation of the different axes of the limb (proximo-distal, antero-posterior and dorso-ventral).

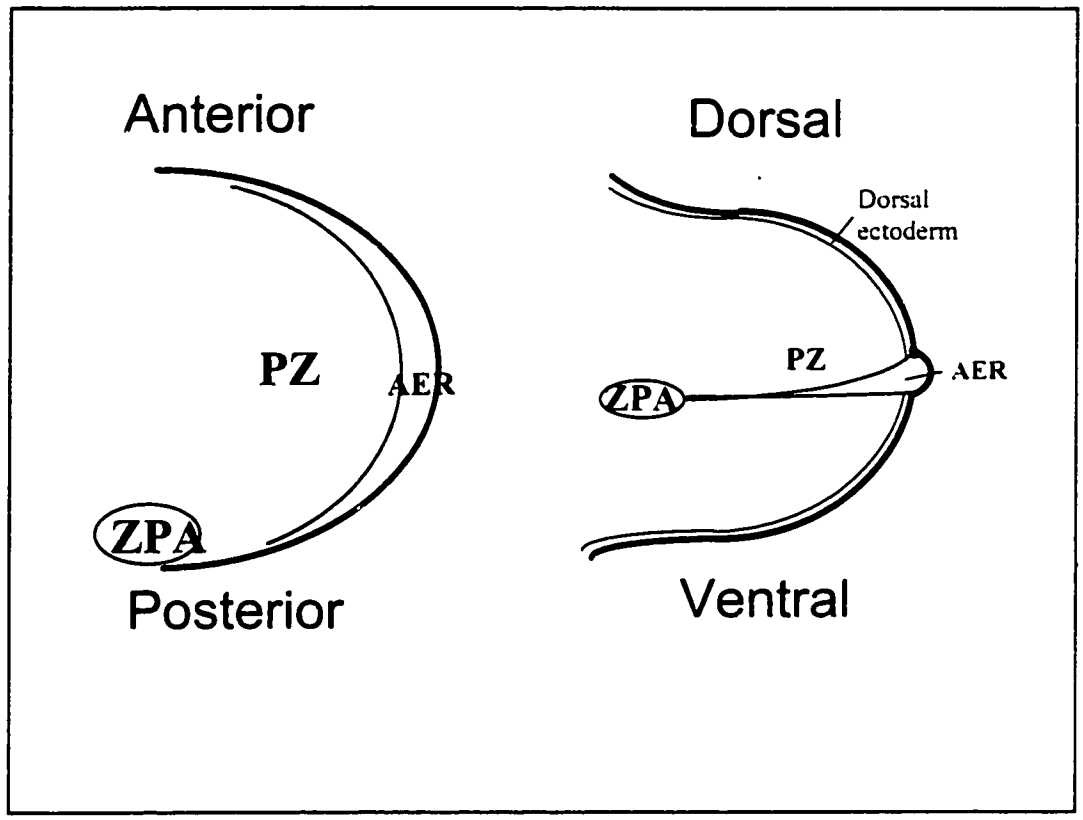
The AER which results from a thickening of the ectoderm of the limb bud, extends anteroposteriorly along the distal margin of the bud. It determines the proximo-distal patterning of the limb by sending mitogenic signals to the subjacent mesodermal tissue, the progress zone (PZ), stimulating its proliferation and maintaining it in an undifferentiated state. Removal of the AER from the limb bud results in cessation of limb

Figure 9. Pectoral and pelvic fin bud formation in zebrafish. **A.** Schematic representation of a zebrafish embryo 31 hours post fertilization (p.f.). The arrow points out to the pectoral fin bud which develops in the dorsal part of the yolk sac at the level of the second and third somite. **B.** Pelvic fins of a 7.7 mm larval developing on both sides of the ventral fin fold (arrows). The staining is the product of a whole mount *in situ* hybridization using the *Shh* probe.
e, eye; o, otic vesicle; h, hindbrain; m, midbrain; f, forebrain; ff, median fin fold; y, yolk sac; a, anus.


A
ff
o
a
h
y
m
f
e


B
ff
a

Figure 10. Diagrams showing the different signaling centres of the tetrapod limb bud. AER, Apical Ectodermal Ridge. ZPA, Zone of Polarising Activity and the Dorsal ectoderm. The three zones are closely interelated; signals from these centres interact with each other in order to pattern the three axes of the limb. The AER, thickening of the ectodermal cells of the limb bud, is involved in the. proximo-distal patterning and outgrowth of the limb. This region produces signals that maintains the cells of the Progress Zone (PZ) undifferentiated and proliferating. The ZPA, subset of mesenchymal cells localised at the posterior margin of the limb bud, participates in anterior-posterior patterning of the limb. The dorsal ectoderm is involved in dorso-ventral patterning of the limb.


Anterior
PZ
AER
ZPA
Posterior
Dorsal
Dorsal
ectoderm
PZ
AER
ZPA
Ventral

outgrowth and patterning. The number and organization of the skeletal components of the limb vary according to the time at which the AER is removed (reviewed by Niswander et al., 1993).

The antero-posterior axis patterning of the limb is regulated by the ZPA, a region located in the posterior mesenchyme of the limb bud (Figure 10). In chicken, grafting of cells from a ZPA in the anterior region of a host limb induces a duplication of digits in a mirror image (Tickle et al., 1975) (Figure 11).

Dorso-ventral patterning is controlled by signals from the ectoderm that covers the limb bud mesenchyme. If the ventral and dorsal limb ectoderm are reversed in a chicken limb bud, dorso-ventral patterning of the limb changes according to the polarity of the ectoderm (MacCabe et al., 1974; Pauto, 1977).

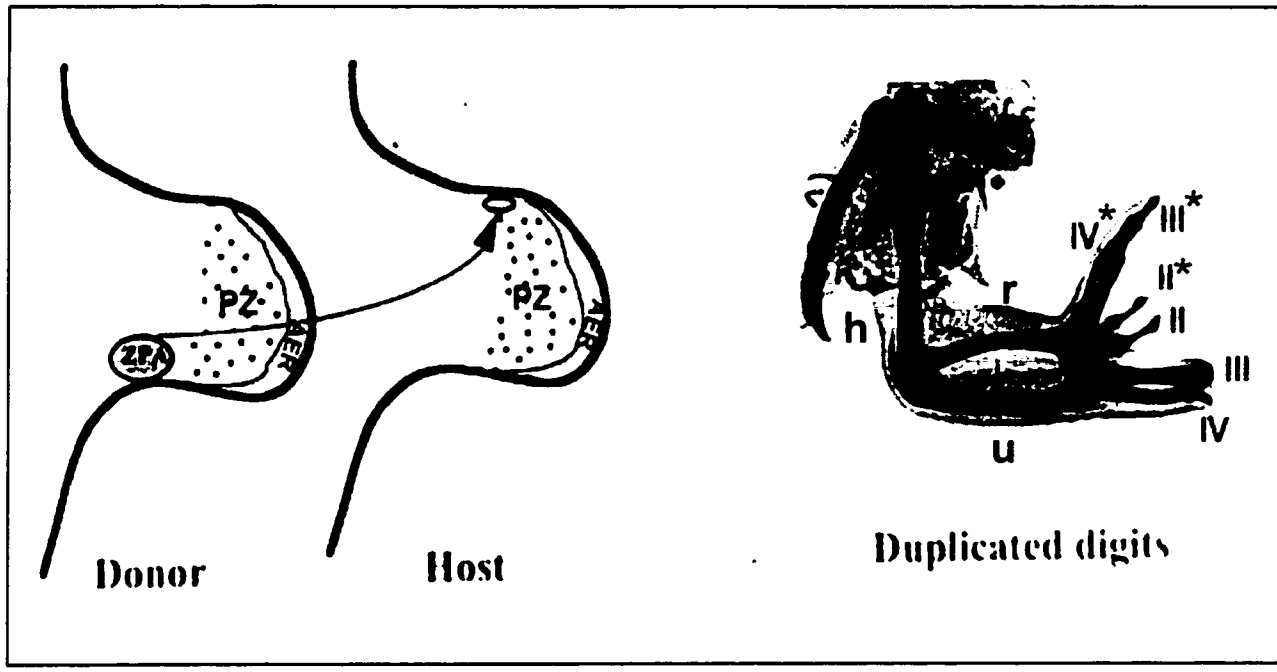
2. Signaling molecules involved in the patterning of each axis

Several studies have been done to identify molecules involved in limb induction, outgrowth and patterning. Different signaling molecules that are expressed within each one of the three signaling centers have been isolated and implicated in the control of one of the limb axis. More recently, experiments have shown that signaling molecules produced by different signaling centers also act together to determine the normal development of the limb suggesting that patterning of all 3 axes is intimately linked.

a. Fibroblast growth factors: Role in initiation and outgrowth of the limb bud

Four of the fifteen different *Fgfs* (*Fgf2*, *Fgf4*, *Fgf8* and *Fgf10*) have been found to be expressed during limb development in higher vertebrates (chicken and mouse). All of them are strongly implicated in limb outgrowth and patterning. Misexpression of any of them in the flank of chick embryos outside of the normal limb field can induce additional limbs, either wings or legs, depending on the somite level at which the misexpression is performed (reviewed in Cohn and Tickle, 1996). Three of these four different fibroblast

Figure 11. The zone of polarising activity (ZPA) is involved in antero-posterior patterning of the limb. The figure illustrates the classical experiment where the ZPA from a chicken wing is grafted to the anterior part of a host wing bud. The grafting experiment results in the duplication, in a mirror image, of the digit of the wing. Similar results are found when Sonic hedgehog is misexpressed in the anterior mesenchyme of the limb bud indicating SHH as a polarizing signal.
u, ulna; h, humerus; r, radius; d, digits II, III and IV correspond to the normal organization of the digits and IV*, III* and II* to the duplicated digits.


PZ
ZPA
AER
PZ
AER
Donor
Host
IV*
III*
II*
II
III
IV
r
h
u
Duplicated digits

growth factors, FGF2, FGF4 (Niswander et al., 1993; Fallon et al., 1994) and FGF8 (Vogel et al., 1996) have been shown to be expressed in the AER in tetrapods. *Fgf8* transcription is detected before the limb bud starts to develop in the pre-limb field ectoderm prior to the formation of the AER. Later, *Fgf8* expression is restricted to the ridge cells (Figure 11). *Fgf4* is expressed in the posterior part of the AER after limb bud outgrowth is initiated and *Fgf2* is expressed widely in the entire ectoderm. Conversely, *Fgf10* is expressed in the mesenchyme prior to limb bud initiation.

However, due to the time and domain of expression, FGF8 together with FGF10 are the proteins implicated in the initiation and outgrowth of the limb (Vogel et al., 1996; Ohuchi et al., 1997). Both of them are expressed prior to limb bud development in areas that classical embryological studies have related to the formation of the limb such as somatic mesoderm, intermediate mesoderm, lateral plate and pre-limb ectoderm (reviewed in Stephens et al, 1991; Vogel et al.,1996; Dealy, 1997).

Fgf8 expression seems to be regulated by FGF10, because *Fgf10* expression in the presumptive limb mesenchyme precedes that of *Fgf8* and the ectopic application of FGF10 to the chick embryonic flank can induce *Fgf8* expression in the adjacent ectoderm (Ohuchi et al., 1997). It is thought that this regulation is mediated by two different isoforms of the FGFR2 receptor (FGFR2c and FGFR2b) working in a reciprocal loop between the mesoderm and the ectoderm (Xu et al., 1998). During limb bud formation, *FGFR2b* is found only in the AER. Cell culture studies have shown that it can only be activated by FGF10. Conversely, the *FGFR2c* domain of expression is localized in the underlying mesenchyme and can only be activated by FGF8. This reciprocal loop generates an epithelial-mesenchymal interaction required for limb induction (Figure 12A). The proposed model is as follows: once FGF10 is produced in the presumptive limb mesenchyme, it binds to the FGFR2b and induces *Fgf8* expression in the ectoderm. The FGF8 signal interacts with the FGFR2c to maintain *Fgf10* expression (Xu et al., 1998). The importance of the regulatory loop between *Fgf8* and *Fgf10* and their receptors is demonstrated by the analysis of mice embryos carrying a deletion in the *FGFR2* gene.

These mutants are limbless; they lack *Fgf8* expression in the presumptive ectoderm and *Fgf10* expression decreases gradually in the presumptive limb mesoderm (Xu et al., 1998).

This finding suggests that FGF factors are important molecules involved in the initiation, outgrowth and proximo-distal patterning of the limb bud. FGF factors are also implicated in patterning of the antero-posterior axis as described below.

b. Sonic hedgehog and antero-posterior patterning

One of the signals that appears to mediate the antero-posterior patterning of the vertebrate limb bud is the product of the *Sonic hedgehog* gene (*Shh)*(Riddle et al., 1993), a secreted protein found in other tissues associated with polarizing activity such as the notochord and floor plate (Echelard, et al., 1993; Krauss et al., 1993). The expression of *Shh* is confined to the ZPA of the limb/fin bud of the mouse, chick and zebrafish. Ectopic expression of *Shh* in the anterior part of the chick wing bud causes the respecification of anterior cells resulting in the duplication of the digits in a mirror image giving a result similar to that obtained after grafting a ZPA in the anterior limb bud (Riddle et al., 1993). FGF8 signal is thought to induce *Shh* expression in the ZPA (Xu et al., 1998) which in turn activates *Fgf4* expression at the posterior part of the AER. Once induced, *Shh* expression is maintained by FGF4, thus establishing a positive feedback loop between the ZPA and the ridge (Niswander et al., 1994). These two signaling molecules have been suggested to be responsible for the activation of downstream genes such as *Msx1*, *Bmp2*, *Hoxd13* (Niswander and Martin, 1993; Fallon et al., 1994; Vogel et al., 1995) and *Evx-1* (Niswander and Martin, 1993) (Figure 12C).

c. Dorso-ventral patterning

Wnt-7a and *Lmx-1* genes, which are expressed in the dorsal limb ectoderm and mesoderm, respectively, have been shown to be involved in the dorso-ventral patterning (Parr et al., 1993; Vogel et al., 1995). The *Wnt* genes are the vertebrate homologues of the

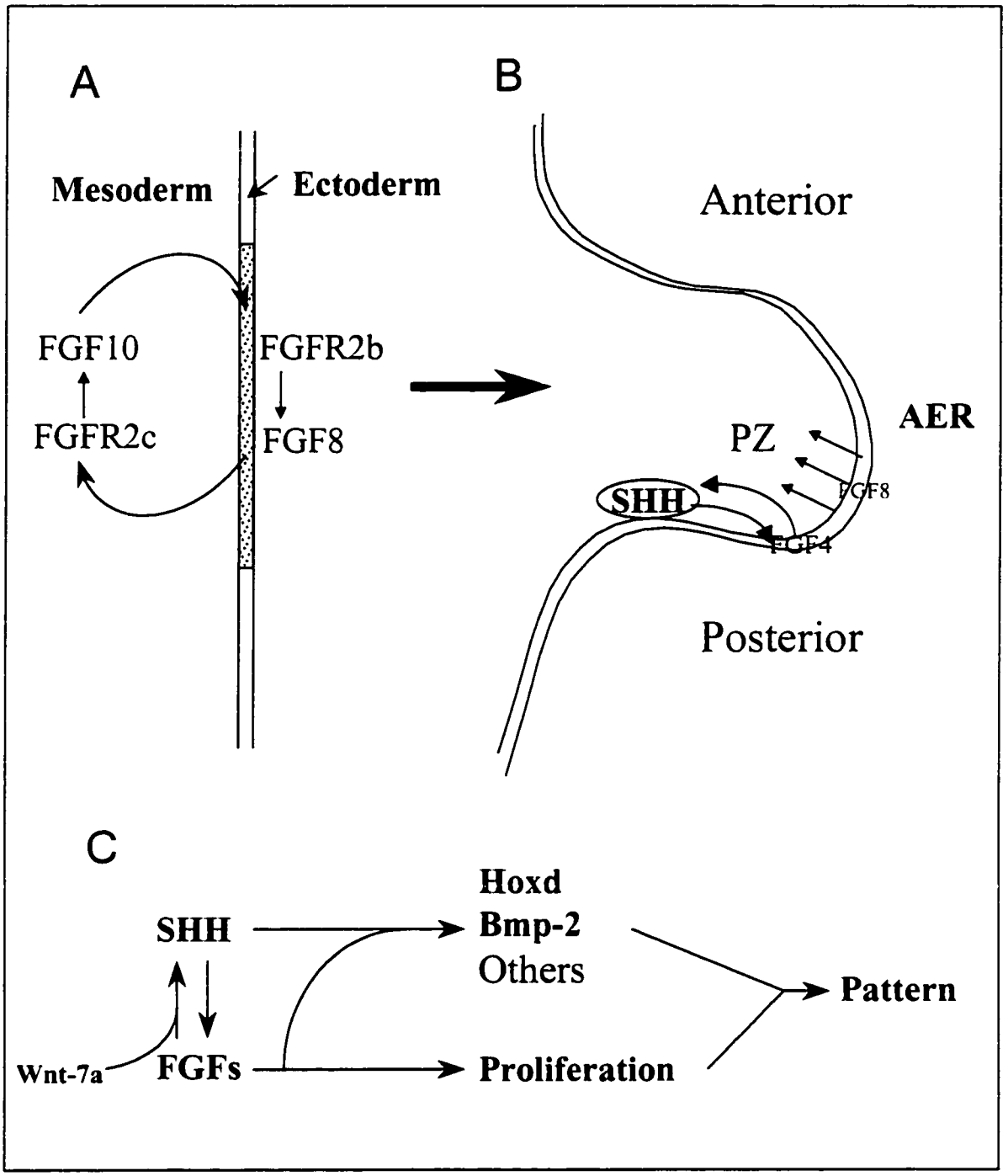
wingless Drosophila (*wg*) gene and encode secreted proteins associated with regulation of cell fate and pattern formation (Nusse and Varmus, 1992). Administration of WNT7A to a limb after removal of the dorsal ectoderm can rescue normal limb development suggesting that *Wnt-7a* can substitute for the dorsal ectodermal signal in chicken (Yingzin and Niswander, 1995). *Wnt-7a* knockout mice show a marked change in dorso-ventral patterning, where characteristics of the skin found only in the sole, are found on both sole and upper paw surfaces (Parr and McMahon, 1995). This result demonstrates that *Wnt-7a* is required for the normal development of the dorsal axis. Wnt-7a regulates *Lmx1* expression in the dorsal mesoderm of the limb. *Lmx1* is a homeobox containing gene that, as the *Drosophila LIM* gene, specifies dorsal cell fate. The ectopic expression of *Lmx1* in the ventral limb mesenchyme is sufficient to generate double-dorsal limbs (Riddle et al., 1995; Vogel et al., 1995). It has been demonstrated that removal of the dorsal ectoderm not only results in the arrest of limb development but also leads to the loss of posterior skeletal elements suggesting that dorsal signaling is also required for antero-posterior patterning. This hypothesis was confirmed by showing that *Wnt-7a* as *Fgf4* is required to maintain *Shh* expression in the posterior mesenchyme (Yingzin and Niswander, 1995). These results show that patterning of all three axes (dorso-ventral, proximo-distal, and antero-posterior) is linked during limb outgrowth and patterning.

Ventral limb development seems to be controlled by the homeodomain-containing transcription factor Engrailed-1 (En-1) expressed in the embryonic ventral limb ectoderm. Loss of *En-1* function in mice results in dorsal transformations of ventral paw structures, and in subtle alterations along the proximo-distal limb axis. Engrailed-1 seems to act in part by repressing dorsal differentiation induced by WNT-7a, and is essential for proper formation of the apical ectodermal ridge (Loomis et al., 1996).

One of the mechanisms proposed for the signaling cascade leading to limb bud formation is as follows:

Under the influence of FGF factors, cells of the lateral plate mesoderm proliferate at the level of the future limb bud. The expression of *Shh* in the ZPA is activated by FGF

Figure 12. Model of limb bud initiation and patterning. The cartoon shows some of the molecules that may act in the initiation and development of the limb bud.
A. FGF8 and FG10 mediate the epithelial-mesenchymal interactions required for limb bud induction. FGF10 activates the expression of *Fgf8* by binding to the FGFR2b receptor isoform and FGF8 signals maintain *Fgf10* expression by binding to the FGFR2c isoform. *Fgf8* also activates the proliferation of mesenchymal cells which results in growth of the bud. *Fgf8* may activate the expression of other patterning genes such as *Shh*. **B** and **C.** Signals involved in antero-posterior patterning. SHH signal induces the expression of *Fgf4* in the posterior part of the AER and FGF4 together with WNT-7a, in the dorsal ectoderm, maintains *Shh* expression in the ZPA. Both, FGF4 and SHH activate downstream genes such as *Hox* and *Bmp* genes.


A
Mesoderm
Ectoderm
FGF10
FGFR2b
FGFR2c
FGF8
B
Anterior
AER
PZ
FGF8
SHH
FGF4
Posterior
C
SHH
Hoxd
Bmp-2
Others
Pattern
Wnt-7a
FGFs
Proliferation

factors and by WNT-7a. In turn, the SHH signal is required to maintain the expression of *Fgf4* in the AER (Niswander et al., 1994). FGF4 together with SHH, indirectly activate the expression of other genes such as: *Bmp-2*, a gene coding for another signaling molecule, member of the transforming growth factor β family (Laufer et al., 1994), and homeobox genes such as members of the *Hoxd* complex (Laufer et al., 1994; Niswander et al., 1994) (Figure 12C).

3. Paired fins development in zebrafish (*Danio rerio*)

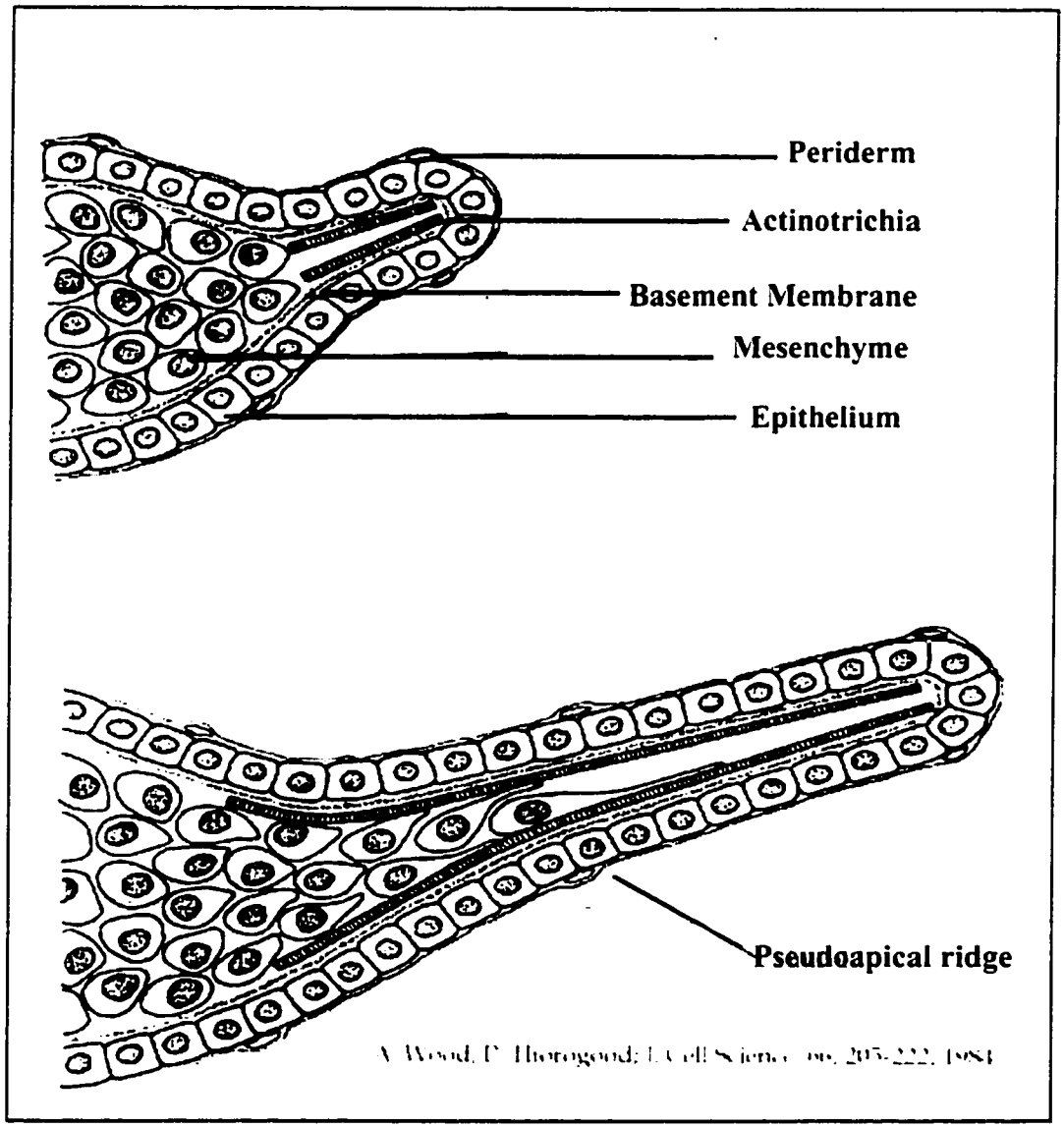
a. Morphological aspects of fin development

In teleosts, the formation of the pectoral and pelvic fins (paired fins) begins with the formation of a bud, which in the early steps of its development is morphologically similar to the tetrapod limb buds. The bud that will eventually form the pectoral fins in zebrafish can be seen as a protuberance of the epidermal tissue at 28 hours post fertilization (p.f.) on the surface of the dorsal part of the yolk sac, on each side of the trunk, at the level of the third somite (Figure 9A). The pelvic fins appear later, during larval development, on each side of the ventral median fin fold when the fish reaches about 7.4 mm in length, approximately four weeks p.f. (Figure 9B). Buds of both paired fins are constituted of mesodermal cells covered by the epidermis which forms a fold at its apex named, the "pseudoapical ridge" (Géraudie and François, 1973) (Figure 13). The fold of the pseudoapical ridge differs from the apical ectodermal ridge of limb buds of tetrapod species which appears as a thickening of the ectoderm in anurans and humans or as a pseudostratified epithelium in chick and reptiles (Reviewed in Géraudie, 1980).

In contrast to the AER of tetrapod limbs which regresses as the limb grows, the fin fold elongates distally taking a hairpin shape (Figure 13). Inside the fold, the dermoskeleton begins to develop first by polymerization of the actinotrichia, and then, as the fin develops, scleroblasts synthesize the lepidotrichia.

The deposition of actinotrichia occurs by an unknown set of cells at the apex of the

Figure 13. Schematic representation of the development of the fin fold. Fin bud begins as an outgrowth of the epidermal tissue that folds at its apex forming the "pseudo apical ridge". The actinotrichia, first component of the dermal skeleton of the fin ray, are polymerised beginning at the apex of the subepidermal space of the fold. The actinotrichia made of elastoidin, are used by mesenchymal cells as substrate for migration during distal growth of the bud. Later on, the lepidotrichia, second and major component of the dermal skeleton of the fin ray, are synthesized by scleroblasts or lepidotrichia forming cells.


Periderm
Actinotrichia
Basement Membrane
Mesenchyme
Epithelium
Pseudoapical ridge

subepidermal space of the fold along the proximo-distal axis. Within the fold, mesenchymal cells migrate distally to the apex using the actinotrichia matrix as support substrate (Wood and Thorogood, 1984). The lepidotrichial matrix is secreted close to the epidermal basal lamina by scleroblasts or lepidotrichia forming cells. This matrix is principally composed of chondroitin sulfate glycosaminoglycans, which show high levels of interaction with collagen, a characteristic feature of cartilage tissues (Montes et al., 1982).

b. Conservation of the molecular network involved in early fin and limb development

Although fins and limbs are morphologically different, the early steps of development seem to be controlled by similar mechanisms (Sordino et al., 1995; Wood, 1982; Akimenko and Ekker, 1995). This is suggested by the similarity in the pattern of expression of several genes involved during limb and fin bud development (Table 1 shows the domain of expression of several genes in the limb and fin buds).

Some of these genes are members of the *HoxD* homeobox gene complex which are involved in patterning of the vertebrate axial skeleton. They show similar patterns of expression to their homologues in zebrafish only during early pectoral and pelvic fin bud development. During early fin bud and limb bud formation, the 5' genes of the *HoxD* gene complex (*Hoxd-10*, *Hoxd-11*, *Hoxd-12* and *Hoxd-13*) are transiently expressed in nested domains in the posterior mesenchyme of the pectoral fin/limb buds (Reviewed by Sordino et al., 1995). Later a second phase of *HoxD* expression occurs only in the distal part of the tetrapod limb buds, not in the pectoral fin buds. This late phase of expression during limb development has been related to the formation of the autopod ("hand or foot") in tetrapods (Sordino et al., 1995), a structure not found in teleost fishes. Based on the comparison of *HoxD* gene expression in zebrafish and tetrapod fin/limb buds, Sordino suggests that the late expression of *HoxD* genes in limbs is a "de-novo activation in a new

domain, in which case the digits would be a completely novel tetrapod invention not homologous to posterior fin radials".

Other homeobox genes members of the *msx* family (*msx A, B, C,* and *D*) are expressed during fin bud development. Observation on *Msx* expression and studies of their function in tetrapod limbs indicate that these genes are involved in epithelial-mesenchymal interactions inducing outgrowth and proximo-distal pattern formation. In mouse and chicken, *Msx1* is mainly expressed in the progress zone whereas cells of the AER express *Msx2* (Robert et al., 1991). The loss of influence of signals produced by the AER, either by normal growth of the limb bud or by AER removal, is followed by a rapid decline in *Msx1* transcripts in the mesenchymal cells (Ros et al., 1992). This can also be seen in *limbless* chicken mutants which do not form an AER. In these animals, *Msx1* expression in the mesoderm is not maintained. Limb growth in *limbless* mutants can be rescued by grafting normal ectoderm to the limb field, leading to the expression of *Msx1* in the mesoderm underneath the induced ridge (Coelho et al., 1991). This result suggests that *Msx1* expression in the progress zone depends upon a signal provided by the AER.

In zebrafish four different *msx* genes (*msxA*, *msxB*, *msxC* and *msxD*) have been described to be involved during paired and unpaired fin development (Akimenko et al., 1995). In paired fin development, cells of the AER express three of these genes; *msx A*, *msxB* and *msxD*. *msxB*, expression, as the mouse *Msx1* gene, is confined to the AER, while *msxA* and *msxD* transcripts are found in the entire ectoderm. Cells proximal to the AER express all four *msx* genes. Among them, *msxC* pattern of expression closely resembles that of the mouse *Msx2* since its expression is restricted to the mesenchymal cells corresponding to the PZ, underlying the AER. A similar pattern of expression is observed during unpaired fin development, where the most distal cells of the median fin fold express *msxA*, *msxB* and *msxD* and the underlying cells express the four *msx* genes. This suggests that the mechanism that involves *msx* genes during paired and unpaired fin development is similar. In addition their function may be comparable to that of the *Msx1* and *Msx2* genes during formation of the tetrapod limb bud which may include patterning along the

proximo-distal axis (Akimenko et al., 1995).

Shh is one of the signaling molecules which mediates the antero-posterior patterning of tetrapod limb buds. The zebrafish homologue is expressed during the development of the fin bud in a region analogous to the ZPA in tetrapods (Krauss et al., 1993), suggesting the presence of a ZPA in the pectoral fin buds. This hypothesis is further supported by the finding that global administration of retinoic acid (RA) induces ectopic expression of *Shh* in the mesenchymal cells at the anterior margin of the bud (Akimenko and Ekker, 1995) in the same way as local application of RA to the anterior part of the chicken limb bud induced expression of *Shh* in an anterior subset of mesenchymal cells. Furthermore, at least part of the SHH signaling cascade is conserved during both tetrapod limb and pectoral fin bud development since *Patched1*(*Ptc-1*) and a downstream gene *Bmp2* are expressed in a similar fashion (Table 1).

Altogether, these data suggest that the molecular network involved in the regulation of limb and paired fin morphogenesis is similar and has been conserved through evolution. As previously mentioned, FGF molecules participate in this network during limb development and urodele limb regeneration (see Chapter I) but it is only recently that some of these genes have been characterised in zebrafish. The important functions ascribed to FGFs during limb development and regeneration in tetrapods make them likely candidates for similar roles during fish fin development and regeneration.

In this work, I focused my attention on two members of the *Fgf* family (*Fgf4* and *Fgf8*) directly involved in limb development in order to analyze their function during embryonic fin development and fin regeneration of adult fish.

4. Characteristics of the fibroblast growth factor 4 and 8

Fifteen members of the *Fgf* family (*Fgf1- Fgf15*) have been described in different vertebrates (Goldfarb, 1990; Crossley and Martin, 1995; Ohuchi et al., 1997; McWhirter et al., 1997). All of them encode monomeric proteins that are homologous to one another

Table 1. Comparison of the domains of gene expression during early stages of mouse limb and zebrafish pectoral fin bud development. The table shows several genes found expressed in similar fashion during limb and fin bud development, indicating that similar patterning mechanisms are taking place at least during the early stages. A difference is observed in the expression of *Hoxd* between fin and limb buds. In limb *HoxD* genes are expressed in two different phases whereas in fins only the first phase during early fin bud formation is observed. The formation of the digits in tetrapods is attributed to the second phase of *HoxD* gene expression.

AER, Apical ectodermal ridge; PZ, Progress zone; ZPA, Zone of polarizing activity; nd, not determined.

Gene	Limb bud expression		Fin bud expression
Msx1(mouse)	PZ		msxC (PZ)
Msx2(mouse)	AER		*msxA, msxB and msxD* (AER)
Fgf-4	Posterior AER		nd
Fgf-2	AER		nd
Fgf-8	AER		AER
Bmp-2	ZPA and AER		AER
Bmp-4	AER		AER
Hoxd-13	Posterior and distal mesoderm	1phase	Posterior mesoderm
Hoxd-11	Distal mesoderm	2phase	
Shh	ZPA		ZPA
ptc1	Posterior mesoderm encompassing the ZPA		Posterior mesoderm encompassing the ZPA
Wnt 7a	Dorsal ectoderm		nd
en-1	Ventral ectoderm		Ventral ectoderm

within a core of 120 amino acids. This core shows homology between the different family members ranging from 33 to 69%. FGF signals act through a pathway mediated by four high affinity tyrosine kinase transmembrane receptors (FGFr1-FGFr4) (Dionne et al., 1990; Keegan et al., 1991; Partanen et al., 1991). Each FGF signal is capable of interacting with more than one FGF receptor (Blunt et al., 1997). All the FGFs, with the exception of FGF1 and FGF2, acid and basic FGFs respectively, contain a similar signal peptide involved in the transport and processing of the molecules for export from the cell (Goldfarb, 1990; Brookes et al., 1989). The mechanism by which the basic and acidic FGFs are exported is still unclear (Goldfarb, 1990).

The fibroblast growth factor 8 gene (*Fgf8*) has been identified in many vertebrates, including *Xenopus* (Christen and Slack, 1997), mouse (Crossley and Martin, 1995), chicken (Crossley et al., 1996b) and human (Gemel et al., 1996). Studies in humans and mouse show that alternative splicing of *Fgf8* mRNAs results in the synthesis of multiple protein isoforms. *Fgf8* is the only member of the *Fgf* family which produces multiple products.

In the mouse genome, the *Fgf8* gene is localized in the distal portion of chromosome 19 and can encode at least eight different secreted protein isoforms. The different isoforms contain a common carboxyl region originating from two exons (exon 2 and 3) and variable amino termini encoded in at least three separate exons (exons 1A, 1B, 1C)(Figure 14.1). In humans, *Fgf8* can encode for 4 potential protein isoforms, formed from two constant exons, as in mouse, and four variable 5' exons (1A, 1B, 1C and 1D).

The functional significance of the different FGF8 isoforms is not yet understood. Crossley and Martin (1995) suggest that as the different isoforms contain different glycosylation sites, this could be important for the regulation of each isoform activity. However, Kuhlman and Niswander (1997) suggest that the overlapping expression of some of these isoforms at multiple sites during embryonic development are functionally redundant.

The *Fgf4* gene has been identified in human (Miyagawa et al., 1988), mouse (Brookes et al., 19989) and chicken (Niswander et al., 1994). The FGF4 protein is encoded by a gene consisting of three exons and contains, as do most of the FGFs, an amino terminus signal peptide and a conserved central core.

The amount of data showing the importance of FGF factors in different signaling centers of various vertebrate species during embryogenesis, including limb bud development, is overwhelming, suggesting that such signals have been conserved and are important in limb development and regeneration. FGF signals (FGF2, FGF4, FGF8 and FGF10) are part of the molecular network essential for the initiation, growth and patterning of embryonic limbs. The data so far collected in zebrafish suggest that the network of molecules involved in limb development is also present during development of the paired fins. Consequently, because of these parallels, we anticipated the participation of FGF factors in zebrafish embryogenisis.

My work focused in the characterization of two members of the *Fgf* family of genes (*Fgf4* and *Fgf8*) in zebrafish by sequencing, mapping and following their expression pattern during embryonic development. I also examined the possible participation of *Fgf8* during fin regeneration since participation of FGF as signaling factors involved in proliferation and differentiation of different tissues, makes them likely candidates as regulators in the regeneration process.

II. MATERIAL AND METHODS

1. Animals

Adults fish purchased from a local supplier, were maintained in aquaria at 28.5oC with a photo period of 14 hours light and 10 hours darkness and fed daily (Westerfield, 1993).

Embryos were obtained by placing 3 females and 2 males in breeding cages the evening prior to the day of collection. Spawning was induced by the onset of the photo period. The fertilized eggs were collected shortly after spawning.

Embryos were maintained at 28.5oC in 100 mm petri dishes and staged according to hours of post fertilization.

Some embryos were raised until late larval stage. Since development rapidly becomes asynchronous a few days p.f. larvae were stages according to their size. Standard length (SL) measurement were made from the tip of the mouth to the beginning of the caudal fin at the hypural bone.

2. DNA sequencing and analysis

The *Fgf8* cDNA insert within Bluescript SK- was sequenced using a Thermo Sequenase Fluorescent Labeled Primer Cycle Sequencing kit (Amersham) and subjected to electrophoresis in a Li-Cor automated sequencing machine (Model 4000L). The M13 reverse primer (5'-AGCGGATAAC AATTTCACACAGG-3') and the T7 forward primer (5'-AATACGACTCACTATAG-3') were used to sequence both strands. The internal sequence was sequenced by the dideoxy-nucleotide termination method using a Sequenace Version 2.0 Kit (Amersham) and a primer synthesized from known sequences within the cDNA clone (5'-CATGAGACTC ATACCTTCAC-3').

The sequence was compared to sequences stored in a number of data banks using the BLAST Network Service (Altschul et al., 1990) available via e-mail at the National Center for Biotechnology Information (NCBI). The entire sequence was submitted, at the

nucleotide level, using the BLASTn program, and at the protein level using the BLASTx program which automatically translates the query in all six reading frames.

3. Genomic mapping of the zebrafish *Fgf8* gene

The genomic position of the *Fgf8* gene was determined by PCR using two specific sets of primers for the zebrafish *Fgf8* cDNA sequence taking into consideration the genomic structure of the mouse *Fgf8* gene (Figure 16.1). DNA prepared from a panel of zebrafish/mouse somatic cell hybrids (Ekker et al., 1996) was used as a template. Two Polymerase Chain Reactions (PCR) were carried out using primers from different positions. The primers and conditions were as follows: PCR I primers 5'-GTA GAG ACG GAC ACA TTT GGG-3' and 5'-CAA CGC TCT CCT GAG TAG CG-3' (see *Fgf8* nucleotide sequence, Figure 14), denaturing step, 50 sec at 92°C; annealing step, 90 sec at 63°C; extension step, 2 min at 72°C. PCR II primers 5'-TTT TCA CAG AGA TAG TCC TG-3' and 5'-TGT TTG AGA AAG TCT CTG GC-3' (see *Fgf8* nucleotide sequence, Figure 14), denaturing step 50 sec at 92°C, annealing step, 90 sec at 58°C; extension step, 2 min at 72°C.

4. Fin amputation

Adult zebrafish were anaesthetized by immersion in water containing 0.17 mg/ml tricaine (ethyl-m-aminobenzoate, MS 222, Sigma). Caudal fins were then amputated proximal to the first fin ray bifurcation, using a scalpel and the fish were returned to their tank. After different times of regeneration, depending on the experiment, the fins were cut off again and fixed in phosphate buffer saline solution (PBS) containing 4% paraformaldhehyde (PFA) (Westerfield, 1993).

5. Whole mount *in situ* hybridization

in situ hybridization on whole-mount embryos or on whole-mount fins were performed as described in Akimenko et al., (1994). The probe consisted of an antisense RNA made from the full length (1.9 kb) zebrafish *Fgf8* cDNA.

Probe Synthesis

The antisense RNA probe was produced by *in vitro* RNA synthesis from a linearized DNA template. The probe was labelled by incorporation of UTP nucleotides coupled to digoxygenin (DIG), a plant steroid. The synthesis reaction contained 1 μg of linearized template DNA, NTPs (10 mM ATP, 10 mM CTP, 10 mM GTP, 6.5 mM UTP, 3.5 mM DIG-11-UTP (Boehringer Mannheim)), transcription buffer (40 mM Tris pH 8.0, 6 mM $MgCl_2$, 10mM DTT, 10 mM NaCl, 2 mM spermidine), 20 units of RNasin (Promega) and 20 units of T7 RNA polymerase (Gibco). After 1 hour of incubation at 37°C another 20 units of T7 RNA polymerase were added to the mixture and reincubated at 37°C for an additional hour. This reaction produced approximately 10 μg of RNA. The synthesized probe was precipitated by addition of lithium chloride (LiCl) to a final concentration of 0.4 M and ethanol to a 75% final volume. After 30 minutes at -70°C, the synthesized probe was pelletted for 30 minutes in a microcentrifuge (Baxter) at high speed, then washed with 70% ethanol and dried. The precipitated probe was resuspended in DEPC treated water to a final concentration of approximately 100 ng/μl. 50 μl of the synthesized product was hydrolyzed at 60°C with the hydrolysis mix (0.4 M Na Bicarbonate, 0.6 M Na Carbonate) for 1 hour. Hydrolysis was stopped by addition of 400 μl of H_2O, 34 μl 3M Na acetate pH4.5, 2.6 μl acetic acid and 1100 μl of ethanol. After 30 minutes at -70°C, the hydrolysis product was pelleted for 30 minutes in a microcentrifuge (Baxter) at high speed, then washed with 70% ethanol, dried and resuspended in 50 μl of DEPC water.

Embryos and fins were fixed in a solution of PBS containing 4% PFA at 4°C for 16 hours. The samples were washed twice in PBS, followed by 2 washes in MeOH and kept

at least 2 hours in methanol at -20°C. The samples were subsequently rehydrated in a series of dilutions of methanol and PBS (75% MeOH/25% PBS; 50% MeOH/50% PBS; 25% MeOH/ 75% PBS) for 5 minutes each. The samples were washed 3 times for 5 minutes each in PBST (1XPBS, 0.1% Tween-20). Two day and three day old embryos were permeabilized by incubation in 20 μg/ml proteinase K (Prot.K)(Boehringer Mannheim) solution for two minutes. Three week old larvae and caudal fins of adult fish were incubated in 50 μg/ml Prot.K solution for 10 minutes. Embryos younger than 48 h did not require the permeabilization step.

After proteinase K treatment, samples were washed twice in PBS for 5 minutes and fixed for 20 minutes in 4% PFA. Following fixation, samples were washed in PBS and acetylated by incubation in a solution containing 125 μl triethanolamine and 27 μl acetic anhydride in 10 ml of DEPC water for 10 minutes.

After 2 washes of 10 minutes in PBST, embryos were prehybridized at 65°C for 3 to 4 hours in a solution containing 50% deionized formamide, 5X SSC (20X SSC stock solution; 3.0 M NaCl, 0.3 M citric acid), 0.1% Tween-20, 50 μg/ml heparin, 10 mM citric acid and 100 μg/ml yeast tRNA. The prehybridization solution was replaced by a fresh prehybridization solution containing 1 ng/μl of DIG-labelled antisense RNA probe and the samples were hybridized 16 hours at 65°C. The next day, the hybridized samples were washed at 65°C for ten minutes in a series of dilutions of hybridization mix (hyb.mix) and twice SSC (75% hyb.mix/25% SSC; 50% hyb.mix/50%SSC; 25%hyb.mix/75% SSC and 100% SSC), 2X for 30 minutes at 60°C in 0.2XSSC and for 5 minutes in a serial dilution of 0.2X SSC and PBST (75% 0.2X SSC/25% PBST; 50% 0.2XSSC/ 50% PBST; 25% 0.2XSSC/ 75% PBST; 100% PBST) at room temperature.
The samples were preincubated for 1 hour at room temperature in PBST containing 0.2% calf serum and 2mg/ml BSA. Embryos were then incubated at room temperature for 4 hours in the solution containing a preabsorbed anti-DIG antibody conjugated to Alkaline Phosphatase (AP)(Boehringer Mannheim). Preabsorption of the antibody was performed

by incubating for 16 hours 1/6 of the total non hybridized embryos in a solution containing: 2 mg/ml of BSA in a final volume of 1030 µl of PBST at 4°C. After incubation with the preabsorbed anti-DIG AP antibody hybridized embryos were washed overnight at 4°C in PBST followed by 6 washes of 15 minutes in PBST. Prior to the chromogenic color reaction, the embryos were equilibrated for 5 minutes in reaction buffer (100 mM Tris pH9.5; 50 mM $MgCl_2$; 100 mM NaCl; 0.1% Tween-20; 1 mM levamisol) at room temperature. Embryos were stained in the same reaction buffer containing 0.175 mg/ml of 5-Bromo-4-Chloro-3-Indolyl phosphate (BCIP) and 0.337mg/ml of Nitro Blue Tetrazodium (NBT) at room temperature. The staining was carried out until a suitable color was visualized. After staining, the samples were washed in PBST, post-fixed for 2 hours in 4% PFA and stored in PBS containing 5mM sodium azide.

The entire whole *in situ* hybridization procedure was done in six well plates (Corning); the embryos being placed in plastic "Baskets" except for the prehybridization steps which were performed in eppendorf tubes.

Embryos, larvae and fins were observed and photographed using a Nikon camera FX-35DX linked to a Leica WILD M10 stereo microscope.

5. Cryostat sectioning and slide mounting

Sectioning and mounting were performed as described in Westerfield et al, 1994. After *in situ* hybridization, fixed embryos or fins were embedded in a molten 50°C solution of PBS containing 1.5% agar and 5% sucrose. After solidification, a block containing the material to be sectioned was cut with a scalpel. These blocks were placed in a 30% sucrose PBS solution and stored at 4°C overnight. The blocks were mounted on the cryostat chuck in a layer of OCT compound (Tissue Tek, Miles) and then frozen with 2-methyl butane (-50°C). The block was sectioned 16 µm thick using a LKB cryostat. Sections were collected with glass slides and allowed to dry for 24 hours.

Slides carrying sectioned tissues from *in situ* hybridization assays were then prepared for mounting by dehydration for 5 min in a series of dilutions of ethanol (20%, 40%, 60%, 80%, 90%,95% and twice in 100% EtOH). Sections were cleared in two washes of xylene for 1 min. A drop of Cytoseal 60 mounting media (Stephens Scientific) was applied onto the tissue sections and a cover glass was laid over the sections. Slides were allowed to dry overnight. Sections were observed using a Zeiss microscope.

6. RNase protection assay

Total RNA prepared from embryos at different developmental stages (6, 10, 24, 30, 48, 72 hours p.f.) was isolated using TRIzol reagent following manufacturers recommendations (Gibco BRL).

Two different radioactive antisense RNA probes, corresponding to a fragment of the *Fgf* PCR fragment of 244 bases and a 200 bases fragment of the *MAX* gene cDNA, were synthesized.

The synthesis reaction contained 1 µg of linearized DNA template, NTPs (1,5 mM ATP, 1,5 mM CTP, 1,5 mM GTP), 40 µCi/µl of ^{32}P-UTP 10 mCi/µl (Amersham), transcription buffer (40 mM Tris pH 8.0, 6 mM $MgCl_2$, 10mM DTT, 10 mM NaCl, 2 mM spermidine), 20 units RNasin (Promega), 20 units of T7 or T3 RNA polymerase (Gibco) and DEPC water to 19 µl. The enzyme was added last. The reaction was incubated at 37°C for 1 hour. After 1 hour of synthesis, the reaction was stopped by addition of 1 µl of DNase I (10000 u/ml, Pharmacia) and incubation at 37°C for 20 minutes. After addition of 1µl of 10 mg/ml tRNA, 100 µl DEPC treated water, the probe was extracted by 100µl water saturated phenol:chloroform (1:1). The solution was mixed on vortex. The aqueous phase was recovered after 5 minutes centrifugation at 14000rpm and precipitated with 9µl of 10M Ammonium acetate, 322µl 100% EtOH at -20°C for 30 minutes. After centrifugation for 20 minutes at 14000 rpm, the supernatant was discarded and 30µl of RNA loading buffer (RLB)(4 ml formamide, 0.1 mM EDTA, 0.02% Dye

Solution (1mg/ml xylene cylanol, 1mg/ml bromophenol blue in DEPC-treated water)) was added.

Probes were purified on a 7.5 % polyacrylamide gel (60 ml gel: 15 ml of 30% stock solution of acrylamide, 4 ml 10XTBE, 19.2 g of Urea, DEPC water. Polymerization was catalyzed by addition to the gel solution of 400µl 10% ammonium persulfate (APS) and 16µl TEMED).

The solution containing the probe was pipetted up and down 35 times and denatured for 2 to 5 minutes at 85°C before loading on the polyacrylamide gel. Electrophoresis was performed at 230V for 90 min. After electrophoresis, the gel was transfered to Whatman's 3M paper, covered with Saran Wrap and exposed to a X-ray film for 35 to 45 seconds (during exposure, the gel contour was marked on the film). After developing, the film was aligned with the gel and the gel region containing the labelled bands isolated. The pieces of gel containing the probe were transfered into eppendorf centrifuge tubes with 600 µl of elution buffer (2M ammonium acetate, 1% SDS, 0,04 mg/ml tRNA in DEPC-treated water) and incubated at 37°C overnight with shaking. The next day, the eluate was transfered to a new tube with an equal volume of 100% ETOH and vortexed for 15 seconds (2 µl of the eluate were taken for radioactivity counting in a BECKMAN, scintillation analyser, model LS 1701). For the hybridization step, 100 µg of total RNA and 1000 counts per minute (cpm) of the probe eluate were mixed and centrifuged at 14000 rpm for 20 minutes. The EtOH was then removed. The pellet was resuspended by pipetting it up and down 35 times in 30 µl of hybridization mix (24µl deionized formamide, 3µl DEPC water, 1X hybridization buffer (10X: NaCl, 0.01 m M EDTA in 400 mM PIPES pH 6.4)) and incubated at 60°C overnight.

The next day 350 µl of RNase digestion buffer (0.3 M NaCl, 10 mM Tris pH 7.5, 5mM NaEDTA pH 8.0, 2µl of RNase T1 500000 U(Sigma) in distilled water) were added to the tube, vortexed and incubated at 37°C for 15 min. Digestion was stopped with 0.125 mg/ml of proteinase K (Sigma) and 0.5% SDS. The protected RNA was extracted with

water saturated with phenol:chloroform (1:1), 1µl tRNA(10 mg/ml). The protected RNA fragment was precipitated by addition of twice the volume of 100% EtOH and kept at -20°C for 45 minutes. After centrifugation, the protected RNA pellet was resuspended in 30µl of RNA loading buffer (RLB), denatured at 85°C for 5 minutes and loaded on a 7.5% polyacrylamide gel. Electrophoresis was performed at 230V for 2 hours. The gel was transfered to a Whatman's 3M paper, covered with saran wrap, dried and exposed to X-ray film for 7 days.

III. RESULTS

1. Sequence analysis of the zebrafish *Fgf8* cDNA.

We received a cDNA clone partially identified which presumably contained the sequence of zebrafish *Fgf8* cDNA from Dr. Stainier's laboratory, University of California, San Francisco. This clone was isolated from a zebrafish embryonic cDNA library made in the phagemid vector λZAP. To fully characterize this clone, the cDNA inserted into the pBluescript SK +/- phagemid, was sequenced from both extremities using the T7 forward (5'-AATACGACTCACTATAG-3') and reverse primers (5'-AGCGG ATAAC AATTT CACAC AGG-3') of the pBluescript vector as well as a primer synthesised from a known sequence within the cDNA clone (5'-CATGAGACTC ATACCTTCAC-3'). The sequence analysis revealed that the cDNA is 1959 base pairs long plus a 21 nucleotide poly A tail (Figure 14). The sequence contains an open reading frame (ORF) beginning at nucleotide position 468. Based on amino acid sequence comparison with other FGF8 proteins the first methionine codon encountered at position 537 is probably the translation initiation codon. The reading frame of 233 amino acid residues ends at the stop codon at position 1166 (Figure 14).

The sequence was used to do a search using the BLAST (local alignment search tool) program in the National Center for Biotechnology Information (NCBI), revealing a very high sequence identity with all previously described eukaryotic *Fgf8* sequences (Table 2).

Figure 14. Nucleotide sequence and predicted amino acid sequence of the zebrafish *Fgf8* cDNA. The sequence is 1959 bp long. The open reading frame starts at the nucleotide position 468 and stops at nucleotide position 1166. The predicted amino acid sequence starting at position 536 is presented in uppercase, using the single letter code. Underlined sequences correspond to the primers used in *Fgf8* gene.mapping. PCRI primers: single line; PCRII primers: double line (See methods)

```
                                    35                                  70
          .         .         .         .         .         .         .
CCTGACTGGCAATGGGAGAAGAGGAGGGGATAAGAGAGTCCAGCAGCACTGCTTCTGGAAGAAAGCTCAT

                                   105                                 140
          .         .         .         .         .         .         .
CAGATGCACTGTCCTGCCTCCTCTGACTGACACTCACACAGGACTCACTGGATCAAACGTCAAAGGATCT

                                   175                                 210
          .         .         .         .         .         .         .
ATTTGTGCTGCGCTCCTCACCTTCATGGACAGCTCGGGATTTCCTCGGGCTGCATGATTACAGGCGCATC

                                   245                                 280
          .         .         .         .         .         .         .
AGAGGCCTTTCGCTTAAACTTGAAAAGTAAATCGTTTACATGCCGCGAGTTTTGCGTAAAGTGCACTTAT

                                   315                                 350
          .         .         .         .         .         .         .
TTGTGGTTCTCGTTGTAATGAAGGAAATGCAGGTAGTGCATGTACTTGTCAGCGGTGTCAGTCTGAAAAA

                                   385                                 420
          .         .         .         .         .         .         .
AACCTTGGACATTTCTTTCTGCTTCTGAGCAGCAACAGATCTTGTAATTTGCCAGTGGACCTGTTCATCA

                                   455                                 490
          .         .         .         .         .         .         .
TATGTTGCGAGTCACAGTGTGGATACAAACGCAGGACTTTACACATTTGAGCGGGAAAACCTGATATTAT

                                   525                                 560
          .         .         .         .         .         .         .
TCTAAATACACTTATTTTTAACCACTTTTTTACTGAGGCTATAAACATGAGACTCATACCTTCACGGTTG
                                                  M   R   L   I   P   S   R   L>

                                   595                                 630
          .         .         .         .         .         .         .
AGTTATCTATTCCTTCACCTCTTTGCGTTTTGCTACTATGCTCAGGTAACCATTCAGTCCCCGCCTAATT
 S   Y   L   F   L   H   L   F   A   F   C   Y   Y   A   Q   V   T   I   Q   S   P   P   N>

                                   665                                 700
          .         .         .         .         .         .         .
TTACACAGCATGTGAGTGAGCAAAGTAAGGTGACGGACCGGGTGAGCCGTAGACTAATCCGGACCTACCA
F   T   Q   H   V   S   E   Q   S   K   V   T   D   R   V   S   R   R   L   I   R   T   Y   Q>

                                   735                                 770
          .         .         .         .         .         .         .
GCTTTACAGCCGAACCAGTGGCAAGCACGTGCAAGTTCTGGCCAACAAGAAAATCAACGCCATGGCCGAA
  L   Y   S   R   T   S   G   K   H   V   Q   V   L   A   N   K   K   I   N   A   M   A   E>

                                   805                                 840
          .         .         .         .         .         .         .
GATGGCGACGTTCATGCCAAGCTTATAGTAGAGACGGACACATTTGGGAGTCGAGTTCGAATTAAAGGAG
 D   G   D   V   H   A   K   L   I   V   E   T   D   T   F   G   S   R   V   R   I   K   G>

                                   875                                 910
          .         .         .         .         .         .         .
CTGAAACAGGCTTTTACATCTGTATGAACAGGAGGGGGAAACTGATTGGCAAGAAAAACGGTCTGGGAAA
A   E   T   G   F   Y   I   C   M   N   R   R   G   K   L   I   G   K   K   N   G   L   G   K>

                                   945                                 980
          .         .         .         .         .         .         .
AGACTGCATTTTTCACAGAGATAGTCCTGGAGAACAACTACACGGCTCTGCAGAATGTGAAGTACGAAGGC
 D   C   I   F   T   E   I   V   L   E   N   N   Y   T   A   L   Q   N   V   K   Y   E   G>
```

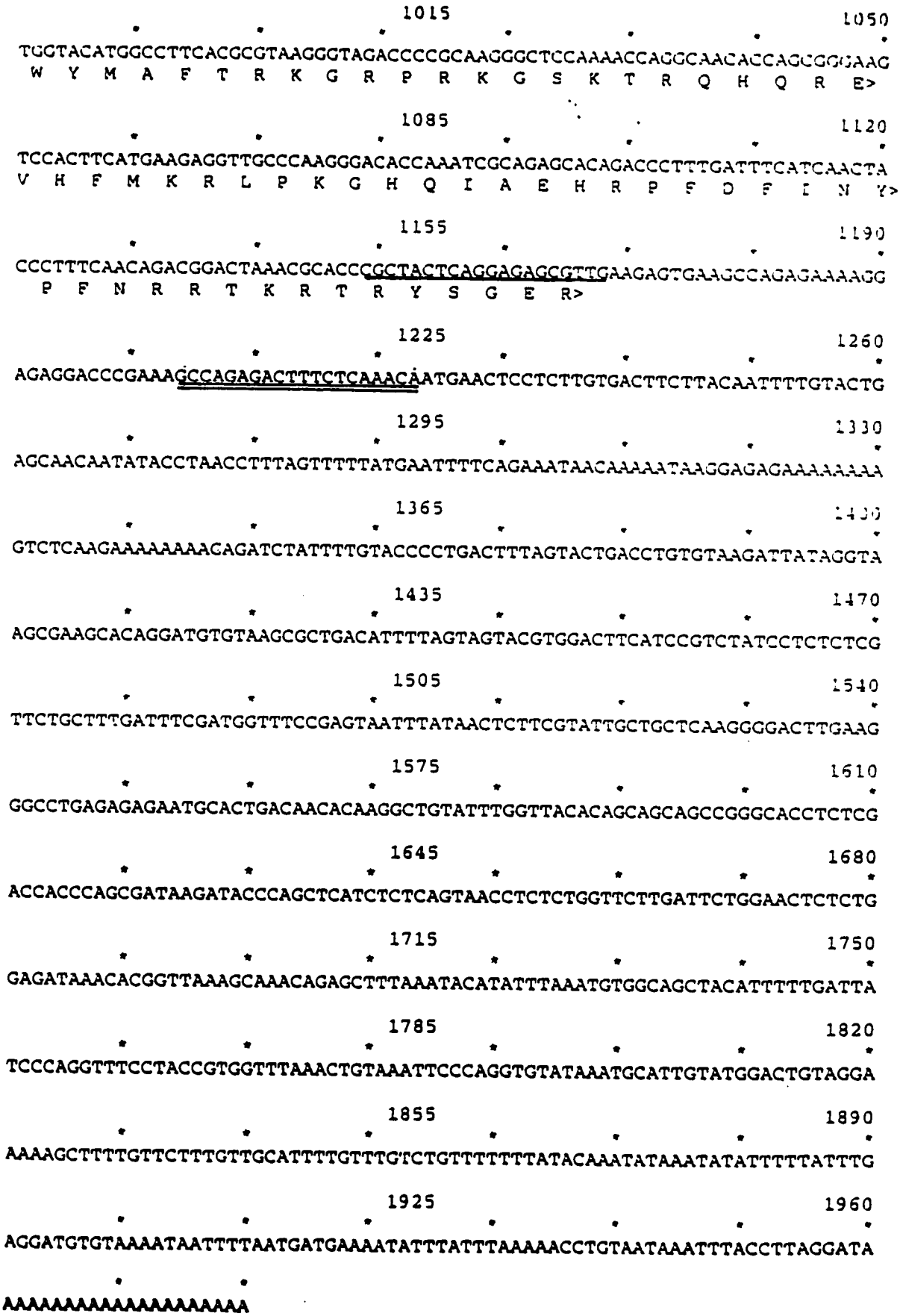

1015
1050
TGGTACATGGCCTTCACGCGTAAGGGTAGACCCCGCAAGGGCTCCAAAACCAGGCAACACCAGCGGGAAG
W Y M A F T R K G R P R K G S K T R Q H Q R E>
1085
1120
TCCACTTCATGAAGAGGTTGCCCAAGGGACACCAAATCGCAGAGCACAGACCCTTTGATTTCATCAACTA
V H F M K R L P K G H Q I A E H R P F D F I N Y>
1155
1190
CCCTTTCAACAGACGGACTAAACGCACCCGCTACTCAGGAGAGCGTTGAAGAGTGAAGCCAGAGAAAAGG
P F N R R T K R T R Y S G E R>
1225
1260
AGAGGACCCGAAAGCCAGAGACTTTCTCAAACAATGAACTCCTCTTGTGACTTCTTACAATTTTGTACTG
1295
1330
AGCAACAATATACCTAACCTTTAGTTTTTATGAATTTTCAGAAATAACAAAAATAAGGAGAGAAAAAAAA
1365
1400
GTCTCAAGAAAAAAAACAGATCTATTTTGTACCCCTGACTTTAGTACTGACCTGTGTAAGATTATAGGTA
1435
1470
AGCGAAGCACAGGATGTGTAAGCGCTGACATTTTAGTAGTACGTGGACTTCATCCGTCTATCCTCTCTCG
1505
1540
TTCTGCTTTGATTTCGATGGTTTCCGAGTAATTTATAACTCTTCGTATTGCTGCTCAAGGGGACTTGAAG
1575
1610
GGCCTGAGAGAGAATGCACTGACAACACAAGGCTGTATTTGGTTACACAGCAGCAGCCGGGCACCTCTCG
1645
1680
ACCACCCAGCGATAAGATACCCAGCTCATCTCTCAGTAACCTCTCTGGTTCTTGATTCTGGAACTCTCTG
1715
1750
GAGATAAACACGGTTAAAGCAAACAGAGCTTTAAATACATATTTAAATGTGGCAGCTACATTTTTGATTA
1785
1820
TCCCAGGTTTCCTACCGTGGTTTAAACTGTAAATTCCCAGGTGTATAAATGCATTGTATGGACTGTAGGA
1855
1890
AAAAGCTTTTGTTCTTTGTTGCATTTTGTTTGTCTGTTTTTTTATACAAATATAAATATATTTTTATTTG
1925
1960
AGGATGTGTAAAATAATTTTAATGATGAAAATATTTATTTAAAAACCTGTAATAAATTTACCTTAGGATA
AAAAAAAAAAAAAAAAAAAA

Figure 15 Alignment of the predicted amino acid sequences of zebrafish, *Xenopus*, chicken, mouse and human FGF8 proteins. Dashes indicate amino acid identity while non-conserved residues are shown in capital letters. The large rectangle indicates the signal peptide and the small box the consensus N-linked glycosylation sites. The amino and carboxyl termini are the least conserved regions. The conserved core sequence is shown between the arrowheads.

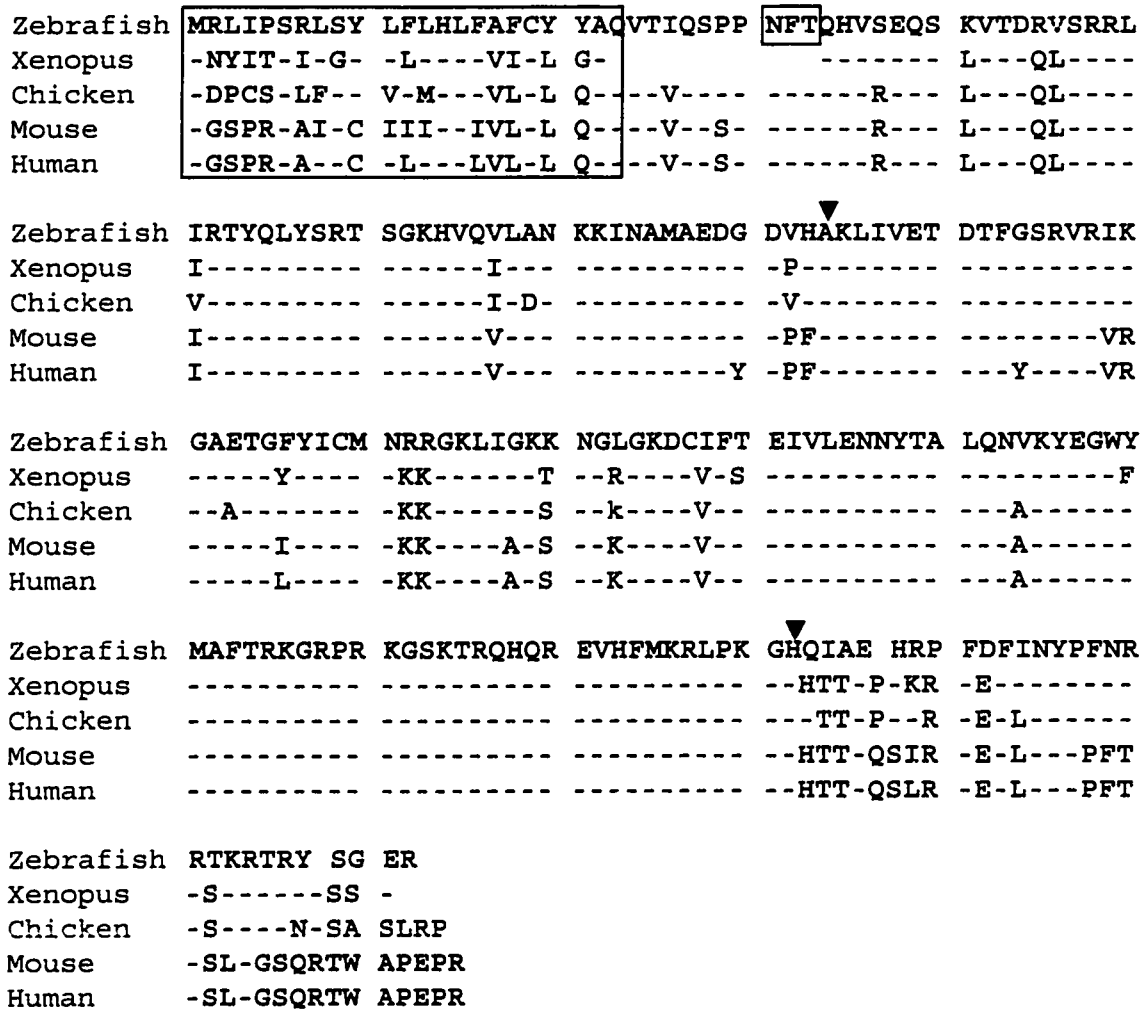

Zebrafish MRLIPSRLSY LFLHLFAFCY YAQVTIQSPP NFTQHVSEQS KVTDRVSRRL
Xenopus -NYIT-I-G- -L----VI-L G- ------- L---QL----
Chicken -DPCS-LF-- V-M---VL-L Q--V---- ------R--- L---QL----
Mouse -GSPR-AI-C III--IVL-L Q--V--S- ------R--- L---QL----
Human -GSPR-A--C -L---LVL-L Q--V--S- ------R--- L---QL----
Zebrafish IRTYQLYSRT SGKHVQVLAN KKINAMAEDG DVHAKLIVET DTFGSRVRIK
Xenopus I--------- ------I--- ---------- -P-------- ----------
Chicken V--------- ------I-D- ---------- -V-------- ----------
Mouse I--------- ------V--- ---------- -PF------- --------VR
Human I--------- ------V--- ---------Y -PF------- ---Y----VR
Zebrafish GAETGFYICM NRRGKLIGKK NGLGKDCIFT EIVLENNYTA LQNVKYEGWY
Xenopus -----Y---- -KK------T --R----V-S ---------- ---------F
Chicken --A------- -KK------S --k----V-- ---------- ---A------
Mouse -----I---- -KK----A-S --K----V-- ---------- ---A------
Human -----L---- -KK----A-S --K----V-- ---------- ---A------
Zebrafish MAFTRKGRPR KGSKTRQHQR EVHFMKRLPK GHQIAE HRP FDFINYPFNR
Xenopus ---------- ---------- ---------- --HTT-P-KR -E--------
Chicken ---------- ---------- ---------- ---TT-P--R -E-L------
Mouse ---------- ---------- ---------- --HTT-QSIR -E-L---PFT
Human ---------- ---------- ---------- --HTT-QSLR -E-L---PFT
Zebrafish RTKRTRY SG ER
Xenopus -S------SS -
Chicken -S----N-SA SLRP
Mouse -SL-GSQRTW APEPR
Human -SL-GSQRTW APEPR

Table 2. Comparison of the *Fgf8* nucleotide sequence and deduced amino acid sequence from zebrafish, chicken, mouse, human and *Xenopus*. The percentage identity was conducted using BLAST program.

Species	DNA	Amino Acid
Xenopus	78%	83%
Chicken	77%	81%
Human	77%	74%
Mouse	76%	73%

The zebrafish cDNA had the highest identity, 78% with *Xenopus laevis* (Genebank # U55189), and slightly less sequence identity with *Gallus gallus*, 77% (Genebank #Y10312), human 77% (Genebank # D12482) and mouse 76% (Genebank # U36223).

The high degree of sequence identity between the zebrafish cDNA and that of other vertebrate species suggests that this clone contains the zebrafish *Fgf8* cDNA.

The deduced zebrafish amino acid sequence was aligned with sequences from the species with which previous alignments at the nucleotide levels were performed (*Xenopus*, chicken, mouse and human). The *Xenopus* sequence presented the highest degree of identity, 83% and the mouse, the lowest degree of identity, 73%, with an overall amino acid identity between all species of 70% (Table 2).

The zebrafish FGF8 predicted protein shares with its homologues in other species, a hydrophobic signal sequence at the NH_2-terminal part, a consensus N-linked glycosylation site as well as a central "core" region of 100 amino acids with an overall identity between all species of 87% (Figure 15). The carboxyl termini as well as the amino termini are less well conserved (Figure 15). The core region presents an overall amino acid identity of 84%.

By the time I performed the sequence of the zebrafish *Fgf8* cDNA, two other zebrafish sequences have been submitted to the data gene bank at the NCBI (Genebank #AF030560 and #AF034264 (Fuerthauer et al., 1997)). Both of them have 100% identity in the ORF but are shorter either in the 5' and 3' untranslated sequences.

2. *Fgf8* gene mapping.

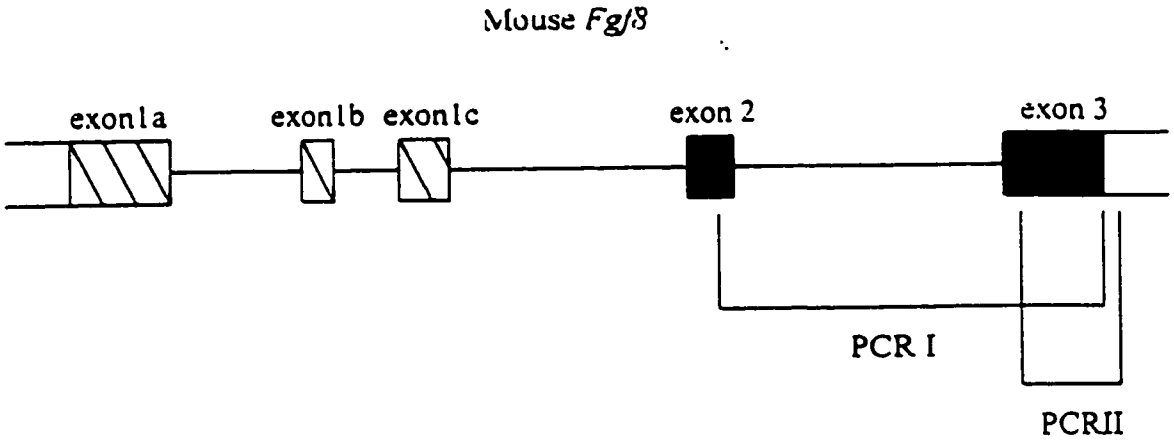
We have determined the approximate genomic position of the zebrafish *Fgf8* gene by PCR using a panel of zebrafish/mouse somatic whole cell hybrids (Ekker et al., 1996) and specific primers which were synthesized based on the zebrafish *Fgf8* sequence and the genomic organization of the mouse *Fgf8* gene (Crossley and Martin, 1995)(Figure 16.1).

The zebrafish/mouse cell hybrid panel was created by whole cell fusion between zebrafish fibroblast-like ZF4 cells and mouse B78 melanoma cells. The resulting cell hybrids contain one or a few zebrafish chromosomal segments in a mouse genomic background. This panel of hybrids allows for the rapid assignment of known genes and anonymous markers to individual zebrafish linkage groups by PCR analysis (there is one linkage group for each of the 25 zebrafish chromosomes). A first PCR reaction, using primers localized in the region that encodes for the most conserved core region in all FGF family members, which is encoded by exons 2 and 3 in mouse (see Figure 16.1), amplified a band of 1500 bp in size. The 1500 bp band was colocalized in ZFB205 and ZFB225 hybrid cell lines, both of which containing fragments of linkage group XIII. The amplified fragment suggests the presence of an intron of approximately 1000 bp. To confirm the first PCR result, a new set of primers localized in the region corresponding to the mouse exon 3 (see Figure 16.1) were made. PCR reaction using the second set of primers amplified a fragment of 300 bp that colocalized with ZFB43, ZFB205 and ZFB225 hybrid cells, all of which have in common linkage group XIII fragment. This result indicated that the zebrafish *Fgf8* gene is present on linkage group XIII near the centromere in the vicinity of the paired box containing gene *Pax2* (Figure 16.2.B). In addition, it appears that the zebrafish *Fgf8* gene may have a similar exon/intron genomic organization to that in mouse, at least in the two exons that encode the most highly conserved region of the protein. In comparison, the mouse *Fgf8* gene is localized in the distal portion of chromosome 19 flanked by the *lipocortin 1* (*Lpc1*) and *insulin 1* (*Ins1*) genes (Crossley and Martin, 1995), while the human *Fgf8* gene maps to 10q24 (Yoshiura et al., 1997) in the same chromosomal region as *Pax2* (Narahara, et al., 1997) and *FGFR2* which has been shown to act as the FGF8 receptor (Xu et al., 1998). This suggests a region of synteny between the human and zebrafish genomes. The chromosomal region where *Fgf8* is found in both mouse and humans has been linked to limb malformations such as split hand-split foot (Raas-Rothschild et al., 1996), and acrocephalosyndactyly (White et al., 1995) in humans and dactylaplasia (Seto et al., 1997) in mouse. The

Figure 16.1. Genomic organization of the mouse *Fgf8* gene (Crossley and Martin, 1995). The structure of the gene is illustrated with exons indicated by boxes and introns by horizontal lines. Black and hatched boxes indicate the coding sequence. The amino terminal region of the FGF8 protein is encoded in at least three exons labeled 1a,1b and 1c. The black boxes indicate the sequence that encodes the amino acid core conserved in all FGF family members and the clear boxes show the 5' and 3' unstranslated regions.
Taking into consideration the genomic structure of the mouse *Fgf8* gene and the zebrafish cDNA sequence, two sets of oligonucleotide primers were made to map the zebrafish *Fgf8* gene using a somatic zebrafish/mouse cell hybrid panel. The first set of the oligonucleotide primers (PCRI) are localized in exons 2 and 3, respectively. The second set of primers (PCRII), used to verify the result of PCRI, are localized within exon 3 and the 3' untranslated region, respectively.

Figure 16.2. Genetic mapping of the zebrafish *Fgf8* gene. A. Table representing the different somatic cell hybrids of the zebrafish/mouse panel used for PCR analysis. PCRI amplified a 1500 bp fragment in ZFB205 and ZF225 hybrid cells. Primers used in PCRII amplified a 300 bp fragment in ZFB43, ZF205 and ZF225 hybrid cells, all of them have fragments of chromosome XIII in common. B. Partial consensus linkage map of the zebrafish chromosome XIII showing location of different markers and the position of the *Fgf8* and *Pax2* genes. Genetic distance is shown to the left in cM units.

14.1

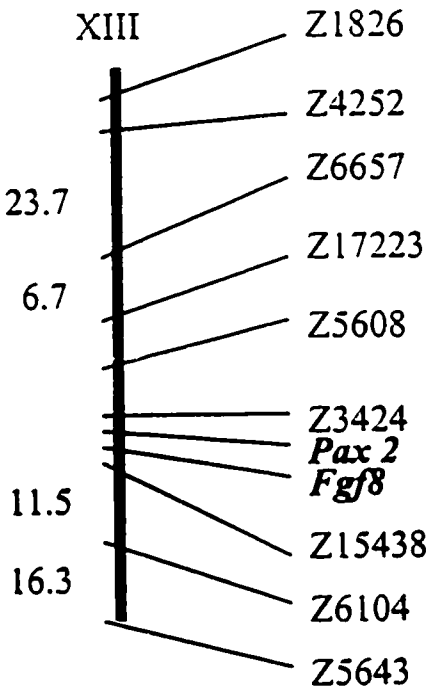


14.2

A

ZFB8	ZFB9	ZFB10	ZFB11	ZFB14	ZFB27	ZFB28	ZFB31
ZFB32	ZFB33	ZFB35	ZFB36	ZFB37	ZFB40	ZFB42	ZFB43
ZFB44	ZFB47	ZFB48	ZFB49	ZFB50	ZFB55	ZFB54	ZFB56
ZFB57	ZFB58	ZFB59	ZFB60	ZFB61	ZFB62	ZFB63	ZFB64
ZFB65	ZFB66	ZFB67	ZFB72	ZFB73	ZFB74	ZFB201	ZFB203
ZFB204	ZFB205	ZFB206	ZFB207	ZFB208	ZFB209	ZFB210	ZFB211
ZFB212	ZFB214	ZFB215	ZFB216	ZFB217	ZFB219	ZFB220	ZFB221
ZFB222	ZFB223	ZFB224	ZFB225	ZFB226	ZFB227	ZFB228	ZFB229
ZFB232	ZFB234	ZFB235	ZFB236	ZFB238	ZFB240	ZFB242	ZFB243
ZFB244	LFFB1	LFFB2	LFFB3	LFFB4	LFFB5	LFFB7	LFFB8
LFFB9	LFFB10	LFFB12	LFFB13	LFFB14	LFFB15	LFFB17	LFFB19
LFFB20	LFFB22	LFFB26	LFFB27	B78.	ZF4-NEO	LFF-NEO	1:10 MIX

B



functions attributed to *Fgf8*, *FGFR2* and *Pax2* during limb development make them good candidates for such malformations (Raas-Rothschild et al., 1996).

The information resulting from the genomic mapping is important as it can be eventually correlated to the position of mutations affecting zebrafish development (Mullins and Nüsslein-Volhard, 1993; Driever et al., 1994).

The genomic structure of the zebrafish *Fgf8* gene has yet to be determined and it is therefore not known if different isoforms exist in this species as has already been found to be the case in human and mouse.

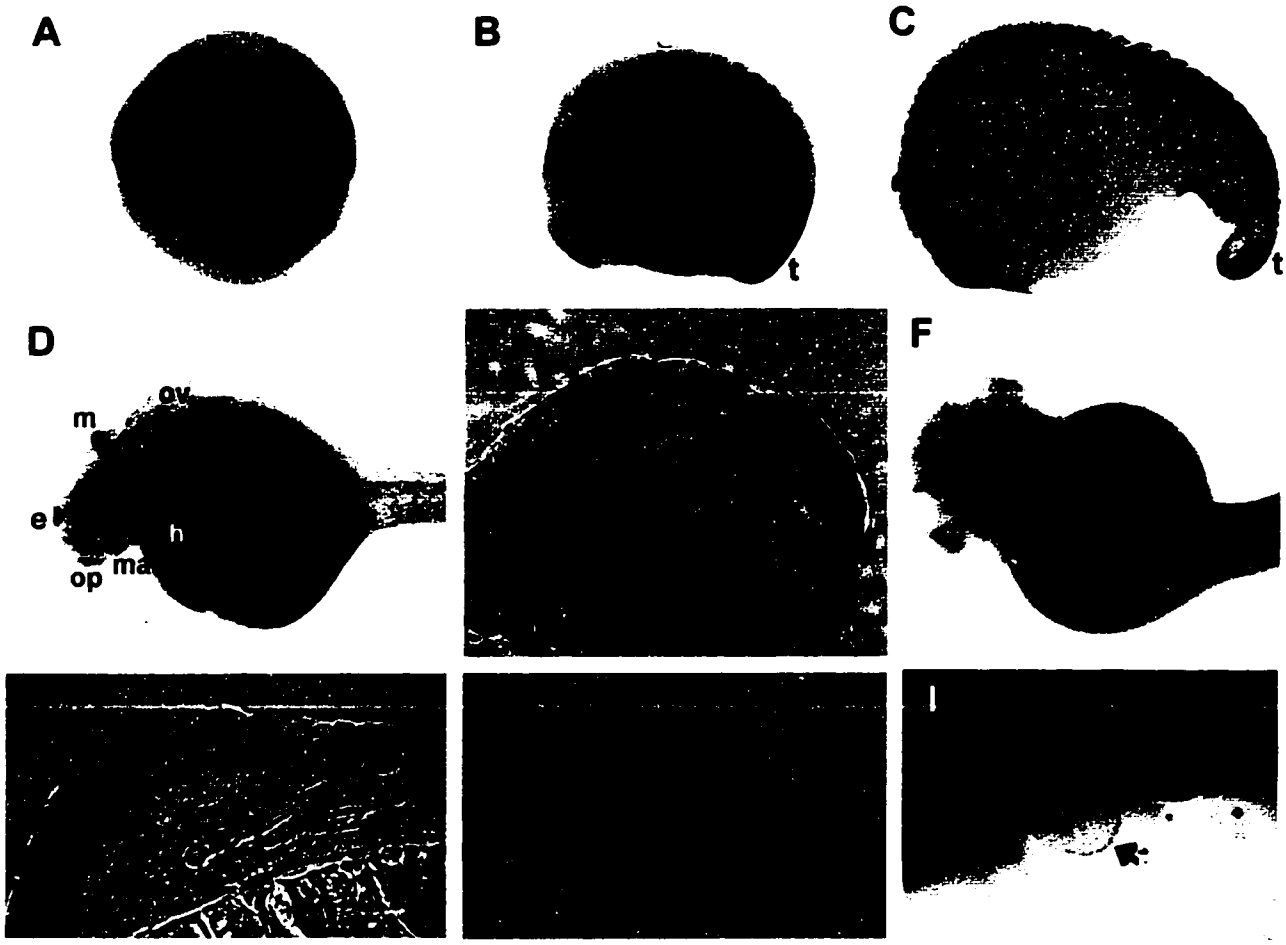
3. *Fgf8* expression during zebrafish embryogenesis

In order to analyze the pattern of expression of *Fgf8* during embryonic development, *in situ* hybridization on whole mount zebrafish embryos at various developmental stages, were performed. The *Fgf8* full length cDNA (1945 bp) was used to synthesize an antisense RNA probe.

The pattern of expression of *Fgf8* in zebrafish embryos (Figure 17) resembles that described in chicken (Vogel et al., 1996) and mouse (Crossley and Martin, 1995) with slight variations, especially during fin bud development. The significance of the pattern of expression of *Fgf8* observed at each developmental stages will be discussed in section 2 in the "Discussion" part.

Transcripts of *Fgf8* are first observed at the blastula stage (30% epiboly) surrounding the margin of the blastoderm (not shown). During early gastrula stages (50% epiboly), *Fgf8* RNAs are restricted to the dorsal part of the marginal region. At mid gastrula stage, *Fgf8* transcripts are confined to epiblast cells in a dorsoventral gradient at the margin of the embryo (Figure 17A arrow). *Fgf8* transcripts are also found in two stripes of cells that will eventually become part of the hindbrain (Figure 17A, arrowhead). During somitogenesis (12 hr p.f.)(Figure 17B), *Fgf8* expression extends to the region that will eventually form the head. It is also expressed in the forming somites and at the most

Figure 17. Expression of *Fgf8* during zebrafish embryonic and larval development. Expression was determined by whole mount *in situ* hybridization. **A.** *Fgf8* expression is first detected during gastrulation in the germ ring (arrow) and in the epiblast layer, a region that will eventually become part of the hindbrain (arrowhead). **B.** At fourteen hours p.f. (10 somites stage), *Fgf8* is strongly expressed in the tail bud (t), in the developing somites, in the rostral head region and in a stripe at the hindbrain-midbrain junction. **C.** 20 hours p.f., *Fgf8* transcripts are localized in the tail bud, in the developing somites, in the rostral head region and in the midbrain-hindbrain boundary. **D.** 24 hour p.f., *Fgf8* transcription is localised in different regions of the head: otic vesicle (ov), midbrain-hindbrain junction (mh), epiphysis (e), the olfactory placode (op) and the mandibular arch (ma). A Strong *Fgf8* expression is seen in the hatching gland (h). **E.** Pectoral fin bud from a 31 hours p.f. zebrafish embryo. *Fgf8* is expressed in the AER in a layer of cells close to the mesenchyme **F.** Dorsal view of a 48 hour old embryo, *Fgf8* is expressed in the AER of the pectoral fin bud and in a patch of dorsal ectodermal cells close to the yolk sac(arrow). **G.** Sagittal section of a pectoral fin bud of a 48 hour embryo. The arrows show ectodermal cells expressing *Fgf8* on the dorsal and ventral sides of the pectoral fin in a region where the muscle fibers appear. The arrowhead shows *Fgf8* expression at the AER. **H.** *Fgf 8* is expressed in the distal cells of the medial fin fold of a 30 hour p.f. embryo **I.** Pelvic fin of a 7.5 mm larval showing *Fgf8* expression at the distal edge of the fin (arrow).


A
B
C
t
t
D
ov
m
e
h
op
ma
F
I

posterior region of the embryo which will generate the tail bud. At 24 hours p.f. (Figure 17C), a strong expression of *Fgf8* is seen in the tail bud, in the anterior border of the forming somites of the tail bud region, and in the head. At 30h p.f. (Figure 17D), *Fgf8* expression is dispersed in different regions of the embryo including: the midbrain-hindbrain junction; the epiphysis; the otic vesicle; the forebrain; the mandibular arch; and the olfactory placodes. Later (32 hr p.f.), following the initiation of pectoral fin bud outgrowth, *Fgf8* RNA is found in the AER, in a layer of cells close to the underlying mesoderm (Figure 17E, arrow) and in the most distal cells of the median fin fold (Figure 17H), tissues that will ultimately give rise to the unpaired fins (dorsal, anal, and caudal fins). At this time, *Fgf8* is still present in the midbrain-hindbrain junction, in the epiphysis, in the anterior forebrain and in the rostral region of the embryo (data no shown). A few hours later (48 hours p.f.)(Figure 17F), *Fgf8* is expressed in the AER and in a patch of cells on the dorsal and ventral sides of the pectoral fin. This later site of expression has not been reported in other species. Longitudinal sections of the pectoral fin, at this stage, show expression of *Fgf8* in the dorsal and ventral mesenchyme, in a region related to the apparition of skeletal muscle fibers (Figure 17G, arrows) and in the first cell layer of the AER (Figure 17G, arrowhead). After 48 hours p.f., the expression of *Fgf8* gradually decreases and is no longer seen after 3 day p.f..

The pelvic fins in zebrafish develop late during the larval stage, when the fish reaches a size of around 7.4 mm. At this stage, *Fgf8* is also transiently expressed in the apical ectodermal ridge of the pelvic fin bud (Figure 17I, arrow).

As in mice and chicken, *Fgf8* expression was colocalized in regions thought to act as signaling centers including, somites, tail bud, midbrian-hidbrain junction and AER, which directs outgrowth and patterning during embryonic development.

4. *Fgf8* expression during fin regeneration

Genes involved in the development of embryonic fins are often re-expressed during fin regeneration, sometimes with patterns that suggest roles different from those played during development. In order to determine the participation of *Fgf8* during the regeneration process, caudal fins of adult fish were amputated proximal to the level of the first bifurcation and allowed to regenerate for different lengths of time (1, 2, 4 and 6 days). After fixation, the fins were submitted to the whole mount *in-situ* hybridization procedure using a synthetic antisense RNA probe prepared from the full length *Fgf8* cDNA.

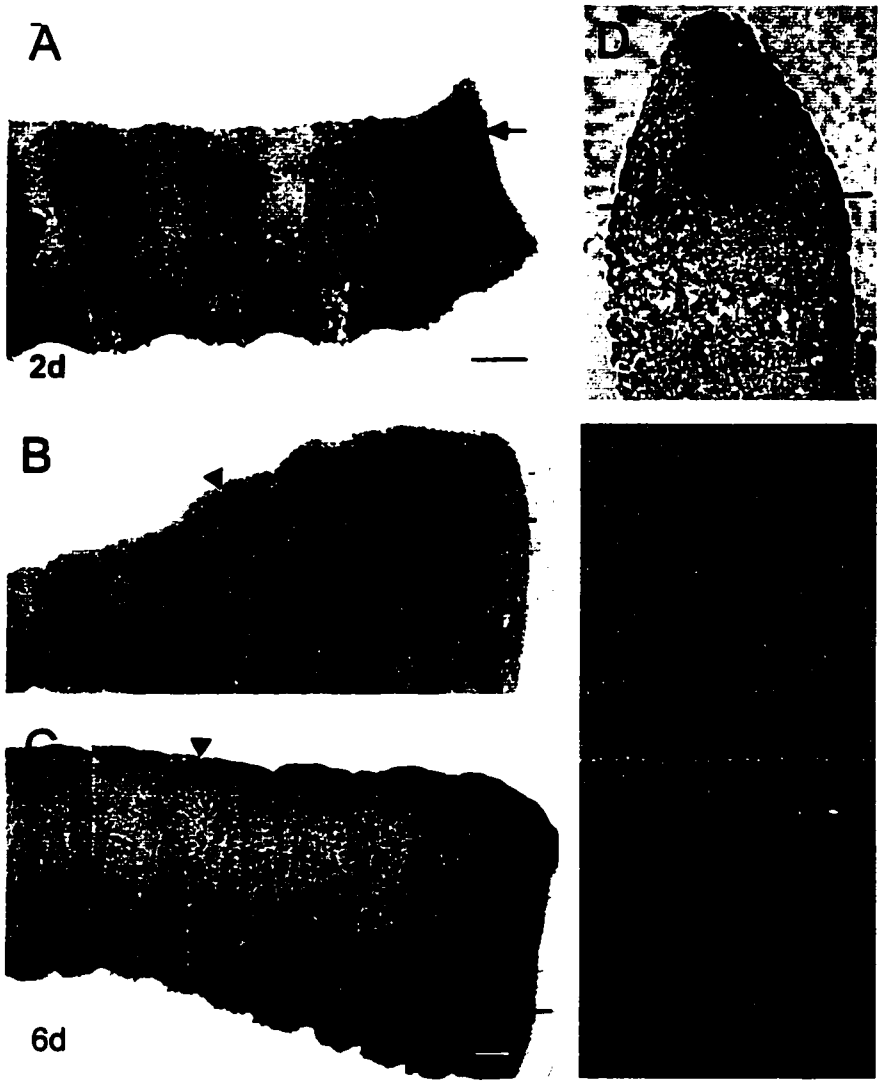
As shown in the previous chapter, 6 hours after amputation, the wound is already covered by epithelial tissue and 24 hours after amputation, the epidermal cap is established but the blastema has yet to be formed. I did not observe *Fgf8* expression in fins 24 hours after amputation. In 48 hour fin regenerates, a small blastema is present and it is at this time that *Fgf8* transcripts are first observed in a patch of cells at the tip of each fin ray (Figure 18A). Sections of hybridized fins showed that *Fgf8* expression is localized in the most distal part of the blastema. Subsequently, the domain of expression increased and remained stable until at least 6 days after amputation (Figure 18A,B,C).

Initiation of *Fgf8* expression correlates with the initiation of formation of the blastema, as shown in BrdU incorporation experiments in Chapter 1, suggesting a role for *Fgf8* in cell proliferation.

We also observed that *Fgf8* expression is not uniform in all rays of a given caudal fin. 48 hours after fin amputation, the domain of *Fgf8* expression increases from the medial to the lateral rays (Figure 18B). This variation of the size of the domain of expression of *Fgf8* correlates with the growth rate of the regenerating fin rays; the most medial rays of a caudal fin develop and regenerate less fast than the most lateral rays. As a mitogenic factor, FGF8 could be influencing the proliferation rate of the tissue in which it is found.

Figure 18. *Fgf8* expression during caudal fin regeneration. Expression was determined by whole mount *in situ* hybridization **A.** 48 hours after amputation, *Fgf8* expression is detected at the tip of each fin ray. **B.** 4 day regenerated fin showing differential expression of *Fgf8* from the lateral to the medial rays. *Fgf8* expression is stronger in the lateral than in the medial caudal fin rays. **C.** *Fgf8* transcripts can be detected until at least 6 days after amputation. **D** and **E.** Proximo-distal section of hybridized fin showing the blastema of a 2 day (D) and 6 day (E) caudal fin as the region that expresses *Fgf8*. Arrows in A,B and C and dashes in D and E represent the level of amputation. Arrowheads in A, B and C show *Fgf8* expression at the tip of a fin ray. In D and E the color at the border of the section represents background due to the long incubation during color reaction.

Scale bars: A, B and C 80 mm, D and E 15 mm.


A
2d
B
6d
D

Therefore, the stronger expression of *Fgf8* in both the dorsal and ventral region of the caudal fin could be influencing its characteristical "V" shape.

5. Towards *Fgf4* cloning

As previously mentioned in the introduction, FGF4, plays an important role in limb patterning. *Fgf4* is expressed in the limb bud of chicken and mouse in the posterior part of the AER and the gene product is required for the continuous expression of *Shh* in the ZPA.

Fgf4 has been shown to rescue the development of limbs after AER removal; probably by stimulating mesenchymal cell proliferation (Niswander et al., 1993). More recently, Kostakopoulou et al. (1996) have shown that FGF4 can induce regeneration of amputated chick wing buds, which do not normally have regeneration capabilities. This implicates *Fgf4* as a possible candidate in the regeneration process of fins. Due to the conservation of the expression pattern of other genes involved in fin/limb development, we anticipated that *Fgf4* would also be involved in zebrafish development and presumably fin regeneration.

The first step to confirm this hypothesis was to try to clone the zebrafish *Fgf4* cDNA. We received from Dr. Tohru Suzuki's laboratory at the National Research Institute of Aquaculture, Japan, a 244 bp PCR fragment amplified from a cDNA obtained from an RNA preparation of the zebrafish swim bladder. A BLAST search of the 244 bp fragment revealed that it is localized in the most conserved region of the *Fgf* genes and presented the highest percentage of identity with *Fgf4* and *Fgf6* from other vertebrates (Figure 19). However, based on nucleotide and amino acid sequence comparisons, it was not possible to determine whether this fragment corresponded to a zebrafish *Fgf4* or to a *Fgf6* sequence. I used this fragment as a probe to screen a λZAP cDNA library prepared from 9 to 28 hours p.f. zebrafish embryos using various stringency conditions. Such a screening did not yield any positive clones. In parallel, I used various PCR approaches to

try to isolate the full length cDNA corresponding to the *Fgf* fragment we had, but none of them gave any satisfactory results.

To determine the stages at which the FGF factor corresponding to the *Fgf* PCR fragment was expressed, Northern blots using total RNA extracted from different zebrafish embryonic stages (10, 24, 30, 48 and 72 hours p.f.) were carried out using the 244 bp fragment as a probe. No hybridization signal was observed (data not shown), suggesting a very low level of expression. The integrity and equal amount of RNA used in the Northern blot analysis were confirmed by hybridizing the blot with a probe which recognized the *Max* gene. This gene is expressed at a relatively uniform level during zebrafish embryonic development. I decided to use the RNAse protection assay, a more sensitive method, to detect low levels of mRNA. In this assay, a synthetic antisense RNA made from the 244bp PCR fragment, was incubated with RNA collected from different embryonic stages (10, 24, 30, 48 and 72 hours p.f.). After RNAse treatment (see methods), the probe was able to protect a fragment of 244 bp giving a faint signal after 7 days of exposure with RNA from embryos 30 hours p.f. (Figure 20). The intensity of the signal increased gradually in subsequent embryonic stages (48 and 72 hours p.f.). On the same gel, RNA from the same source, was probed with a fragment of the *Max* gene to confirm equal loading of RNA and RNA integrity.

These results show that the *Fgf* PCR fragment is expressed at very low levels starting at 30 hours p.f. during zebrafish embryonic development. The fact that the cDNA library that was screened and that the DNA used as PCR template came from embryonic stages prior to 30 hours may explain the difficulty I encountered to isolate the zebrafish *Fgf* gene. It also suggests that the *Fgf* PCR fragment may correspond to the *Fgf6* gene whose expression in mouse is restricted to the muscle cell lineage at 9.5 days post-coitum and at later stages to the developing skeletal muscles (deLapeyriere et al., 1993).

Figure 19. Nucleotide sequence comparison of the zebrafish *Fgf* PCR fragment with the *Fgf4* and *Fgf6* sequences from different species. The fragment presents a high percentage of identity with *Fgf 4* of *Gallus gallus* (70%), *Xenopus* (71%) as well as with *Fgf6* of human (71%), mouse (69%). The alignment was carried out using the BLAST Network service (Altschul et al., 1990) at the National Centre for Biotechnology Information (NCBI).
Xenopus laevis Fgf4 accession number X62594; *Gallus gallus Fgf4* accession number U14654; *Homo sapiens Fgf6* accession number X63454 and *Mus musculus Fgf6* accession number M92416.

gb|U14654|GGU14654 Gallus gallus fibroblast growth factor 4 (FGF4) gene, complete cds.
Length = 585

Plus Strand HSPs:

Score = 581 (160.5 bits), Expect = 1.9e-38, P = 1.9e-38
Identities = 173/244 (70%), Positives = 173/244 (70%), Strand = Plus / Plus

```
Query:      1 ATGTCGGCATTGGGTTTCACCTGCAGGTCCTGCCAGACGGAAGGATAAACGGAGTCCATA 60
              | || ||||| || || ||| | |||||||||| |||||  ||||  ||||  |||| |
Sbjct:    230 ACGTGGGCATCGGCTTCCACATCCAGGTCCTGCCCGACGGCCGGATCGACGGGATCCACA 289

Query:     61 ATGAGAACCAGTACAGTCTAATTGAAATCTCAGCGGTAGAGAGAGGAGTGGTTAGTCTAT 120
             |||||||  |||||||| | |||||||| | || || |||||||||| ||  |||
Sbjct:    290 GCGAGAACCGATACAGTCTGCTGGAAATCTCCCCTGTGGAAAGAGGAGTGGTGAGCATAT 349

Query:    121 ACGGCGTGAAAAGCGGGCTGTTTGTCGCAATGAGCAGCCGCGGGAGGTTATACGGAACGA 180
              || || | ||| || || || || || |||| |||  || |  | || ||| | |
Sbjct:    350 TTGGTGTTAGAAGTGGACTCTTCGTGGCCATGAATAGCAAAGGCAAACTCTATGGATCTA 409

Query:    181 GGGCCTTCCGTGACGAGTGCAAGTTCAAGGAGACACTGCTGCCCAACAACTACAACGCCT 240
                ||  ||| |||||||| ||||| |||| || ||||| |||||||||| || |
Sbjct:    410 CCCATGTCAATGATGAGTGCAAATTCAAAGAGATCCTCCTGCCAAACAACTACAATGCTT 469

Query:    241 ACGA 244
              ||||
Sbjct:    470 ACGA 473
```

emb|X62594|XLFGFII X.laevis XeFGF(ii) mRNA for embryonic fibroblast growth factor
Length = 589

Plus Strand HSPs:

Score = 572 (158.1 bits), Expect = 1.1e-37, P = 1.1e-37
Identities = 172/244 (70%), Positives = 172/244 (70%), Strand = Plus / Plus

```
Query:      1 ATGTCGGCATTGGGTTTCACCTGCAGGTCCTGCCAGACGGAAGGATAAACGGAGTCCATA 60
              | || ||||| ||||||||  | |||||  | || ||||||||||||||| ||  | || |
Sbjct:    234 ACGTGGGCATCGGGTTTCATATCCAGGTTTTACCGGACGGAAGGATAAATGGCATGCACA 293

Query:     61 ATGAGAACCAGTACAGTCTAATTGAAATCTCAGCGGTAGAGAGAGGAGTGGTTAGTCTAT 120
             | || || |  |||||| || | || || ||  | || ||     ||||| || ||| | |
Sbjct:    294 ACGAAAATCGATACAGTTTACTGGAGATTTCCCCAGTGGAAGTGGGAGTCGTGAGTTTGT 353

Query:    121 ACGGCGTGAAAAGCGGGCTGTTTGTCGCAATGAGCAGCCGCGGGAGGTTATACGGAACGA 180
              ||||| | |||||||   ||||||| || ||||         ||||  |  |  ||  ||| | |
Sbjct:    354 ACGGCATTAAAAGCGCCATGTTTGTTGCTATGAATGCAAAGGGGAAGCTGTATGGATCAA 413

Query:    181 GGGCCTTCCGTGACGAGTGCAAGTTCAAGGAGACACTGCTGCCCAACAACTACAACGCCT 240
             |     |||    || || ||||| ||||| |||||||| ||||||||||||||| ||||| |
Sbjct:    414 GATATTTCAACGAGGAATGCAAATTCAAAGAGACACTTCTGCCCAACAACTATAACGCTT 473

Query:    241 ACGA 244
              ||||
Sbjct:    474 ACGA 477
```

emb|X63454|HSHST2 H.sapiens hst-2 (FGF-6) mRNA
Length = 744

Plus Strand HSPs:

Score = 599 (165.5 bits), Expect = 6.0e-40, P = 6.0e-40
Identities = 175/244 (71%), Positives = 175/244 (71%), Strand = Plus / Plus

```
Query:     1 ATGTCGGCATTGGGTTTCACCTGCAGGTCCTGCCAGACGGAAGGATAAACGGAGTCCATA 60
             | || ||||| || |||||||| ||||| || || |||||  |||| | |||   |||
Sbjct:   316 ACGTGGGCATCGGCTTTCACCTCCAGGTGCTCCCCGACGGCCGGATCAGCGGGACCCACG 375

Query:    61 ATGAGAACCAGTACAGTCTAATTGAAATCTCAGCGGTAGAGAGAGGAGTGGTTAGTCTAT 120
             | |||||||  ||||| ||  | |||||| ||  | || ||| |||| ||||| ||||| |
Sbjct:   376 AGGAGAACCCCTACAGCCTGCTGGAAATTTCCACTGTGGAGCGAGGCGTGGTGAGTCTCT 435

Query:   121 ACGGCGTGAAAAGCGGGCTGTTTGTCGCAATGAGCAGCCGCGGGAGGTTATACGGAACGA 180
               || |||| ||| |  || || || || |||| |||    || || || |||| ||||
Sbjct:   436 TTGGAGTGAGAAGTGCCCTCTTCGTTGCCATGAACAGTAAAGGAAGATTGTACGCAACGC 495

Query:   181 GGGCCTTCCGTGACGAGTGCAAGTTCAAGGAGACACTGCTGCCCAACAACTACAACGCCT 240
               |||||  || || |||||||||| || || || |||||||||||| ||||| ||||
Sbjct:   496 CCAGCTTCCAAGAAGAATGCAAGTTCAGAGAAACCCTCCTGCCCAACAATTACAATGCCT 555

Query:   241 ACGA 244
             ||||
Sbjct:   556 ACGA 559
```

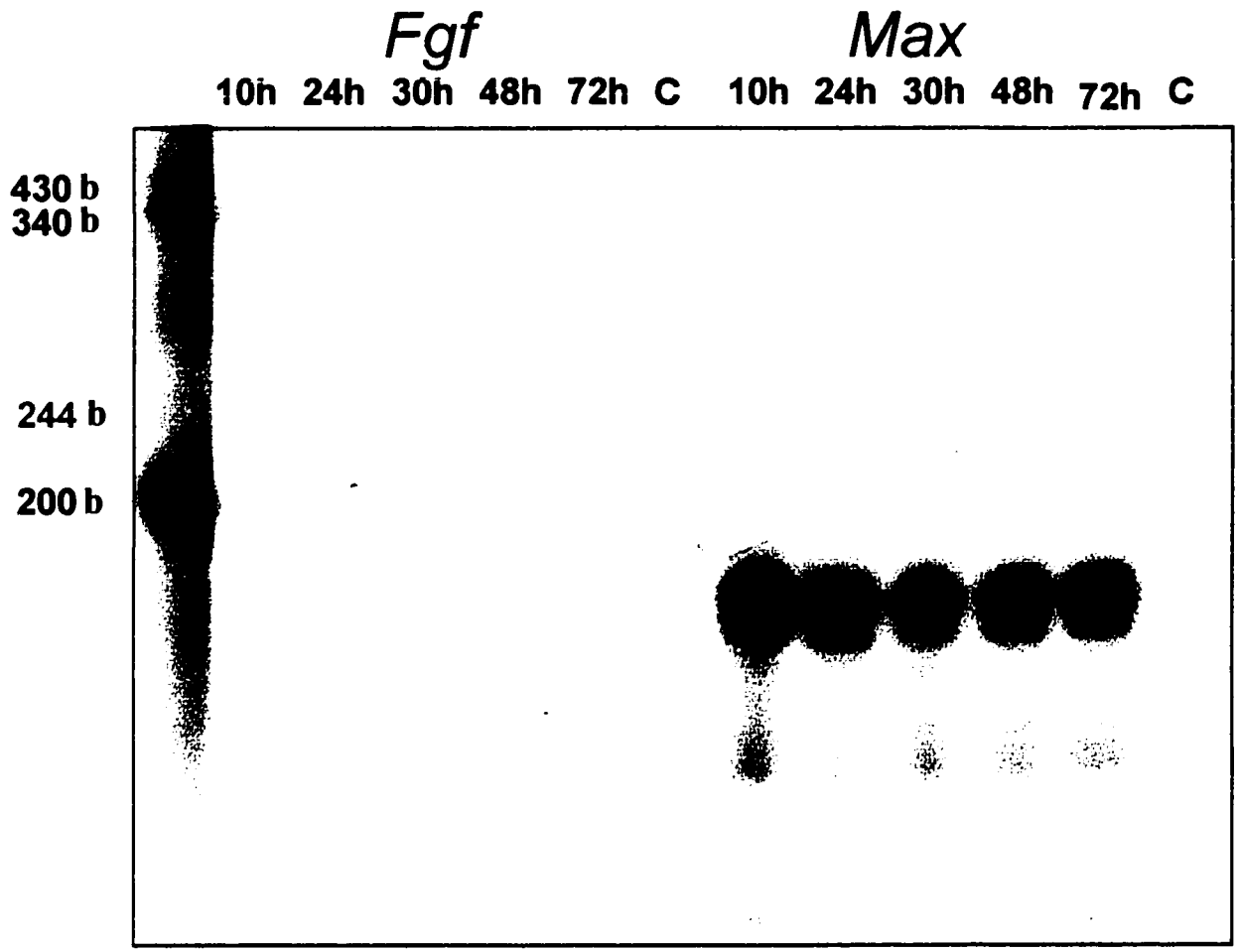
gb|M92416|MUSFGFAB Mus musculus fibroblast growth factor (Fgf6) mRNA, 3' end.
Length = 4607

Plus Strand HSPs:

Score = 554 (153.1 bits), Expect = 3.3e-36, P = 3.3e-36
Identities = 170/244 (69%), Positives = 170/244 (69%), Strand = Plus / Plus

```
Query:     1 ATGTCGGCATTGGGTTTCACCTGCAGGTCCTGCCAGACGGAAGGATAAACGGAGTCCATA 60
             | || |||||||| || ||||| ||||| |  || |||||  | || |  |||   ||
Sbjct:   218 ACGTGGGCATTGGCTTCCACCTCCAGGTGCCTCCTGACGGCCGCATCAGTGGAACACACG 277

Query:    61 ATGAGAACCAGTACAGTCTAATTGAAATCTCAGCGGTAGAGAGAGGAGTGGTTAGTCTAT 120
             | |||||||  ||||| ||  | || ||||||  |||||||  | || ||||| || || |
Sbjct:   278 AGGAGAACCCCTACAGCCTGCTGGAGATCTCCACGGTAGAACGGGGTGTGGTGAGCCTCT 337

Query:   121 ACGGCGTGAAAAGCGGGCTGTTTGTCGCAATGAGCAGCCGCGGGAGGTTATACGGAACGA 180
               ||| ||||| || |  || ||  | || |||| |||    || ||  | |||  ||||
Sbjct:   338 TCGGGGTGAAGAGTGCTCTCTTCATTGCCATGAACAGTAAAGGAAGACTGTACACAACGC 397

Query:   181 GGGCCTTCCGTGACGAGTGCAAGTTCAAGGAGACACTGCTGCCCAACAACTACAACGCCT 240
               ||||| |||||| |||||||||  || || || || || ||||||||||||||||
Sbjct:   398 CCAGCTTCCATGACGAATGCAAGTTCCGAGAAACCCTCCTTCCAAACAACTACAACGCCT 457

Query:   241 ACGA 244
             ||||
Sbjct:   458 ACGA 461
```

Figure 20. RNase protection assay.on zebrafish embryonic RNA. Total RNA prepared from zebrafish embryos at 10, 24, 30, 48 and 72 hours post fertilization were incubated with radioactive labeled synthetic RNA antisense probe made from a *Fgf* PCR fragment. After Rnase treatment, electrophoresis was done in a 7.5 % acrylamide gel which was exposed to X-ray film for 7 days. Lane 1 shows different RNA probes used as size markers (430, 340, 244, 200 b). Lane C, probe incubated without RNA, was the control (see methods). The *Fgf* probe protected a fragment of 244 RNA bases from embryos at 30 hours p.f. A slight increase in RNA transcription was seen in successive stages (48 and 72 hours p.f.). Max gene was used to show RNA integrity and equal loading.


Fgf
Max
10h 24h 30h 48h 72h C
10h 24h 30h 48h 72h C
430 b
340 b
244 b
200 b

IV. DISCUSSION

1. Zebrafish *fibroblast growth factor 8*

I have sequenced a zebrafish *Fgf8* clone containing the zebrafish *Fgf8* cDNA isolated from a zebrafish embryonic cDNA library. The DNA and the predicted amino acid sequences showed a high level of identity to the sequence of *Fgf8* genes from different vertebrate species (Crossley et al.,1995; Gemel et al., 1996; Vogel et al., 1996).

Our zebrafish *Fgf8* cDNA corresponds to the mouse *Fgf-8b* isoform (Crossley and Martin, 1995). The 1.9 kb zebrafish *Fgf8* gene encodes for a 211 amino acid protein that shares with other FGF8 proteins in different species both a NH2-terminal signal peptide motif constituted by 22 hydrophobic amino acids and a highly conserved internal core region. The signal peptide directs the protein into the secretory pathway. The predicted protein shows a consensus N-linked glycosylation site. The glycosylation sites have been suggested to regulate FGF activity. Different FGF8 isoforms in human and mouse, can contain 0, 1 or 2 N-linked glycosylation sites (Crossley and Martin, 1995; Gemel et al., 1996) which can provide them different properties *in vivo*. Indeed, in FGF4, the cleavage of the N-terminal signal peptide causes an increase in the mitogenic activity of this factor and it has been shown that glycosylation of this region appears to inhibit that cleavage (Bellosta et al. 1993).

2. *Fgf 8* expression during embryogenesis in zebrafish

a. *Fgf8* expression during early embryonic stages

The domains of *Fgf8* expression during zebrafish embryonic development are localized in signaling centres that direct outgrowth and patterning in embryos of different species (Chicken, Mouse and *Xenopus*). The expression of *Fgf8* is not restricted to a particular cell type since they can be seen in mesoderm and ectoderm derived structures. The expression of *Fgf8* observed during early zebrafish embryonic developmental stages correlates with that already described (Furthauer et al., 1997). The expression of *Fgf8*

restricted to the dorsal epiblast cells at the gastrula stage suggests a role in dorso-ventral patterning of the embryo. This hypothesis was confirmed since injections of a synthetic *Fgf8* RNA in one cell stage zebrafish embryos induced an expression of the dorsolateral structures at the expense of ventral domains and also the formation of a secondary axis (Furthauer et al., 1997). It was found that the dorsalization induced by overexpression of *Fgf8* produced the inhibition of BMP2 and BMP4 which are, at this stage, ventralizing factors. During somitogenesis the expression of *Fgf8*, in the tail bud, suggests that this signal is regulating genes that are involved in the antero-posterior elongation of the body axis. This has been confirmed in experiments where blocking the FGF signal by injection of a dominant negative form of the *Fgf* receptor to zebrafish embryos results in embryos that lack trunk and tail structures (Griffin et al, 1995). This result suggests that FGF signals are essential for the formation of two different posterior domains, the tail and trunk, by regulating downstream genes that are involved in body axial patterning. (Griffin et al., 1995).

During somitogenesis, *Fgf8* transcripts in fish, as previously described in chicken (Vogel et al., 1996), is detected in the anterior half of the somites following their direction of differentiation in an anterior to posterior wave. *Fgf8* expression in the somites of zebrafish increases as the embryo develops. However, this expression differs from the expression reported in mouse where it is detected in the anterior and posterior part of the somites (Crossley and Martin, 1995). The function of the FGF8 signal during somitogenesis remains to be determined. In vertebrates, muscles of the back, abdominal wall and limbs are derived from a portion of the somite denominated myotome. It has been shown in different vertebrates that members of the *Fgf* family and their receptors, during somitogenesis, may be playing an important role in muscle development (Templeton et al., 1992; Patapoutian et al., 1995; Itoh et al., 1996; Grothe et al., 1996; Marcelle et al., 1995; Thisse et al., 1995). *Fgf6* transcripts are detected in the somites of the mouse and its expression continues in the developing skeletal muscles. At the same time, the putative receptor (FGF4R) is expressed in the same tissue but in a different

population of cells (deLapeyriere et al., 1993). FGF6 factor apparently acts by blocking the terminal differentiation of myogenic cells within the somites which then differentiate into postmitotic muscle fibers when the concentration of the mitogen decreases or the levels of FGFR drop (Itoh et al, 1996). Interestingly enough, the levels of *Fgf8* expression in zebrafish decrease as the muscle fibers develop in the somites, suggesting a possible participation in muscle development .

b. *Fgf8* expression in the developing brain

Fibroblast growth factor 8 plays an important role during the organization of the midbrain and forebrain in higher vertebrates. In chick its expression is localized in the isthmus, a constriction in the embryonic mid-hindbrain region that is thought to be an organizing centre. An ectopic expression of FGF8 in the forebrain, has the same midbrain inducing and polarizing effect, as a graft of isthmic tissue (Crossley et al, 1996a). In zebrafish, *Fgf8* expression close to the mid/hindbrain junction during early embryonic stages may indicate a similar function for this factor.

c. *Fgf8* function in the developing fin

During fin bud formation, *Fgf8* transcription follows a characteristic pattern of expression. It is detected after the fin bud starts to grow in a distal layer of cells, analogous to the AER in tetrapod limbs. However, there is no detectable expression by *in situ* hybridization before the fin bud appear. This result differs from *Fgf8* expression in chicken where *Fgf8* transcripts can be seen in the pre-ridge ectoderm, suggesting its involvement during limb bud initiation together with *Fgf10*. The expression of *Fgf8* in the AER of the fin suggests a similar role to that proposed for FGF8 factors in other vertebrate species. Expression of *Fgf8* in the AER may keep the underneath mesenchymal cells undifferentiated and proliferating and may probably act as a signal to activate the expression of other genes involved in fin patterning.

Fgf8 is also expressed in the most distal cells of the median fin fold. This expression supports the idea that at least in some aspects, the cells of the median fin fold correspond to the AER of paired fin buds and that *Fgf8* plays a similar role during the early stages of both paired and unpaired fins. The same observation has been previously reported for the members of the *msx* homeobox gene family. Each member, *msxA*, *msxB*, *msxC*, and *msxD* are expressed in very specific pattern, which are similar in the pectoral fin buds and the median fin fold (Akimenko et al., 1995).

Twenty four hours after the fin bud starts to develop (48 hours p.f.) an expression, not seen in other vertebrates, appears in the dorsal and ventral sides of the fin bud. Sections of the fin bud show that *Fgf8* is expressed in the newly forming muscle of the dorsal and ventral sides of the pectoral fin, suggesting that FGF8 can be one of the signals involved in migration and terminal differentiation of the limb muscle in zebrafish as it has been suggested in other vertebrate species (deLapeyriere et al., 1993; Itho et al., 1996). The muscles in the tetrapod limb and in the ventral body are formed from pre-muscle cells that migrate from the lateral half of the somites or myotomes. The pre-muscle cells migrate as undifferentiated cells which differentiate into muscle fibers when reaching the limb bud. The muscle cell differentiation is dependent on FGFR levels and not on the exogenously expressed ligand (Itoh et al., 1996). Indeed, cells overexpressing *FGFR1*, the predominant FGFR receptor in myogenic cell lineage (Sato et al., 1993), migrate into the limb muscle mass but remain as undifferentiated myoblasts. In contrast, cells infected with a dominant negative mutant form of *FGFR1* cannot migrate but differentiate into myotomal muscle within the somites. It is interesting to note that FGFR1 is known to interact with FGF8, which suggests that the presence of *Fgf8* transcripts in the dorsal and ventral ectoderm of the pectoral fin bud can be playing a role in the establishment of the striated muscle in the fin.

3. *Fgf8* expression during fin regeneration

In other species having regenerating capabilities, such as the newt and axolotl, the presence of different FGF and their receptors either in the blastema or in the epidermal cap has been reported. Another interesting observation that points out to the importance of FGF factors in regeneration is that they can rescue amputated limbs of animals without regenerating capability (Kostakopoulou et al.,1996). Beads loaded with FGF4 and implanted posteriorly or apically on chick limb bud stumps were sufficient to induce the regeneration of distal structures in the chicken wing. In this experiment, *Fgf4* also reactivates the expression of genes that are normally expressed during limb development as *Msx* and *Shh*.

Fgf8 expression in fin regeneration is correlated with the establishment of the blastema at the tip of each fin ray. This tissue shows a high proliferation rate (shown by the incorporation of BrdU in Chapter 1) suggesting that FGF8 may be acting as a signal in the proliferation of blastemal cells. This hypothesis is further supported by the correlation between the growth rate of the fin rays and the levels of *Fgf8* expression observed from the lateral to the medial rays (Figure 18B).

Han and Kim (1998), recently cloned the axolotl *Fgf8* cDNA and found similar patterns of expression during limb development and regeneration as those described here in the zebrafish fins. *Fgf8* expression was detected in the AER of developing limb buds and in the most distal part of the blastema, during limb regeneration. This result indicates that *Fgf8* may be playing similar roles in both fish and urodeles. It is interesting to note the change in tissue expression of *Fgf8* during embryonic development and regeneration. During embryonic development *Fgf8* is expressed in ectodermal cells, while during regeneration it is expressed in cells of the mesenchyme. Such change in expression domains has been observed for other genes involved in patterning. One of the most striking being *Shh*, which has been found expressed in many different organs in epithelial as well as in mesenchymal cells. For example, during fin development, *Shh* is expressed

in the posterior mesoderm of the paired fin bud and it is thought to play a role in the antero-posterior patterning of the fin. In contrast, during fin regeneration, *Shh* is expressed in epithelial cells (basal epidermal layer) close to the newly forming lepidotrichia suggesting its involvement in dermal bone formation (Akimenko et al., submitted). The change in domain suggests that these genes may have different functions within the tissue where they are expressed. However, the signalling pathways remain conserved since many transcription factors and signalling molecules are shared between the different processes.

V. CONCLUDING REMARKS

We have shown high homology between FGF8 proteins of different species and similarities in the expression pattern of the gene, which may indicate a conservation of the function of this signal in the developmental process of different tissues during vertebrate development.

So far, possible functions for the *Fgf8* gene during fin development and regeneration can only be allocated through comparison of its pattern of expression with that of other studied vertebrates.

To further establish the interactions of the *Fgf8* during fin regeneration and determine the interactions of *Fgf8* with other signaling molecules as well as transcriptional factors involved in this process, fuctional studies have to be done in this system. This is the current goal of our lab where, for example, FGF activity is blocked using sulphated polysaccharides. These polysaccharides seize FGF proteins preventing them from binding to the receptors. This will probably result in the decrease of tissue growth as a consequence of the decline in mitotic activity of blastema cells.

GENERAL CONCLUSIONS

Although we are still far from be able to induce regeneration of a missing limb or organ in higher vertebrates, studies in regeneration have great repercussion in the medical field.

Insights into the molecular bases of the cell cycle will provide fundamental knowledge in understanding with more detail the mechanisms that after an injury, trigger mature cells to restart the process of proliferation, patterning and differentiation. This will be of great relevance for cancer research where mutated cells reenter the cell cycle and proliferate indefinitely without differentiation.

At present this research in regeneration has been applied in the process of skin healing. Processes such as early wound closure, permanent skin replacement and management of postburn deformities are still unsolved problems in the treatment of burns. However, developments in biology and molecular biology provide new possibilities for improved therapy. In fact, the knowledge that factors such as FGF, IGF and EGF play fundamental roles during skin regeneration, has been used for the treatment of wound healing after burn.

The studies of genes involved in embryonic development have revealed important information that can be useful in the future in the design of new molecular approaches for the treatment of many developmental human defects. The mapping of the genes involved in tissue outgrowth and patterning in zebrafish will be very useful for the localization of mutations affecting fins which can be extrapolated to humans. The delineation of a conserved chromosome region, as shown here, has important implications for the understanding of genome evolution in vertebrate species and for the development of animal models of human genetic.

On the other hand, in evolutive terms, a better knowledge of the process of regeneration will probably give us the answer of why higher vertebrates possess restricted regenerative ability as adults. However recent works have shown that members of the

FGF family can induce regeneration in vertebrates with non regeneration capability suggesting their importance in this process and that the mechanism of regeneration is not completely lost.

REFERENCES

Aberg, T., Wozney, J., and Thesleff, I. (1997). Expression pattern of bone morphogenetic proteins (*Bmps*) in the developing mouse tooth suggest roles in morphogenesis and cell differentiation. *Dev. Dyn.* 210:383-396.

Albert P, Boilly B, Courty J, Barritault D. (1987). Stimulation in cell culture of mesenchymal cells of newt limb blastemas by EDGF I or II (basic or acidic FGF). *Cell Differ.* 21:63-68.

Altschul, S., Gish, W., Miller, W., Myers, E., and Lipman, D. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403-410.

Akimenko, M.-A., and Ekker, M. (1995). Anterior duplication of sonic hedgehog expression pattern in the pectoral fin buds of zebrafish treated with retinoic acid. *Dev. Biol.* 170, 243-247.

Akimenko, M.-A., Ekker, M., Wegner, J., Lin, W. and Westerfield, M. (1994). Combinatorial expression of three zebrafish genes related to distal-less: part of homeobox gene code for the head. *J. Neurosci.* 14, 3475-3486.

Akimenko, M.-A., Johnson, S. L., Westerfield, M., and Ekker, M. (1995). Differential induction of four *msx* homeobox genes during fin development and regeneration in zebrafish. *Development.* 121, 347-357.

Akiyama, S.K. (1996). Integrin in cell adhesion and signaling. *Human Cell.* 9:181-186.

Beauchemin, M., Noiseux, N., Tremblay, M., and Savard, P. (1994).Expression of Hox A11 in the limb and the regeneration blastema of adult newt. *Int J Dev Biol* 1994 38:641-649.

Beauchemin, M., And Savard, P. (1993). Expression of five homebox genes in the adult newt appendages and regeneration blastemas. In Limb development and Regeneration, eds. J.F. Fallon et al., New York: Wiley-Liss. pp 41-50.

Becerra, J., Junqueira, L. C. U., Bechara, I. J., and Montes, G. S. (1996). Regeneration of fin rays in teleosts: a histochemical, radioautographic, and ultrastructural study. *Arch.Histol.Cytol.* 59:15-35.

Belleville, S., Beauchemin, M., Tremblay, M., Noiseux, N., and Savard, P. (1992). Homebox-containing genes in the newt are organized in clusters similar to other vertebrates. *Gene.* 114:170-186.

Bellosta, P., Talarico, D., Rogers, D., and Basilico, C. (1993). Cleavage of K-FGF produces a truncated molecule with increase biological activity and receptor binding affinity. *J.Cell Biol.* 121:705-713.

Bernhard, Y. M., and Wagner, G. P. (1992). Regeneration in *Salaria pavo* (*Blenniidae, Teleostei*). *Anat. Embryol.* 186:153-165.

Blunt, A. G., Lawshe, A., Cunningham, M. L., Seto, M. L., Ornitz, D. M., and MacArthur, C. A. (1997). Overlapping expression and redundant activation of mesenchymal fibroblast growth factor (FGF) receptors by alternatively spliced ligands. *J.Biol. Chem.* 272:3733-3738.

Boilly, B., Cavanaugh, K. P., Thomas, D., Hondermarck, H., Bryant, S. V., and Bradshaw, R. A. (1991). Acidic fibroblast growth factor is present in regenerating limb blastema of axolotls and binds specifically to blastema tissues. *Dev. Biol.* 145:302-310.

Borday, V., Brulfert, A., and Géraudie, J. Hox gene induction in regenerating fins. Abstract presented at the 1998 meeting on zebrafish development and genetic. Cold Spring Harbor, New York. pp

Brockes, J. P. (1987).The nerve dependence of amphibian limb regeneration. *J.Exp. Biol.* 132:79-91.

Brockes, J. P. (1990). Retinoic acid and limb regeneration. *J.Cell Sci. Suppl.* 13:191-198.

Brockes, J. P. (1996). Retinoid signalling and retinoid receptors in amphibian limb regeneration. *Biochemical Society Symposia* 1996:137-142.

Brockes, J. P. (1997). Amphibian Limb Regeneration Rebuilding a Complex Structure. *Science.* 276: 81-87.

Brookes, S., Smith, R., Thurlow, J., Dickson, C., and Peters, G. (1989). The mouse homologue of *hst/* k-FGF: sequence, genome organization and location relative to int-2. *Proc. Natl. Acad. Sci. USA.* 86:5678-5682.

Brown, R., and Brockes, J. P. (1991). Identification and expression of a regeneration-specific homebox gene in the newt limb blastema. *Development.* 11:489-496.

Buck, C. A., and Horwitz, A. F. (1987). Integrin, a transmembrane glycoprotein complex mediating cell-substratum adhesion. *Cell. Sci. Suppl.* 8:231-250.

Blunt, A. G., Lawshé, A., Cunningham, M. L., Seto, M. L., Ornitz, D. M., and MacArthur, C. A. (1997). Overlapping expression and redundant activation of mesenchymal fibroblast growth factor (FGF) receptors by alternatively spliced FGF-8 ligands. *J.Biol.Chem.* 272:3733-3738.

Bhora, F. Y., Dunkin., B. J., Batzri, S., Aly, H. M., Bass, B. L., Sidawy, A. N., and Harmon, J. W. (1995). Effect of growth factors on cell proliferation and epithelialization in human skin. *J Surg Res.* 59:236-244

Chandross, R. J., and Bear, R. S. (1979). Comparison of mammalian collagen and elasmobranch elastoidin fiber structures, based on electron density profiles. *J.Mol.Biol.* 130:215-229.

Chang, B. E., Blader, P., Fischer, N., Ingham, P. W., and Strähle, U. (1997). Axial (HNF3β) and retinoic acid receptor are regulators of zebrafish *sonic hedgehog* promoter. *EMBO J.* 16:3955-3964.

Chen, Q., Johnson, D. M, Haudenschild, D. R, and Goetinck, P. F. (1995). Progression and recapitulation of the chondrocyte differentiation program: cartilage matrix protein is a marker for cartilage maturation. *Dev. Biol.* 172:293-306.

Christen, B., and Slack, J. M. W. (1997). FGF-8 Is Associated with Anteroposterior Patterning and Limb Regeneration in *Xenopus*. *Dev. Biol.* 192: 455-466.

Coelho, C. N. D., Krabbenhoft, K. M., Upholt, W. B., Fallon, J. F., and Kosher, R. A. (1991). Altered expression of the chicken homebox-containing genes Ghox-7 and Ghox-8 in the limb buds of limbless mutant chick embryos. *Development.* 113:1487-1493.

Cohn, M. J., and Tickle, C. (1996). Limbs: a model for pattern formation within the vertebrate body plan. *Trends in Genetics.* 12: 253-257.

Crawford, K., and Stocum, D. L. (1988). Retinoic acid proximalizes levels-specific properties responsible for intercalary regeneration in axolotl limbs. *Development.* 104:703-712.

Crews, L., Gates, P. B., Brown, R., Joliot A, Foley, c., Brockes, J. P., and Gann A. A. (1995). Expression and activity of the newt *Msx-1* gene in relation to limb regeneration. *Proc. R. Soc. Lond. B. Biol. Sci.* 259:161-171.

Crossley, P. H., and Martin, G. R. (1995). The mouse *Fgf8* gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. *Development.* 121:439-451.

Crossley, P. H., Martinez, S., and Martin, G. R.(1996a). Midbrain development induced by FGF8 in chick embryos. *Nature.* 7:66-668.

Crossley, P. H., Minowada, G., MacArthur, C. A., Martin, G. R. (1996b) Roles for FGF8 in the induction, initiation, and maintenance of chick limb development. *Cell.*84:127-136.

Davidson, D. R., Crawley, A., Hill, R. E., and Tickle, C. (1991). Position-dependent expression of two related homebox genes in developing vertebrate limbs. *Nature.* 352:429-431.

Davis, A. P., and Capecchi, M. R. (1994). Axial homeosis and appendicular skeleton defect in mice with a target disruption of Hox D11. *Development.*120:2187-2198.

Davis, A. P., Witte, D. P., Hsie-Li, H. M., Potter, S. S., and Capecchi, M. R. (1995). Absence of radius and ulna in mice lacking Hox A11 and Hox D11. *Nature.* 375:791-795.

Dealy, C. N. (1997). Hensen's node provides an endogenous limb-forming signal. *Dev.Biol.* 188:216-223.

Del Rio-Tsonis, K., Washabaugh, C. H., and Tsonis, P. A. (1992). The mutant axolotl Short toes exhibits impaired limb regeneration and abnormal basement membrane formation. *Proc. Natl. Acad. Sci.* USA 89:5502-5506.

Dinsmore, C. E., and Mescher, A. L. (1998). The role of the nervous system in regeneration. In "Cell and Molecular Biology of Regeneration" (P. Ferretti and J. Géraudie, Eds.), pp. 79-108. Wley, Chichester, UK.

Dionne, C. A., Crumley, G., Bellot, F., Kaplow, J. M., Searfoss, G., Ruta, M., Burgess, W. H., Jaye, M., and Schlessinger, J. (1990). Cloning and expression of two distinct high-affinity receptors cross-reacting with acidic and basic fibroblast growth factors. *EMBO J.* 9:2685-2692.

deLapeyriere, O., Ollendorff, V., Planche, J., Odile, O., Pizette, S., Coulier, F., and Birnbaum, D. (1993).Expression of the *Fgf6* gene is restricted to developing skeletal muscle in the mouse embryo. *Development.*118:601-611.

Dike, L. E., and Ingber, D. E. (1996). Integrin-dependent induction of early growth response genes in capillary endothelial cells. *J. Cell. Sci.* 109:2855-2863.

Dollé, P., Izpisua-Belmonte, J. C., Brown, J., Tickle C., and Duboule, D. (1993). Hox genes and the morphogenesis of the vertebrate limb. *Progress in Clinical & Biological Research.* 383:11-20.

Dollé, P., Izpisua-Belmonte, J. C., Tickle, C., Brown, J., and Duboule, D. (1989). Coordinate expression of the murine Hox-5 complex homeobox-containing gene during limb pattern of formation. *Nature.* 342:767-772.

Donaldson, D.J., and Dunlap, M.K. Epidermal cell migration during attempted closure of skin wounds in the adult newt: observations based on cytochalasin treatment and scanning electron microscopy. *J. Exp. Zoo.* 217:33-43.

Duboule, D. (1992). The vertebrate limb: A model system to study the HOX/HOM gene network during development and evolution. *BioEssays.* 14:375-384.

Duellman , W. E., and Trueb, L. (1986). "Biology of amphibians" McGraw-Hill, New York.

Dunkin, B. J., Batzri, S., Aly, H. M., Bass, B. L., Sidawy, A. N., and Harmon, J. W. (1995). Effect of growth factors on cell proliferation and epithelialization. Human skin *J. Surg. Research.* 59:236-244.

Driever, W., Stemple, D., Schier, A., and Solnica-Krezel, L. (1994). Zebrafish: genetic tools for studying vertebrate development. *Trends Genetics.* 10:152-159.

Dresden, M. H., and Gross, J. (1970). The collagenolytic enzyme of regenerating limb of the newt *Triturus viridescens. Dev. Biol.* 22:129-137.

Drucker, B. J., and Goldfarb, M. (1993). Murine FGF-4 gene expression is spatially restricted within embryonic skeletal muscle and other tissues. Mechanisms of *Developmen*t. 40:155-163.

Ekker, M., Speevak, M. D., Martin, C. C., Joly, L., Giroux, G., and Chevrette, M. (1996). Stable transfer of zebrafish chromosome segments into mouse cells. *Genomics.* 33:57-64.

Echelar, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A., and McMahon, A. P. (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell.* 75:1417-1430.

Faber, J. (1971). Vertebrate limb ontogeny and limb regeneration: morphogenetic parallels. *Adv. Morphog.* 9:127-147.

Fallon, J. F., Lopez, A., Ros, M. A., Savage, M. P., Olwin, B. B., and Simandl, B. K. (1994). FGF-2: apical ectodermal ridge growth signal for chick limb development. *Science.* 264, 104-107.

Ferretti , P., and Brockes, J. P. (1991). Cell origin and identity in limb regeneration and development. *Glia.* 4: 214-224.

Fuerthauer, M., Thisse, C., and Thisse, B. (1997). A role for FGF-8 in the dorsoventral patterning of the zebrafish gastrula. *Development.* 124:4253-4264.

Gardiner, D. M., Blumberg, B., and Bryant, S. V. (1993). Expression of homeobox genes in limb regeneration. In Limb Development and Regeneration, eds. J. F. Fallon et al., pp.31-40. New York: Wiley-Liss.

Gardiner, D. M., Blumberg, B., Komine, Y., Bryant, S. V. (1995). Regulation of *HoxA* expression in developing and regenerating axolotl limbs. *Development.* 121: 1731-1741.

Gemel, J., Gorry, M., Ehrlich, G. D., and MacArthur, C. A. (1996). Structure and sequence of human *Fgf8. Genomic.* 35:253-257.

Géraudie, J., and François, Y. (1973). Les premiers stades de la formation de l'ébauche de nageoire pelvienne de la truite (*Salmo fario* et *Salmo gairdneri). J. Embryol. Exp. Morphol,* 29:221-237.

Géraudie, J., and Singer, M. (1979). Nerve dependent macromolecular synthesis in the pectoral fin regenerate of the fish *Fundulus heteroclitus. J. Exp. Zool.* 208:281-286.

Géraudie, J. (1980). Mitotic activity in the pseudoapical ridge of the trout pelvic fin bud, *Salmo gairdneri. J. Exp. Zool.* 214:311-316.

Géraudie, J. (1983). Morphogenesis of the dermal skeleton in the teleosts fin. In: Limb Development and Regeneration. Part A. Liss, New York, pp.289-297.

Géraudie, J., and Singer, M. (1992). The fish fin regeneration. *Monogr. Devel. Biol.* 23:62-72.

Géraudie, J., Brulfert, A., Monnot, M. J., and Ferretti, P. (1994). Teratogenic and morphogenetic effects of retinoic acid on the regenerating pectoral fin in zebrafish. *J. Exp. Zool.* 269:12-22.

Géraudie, J., Monnot, M. J., Brulfert, A., and Ferretti, P. (1995). Caudal fin regeneration in wild type and long-fin mutant zebrafish is affected by retinoic acid.

Géraudie, J., and Ferretti, P. (1998). Gene expression during amphibian limb regeneration. *Int. Rev. Cytol.* 180:1-50

Gibbins, J. R. (1978). Epithelial migration in organ culture. A morphological and time lapse cinematographic analysis of migrating aquamous epithelium. *Pathology.* 10:207-218.

Goldfarb., M. (1990). The fibroblast growth factors family. *Cell Growth Differ.*1: 439-445.

Goss, R. J. (1969). Principles of regeneration. Academic Press, NewYork. London

Greenberg, G., and Hay, E. D. (1982). Epithelia suspended in collagen gels can lose polarity and express characteristics of migrating mesenchymal cells. *J. Cell Biol.* 95:333-

Griffin, K., Patient, R., and Holder, N. (1995). Analysis of FGF function in normal and no tail zebrafish embryos reveals separate mechanisms for formation of the trunk and tail. *Develoment.*121:2983-2994.

Grillo, H. L., Lapiere, C. M., Dresden, M. H., and Gross, J. (1968). Collagenolytic activity in regenerating forelimbs of the adult newt (*Triturus viridescens*). *Dev.Biol.* 17:571-583.

Grothe, C., Brand-Saberi, B., Wilting, J., and Chris, B. (1996). Fibroblast growth factor receptor 1 in skeletal and heart muscle cell: expression during early avian development and regulation after notochord transplantation. *Dev.Dyn.*206:310-307.

Gulati, A. K., Zalewski, A. A., and Reddi, A. (1983). An immunofluorescent study of the distribution of fibronectin and laminin during limb regeneration in the adult newt. *Dev.Biol.* 46:355-365.

Han, M. J., and Kim, W. S. Cloning and Expression pattern of *Fgf8* in Mexican Axolotl, *Ambystoma mexicanum.* (1998) 6th International Limb Development and Regeneration Conference. Sun Valley, Idaho.

Hatta, K., Bremiller, R., Westerfield, M., and Kimmel, C. B. (1991). Diversity of expression of engrailed-like antigens in zebrafish. *Development.* 112:821-832.

Hay, E. D., and Fishman, D. A. (1961). Origin of blastema in regenerating limbs of the newt *Triturus viridescens. Dev. Biol.* 3:26-59.

Hay, E. D. (1989). Extracellular matrix, cell skeletons, and embryonic development. *Am. J. Medical Genetics.* 34:14-29.

Imokawa, Y., and Yoshizato, K. (1997). Expression of Sonic hedgehog gene in regenerating newt limb blastemas recapitulates that in developing limb buds. *Proc. Natl. Acad. Sci. USA.* 94:9159-9164.

Ingber, D. E. (1997). Tensegrity: the architectural basis of cellular mechanotransduction. *Ann. Rev. Physiol.* 59:575-599.

Isaacs, H. V., Tannahill, D., and Slack, J. M. V. (1992). Expression of novel FGF in the Xenopus embryo. A new candidate inducing factor for mesoderm formation and anterioposterior specification. *Development.* 114:711-720.

Itoh, N., Mima, T., and Mikawa, T. (1996) Loss of fibroblast growth factor receptor is necessary for terminal differentiation of embryonic limb muscle. *Development.* 122:291-300.

Izpisua-Belmonte, J. C., Falkenstein, H., Dollé, P., Renucci, A., and Duboule, D. (1991). Murine gene related to the Drosophila *AbdB* homeotic are sequentially expressed during development. of the posterior part of the body. *EMBO J.* 10:2279-2289.

Izpisúa-Belmonte, J. C., and Duboule, D. (1992). Homeobox and pattern formation in the vertebrate limb. *Dev. Biol.* 152:26-36.

Jabaily, J., and Singer, M. (1977). Neurotrophic stimulation of DNA synthesis in the regenerating forelimb of the newt, *Triturus. J.Exp. Zool.* 199:251-256.

Jones, B. B., Ohno, C. K., Allenby, G., Boffa, M. B., Levin, A. A., Grippo, J. F., and Petkovich, M. (1995). New retinoid X receptor subtypes in zebrafish (*Danio rerio*) differentially modulate transcription and do not bind 9-*cis* retinoic acid. *Mol.Cell. Biol.* 15:5226-5234.

Kanazashi, S. I., Sharma, C. P., and Arnaout, M. A. (1997). Integrin-ligand interaction: scratching the surface. *Curr. Opin. Hematology.* 4:67-74.

Keegan, K., Johnson, D. E., Williams, L. T., and Hayman, M. J. (1991). Isolation of an additional member of the fibroblast receptor family, FGFR-3. *Proc. Natl. Acad. Sci.* USA. 88:1095-1099.

Kettunen, P., and Thesleff, I. (1998). Expression and functional of FGFs-4, -8, -9 suggest functional redundancy and repetitive use as epithelial signals during tooth morphogenesis. *Dev. Dyn.* 211:256-268.

Krauss , S., Concordet, J. P., and Ingham, P. W. (1993). A functionally conserved homolog of the Drosophila segment polarity gene *hh* is expressed in tissues with polarizing activity in Zebrafish embryos. *Cell.* 75:1431-1444.

Kuhlman, J., and Niswander, L. (1997). Limb deformity proteins: role in mesodermal induction of the apical ectodermal ridge. *Development.* 124:133-139

Laufer, E., Nelson, C. E., Johnson, R. I., Morgan, B. A., and Tabin, C. (1994). Sonic hedgehog and *Fgf-4* act through a signalling cascade and feedback loop to integrate growth and patterning of the developing limb bud. *Cell.* 79:993-1003.

Leliévre, S., Weaver, V. M., and Bisell, M. J. (1996). Extracellular matrix signaling from the cellular membrane skeleton to the nuclear skeleton: a model of gene regulation. *Recent. Progress. Hormon. Research.* 51:417-432.

Lewis, E. B. (1978). A gene complex controlling segmentation in *Drosophila. Nature.* 276:565-571.

Liversage, R. A. (1991). Origin of the blastema cells in epimorphic regeneration of urodele appendages: A history of ideas. In "A History of regeneration research" (C.E. Dinsmore, Ed.), Cambridge Univ. Press, Cambridge, UK. pp. 179-199.

Lo, D. C., Allen, F., and Brockes, J. P. (1993). Reversal of muscle differentiation during urodele limb regeneration. *Proc. Natl. Acad. Sci. USA.* 90(15): 7230-7234

Loomis, C. A., Harris, E., Michaud, J., Wurst, W., Hanks, M., and Joyner A. L. (1996). The mouse Engrailed-1 gene and ventral limb patterning. *Nature*. 382:360-3.

Lutzow-Holm, C., De Angelis, P., and Clausen, O. P. (1995). Topical application of retinoic acid induces murine epidermal proliferation without reducing the cell cycle time. A bivariate BrdUrd/DNA flow cytometric epithelial. *Cell Biology*. 4:2-7

MacCabe, J. A., Errick, J., and Saunders, J. W. (1974). Ectodermal control of the dorsoventral axis in the leg bud of the chick embryo. *Dev.Biol.* 39:69-82.

Maier, C. E., Watanabe, M., Singer, M., McQuarrie, I. G., Sunshine, J., and Rutishauser, U. (1986). Expression and function of neural cell adhesion molecule during limb regeneration. *Proc. Natl. Acad. Sci. USA*. 83:8395-8399.

Marí-Beffa, M., Carmona, M. C., and Becerra, J. (1989). Elastoidin turn-over during tail fin regeneration in teleosts. A morphometric and radioautographic study. *Anat. Embryol.* 180:465-470.

Marí-Beffa, M., Santamaría, J. A., Fernández-Llebrez, P., and Becerra, J. (1996). Histochemically defined cell states during tail fin regeneration in teleost fishes. *Differentiation*. 60:139-149.

Marcelle, C., Wolf, J., and Bronner-Fraser, M. (1995).The in vivo expression of FGF receptor FREK mRNA in avian myoblast suggests a role in muscle growth and differentiation. *Dev. Biol.* 172:100-114.

Matsumoto, K., Ziober, B. L., Yao, C. C., and Kramer, R. H. (1995). Growth factor regulation of integrin-mediated cell motility. *Cancer and Metastasis review*. 14:205-217.

McGinnis, W., and Krumlauf, R. (1992). Homeobox genes and axial patterning. *Cell*. 68:283-302.

McWhirter, J. R., Goulding, M., Weiner, J. A., Chun, J., and Murre, C. (1997) A novel fibroblast growth factor gene expressed in the developing nervous system is a downstream target of the chimeric homeodomain oncoprotein E2A-Pbx1. *Development*.124:3221-3232.

Means, A. L., and Gudas, L. J. (1995). The role of retinoids in vertebrate development. *Annu. Rev. Biochem*. 64:201-233.

Mescher, A. L. (1976). Effect on adult newt limb regeneration of partial and complete skin flaps over the amputation surface. *J. Exp. Zool.* 195:117-128.

Mescher, A. L and Munaim, S. I. (1986). Changes in the extracellular and glycosaminoglycans synthesis during the initiation of regeneration in adult newt forelimbs. *Anat. Rec.* 214: 424-431.

Meredith, J. Jr., Takada, Y., Fornaro, M., Languino, L. R., and Schwartz, M. A. (1995). Inhibition of cell cycle progression by alternatively spliced integrin beta1c. *Science.* 269:1570-1572.

Misof, B. Y., and Wagner, G. P. (1992) Regeneration in *Salaria pavo* (*Blenniidae, Teleostei*). *Anat. Embryol.* 186:153-165.

Miyagawa, K., Sakamoto, H., Yoshida, T., Yamashita, Y., Mitsui, Y., Furusawa, M., Maeda, S., Takaku, F., Sugimura, T., and Terada, M. (1988). hst-1 transforming protein: expression in silkworm cells and characterization as a novel heparin-binding growth factor. *Oncogene.* 3:383-389.

Miyazaki, K., Uchiyama, K., Imokawa, Y., and Yoshizato, K. (1996). Cloning and characterization of cDNAs for matrix metaloproteinase of regenerating newt limbs. *Proc. Natl. Acad. Sci. USA.* 93:6819-6824.

Montes, G. S., Becerra, J., Toledo, O. M., and Gordilho, M. A. (1982). Junqueira LC. Fine structure and histochemistry of the tail fin ray in teleosts. *Histochemistry.* 75:363-376.

Mooney, D. J., Langer, R., and Ingber, D. E. (1995). Cytoskeletal filament assembly and the control of cell spreading and function by extracellular matrix. *J. Cell. Sci.* 108:2311-2320.

Morgan, B. A., Izpisúa-Belmonte, J. C., Duboule, D., and Tabin, C. J. (1992). Targeted misexpression of hox-4.6 in the avian limb bud cause apparent homeotic transformations. *Nature.* 358:236-239.

Mullins, M. C., and Nüsslein-Volhard, C. (1993). Mutational approaches to studying embryonic pattern formation in zebrafish. *Curr. Opin. Genet. Dev.* 3:648-654.

Mullen, L. M., Bryant, S. V., Torok, M. A., Blumberg, B., and Gardiner, D. M. (1996). Nerve dependency of regeneration: the role of *Distal-less* and FGF signaling in amphibian limb regeneration. *Development.* 122:3487-3497.

Muneoka, K., Fox, W., and Bryant, S. V. (1986). Cellular contribution from dermis and cartilage to the regenerating limb blastema in axolotl. *Dev. Biol.* 116:256-260.

Narahara, K., Baker, E., Ito, S., Yokoyama, Y., Yu, S., Hewitt, D., Sutherland, G. R., Eccles, M. R., and Richards, R. I. (1997). Localisation of a 10q breakpoint within the PAX2 gene in a patient with a de novo t(10;13) translocation and optic nerve coloboma-renal disease. *J. Medical Genetics*. 34:213-216.

Niazi, I. A., and Saxena, S. (1978). Abnormal hindlimb regeneration in tadpoles of the toad, *Bufo anderosin*, exposed to excess vitamin A. *Folia Biol.* 26:3-11.

Nicolas, S., Massacrier, A., Caubit, X., Cau, P., and Le Parco, Y. (1996). A distal – less-like gene is induced in the regenerationg central nervous system of the urodele Pleurodeles waltl. *Mech. Dev.* 56:209-220.

Neubuser, A., Peters, H., Balling, R., and Martin, G. R. (1997). Antagonistic interactions between FGF and BMP signaling pathways: a mechanism for positioning the sites of tooth formation. *Cell.* 25:247-255.

Niswander, L., Cheryll, T., Vogel, A., Booth, I., and Martin, G. R. (1993). FGF-4 replaces the apical ectoderm ridge and direct outgrowth and patterning of the limb. *Cell.* 75:579-587.

Niswander, L., and Martin, G. R. (1993). FGF-4 regulates expression of *Evx-1* in the developing mouse limb. *Development.* 119:287-294.

Niswander, L., Tickle, C., Vogel, A., Booth, I., and Martin, G. R. (1993). FGF-4 replaces the apical ectodermal ridge and directs outgrowth and patterning of the limb. *Cell.* 75, 579-587.

Niswander, L., Jeffrey, S., Martin, G. R., and Tickle, C. (1994). A positive feedback loop in signalling in the vertebrate limb development. *Nature.* 371, 609-612.

Nusse, R., and Varmus, H. E. (1992). *Wnt* genes. *Cell.* 69:1073-1087.

Onda, H., Poulin, M. L., Tassava, R. A., and Chiu, I. M. (1991). Characterization of a newt tenascin cDNA and localization of tenascin mRNA during newt limb regeneration by *in situ* hybridization. *Dev. Biol.* 148:219-232.

Ohuchi, H., Nakagawa ,T., Yamamoto, A., Araga, A., Ohata, T., Ishimaru, Y., Yoshioka, H., Kuwana, T., Nohno, T., Yamasaki, M., Itoh, N., and Noji, S. (1997). The mesenchymal factor, FGF10, initiates and maintains the outgrowth of the chick limb bud through interaction with FGF8, an apical ectodermal factor. *Development.* 124:2235-2244.

Pautou, M. P. (1977). Dorso-ventral axis determination of chick limb bud development. In Vertebrate Limb and Somite Morphogenesis, D.A. Ede, J.R. Hinchliffe, and M. Balls, eds. (Cambridge, England: Cambridge University Press), pp. 257-266.

Partanen, J., Makela, T. P., Eerola, E., Korhonen, J., Hirvonen, H., Claesson-Welsh, L., and Alitalo, K. (1991). FGFR-4, anovel acidic fibroblast growth factor receptor with a distinct expression pattern. *EMBO J.* 10:1347-1354.

Parr, B. A., Shea, M.J ., Vassileva, G., and McMahon, A. P. (1993). Mouse Wnt genes exhibit discrete domains of expression in the early embryonic CNS and limb buds. *Development.* 119:247-261.

Parr, B. A., and McMahon, A. P. (1995). Dorsalizing signal Wnt-7a required for normal polarity of the D-V and A-P axes of mouse limb. *Nature.* 374:350-353.

Pascolini, R., Tei, S., and Bondi, C. (1984). Epidermal cell migration during wound healing in Dugesia lugrubis. Observation based on scanning electron microscopy and treatment with cytochalasin. *Cell Tissue.* Res. 236:345-329.

Patapoutian, A., Yoon, J. K., Miner, J. H., Wang, S., Stark, K., and Wold, B. (1995). Disruption of mouse MRF4 gene identifies multiple waves of myogenesis in myotome. *Development.* 121:3347-3358.

Pecorino, L. T., Lo, D. C., and Brockes, J. P. (1994). Isoform-specific induction of a retinoid-responsive antigen after biolistic transfection of chimaeric retinoic acid/thyroid hormone receptors into a regenerating limb. *Development.* 120:325-333

Pecorino, L. T., Brockes., J. P., and Entwistle, A. (1996a). Semi-automated positional analysis using laser scanning microscopy of cells transfected in a regenerating newt limb. *J. Histochem. Cytochem.* 44:559-569.

Pecorino, L. T., Entwistle, A., and Brockes, J. P. (1996b). Activation of a single retinoic acid receptor isoform mediates proximodistal respecification. *Curr. Biol.* 6:563-569.

Pescitelli, M. J. Jr., and Stocum, D. L. (1980). The origin of skeletal structures during intercalary regeneration of larval *Ambystoma* Limbs. *Dev Biol.* 79:255-275.

Poulin, M. L., Patrie, K. M., Bothelho, M. J., Tassava, R. A., and Chiu, I. M. (1993). Heterogeneity in the expression of fibroblast growth factor receptor during limb regeneration in newts (*Notophthalmus viridescens*). *Development.* 119:353-361.

Poulin, M. L., and Chiu, I. M. (1995). Re-programming of expression of the KGFR and bek variants of fibroblast growth factor receptors 2 during limb regeneration in newts (*Notophthalmus viridescens*). *Dev. Biol.* 202:378-387.

Raas-Rothschild, A., Manouvrier, S., Gonzales, M., Farriaux, J. P., Lyonnet, S., and Munnich, A. (1996). Refined mapping of a gene for split hand-split foot malformation (SHFM3) on chromosome 10q25. *J. Medical Genetics.* 33:996-1001.

Ragsdale, C. W., Petkovich, M., Gates, P. B., Chambon, P., and Brockes, J. P. (1989) Identification of a novel retinoic acid receptor in regenerative tissues of the newt. *Nature* 341:654-657

Ragsdale, C. W., Gates, P. B., and Brockes, J. P. (1992). Identification and expression pattern of a second isoform of the newt alpha retinoic acid receptor. *Nucleic Acids Res.* 20:5851

Repesh, L. A., and Oberpriller, J. C. (1980). Ultrastructural studies on migrating epidermal cells during the wound healing stage of regeneration in the adult newt, *Notophthalmus viridescens. Am. J. Anat.* 159:187-208.

Riddle, R. D., Johnson, R. L., Laufer, E., and Tabin, C. (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell.* 75, 1401-1416.

Riddle, R. D., Ensini, M., Nelson, C., Tsuchida, T., Jessell, T. M., and Tabin C. (1995). Induction of the LIM homeobox gene Lmx1 by WNT7a establishes dorsoventral pattern in the vertebrate limb. *Cell.* 83:631-40.

Robert, B., Lyons, G., Simandl, B. K., Kuroiwa, A., and Buckingham, M. (1991). The apical ectodermal ridge regulates *Hox-7* and *Hox-8* gene expression in developing limb bud. *Genes Dev.* 5:2363:2374.

Ros, M. A., Lyons, G., Kosher, R. A., Upholt, W. B., Coelho, C. N. D., and Fallon, J. F. (1992). Apical ridge dependent and independent mesodermal domains of Ghox-8 expression in chick limb buds. *Development.* 116:811-818.

Roskelley, C. D., Srebrow, A., and Bissell, M. J. (1995). A hierarchy of ECM-mediated signalling regulates tissue-specific gene expression. *Current. Op. in Cell. Biol.* 7:736-747.

Rudzki, Z., and Jothy, S. (1997). X82p4 and the adhesion of neoplastic cells. *Molecular Pathology.* 50:57-71.

Salpeter, M., and Singer, M. (1960). The fine structure of mesenchymatous cells in the regenerating forelimb of adult newt *Triturus. Dev. Biol.* 2:516-534.

Santamaría, J. A., and Becerra, J. (1991). Tail fin regeneration in teleosts: cell-extracellular matrix interaction in blastemal differentiation. *J. Anat.* 176: 9-21.

Santamaría, J. A., Marí-Beffa, M., and Becerra, J. (1992). Interactions of the lepidotrichia! matrix components during tail fin regeneration in teleosts. *Differentiation.* 49:143-50

Santamaría, J. A., Marí-Beffa, M., Santos-Ruiz, L., and Becerra, J. (1996a). Incorporation of Bromodeoxyuridine in Regenerating Fin Tissue of the Goldfish *Carassius auratus. J. Exp. Zool.* **275**, 300-307.

Santamaría, J. A., Santos-Ruiz, L., Becerra, J. (1996b). An antiserum against ceratotrichia (selachian) recognizes actinotrichia in teleost regenerating fins. *Int J Dev Biol.* 1:175-176.

Santos Ruiz., L., Santamaria, J. A., and Becerra, J. (1996). Cell proliferation in fish regeneration. *Int. J. Dev. Biol.* 1:183-184.

Sato, B., Kouhara, H., Koga, M., Kasayama, S., Saito, H., Sumitami, S., Hashimoto, K., Kishimoto, T., Tanaka, A., and Matsumoto, K. (1993). Androgen-induced growth factor and its receptor: demonstration of the androgen-induced autocrine loop in mouse mammary carcinoma cells. *J.Steroid Biochem. Molec. Biol.* 47:91-98.

Scadding, S. R., and Maden, M. (1994). Retinoic acid gradients during limb regeneration. *Dev.Biol.* 162:608-617.

Schilthuis, J. G., Gann, A. A., and Brockes, J. P. (1993). Chimeric retretinoic acid/thyroid hormone receptors implicate RAR-alpha 1 as mediating growth inhibition by retinoic acid. *EMBO J.* 12:3459-3466.

Schiltz, J. R. (1979). Collagen synthesis during the cell cycle of chick embryo chondroblast population. *Cell Differentition.* 8:83-91.

Schmidt, A. J. (1968)."Cellular Biology of Vertebrate Regeneration and Repair". pp.420.Univ.of Chicago Press, Chicago.

Seto, M. L., Nunes, M. E., MacArthur, C. A., and Cunningham, M. L. (1997). Pathogenesis of ectrodactyly in the Dactylaplasia mouse: aberrant cell death of the apical ectodermal ridge. *Teratology.* 56:262-270.

Singer, M., and Craven, L. (1948). The growth and morphogenesis of the regenerating forelimb of adult *Triturus* following denervation at various stages of development. *J.Exp.Zool.* 108:279-308.

Singer, M., Maier, C. E., and McNutt, W. S. (1976). Neurotrophic activities of brains extracts in forelimb regeneration of urodele, *Triturus. J.Exp.Zool.* 196:131-150.

Simon, H. G., and Tabin, C. J. (1993). Analysis of Hox-4.5 and Hox-3.6 expression uring newt limb regeneration: differential regulation of paralogous Hox genes suggest different roles for members of different Hox clusters. *Development.* 117:1397-1407

Slack, J. M., Isaacs, H. V., Song, J., Durbin, L., and Pownall, M. E. (1996). The role of fibroblast growth factors in early *Xenopus* development. Biochemical Society Symposia. 62:1-12.

Small, K. M., and Potter, S. S. (1993). Homeotic transformations and limb defect in *HoxA11* mutant mice. *Gene Dev.* 7:2318-2328.

Smith, G. N., Toole, B. P., and Gross, J. (1975). Hyaluronidase activity and glycosaminoglycan synthesis in the amputated newt limb: Comparison of denervated, nonregenerating limbs with regenerates. *Dev. Biol.* 43:221-232.

Song, K., Wang, Y., And Sassoon, D. (1992). Expression *Hox-7.1* in myoblast inhibits terminal differentiation and induce cell transformation. *Nature.* 360:477-481.

Sordino, P., van der Hoeven, F., and Duboule, D. (1995). Hox gene expression in teleost fins and the origin of vertebrate digits. *Nature.* 375: 678-681.

Steen, T. P. (1968). Stability of chondrocyte differentiation and contribution of muscle to cartilage during limb regeneration in the axolotl (*Siredon mexicanum*). *J.Exp. Zoo.* 167(1), 49,78

Steen, T. P. (1970). Origin and differentiative capacities of cells in the blastema of the regenerating salamander limb. *American Zoologist* 10:119-32.

Stephens, T. D., Spall, R., Baker, W. C., Hiatt, S. R., Pugmire, D. E., Shaker, M. R., Willis, H. J., and Winger, K. P. (1991). Axial and paraxial influences on limb morphogenesis. *J. Morphol.* 208:367-379.

Tkei, T., Rivas-Gotz, C., Delling, C.A., Koo, J.T., McCarthy, T.L., Centrella, M., Sumpio, B.E. (1997). Effect of strain on human keratinocytes in vitro. *J.Cell. Physiol.* 173:64-72.

Takenaka, H., Kishimoto, S., Tooyama, I., Kimura, H., and Yasuno, H. (1997). Protein expression of fibroblast growth factor receptor-1 in keratinocytes during wound healing in rat skin. *J. Invest Dermatol.* 109:108-112.

Tassava, R. A., and Mescher, A. L. (1975). The role of injury, nerve and the wound epidermis during the initiation of amphibian limb regeneration. *Differentiation.* 4:23-24.

Tassava, R. A., and Lloyd, R. M. (1977). Injury requirement for initiation of regeneration of newt limbs which have whole skin grafts. *Nature.* 268:49-50.

Templenton, T. J., and Hauschka, S. D. (1992). FGF-mediated aspects of skeletal muscle growth and differentiation are controlled by high affinity receptor, FGFR1. *Dev.Biol.*154:169-181.

Thisse, B., Thisse, C., and Weston, J. A. (1995). Novel FGF Receptor (Z-FGFR4) Is dynamically expressed in mesoderm and neurectoderm during early zebrafish embryogenesis. *Dev.Dyn.*203:377-391.

Thoms, S. D., and Stocum, D. L. (1984). Retinoic acid-induced pattern duplication in regenerating urodele limbs. *Dev.Biol.* 103:319-328.

Thornton, C. S. (1957). The effects of apical cap removal on limb regeneration in *Ambystoma* larvae. *J. Exp. Zool.* 134:357:-382.

Thornton, C. S. (1970). Amphibian limb regeneration and its relation to nerves. *Am. Zool.* 10:113-118.

Tickle, C., Summerbell, D., and Wolpert, L. (1975). Positional signaling and specification of digits in chick limb morphogenesis. *Nature.* 254, 199-202.

Toole, B. P., Goldberg, R. L., Chi-Rosso, G., Underhill, C. B., and Orkin, R. W. (1984). Hyluronate cell-interaction. In "The role of Extracellular Matrix in Development" (R.L. Trelstad, Ed.), A.R. Liss, New York.

Tsonis, P. A., and Eguchi, G. (1983). Effect of carcinogens *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine on blastema formation in newt limb regeneration. *Dev. Growth Diffe.* 25:201-210.

Tsonis, P. A. (1996). Limb Regeneration, Developmental and Cell Biology Series.Barlow, P.W., Bard, J.B.L., Green, P.B., and Kirk, D.L.Cambridge University Press, pp 23-26.

Tsonis, P. A., Doane, K., and Del Rio-Tsonis K. (1997). Expression of integrins during axolotl limb regeneration. *Development Growth and Differentiation.* 39:9-14.

Viviano, C. M., Horton, C. E., Maden, M., and Brockes, J. P. (1995). Synthesis and release of *9-cis* retinoic acid by the urodele wound epidermis. *Development.* 121:3753-3762.

Vogel, A., Rodriguez, C., Warnken, W., and Izpisúa-Belmonte, J. C. (1995). Dorsal cell fate specified by chick Lmx1 during vertebrate limb development. *Nature.* 378:716-720.

Vogel, A., Roberts-Clarke, D., and Niswander, L. (1995). Effect of FGF on gene expression in chick limb bud cells in vivo and in vitro. *Dev. Biol.* 171:507-520.

Vogel, A., Rodriguez, C., and Izpisúa-Belmonte, J. C. (1996). Involvement of FGF-8 initiation, outgrowth and patterning of the vertebrate limb. *Development.* 122:1737-1750.

Wagner, G. P., and Misof, B. Y. (1992). Evolutionary modification of regenerative capability in vertebrates: a comparative study on teleost pectoral fin regeneration. *J Exp Zool.* 261:62-78.

Wallace, H. (1981). "Vertebrate Limb Regeneration" Wiley, Chichester, UK.

Westerfield, M. (1995). The Zebrafish Book. Eugene: University of Oregon Press.

White, J. A., Boffa, M. B., and Petkovich, M. (1994). A zebrafish retinoic acid receptor expressed in the regenerating caudal fin. *Development.* 120:1861-1872.

White, R. A., Dowler, L. L., Angeloni, S. V., Pasztor, L. M., and MacArthur, C. A. (1995). Assignment of FGF8 to human chromosome 10q25-q26: mutations in FGF8 may be responsible for some types of acrocephalosyndactyly linked to this region. *Genomics.* 30:109-111.

Wood, A. (1982). Early pectoral fin development and morphogenesis of the apical ectodermal ridge in the killifish, *Aphysosemion scheeli. Anat. Rec.* 204, 349-356.

Wood, A., and Thorogood, P. (1984). An analysis of in vivo cell migration during teleost fin morphogenesis. *J. Cell Sci.* 66:205-22.

Xu, X., Weinstein, C. L., Naski, M., Cohen, R. I., Ornitz, D. M., Leder, P., and Deng, C. (1998). Fibroblast growth factor receptor 2 (FGFR2)-mediated reciprocal regulation loop between FGF8 and FGF10 is essential for limb induction. *Development.* 125:753-765.

Yang, E. V., and Bryant, S. V. (1994). Developmental regulation of a matrix metalloproteinase during regeneration of axolotl and newt limb stump but is absent from the undifferentiated regenerate. *J. Exp. Zool.* 264:337-350.

Yingzin, Y., and Niswander, L. (1995). Interaction between the signaling molecules WNT7a and SHH during vertebrate limb development: Dorsal signals regulate anteroposterior patterning. *Cell.* 80:939-947.

Yokouchi, Y., Nakazato, S., Yamamoto, M., Goto,Y., Kameda, T., Iba, H., and Kuroiwa, A. (1995). Misexpression of Hoxa-13 induces cartilage homeotic transformation and changes cell adhesiveness in chick limb bud. *Genes Dev.* 9:2509-2522.

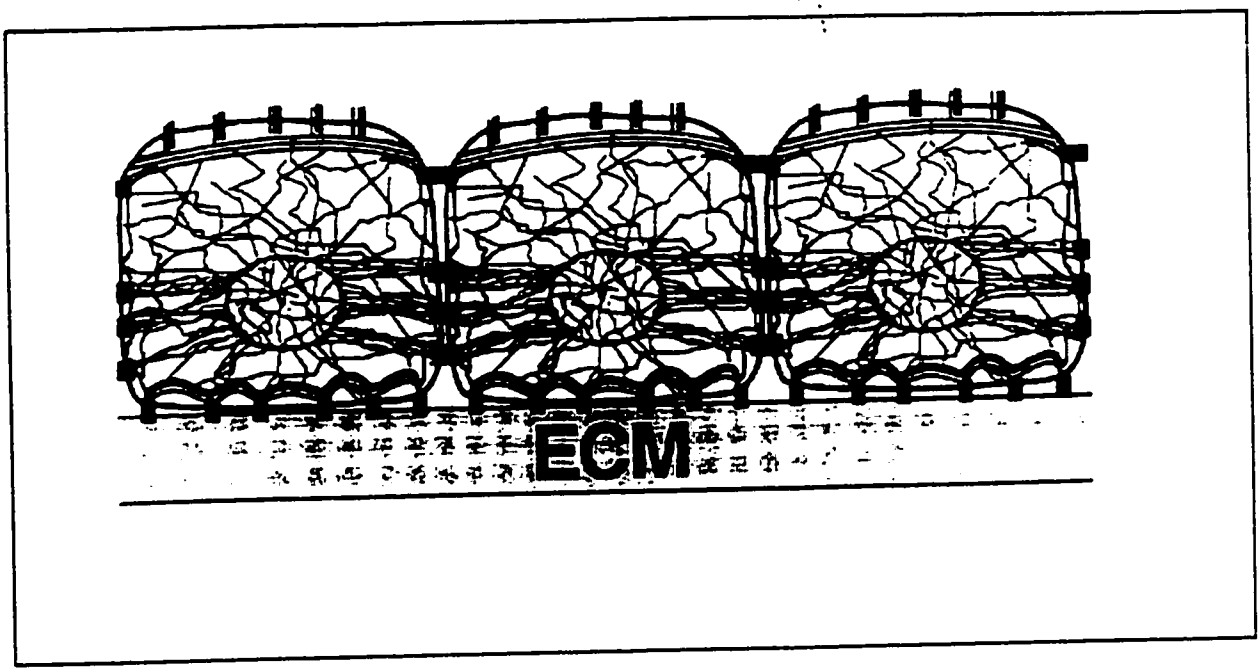
Yoshiura, K., Leysens, N. J., Chang, J., Ward, D., Murray, J. C., and Muenke, M. (1997). Genomic structure, sequence, and mapping of human *Fgf8* with no evidence for its role in craniosynostosis/limb defect syndromes. *Am. J. Med. Genet.* 72:354-362.

Zenjari, C., Boilly-Marer, Y., Desbiens, X., Oudghir, M., Hondermarck, H., and Boilly, B. (1996). Experimental evidence for FGF-1 control of blastema cell proliferation during limb regeneration of the amphibian *Pleurodeles waltl. Int.J.Dev.Biol.* 40:965-971.

which leads to different responses. For example, integrin $\alpha 5\beta 1$ binds fibronectin, whereas $\alpha 2\beta 1$ binds collagen.

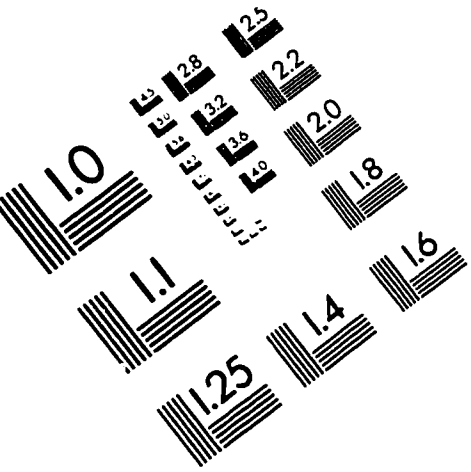
Several examples of signaling by mechanical stresses have been reported. Changes in the cytoskeleton organization modulate Na^+ channels in the epithelia (Cantiello, 1995). Phosphorilation of the focal adhesion kinase (FAK), protein-tyrosine kinase, is implicated in controlling cellular response and changes in cytoplasmic pH and calcium ion concentration can regulate a vast number of cellular functions (reviewed by Akiyama, 1996).

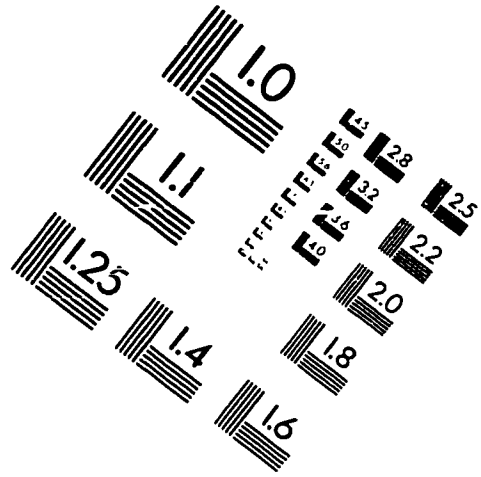
Another family of transmembrane glycoproteins involved in ECM binding are the CD44 (Rudzki and Jothy, 1997). The CD44 are receptors for hyaluronan but also can bind others ECM as chondroitin sulphate, heparan sulphate, fibronectin, serglycin and osteopontin. The interaction of the CD44 protein with the ECM influence cell motility.
"The integrin receptors that bind specific extracellular adhesion molecules, and growth factors that bind their respective ligands can regulate cell locomotion. Cross talk between integrin and growth factor receptors occurs through their common intracellular signaling pathways. In this way, each receptor can either amplify or attenuate the other's signal and down stream response" (Matsumoto et al., 1995).

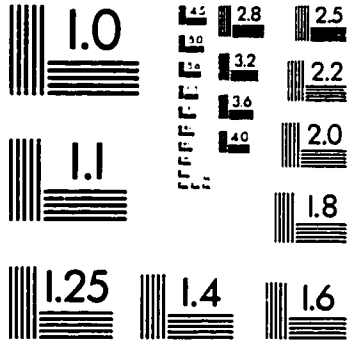


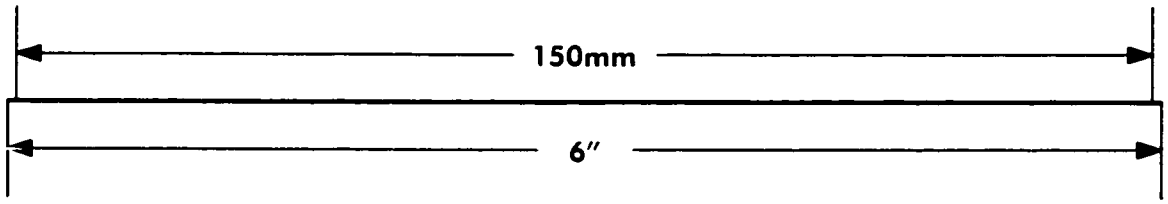
Appendix Figure. The intracellular cytoskeleton interconnects with the underlying extracellular matrix and neighboring cells through focal adhesion complexes at the cell base and specialized junctional complexes at the lateral cell border, respectively. Because of the presence of this molecular continuum, distant molecules in the extracellular matrix, cytoplasm, and nucleus may be mechanically coupled (from Ingber, 1997).

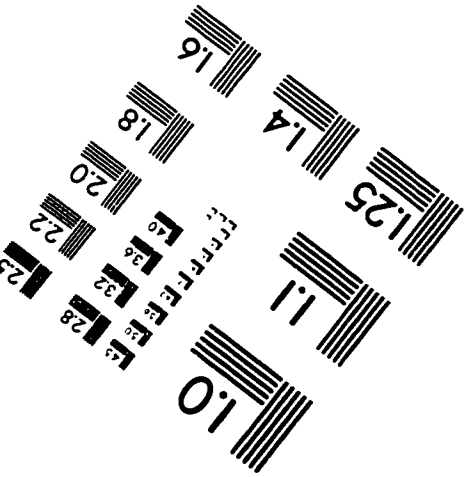
IMAGE EVALUATION
TEST TARGET (QA-3)

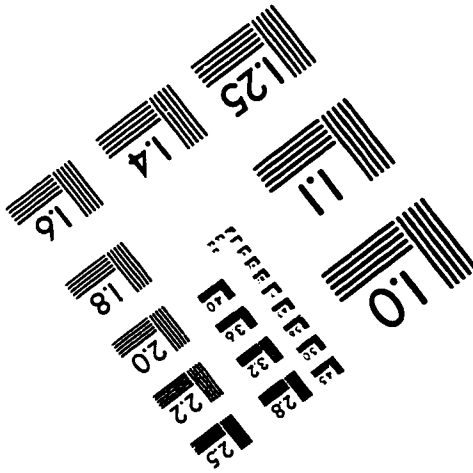












APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989