CELLULAR AND MOLECULAR MECHANISMS OF EPIMORPHIC REGENERATION IN TELEOST FISH: ROLE OF FIBROBLAST GROWTH FACTOR 2

Annasamudram Saradamba

DEPARTMENT OF ZOOLOGY, FACULTY OF SCIENCE

THE MAHARAJA SAYAJIRAO UNIVERSITY OF BARODA

VADODARA – 390 002, INDIA

Ph.D. THESIS **OCTOBER 2012**

A THESIS SUBMITTED TO

THE MAHARAJA SAYAJIRAO UNIVERSITY OF BARODA

BARODA

FOR THE AWARD OF DOCTOR OF PHILOSOPHY

IN

ZOOLOGY

The Maharaja Sayajirao University of Baroda

DEPARTMENT OF ZOOLOGY
FACULTY OF SCIENCE
VADODARA 390 002, INDIA

CERTIFICATE

This is to certify that the thesis "**Cellular and molecular mechanisms of epimorphic regeneration in teleost fish: Role of Fibroblast Growth Factor 2**" incorporate the results of investigation carried out by the candidate herself and analyzed in the Department of Zoology, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara.

Candidate
(Annasamudram Saradamba)

Guiding Teacher
(Dr. B. Suresh)

CONTENTS

ACKNOWLEDGEMENTS

This section is going to be long, I warn.

There is no other way to start this section than to thank the most important person in the development of this thesis work, my Guide, ***Dr. B. Suresh****. He has been my thesis director for a bit more than four years now but he has been my teacher for more than 9 years starting from the first day I stepped into this department; and I have learnt a lot from him, both actively by discussing and passively by just watching him work. I had a great experience working with him and thank him for providing the best guidance and support that helped in both academic as well as my personal development. He is not only one of the most privileged minds I have met so far in the scientific field but his talent on resolving any kind of problems has always kept influencing me. His support, extensive knowledge, patience and good advice assisted me throughout my work. He has taught me everything in the lab. I am truly grateful to you Sir! I can write several other sentences about how beneficial has been his influence in my still short scientific career but he would probably suggest me to cut this off, so I will move on.*

Very special thanks to ***Dr. Isha Desai****... Mam you've been the best academic advisor and a friend. Your cheerful personality has always attracted me and your knowledge about the subject has always exerted a pull on me to work hard. The way you manage things despite your busy schedule have made me learn a lot. I always felt a positive energy talking to you. Thank you for being there these years.*

I owe thanks to my other teachers of the department, ***Dr. Dolly Kumar*** *and* ***Dr. Pragna Parikh****. This journey would not have been so pleasant without the support of teachers like u. Thank you Mam for your affection and love.*

I am grateful to the ***Department of Science and Technology (DST)*** *for the financial funding in the form of fellowship (JRF/SRF).*

My sincere thanks to ***The Head, Department of Zoology*** *for providing me with all the necessary facilities to carry out my research work.*

My warmest acknowledgements are also to the other teachers of the department for their very professional, but joyful companionship.

To my Labmates: ***Pratyush Patankar***, ***Gowri Uggini***, ***Hetal Roy***, ***Shailja Rajaram***, ***Anushree Pillai***, ***Ayaz Rangrez***, ***Tushar Panot***, ***Ruchika Kataria***, ***Bhavika Desai***, ***Shalaka Shadekarpawar***, ***Pranav Buch and Hiral Murawala***, *thank you for the good times that may never turn back again. Exceptional thanks to* ***Shailja***, *for the n number of coffees and chats we had together when get worn-out of the lab work. You have been a very good friend and I will always cherish the time spend with you.*

Very heartfelt and honest thanks to ***Hiral*** *and* ***Pranav*** *for helping me with the gels and aid me wind up with this thesis work. Your hardworking nature with smiling faces has always been making the lab a great environment to work in.* ***Hiral*** *you have been a very sweet friend and* ***Pranav*** *you are an all-rounder! I am sure sir has got gem of the person in the form of u both. Thank you very much and I wish you both a very good luck and success in future.*

To all those who have helped me with the thesis: ***Pranav***, ***Shailja*** *and* ***Hiral*** *for going through the chapters,* ***Pranav*** *again for editing the figures and tables,* ***Gowri*** *for helping me with the graphs and* ***Pratyush*** *for helping me editing the references. Thank you all. Your help counts a lot for this thesis.*

Thanks to my other friends of the department, ***Kala Mokaria, Suchi Gandhi*** *and* ***Reshma Solanki*** *for the warm greetings every morning as I entered the department and* ***Deepa Menon*** *for cheers along the way.*
I am also very grateful to all the nonteaching staff of the department, for their immense cooperation. Everyone has been so accommodating and very approachable. Thank you all... Talking with u people always made my mood light.

To the friends that I have made at Hostel: ***Shilpi***, ***Ankita***, ***Jigna***, ***Akhila***, ***Daxa***, ***Nisha***, ***Meeta***, ***Nancy***, ***Vriti***, ***Apoorva***, ***Hiral***, ***Uloopi***, ***Sweety***. *Thank you for the good times.... There were many!*

Special thanks go to my friends ***Sankari***, ***Jisha***, ***Ruchika***, ***Umesh Varma*** *and* ***Kartika*** *for the friendship that started at my very first days in Baroda*

and never knew would go so stronger. Your e-mails and messages kept me going throughout these years and made my journey very blissful.

To my best friends, ***Gowri Uggini****,* ***Nisha Prajapati*** *and* ***Tushar Panot****. Thank you for being there and for your support. Your friendship remains irreplaceable.*

To my friends from back home, thank you all.

Thanks to all my relatives and cousins for their best wishes.

My brother ***Surya****…You're love, support and encouragement has meant the world to me! Thank you!*

To my ***Parents****, thank you so much for your unconditional love and support. Sometimes it was the only thing that got me through some tough times. I love you more than words can express.*

And finally to the Almighty God..!! Thank you for being there with me… always…

With this ***I fondly dedicate this thesis to my Parents***

A. Saradamba

LIST OF ABBREVIATIONS

AEC	–	Apical epithelial cap
BCA	–	Bicinchoninic acid
BL	–	Blastema
BrdU	–	Bromodeoxyuridine
BSA	–	Bovine serum albumin
COX-2	–	Cyclooxygenase-2
DAB	–	3, 3’ Diaminobenzidine
DF	–	Differentiation
DMSO	–	Dimethyl sulphoxide
dpa	–	Days post amputation
ECM	–	Extra cellular matrix
FGF2	–	Fibroblast growth factor 2
FGFR	–	Fibroblast growth factor receptor
FITC	–	Fluorescein isothiocyanate
hpa	–	Hours post amputation
HRP	–	Horse radish peroxidase
HSPG	–	Heparan sulphate proteoglycan
MMP	–	Matrix metalloproteinase
OCT Medium	–	Optimal cutting temperature Medium
PBS	–	Phosphate buffered saline
PBST	–	Phosphate buffered saline-Tween-20
SDS-PAGE	–	Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis
TIMP	–	Tissue inhibitor of matrix metalloproteinase
VEGF	–	Vascular endothelial growth factor
VEGFR	–	Vascular endothelial growth factor receptor
WE	–	Wound epithelium

INTRODUCTION

Regeneration is a term classically used to denote biological self-repair and it is one of the imperative prerequisite for life (**Goss, 1969)**. There are a number of regenerative mechanisms by which animals are able to replace or restore damaged cells and tissues, ranging from simple wound healing and tissue maintenance to the ability to regenerate limbs, organs and in some cases, entire new organism (**Tsonis, 2000**). Hence a complete understanding of the cellular and molecular mechanisms that control the regenerative process remained ever elusive.

Moreover, the regeneration ability in animals is neither rare nor is it all that common. It is widely distributed among most animal phyla although the degree of regenerative ability varies from species to species (**Alvarado, 2000; Brockes *et al*., 2001; Stocum, 2004**). In most groups of animals with regenerative powers, only certain parts of the body can be regenerated, like the fin of fish or tail of lizard. However, others are super-regenerators. With many sea stars (starfish) not only can any part of a missing arm be regenerated, but if enough of the arm is left, the entire sea star can be regenerated from the piece of arm. In fact, many sea stars reproduce by simply splitting in two, with each piece growing back the missing parts. But some possess a limited power of regeneration, for example, a cockroach can regrow a new appendage, when lost, but the appendage itself cannot generate a new cockroach. This is named as *unidirectional regeneration*; whereas the one showed by the animals of simple body plan like hydras, starfish and anemones is called *bidirectional regeneration*; in other words, they can go both ways. Cut a hydra in half, and you will get two hydras. Cut it into four pieces, and you will get four (**Goss, 1969)**.

Humans are not completely without regenerative abilities. We heal from wounds and fractures and we are always creating new skin, new blood, and new linings for our stomachs, intestines and lungs. To a certain extent, our livers can even regenerate after they have been damaged (**Brockes *et al*., 2001)**.

NATURE OF REGENERATION IN INVERTEBRATES AND VERTEBRATES

It is a general, but not universal rule that less highly evolved species have greater capacities

for regeneration. Many simple organisms such as hydra, starfish and flatworm are highly regenerative, even able to regenerate new individual bodies when cut into pieces. However, the capacity for wide-range regeneration is limited in chordates (**Dinsmore, 1992**). Invertebrates possess much more spectacular regenerative abilities and can readily regenerate whole body parts. In contrast, in vertebrates regenerative abilities have been scaled down and limited to appendages and eyes **(Tsonis, 2000)**. In short, it can be stated that, the more advanced along the evolutionary scale an organism is, the more restricted is its regenerative ability (**Wirth *et al.*, 1992)**.

Invertebrate regeneration has been studied for more than 200 years (**Lenhoff and Lenhoff, 1986**). Two classes of invertebrate that have received the most contemporary attention are: the diploblast *Hydra vulgaris*, and the triploblast, bilaterally symmetrical freshwater planarians such as *Schmidtea mediterranea* and *Dugesia japonica*. Hydra has the distinction of being the first animal in which regeneration was formally described (**Lenhoff and Lenhoff, 1986**). Death due to the loss of essential body regions such as the head is prevented by regeneration in this species. Moreover, because hydra constantly replaces the cells that are lost to normal physiological turnover, these animals can be considered negligibly senescent (**Martinez, 1998**). Another interesting property of hydra is its ability to re-form an animal from dissociated cells (**Hobmayer *et al.*, 2000**) a trait that could serve as a paradigm for understanding the molecular basis of *de novo* organizer formation during non-embryonic processes such as regeneration (**Meinhardt, 2002**). Many large freshwater planarians (flatworms) practice architomy, a form of asexual reproduction where the animal simultaneously fragments into several pieces; each piece growing back into a fully functional flatworm. Planarians can regenerate a complete individual from less than 1/200 of its body (**Morgan, 1898**). Sponges have undifferentiated cells called archeocytes that can regenerate lost tissues. Cnidarians (jellyfish, corals, sea anemones, hydras and others) have undifferentiated interstitial cells packed in under their epidermis. In scorpions, complete regeneration of appendages is either rare or non-existing. They have been known to regenerate parts of legs, but in a non-methodical way (**Lenhoff and Lenhoff, 1986**).

Regeneration of vertebrate appendages has been one of the most extensively studied model systems (**Tsonis, 2000; Brockes and Kumar, 2002**). Among the Vertebrates amphibians are known to possess the highest power of regeneration. Urodeles and zebrafish are among the vertebrate species that retain a significant regenerative ability during adulthood (**Kawakami**

***et al*., 2006)**. Unlike in some invertebrates where regeneration is a mode of reproduction (e.g. Echinoderms), among the vertebrates, the power of regeneration is mainly used to escape from predation e.g. in most lizards, tails break off easily and the detached tail performs lively wriggling contractions that divert the attention of a predator and allow the lizard to escape (**Dial and Fitzpatrick, 1983**). Thus, tail autotomy in lizards is known to be effective for predator defense (**Arnold, 1984, 1988**).

In man, the process is normally considered to be limited to certain tissues and organs, including skin and bone (**Liozner, 1974; Singer *et al*., 1987**). Prior research, however, has indicated that children have the ability to regenerate a functional and morphologically exact replica of a fingertip accidentally severed at the outermost joint (**Douglas, 1972; Illingworth, 1974; Louis *et al*., 1980**). Full repair, including the finger whorls, takes approximately 12 weeks and is best accomplished with a periodic cleansing and dressing of the wound with no additional therapeutic intervention (**Wirth *et al*., 1992**). Although accounts of fingertip regeneration have generally been limited to children 11 years of age or younger, this may be due to the fact that relatively few older children and adults enter hospitals with amputated fingers (**Illingworth, 1974**).

The regenerative capacities possessed by different organisms at different biological level are well depicted in the figure (**Figure 1**) given below:

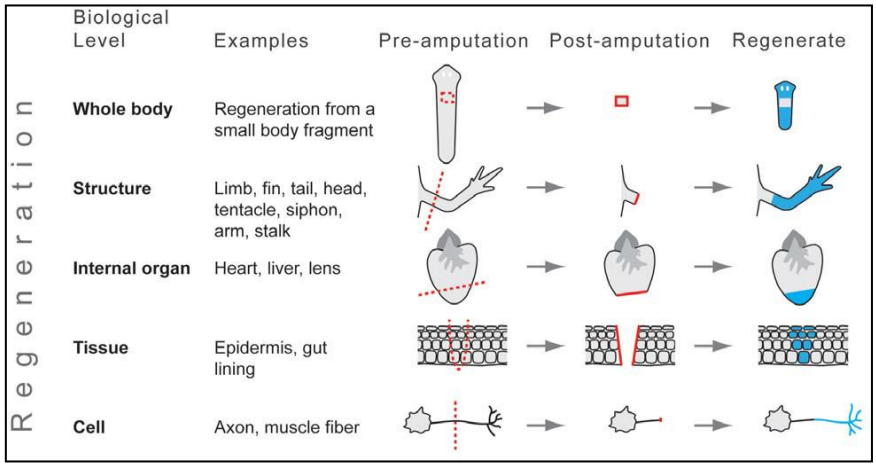


Figure 1: Regeneration at different levels of biological organization (from **Bely and Nyberg, 2010**)
Dashed red lines indicate amputation planes; solid red lines indicate wound surfaces; and blue fill indicates regenerated body parts.

HISTORY AND EVOLUTION

Why does regeneration happen? And when it happen, why does it do so in some but not in all? What are the positive and negative regulatory factors operating behind this phenomenon? As one tries to understand regeneration closely, many such questions remain unanswered. In fact, the problem of animal regeneration has withstood the probing of scientific inquiry for over 250 years and still awaits a satisfactory mechanistic explanation (**Alvarado, 2000**).

For centuries the extensive regenerative abilities possessed by certain species have fascinated mankind. This fascination is reflected in many myths and fictional stories featuring creatures with regenerative powers. Myth of the ability to replace lost body parts has been known since at least 6th century BCE. Hydra was a gigantic, nine-headed water-serpent. Hercules was sent to destroy her as one of his twelve labours (eleventh labour), but for each of her heads that he decapitated, two more sprang forth. Hence, he applied burning brands to the severed stumps, cauterizing the wounds and preventing regeneration. Prometheus was a champion of human-kind known for his wily intelligence who stole fire from Zeus and gave it to mortals. Zeus then punished him for his crime by having him bound to a rock while a great eagle ate his liver every day only to have it grow back to be eaten again the next day (**Okada, 1996**). The following quotation from **Newth (1958)** tells us the excitement very vividly.

> "In 1768 the snails of France suffered an unprecedented assault. They were decapitated in their thousands by naturalists and others to find out whether or not it was true, as the Italian Spallanzani had recently claimed that they would then equip themselves with new heads..... The slaughter went on. But, as Spallanzani had suggested, decapitation did not always bring death. In the hands of some experimenters his results were repeated, the unfortunate animals survived and, after a few weeks, had grown new heads. Thus the study of regeneration in animals which had recently been put on a scientific footing by Reaumur, Bonnet, and Trembley became, perhaps, the first of all branches of experimental biology to be popularized."
>
> ***- from Newth, 1958***

Thus the remarkable phenomenon of regeneration was not just an issue for specialized experts or intellectuals, but a concern of the general public in the period. There were heated philosophical and religious discussions, since the discoverers of a number of examples of regeneration included priests, who, at that time, served to be responsible for comprehensive

intellectual activities. They argued that if each part of an animal could regenerate the entire animal, where was the residence of the 'soul'? In order to see how deeply people were impressed, it may be pertinent to cite the reaction of such a distinguished philosopher like Voltaire after the regeneration experiments by his own hands (**Okada, 1996**).

> "Voltaire marvelled briefly: he saw at once that the loss and replacement of one's head presented serious problems for those who saw that structure as the seat of a unique 'spirit' or soul: and thought of the possible consequences of the experiment for man. Writing at this time to poor blind Madame du Deffand, he lamented that for snails but not for her the replacement of bad eyes by good was a possibility. Later he expressed confidence that men would one day so master the process of regeneration that they too would be able to replace their entire heads. There are many people, he implied, for who the change could hardly be for the worse".
>
> ***-from Newth, 1958***

Since the first scientific publications on regeneration in the early 1700s (**Dinsmore, 1992**), researchers have studied regenerative phenomena in a wide variety of species, ranging from protozoa to humans. The first Experimental Biologist to study regeneration was René Antoine Ferchault de Réaumur. He was the first scientist to perform a serious study of regeneration. In the early 1700's he described limb regeneration in crayfish. He noted that crayfish would regenerate only the lost part of the limb or claw, but often amputations toward the tip of the limb would induce the animal to cast off the rest of the limb at a defined point near its base. This loss is known as autotomy and is caused by the contraction of specialized muscles at the breakage site. Thus he hypothesized that the new limb arose from tiny preformed limbs residing at the base of the limb (**Reaumur, 1712**). This may sound like a surprising view today, but at that time, the philosophical ideas of preformation had a strong influence leading brilliant scientists to believe that each sperm contained a tiny human (**Loof, 2008**).

The Swiss scientist Abraham Trembley began studying the freshwater polyp in the 1740s. This work led to the remarkable discovery that hydra could regenerate their heads and feet and if cut into a few pieces, all of them would regenerate to form new individuals. He was able to split the head of the polyp longitudinally and allow two heads to regenerate. By repeatedly splitting the new heads, he was able to generate a multiheaded animal that he

named ‘Hydra’ in reference to the mythological creature. Thus, he is one of the first scientists to demonstrate that animals could reproduce asexually (**Okada, 1996**).

Distinguished scholars who joined in regeneration studies in that period were truly multitalented. One has to realize that it was a time well before the establishment of science as a profession. Lazzaro Spallanzani (1729-1799), the discoverer of amphibian limb and tail regeneration was a lawyer, mathematician, philosopher, Catholic priest and natural historian. Rene Antoine Frechault Réaumur (1683-1757) who studied the regeneration of crayfish legs scientifically for the first time, was a metallurgist well famed as the inventor of the "Reaumur process" for the steel industry, mathematician. Above all, he generated an encyclopaedia of all the arts, industries and professions. Trembley himself, besides a keen natural historian, was a mathematician and lived as the tutor of the sons of Count William Bentick of The Hague, on whose estate he collected Hydras from the pond together with the young boys. Morgan's life was symbolic. After having started his brilliant scientific career as a regeneration researcher, he left this subject and turned his interest to genetics (**Okada, 1996**). A summary of above contribution by scientists and their work on regeneration is shown in the following table.

Table 1: List of scientists and their respective contribution to the regeneration study

Name of the Scientist	Regeneration study
Abraham Trembley (1744)	First scientific discovery of regeneration using Hydra
Lazzaro Spallanzani (1765)	Announcement of regeneration of tails and limbs in Salamanders; in “Prodromo”
Charles Bonnet (1762)	Reporting several regeneration systems in Salamanders (including Lens regeneration)
Tweedy John Todd (1823)	Discovery of “Neurotrophic effects” on the limb regeneration
Gustav Wolff (1894)	Announcement of the regeneration of the lens from dorsal Iris in Newts
Elmer Grinshaw Butler (1933)	Establishment of de-differentiated mesoderm as a source of regeneration Blastema
Goro Eguchi and T.S. Okada (1973)	Demonstration of Cell transdifferentiation in in vitro systems
I A Niazi and S. Saxena (1978)	Induction of abnormal limb regeneration by Vit. A in Anuran Tadpoles
Susan V Bryant and others (1981)	Interpretation of the pattern formation in the

	regeneration in terms of positional information
Priyambada Mohanti-Hejmadi *et al*., (1992)	Induction of Heteromorphic regeneration by Vit. A and Retinoids in Tadpoles
Brockes *et al.* (2007)	newt Anterior Grade protein secreted by the nerve sheath drives the regeneration of the limb from the stump

TYPES OF REGENERATION

The processes of animal regeneration were categorized into two main groups by the Nobel Prize-winning geneticist, Thomas Hunt Morgan (1866-1945) in 1901 (**Morgan, 1901**). They are: ***Morphallaxis***, i.e. renewal of lost tissue by the remodelling of existing cells or tissues without cell proliferation, and ***Epimorphosis***, i.e. regeneration of a piece of an organism by way of cell proliferation on the cut surface. Currently, epimorphic regeneration is subdivided into two broad categories: non blastemal and blastemal based regeneration (**Alvarado, 2000**). Non-blastemal regeneration occurs as a result of: a) transdifferentiation of the remaining tissue into the missing structure) limited dedifferentiation and proliferation of the surviving cells in the organ after injury or amputation; and c) by the proliferation and differentiation of stem cells already present in the damaged tissue. Blastemal based epimorphosis, however, requires pre-existing stem cells or dedifferentiation generated progenitor cells (blastemal cells) to proliferate, differentiate, and finally replace the lost cells (**Alvarado, 2000**).

During Morphallaxis, the remaining undifferentiated cells simply migrate to the site and differentiate into the specialized cells, with little cellular proliferation. It is observed in species such as the hydra. The morphallactic regeneration involves reorganization of the existing cells to form the lost structure, hence the regenerates are smaller than the original structure or organism, and growth takes place subsequent to regeneration (**Cai *et al*., 2007**).

The other types of regeneration as described in **Stoick-Cooper *et al*. (2007a)** are:

Compensatory Growth where it is not the damaged part of an organ that is restored, but uninjured parts of the organ compensate for the loss by growth (e.g. After removal of the two lobes of the liver, the third lobe grows until the original mass of the liver is stored).

Tissue Regeneration that includes the repair of the limited damage to an organ predominantly via restoration of only one cell type (e.g. skeletal muscle).

EPIMORPHIC REGENERATION

As mentioned above, the term Epimorphic regeneration was coined by **Thomas Hunt Morgan (1901)** to describe "cases of regeneration in which a proliferation of material precedes the development of new part". In the case of limb or tail regeneration, the residual part of the limb/tail remains as it is and a 'blastema' forms at the site of the wound and eventually regenerates the lost tissues and organs (**Agata *et al.*, 2007**). This kind of 'add-on' regeneration is called 'epimorphic regeneration' (**Suzuki *et al.* 2006**). In this type of regeneration, the old stump provides cells participating in blastema formation without drastic rearrangement of the remaining tissues.

Epimorphic regeneration is a widespread phenomenon throughout the animal world. Organisms as divergent as insects (**Bohn, 1976**), starfish (**Thorndyke *et al.*, 2001b**), amphibians (**Brockes and Kumar, 2002**), reptiles (**Alibardi, 2009; Alibardi and Lovicu, 2010; McLean and Vickaryous, 2011; Yadav *et al.*, 2012**) and fishes (**Schebesta *et al.*, 2006, Iovine, 2007; Wills *et al.*, 2008a,b; Anusree *et al.*, 2011**) can regenerate their extremities by epimorphic response. The ability to regenerate a whole body plan, seen in some simple organisms like Planaria, also depends on epimorphosis (**Alvarado, 2000**). Many Vertebrates have the ability to perform epimorphic regeneration, but this ability is more restricted, either to certain structures or to specific phases of the life cycle. e.g., the Anuran Amphibians regenerate their tail and limbs as Tadpoles but not as adults (**Slack *et al.*, 2007**). When it comes to mammals, the examples of epimorphic regeneration are few and in most cases, there is some uncertainty as to whether they are truly epimorphic process (**Loof, 2008**). Digit tips in Infants have been reported to regenerate if not covered by a skin flap, which is a standard treatment for amputed fingertips (**Illingworth, 1974**). The annual regrowth of Deer Antlers (**Price *et al.*, 2005**) and regeneration of earholes in some Mammals (**Heber-Katz *et al.*, 2004**) are other processes that have been suggested to proceed through an epimorphic response.

For mammals, epimorphic regeneration is largely limited by an irreversible differentiation process, although it has been demonstrated that stem cells are activated during the regeneration of muscles, bones, epithelia, and some other tissues (**Ding and Schultz, 2004**). Although they are not endowed with as great an ability to regenerate complex structures, they have the potential to regenerate a surprisingly large array of injured tissues. Unlike that in salamanders, in which regeneration, once started, typically results in the formation of an

almost perfect replica of the structure that was lost, mammalian regeneration proceeds with varying degrees of success. One of the major challenges in the scientific study of regeneration in mammals and its clinical application in humans is to understand why regeneration proceeds very well under some circumstances and very poorly under others (**Ding and Schultz, 2004**).

A number of important principles underlie epimorphic regeneration (**Carlson, 1974; Wallace, 1981; Stocum, 1995; Tsonis, 1996**). Epimorphic regeneration proceeds in a well-defined sequence of stages as explained in the following description.

STAGES OF EPIMORPHIC REGENERATION

Wound Healing and Formation of Wound Epithelium

Within seconds after amputation, bleeding stops because of contractions of the vascular walls. Epidermal cells at the circumference of the stump are then mobilized and start to migrate across the amputation surface. A complete wound epidermis, covering the whole amputation surface is formed within hours to couple of days depending on the species (**Loof, 2008**).

Normal wound repair is highly dynamic, consisting of several overlapping phases (**Schaffer and Nanney, 1996; Singer and Clark, 1999**). In the past 20 years, tremendous progress has been achieved in understanding the cellular and molecular events of wound repair. Tissue injury disrupts capillaries, which immediately triggers activation of platelets to begin the clotting cascade and the events of inflammation. Neutrophils enter the injured tissue with the major function of removing bacteria, but these cells and other leukocytes release a variety of proteases, growth factors, and other cytokines with profound effects on the repair process (**Harty *et al*., 2003**).

Dedifferentiation and Formation of Blastema

From the early days of regeneration study, a major goal has been to characterize the cellular and molecular nature of blastema cells. A blastema can easily be recognized as a ‘white region’ formed on the cut surface of the body (**Agata *et al*., 2007**). The definition of a blastema as given by Agata *et al*. (2007) is as follows “the blastema is the structure that develops at the cut end of an amputated region, having an area of whitish cells that are in an embryonic-like state, filled with pluripotent blastemal cells that are able to become any of a

number of kinds of cells". Over time, these cells will divide, more and more of them will differentiate, and the form of the missing body part will take shape. Some factor(s) maintaining the undifferentiated state of cells in the blastema may also inhibit the differentiation of pigment cells. This may be the reason why the blastema can be recognized as a white area during regeneration, but nobody has succeeded in completely identifying such factor(s). The histological sections of this white region confirm the blastema of being composed of typical morphologically undifferentiated cells (**Agata *et al.*, 2007**). Characteristics of epimorphic regeneration is that much like during embryonic development, epithelial-mesenchymal interactions are essential and within the blastema, a morphogenetic pattern is set up to govern the subsequent development of the regenerating tissue.

Differentiation

Next and the final step in the process of regeneration is differentiation. The key to regeneration is in cell differentiation. Cell proliferation continues with an increase in blastema size matched by the development of new blood vessels and the outgrowth of the tissue. The once uniform population of cells in the blastema begins to differentiate, resulting in the formation of cartilage, muscle, adipose tissue and fibrous connective tissue. Differentiation proceeds in a cranial to caudal axis, with the most mature tissues in the proximal region of the regenerate tail. Although cell proliferation continues during this time the cell population is no longer uniform and the blastema ceases to be discretely recognizable. Congruent with the differentiation of cell types, levels of proliferation are highest distally and lowest proximally (**McLean and Vickaryous, 2011)**.

PHASES OF REGENERATION

PHASE I- WOUND EPITHELIUM

Epidermal healing	Epidermal sheet migrates to cover the wound area within 1-2 hrs
Induction of gene expression	Genes common to wound healing and limb regeneration are expressed (e.g. msx 2 and MMP-9)
Nerve dependency	Not dependent on nerves

PHASE II- DEDIFFERENTIATION

Dedifferentiation	Cells in the stump tissues lose their specialized characteristics and become migratory
Blastema formation	Cells derived from fibroblasts migrate to form the blastema and begin to proliferate
Induction of gene expression	Re-expressed genes show spatial and/or temporal patterns that differ from development
Nerve dependency	No regeneration if nerve supply is interrupted
PHASE III- DIFFERENTIATION	
Growth and pattern formation	Classic responses to grafting are the same as in developing limbs; developing and regenerating limbs can cooperate to form a chimeric limb
Induction of gene expression	Genes show similar expression and function as in developing limbs
Nerve dependency	Continued growth depends on nerves, but differentiation is nerve-independent
Positional dependency	Requirement for a blastema consisting of cells

EPIMORPHIC REGENERATION IN FISH FIN

Caudal fin regeneration in the Teleost fish represents an excellent model to understand epimorphic regeneration (**Poss *et al*., 2000a**). The tail fin of teleost fish is a symmetric organ composed of multiple skeletal rays, the lepidotrichia (**Montes *et al*., 1982; Becerra *et al*., 1983; Santamaría and Becerra, 1991; Géraudie and Singer, 1992**). These rays originate from the base of the fin and they spread distally, towards the edge. Extending from the distal portion of each lepidotrichia towards the edge of the tail fin there is a cluster of small, fusiform, rigid and slender spicules called actinotrichia, which support the border of the tail fin (**Becerra *et al*., 1983**). Both the lepidotrichia and actinotrichia are surrounded by connective tissue containing blood vessels, nerves, pigment cells and fibroblasts and covered by a multistratified epidermis (**Becerra *et al*., 1983**).

The phases of the caudal fin regeneration are: formation of a multistratified epidermal layer (wound epithelium), disorganisation and distal migration of mesenchymal cells near the amputation plane, proliferation of these mesenchymal cells to form the blastema, continuous proliferation of the distal blastema to facilitate growth, and differentiation of the proximal

blastemal cells to replace its lost structures (**Goss and Stagg, 1957; Santamaría and Becerra, 1991; Géraudie and Singer, 1992; Johnson and Weston, 1995; Poss *et al*., 2000a; Santos-Ruiz *et al*., 2002; Akimenko *et al*., 2003**).

Regeneration of the caudal fin occurs in 3 stages: wound healing (wound epithelium), blastema formation and regenerative outgrowth (**Figure 2**). Wound healing involves the covering of the wound by a thin layer of epithelial cells, called the wound epidermis, within 12 hours post amputation (hpa). From 12 to 24 hpa, mesenchymal cells dedifferentiate and move towards the epidermis. During 24-48 hpa there is proliferation of mesenchymal cells forming a blastema. Finally, from 48 hpa forward, the blastema differentiates and develops structures required for fin regeneration, including blood vessels, bony rays, and connective tissue (**Santamaria and Becerra, 1991; Johnson and Weston, 1995; Becerra *et al.*, 1996; Mari-Beffa *et al.*, 1996; Poss *et al.*, 2000b;**).

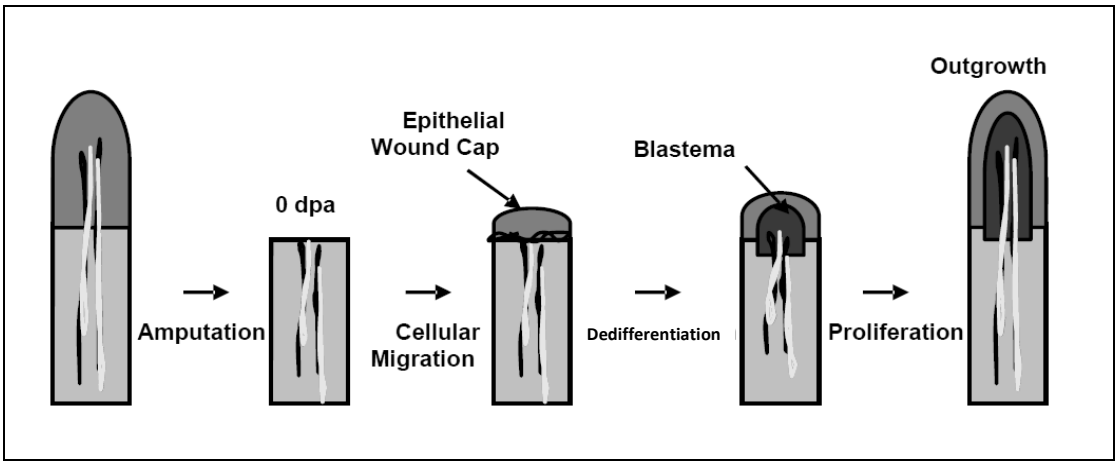


Figure 2: Schematic representation of stages of epimorphic regeneration in fish as adapted from **(Mathew *et al.*, 2007)**

TELEOST FISH AS EXPERIMENTAL MODEL

Scientists believe: *"It's not just a fish... It's a hope...!"*

Teleost Fish, now a days is preferred as alternate vertebrate model of choice for regeneration studies. Teleosts have a high ability to regenerate their wound tissues and organs including heart. They can regenerate almost any part of their body (**Akimenko *et al*., 2003)**. This has made the teleost fish an ideal experimental model for regeneration studies.

More than 200 years ago, **Broussonet (1786**) reported that an adult fish could completely regenerate its fins after amputation. Many studies focusing on the regeneration of the fish fin have also been performed to examine the mechanism of regeneration (**Akimenko *et al*.,**

2003; Poss *et al*., 2003). The teleost fish have remarkable regeneration ability in a variety of tissues and organs including scales, muscles, spinal cord and heart in addition to their fins (**Akimenko *et al*., 2003; Poss *et al*., 2003**). In terms of their tissue structures and cellular origins, the fish fins can be regarded as homologous tissues of vertebrate tails and limbs, and the cellular mechanism of regeneration may be shared between the urodele limbs and fish fins (**Hinchliffe, 2002**). Also, teleostan zebrafish has a fully sequenced genome and therefore genetic manipulations can be done to produce mutant or transgenic lines (**Akimenko et al. 2003, Poss *et al*., 2003**). More interestingly, a comparison of the putative protein sequences between the fish and human showed that the coding region is highly conserved (74.8% identical) (**Hata *et al*., 1998**). Hence the tiny fish can reveal many secrets of regeneration, and take us a step closer to unlock the secrets of body parts regeneration. Therefore, the understanding of the regeneration mechanism in teleost fishes will provide an essential knowledge base for rational approaches to tissue and organ regeneration in mammals including humans (**Masaki and Ide, 2007**). Such knowledge will be of clinical importance for developing a novel therapy using the intrinsic regenerative ability.

As compared to other regenerating structures, fin regrowth can easily be monitored *in vivo* and regenerating tissues can be used to study this dynamic developmental process. Also, a remarkable fact is that many genes involved in fin regeneration are also involved in fin development, and those genes are involved in limb development in other species, including amphibians and possibly humans (**Laforest *et al*., 1998, Ingham and McMahon, 2001; Iovine, 2007**). This has led to an increase in the interest in fin regenerative study throughout.

Fin regeneration has been studied in many different teleost fishes including Tilapia (**Kemp and Park, 1970; Santamaria and Beccerra, 1991; Santamaria *et al.*, 1992**), Minnows and Blennies (**Morgan, 1900, 1902, 1906; Goss and Stagg, 1975; Geraudie, 1977**), *Opalina gouramis* (**Tassavva and Goss, 1966**) Gold fish (**Morgan, 1902; Santamaria *et al*., 1992, 1996; Mari-Beffa *et al*., 1999**), Trout (**Alonso *et al*., 2000**) Sword tail fish (**Zauner *et al*., 2003**) and Zebrafish (**Geraudie *et al*., 1994; Akimenko *et al*., 1995; Johnson and Weston, 1995; Mari-Beffa *et al*., 1999; Poss *et al*., 2003**).

However, considering the easy and ready availability, in the present study, the teleost fish Sailfin Molly, *Poecilia latipinna* (Lesueur, 1821), was used to investigate the role of FGF2 in caudal fin regeneration.

Some other supportive reasons for using *P. latipinna* were as follows:

- Readily available and can be maintained at ambient temperature allowing very large numbers to be housed cheaply.
- Their fins are relatively simple
- Fin amputation is easy and a non-lethal surgery
- The regeneration process is easy to observe from outside as well as under the microscope through transparent regenerate.

The Sailfin Molly - *Poecilia latipinna*

The sailfin molly, *Poecilia latipinna,* formerly described and named *Mollienesia latipinna* by Charles Alexandre Lesueur in 1821 (**Robins, 2003**) is from the family Poeciliidae, comprising over 190 species (**Parenti and Rauchenberger, 1989**). The natural distribution of the sailfin molly is fresh, brackish, and salt waters of Florida, Mexico, Texas, South and North Carolina, and Virginia (**Petrovicky, 1988; Courtenay and Meffe, 1989; Robins, 2003**). Non-indigenous populations are established in the western U.S. (Arizona, California, and Nevada), Hawaii, Canada, Central America, Singapore, Australia, New Zealand, Guam, and the Philippines (**Courtenay and Meffe, 1989**). The sailfin molly prefers lowland areas such as marshes, lowland streams, swamps, and estuaries (**Robins 2003**).

The sailfin molly is a fusiform shaped small fish (15-53 mm total length) with a small head and upturned mouth (**Robins, 2003**). The dorsal fin is greatly enlarged in mature males compared to those of mature females. The gestation period is 3-4 weeks. Females are viviparous and give birth multiple times during the year (**Robins, 2003**).

The sailfin molly is primarily an herbivore, eating plants and algae, but is also opportunistic and will eat other food items including detritus or insect larvae and cannibalism has been reported (**Meffe and Snelson, 1989**). It is a prey item for many predators. Predators that eat this fish include reptiles, birds, other fishes, amphibians, and insects. It has been used for research and for biological control of mosquitoes (**Courtenay and Meffe, 1989**). The molly is also popular in the aquarium trade and is available in a wide variety of colours through domestication . The molly used in the present studies was black molly.

MOLECULAR MECHANISM OF EPIMORPHIC REGENERATION

There are many key regulators which play an important role in epimorphic regeneration. It is well known that limb regeneration requires innervation (**Wallace, 1981**). It has been

suggested that axons secrete what have been called "neurotrophic factors" in the site of amputation and that these factors up-regulate genes important for the regenerative process. **Brockes and Kumar (2005)** have reported that nAG (for newt Anterior Grade) protein is secreted by the nerve sheath at the site of amputation in salamander limbs and it initiates and drives the regeneration of the limb from the stump. It has been shown that innervation is also required for maintenance of expression of genes (including *tbx5* and *prx1*) in the early blastema of the froglet, and for the initiation of expression of *msx1, fgf8, and fgf10* in the late blastema (**Suzuki *et al.*, 2006**). The major signals regulating tail regeneration in vertebrate models are bone morphogenetic protein (BMP) and Notch signalling (**Beck *et al.*, 2003**). Both the signals are required for cell proliferation although BMP pathway is independent of Notch signalling (**Stoick-Cooper *et al.*, 2007b**). **Schnapp *et al.* (2005)** studied that sonic hedgehog (*shh*) signalling besides pattering is required for proliferation of surrounding mesodermal tissue.

Nevertheless, apart from the several putative factors mentioned earlier one important upstream regulatory factors released at the site of amputation are members of a family of growth factors – fibroblast growth factors (**Taylor *et al.*, 1994; Yokoyama *et al.*, 2001; Christensen *et al.*, 2002**). The molecular mechanisms of epimorphic regeneration clearly state the roles of FGFs in regeneration studies of amphibian limb (**Christen and Slack, 1997; Han *et al.*, 2001**). And there are evidences which show that FGFs are also playing role in teleost caudal fin regeneration (**Lee *et al.*, 2005; Poss *et al.*, 2000b**).

THE FGF FAMILY

Fibroblast growth factors (FGFs) constitute a large family of signalling polypeptides that are expressed in various cell types from early embryos to adults (**Dvorak and Hampl, 2005**). Since the first discovery of FGF in 1974, 23 distinct members of the FGF family have been described (**Gospodarowitz, 1974**). FGF family members range from 16 to 34 kDa and share 13-71% amino acid identity (**Dvorak and Hampl, 2005**). A core of 155 amino acids remains highly homologous throughout the FGF family (**Pazmany, 2003**). These conserved amino acids form 12 anti-parallel beta-strands that create a cylindrical barrel, a defining feature of the FGF family (**Zhang *et al.*, 1991**). It is this topological similarity that unites the FGFs, not their biological function.

The two FGFs, acidic FGF (FGF-1) and basic FGF (FGF-2) [the term acidic and basic was given based on their isoelectric point] were first to be identified and they were originally identified as growth factors for fibroblasts. However, FGFs are now recognized as polypeptide growth factors with diverse biological activities and expression profiles. The various FGFs have been reported to regulate complex biological processes such as embryonic development, angiogenesis, wound healing, nerve regeneration, chronic inflammation and cancer. The FGF family members mediate diverse biological responses in a number of different tissues. FGF1 and FGF2 are detectable in a variety of tissues during both development and adulthood (**Baird and Klagsbrun, 1991; Yamagushi and Roussant, 1995**). FGF-1, FGF-2, FGF-4, FGF-5, FGF-6, FGF-7 and FGF-8 are expressed in developing skeletal muscle (**Mason, 1994; Grass *et al*., 1996**); Whereas FGF-4, FGF-7, and FGF-8 are expressed transiently in myotomes (**Floss *et al*., 1997).** FGF-10 is implicated in the differentiation processes in white adipose tissue (**Sakaue *et al*., 2002**) and pancreas (**Ohuchi *et al*., 2000; Bhushan *et al*., 2001**), while FGF16 (**Konishi *et al*., 1999**) is considered to be a specific factor for brown adipocytes. FGF19 is considered as a regulator of energy expenditure (**Tomlinson *et al*., 2002; Fu *et al*., 2004**). FGF21 is a potent activator of glucose uptake on adipocytes, protects animals from diet-induced obesity when over expressed in transgenic mice, and lowers blood glucose and triglyceride levels when therapeutically administered to diabetic rodents (**Kharitonenkov *et al*., 2005**). Fibroblast growth factor 23 (FGF-23) has recently been shown to be involved in phosphate regulation and bone mineralization (**Pazmany, 2003**).

In the adult organism, FGFs represent important homeostatic factors and play a role in response to injury and tissue repair (**Cutroneo, 2003**). Immediately after injury, during the inflammatory phase, which is a hallmark of wound healing stage, there is release of cytokines and growth factors ensuring permeability of blood vessels and chemotaxis of inflammatory cells. Each process is regulated by many bioactive substances, including growth factors (**Sharma and Suresh, 2008**). These processes require spatial and temporal integration of several cell responses, including cell survival, proliferation, migration and invasion, and cell differentiation.

All these responses or functions are induced or modulated by the interaction of FGFs with receptors. In fact all growth factors function as *ligands* that bind to specific *receptors*, which deliver signals to the target cells.

FGF RECEPTORS

By immobilizing FGF in affinity chromatography, **Lee *et al.* (1989)** first purified a receptor tyrosine kinase, called FGFR1. Soon, FGFR1 as well as several related receptors now denoted FGFR2, 3, 4, were identified in a number of different species (**Jianwu *et al*., 2003**).

Four specific high-affinity FGF receptors (FGFRs1, 2, 3, 4) have been described, that are present in all vertebrate taxa (**Szebenyi and Fallon, 1999**).These consist of three extracellular immunoglobulin (Ig) domains, two of which are involved in ligand binding, a single transmembrane domain with a long juxtamembrane region, and an intracellular cytoplasmic domain that contain the tyrosine kinase activity (**Figure 3a**). A row of eight consecutive acidic residues (the "acidic box") (**Figure 3a**) is situated between the first and the second immunoglobulin like fold in all FGF receptor types; the acidic box is the unique feature of the FGFR and appears to be important for FGFR function. The tyrosines are phosphorylated upon ligand binding and recruit intracellular signalling proteins (**Givol and Yayon, 1992**). Ligand binding appears to depend on the interaction of FGFs with cell surface heparan sulfate proteoglycans (**Roghani *et al*., 1994**) (**Figure 3b**). A fifth FGFR (FGFR5) has also been described that is structurally distinct from the other FGFRs (**Burrus *et al*., 1992**). It is an integral membrane protein containing an extracellular domain with 16 cysteine-rich repeats. The ligand specificity of this receptor subtype is not known although it can bind FGF1, 2, 3, and 4. However, most FGF action is mediated through FGFRs1-4 (**Lee *et al*., 1989; Powers *et al*., 2000; Schlessinger *et al*., 2000; Sleeman *et al*., 2001**). For the most part, there is no one-to-one correspondence between FGF ligands and receptors. A given FGF may be capable of multiple receptor isoforms; conversely, any receptor variant may bind multiple FGFs (**Burgess and Maciag, 1989; Basilico and Moscatelli, 1992; Galzie *et al*., 1997).**

FGFs have two different possible receptors: cell-surface bound tyrosine kinase receptors (designated as FGFRs) as high affinity receptors (**Coughlin *et al*., 1988**) and heparan-like glycosaminoglycans, such as Heparan sulphate proteoglycans (designated as HSPGs) as low-affinity receptors (**Venkatamaran *et al*., 1999; Stauber *et al*., 2000**). The binding of FGF to the low affinity receptors do not transmit a biological signal; rather they function as accessory molecules that regulate FGF-binding and activation of the FGFRs (**Yayon *et al*., 1991; Ornitz *et al*., 1992; Spivak-Kroizman *et al*., 1994; Lin *et al*., 1999**). HSPG binding of FGF induces FGFR dimerization, which is followed by the transphosphorylation of

receptor subunits and the initiation of intracellular signalling events (**Robinson *et al*., 1995; Ornitz *et al*., 1996; Powers *et al*., 2000**).

Two FGF molecules can form a complex bound to one FGFR, which is connected by a heparan sulfate proteoglycan. The receptor complex can trigger phosphorylation of receptors, causing recruitment and activation of intracellular signalling pathways. Heparan sulphate (HS) is required for activation of FGFRs *in vitro* and *in vivo* (**Rapraeger *et al*., 1991; Yayon *et al*., 1991; Ornitz *et al*., 1992; Lin *et al*., 1999**). In the absence of HS, FGF cannot bind FGFRs; on the other hand, addition of HS can reconstitute FGF-FGFR complex formation (**Yayon *et al*., 1991**). The HS chains play a key role in orchestrating the formation and stabilization of the FGF:FGFR signalling complex (**Guimond and Turnbull., 1999; Guimond *et al*., 2001**) (**Figure 3c**).

In brief, it can be said that FGFRs are receptor tyrosine kinases that are activated by ligand-induced dimerization and subsequent auto-phosphorylation. The FGFs bind to FGFRs in a 1:1 complex that is facilitated by heparan sulfate, which makes numerous contacts with both the FGF and FGFR molecule. Heparan sulfate also interacts with an adjoining FGFR to promote FGFR dimerization. The activated FGFR interacts with intracellular signalling molecules allowing it to couple to several signal transduction pathways during development and regeneration (**Ornitz *et al*., 1992; Lin *et al*., 1999**).

All the four FGF receptors (FGFR1, 2, 3, 4) have been identified in the current regeneration model *viz*., caudal fins of teleost fish. However, they displayed different distribution patterns in mature and regenerating fin (**Santos-Ruiz *et al*., 2001**). This is indicative of a distinct regulation of these molecules during regeneration with respect to quiescent ray, and is, so suggestive of a role of FGF receptors in regeneration. Furthermore, differential expression of each receptor suggests each of them may be implicated in different functions. The expression of these receptors in the mature ray and regenerating ray is shown in **Table 2**

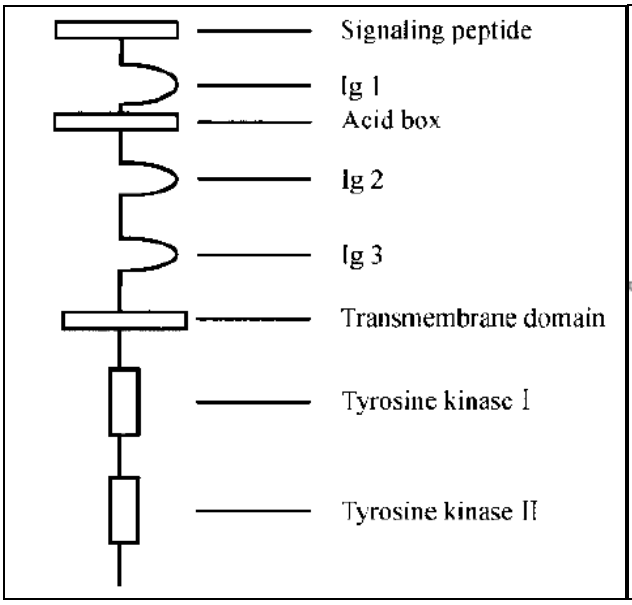


Figure 3a: Sketch map of FGFR (**Turner and Grose, 2010)**

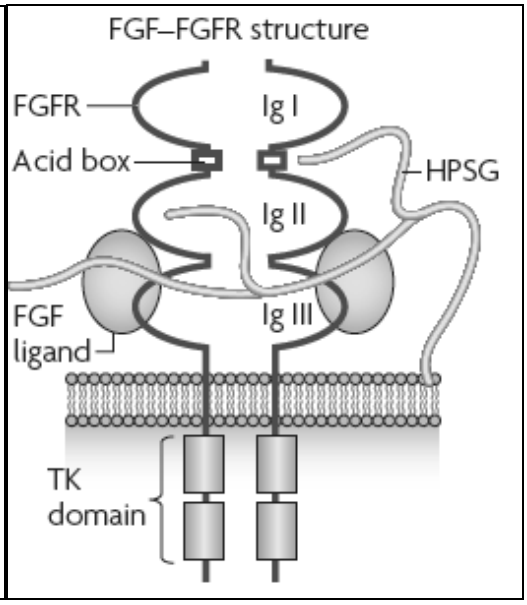


Figure 3b: The basic structure of FGF-FGFR complex **(Turner and Grose, 2010)**

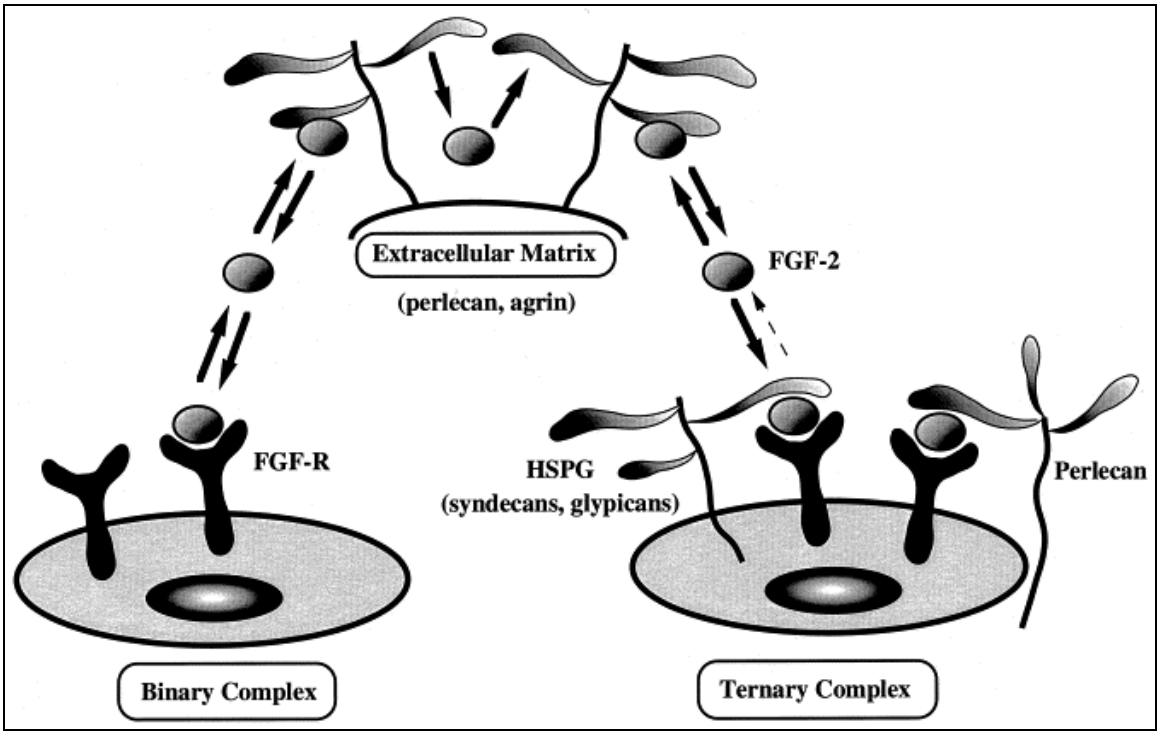


Figure 3c: Model of HSPG regulation of FGF2-FGFR complex (**Nugent and Iozzo, 2000**)

Table 2: Differential expression of FGFR in fish fin

RECEPTOR	EXPRESSION IN MATURE RAY	EXPRESSION IN REGENERATED RAY
FGFR1	Epidermis and mesenchyme	Mesenchymal intra to inter-ray transition area
FGFR2	No mesenchymal immunoreactivity Certain phenotypically distinguishable	Strong in outer LFCs No epidermal staining epidermal cells
FGFR3	Some mesenchymal cells surrounding the lepidotrichia lateral edge Slight at the epidermis of the ray area	Distal blastema and epidermis Acquires strong intensity in cells that contact actinotrichia and lepidotrichia. Disappears when these are restored
FGFR4	Not detected	Distal epidermis, in contact with a proliferative blastema

Among all the factors and neurotrophins that have been implicated, FGF2 is regarded as one of the most potent (**Ghosh and Greenberg, 1995; Temple and Qian, 1995; Vicario-Abejon *et al*., 1995; Kuhn *et al*., 1997; Vaccarino *et al*., 1999**). FGF-2 is released from cells in response to injury (**Ku and D'Amore, 1995**). FGF-2 and its family of receptors are widely distributed in the adult CNS (**Pettmann *et al*., 1986; Walicke *et al*., 1989; Wanaka *et al*., 1990**). Hippocampal neural progenitor cells in culture proliferate with only FGF2 supplementation (**Ray *et al*., 1993; Ghosh and Greenberg, 1995; Temple and Qian, 1995; Vicario-Abejon *et al*., 1995**). In addition, FGF-2 has been shown to reduce neuronal death after traumatic brain injury (TBI), cerebral ischemia, and seizures (**Mocchetti and Wrathall, 1995; Dietrich *et al*., 1996; Cuevas, 1997; Tretter *et al*., 2000**).

FGF2

In 1974, FGF2 was first identified as a partially purified activity (a 146-amino acid protein (**Bohlen *et al*., 1984**)) from bovine pituitary (**Jianwu *et al*., 2003**). In 1986, firstly, Abraham sequenced the cDNA of human FGF2 (**Jianwu *et al*., 2003)**.

Structure of FGF2

Growth factor FGF2 exists in several isoforms differing in their N-terminal extensions, subcellular distribution and function. The smallest, an 18kDa FGF2 low molecular weight (LMW) variant is released by cells and acts through activation of cell surface FGF-receptors,

whereas the high molecular weight (22, 22.5, 24 and 34 kDa), FGF2s localize to the nucleus and signal independently of FGFR (**Sorensen *et al*., 2006; Yu *et al*., 2007**). In short, it can be said that these multiple FGF2 isoforms localize to different subcellular compartments: the high molecular weight forms are nuclear, while the endogenous LMW form is primarily cytoplasmic (**Renko *et al*., 1990; Quarto *et al*., 1993**), and their different subcellular localizations might account for different functional roles (**Quarto *et al*., 1991**).

Using recombinant protein, several groups have determined the three-dimensional structure of crystalline 18-kDa FGF2 (**Ericksson *et al*., 1991; Zhu *et al*., 1991**). FGF2 contains 12 anti-parallel b-sheets organized into a trigonal pyramidal structure. Several domains may be important for FGF2 function. Residues 13–30 and 106–129 are believed to represent the receptor-binding sites (**Baird *et al*., 1988**, **Yayon, *et al*., 1993**).

Biological Roles of FGF2

FGF2 has been proposed to have an important function in the development and function of numerous organ systems **(Bikfalvi *et al*., 1997)**. FGF2 is a potent angiogenic molecule *in vivo* and *in vitro* stimulates smooth muscle cell growth, wound healing, and tissue repair (**Basilico and Moscatelli, 1992; Schwartz and Liaw, 1993**). In addition, FGF2 may stimulate hematopoiesis (**Bikfalvi and Han, 1994; Allouche and Bikfalvi, 1995**) and may play an important role in the differentiation and/or function of the nervous system (**Logan *et al*., 1991; Unsicker *et al*., 1992; Baird, 1994**), the eye (**McAvoy *et al*., 1991**), and the skeleton (**Riley *et al*., 1993; Fallon *et al*., 1994**). It has been isolated from various normal and malignant tissues (**Burges and Maciag, 1989; Rifkin and Moscatelli, 1989; Gospodarowicz, 1990; Bikfalvi *et al*., 1997**). This growth factor is one of the most potent inducers of formation of mesenchyme (**Slack *et al*., 1987**), angiogenesis (**Montesano *et al*., 1986; Folkman *et al*., 1988**), and caused rapid neovascularization in various tissues (**Davidson *et al*., 1985; Folkman and Klagsbrun 1987; Hayek *et al*., 1987; Rifkin and Moscatelli, 1989**). It stimulates the proliferation of all cell types involved in the wound healing process both *in vivo* and *in vitro* (**Wahl *et al*., 1989; Gospodarowicz, 1990**), and it increases the formation of granulation tissue *in vivo* (**Buntrock *et al*., 1982a; Buntrock *et al*., 1982b; Buntrock *et al*., 1984**). A brief summary of the functions of FGF2 in the development and function of numerous organ systems is outlined in the table (**Table 3**) below:

Table 3: Putative functions of FGF2 in different organ systems

ORGAN	PUTATIVE FUNCTION OF FGF2
Brain	Neuronal differentiation and survival
Blood vessel	Angiogenesis, smooth muscle cell proliferation, atherogenesis, blood pressure control
Lung	Branching morphogenesis, fibrosis
Limb	Limb development
Muscle	Myogenesis
Bone	Osseous healing, chondrogenesis
Eye	Photoreceptor survival and transduction
Skin	Melanogenesis, morphogenesis of the keratinocytes, tissue repair

FGF2-FGFR1 INTERACTIONS

FGF2 binds to FGFR1, a member of the tyrosine kinase family (**Simon, 2000**). FGF2 and FGFR1 form a dimeric complex which plays an important role in trans-membrane signal transduction. The binding of FGF2 to its receptors is enhanced by HSPG in cell surface and leads to activation of autophosphorylation of the FGFR on several tyrosine residues (**Jianwu *et al.*, 2003**). According to **Plotnikov *et al.*, 1999**, FGF2-FGFR1 forms a 2-fold (4-chain) symmetric dimeric complex, which is essential for activating the tyrosine-kinase functionality of FGFR1 in transmembrane signal transduction. FGFR1 contains two Ig-like domains as shown above in **Figure 3a** (say D2 and D3). Within each complex, FGF2 interacts extensively with D2 and D3 as well as with the linker between the two domains. The dimer is stabilized by interactions between FGF2 and D2 of the adjoining complex and by a direct interaction between D2 of each receptor. **Pellegrini *et al.*, 2000** further studied the crystal structure of the FGFR1 ectodomain in a dimeric form that is induced by simultaneous binding to FGF2 and a heparan decasaccharide. The complex is assembled around a central heparin molecule linking two FGF2 ligands into a dimer that bridges between two receptor chains. The asymmetric heparin binding involves contacts with both FGF2 molecules but only one receptor chain. The structure of the FGF2-FGFR1-heparin ternary complex provides a structural basis for the essential role of heparan sulphate in FGF signalling.

FGF2 IN EPIMORPHIC REGENERATION

Epimorphic regeneration in lizard is considerably regulated by fibroblast growth factor 2 **(Yadav *et al.*, 2008)**. As per **Bikfalvi *et al.*, 1997**, endogenous FGF2 plays role in cell growth, migration, and differentiation; important events during the process of epimorphic regeneration. FGF2 exerts pleiotropic effects during limb regeneration in urodele that includes angiogenesis and matrix remodelling. FGF2 may also play an important role in regeneration after injury of the CNS **(Logan and Berry, 1993)**. The proliferation and differentiation of normal human melanocytes are dependent on FGF2 **(Halaban *et al.*, 1992)**. Thus, FGF2 seems to be good evocate of wound healing and regeneration.

In one of the experiments carried out in our laboratory, it was found that FGF2 plays a vital role in the process of caudal regeneration in northern house gecko (**Yadav, 2005**). The follow up studies were undertaken in which, the signalling pathway of FGF2 was implicated in the regulation of early events of regeneration in lizards (**Anusree, 2012**). It was therefore thought worth investigating the role of FGF2 signalling in a regeneration model which is of a different evolutionary lineage. Hence, selected an anamniote model the teleost fish for the current study. The rationale was to understand whether the signalling pathways involved in epimorphic regeneration amongst vertebrates are evolutionarily conserved or not. In order to achieve this notion one targeted the FGF2 signalling.

Blocking the FGF2 signalling pathway via inhibition of tyrosine activity of its receptor would be of great restorative value. Studies by **Lefevre *et al.* (2009)** shown that FGF2, and their FGFR1 receptor were strongly expressed in the cell lines of primary uveal melanoma and cell proliferation in these lines were strongly reduced on experimental depletion of endogenous FGF2, immunoneutralization of secreted FGF2 and pharmacologic inhibition of FGFR1 (**Lefevre *et al.*, 2009**).

Based on the crystallographic studies of the catalytic domain of FGFR1 with indolinones (**Mohammadi *et al.*, 1997; Laird *et al.*, 2000; Sun *et al.*, 2000)** several classes of indolinones have emerged as inhibitors of various split kinases. SU5402 (**Fig: 4**) is one such indolinone that inhibits the tyrosine kinase activity of FGFR1 by interacting with its catalytic domain. SU5402 acts by binding specifically to the entire family of trans-membrane fibroblast growth factor receptors (FGFR). This binding prevents the function of all FGF ligands from activating their respective signal transduction pathways. SU5402 directly

interacts to the catalytic domain of FGFR1, a member of the receptor tyrosine kinase family and inhibits the phosphorylation activity of the receptor (**Simon, 2000**).

SU5402

SU5402 is a drug discovered at SUGEN, a biotechnology company which pioneered protein kinase inhibitors with compounds like SU5416, SU6668 and SU11248. The concept was of an ATP analog that would compete with ATP for binding to the catalytic site of receptor tyrosine kinases. This concept led to the invention of many small-molecule tyrosine kinase inhibitors including many cancer drugs. Also, considering the fact that it does not inhibit the phosphorylation of insulin receptors and exhibits no inhibitory effects on EGF receptor kinase, it is possible to hypothesize the use of SU5402 as antiproliferative and/or antiangiogenic agent to counteract the uncontrolled proliferation and neoangiogenesis in cancer. In perspective, an evaluation of the biological action of this drug is therefore, an essential prerequisite for its potential use in clinical applications. Hence, it is hoped that the results of the current study, in addition to the proposed objectives, might also help in evaluating the compound's (SU5402) impending use as an anticancer drug in time to come.

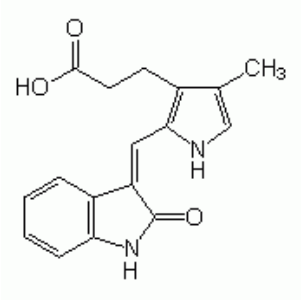


Figure 4: SU5402 (3-[3-(2-Carboxyethyl)-4-methylpyrrol-2-methyllidenyl]-2-indolinone)

PURPOSE OF THE STUDY

While observations of regeneration have been made in many species, the cellular and molecular mechanisms by which animals are able to successfully compensate for lost body parts are still poorly defined. Several regulatory molecules have been proposed to modulate the process of epimorphosis in vertebrates. However, studies conducted in our lab for the past decade points to a possible master regulator role in FGF2 signalling at least in an amniote model - the northern house gecko, *Hemidactylus flaviviridis* (**Yadav *et al.*, 2005**). Using this as a baseline, the influence of COX-2-Induced PGE_2 the upstream modulator of FGF2 on tail regeneration of *H. flaviviridis* was carried out, that proposed that the impairment of PGE_2 significantly hampered the recruitment and proliferation of blastemal cells (**Sharma and**

Suresh, 2008). The follow up studies proved that FGF2 indeed is initiated by PGE_2 and the FGF2 signalling indeed has a stage specific influence on the progress of tail regeneration in the lizard significantly influencing a whole gamut of events during the wound epithelium and blastemal stages (**Anusree**, **2012**). In order to test whether FGF2 plays a similar role in other group of vertebrates with regenerative abilities, the study was extended to teleost fish. This comparative study will reveal whether the mechanism of epimorphic regeneration is evolutionarily conserved or not. Also understanding the finer mechanisms by which FGF2 holds the process of epimorphic regeneration in such a controlled manner and can be targeted for novel drug discovery programs.

As mentioned elsewhere, FGF2 transduce their signal by binding to a respective FGFR1 which has an intracellular protein tyrosine kinase domain. The tyrosine kinase domain is activated upon FGF binding, resulting in the activation of a transcription factor by means of a signal transduction cascade. To study the effect of FGF2 signalling during regeneration, we interfered with FGF2 signalling by treating the amputated fins of teleost fish *P. latipinna*, with SU5402, a FGF2 receptor inhibitor. SU5402 directly interacts to the catalytic domain of FGFR1 and inhibits the phosphorylation activity of the receptor (**Edel *et al.*, 2010, Lamont *et al.*, 2011**). The interaction of FGF2 and SU5402 with FGFR1 is depicted in **Fig: 5**.

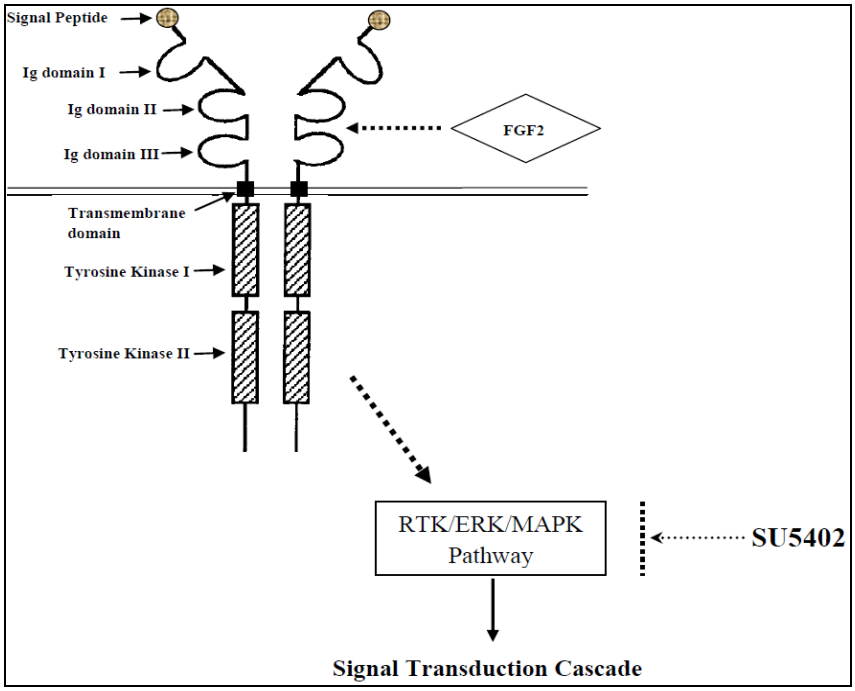


Figure 5: FGFR1 inhibition by synthetic drug SU5402

OBJECTIVE OF THE STUDY

The overall objective of the present work was to elucidate whether FGF2 signalling evoke a comparable response during fish fin regeneration as that of other vertebrate models and if yes then the possible role(s) the FGF2 signalling play in the initiation and progression of caudal fin regeneration in teleost fish *Poecilia latipinna*.

This was achieved through the following five specific aims using *P. latipinna* wherein the tail was partially amputated as described in the section **material and methods** and treated with SU5402 to suppress the FGF2 signalling.

1. Identify the spatial and temporal expression pattern of FGF2 in the regenerate.
2. Find out stage specific role of FGF2 in the initiation and progression of epimorphic regeneration.
3. Evaluate the existence of any regulatory role the FGF2 play in upregulating the protease activity at the site of amputation.
4. Unearth its role on cellular synthetic and proliferative activities during regeneration.
5. Study the role of FGF2 signalling in the growth and differentiation of various tissues in the regenerate.

MATERIAL AND METHODS

MAINTENANCE OF TELEOST FISH *POECILIA LATIPINNA*

Sailfin Molly, *Poecilia latipinna* (Lesueur, 1821), of both the sexes of same age (size 4-5cm), weighing about 4-5g were purchased from a commercial supplier and maintained in glass aquariums containing sterile dechlorinated water with constant aeration at the animal facility of Department of Zoology, The M.S. University of Baroda (827/ac/04/CPCSEA). All the animals were initially quarantined, screened for parasitic infections and only the healthy ones were used for the experiment after at least one week of purchase. The daily photoperiod was 12h (hours) of light and 12h of darkness, and the water temperature was kept in the range of 26±2°C. About 10% of the aquarium water was changed every day and replaced with fresh charcoal and UV purified water (with Aquaguard). Handling and processing of fish were carried out according to the ethical principles (Drugs and Cosmetics Rules, 2005) approved by the Institutional Animal Ethics Committee (IAEC) [Form B No. ZL/IAEC/15-2010] constituted as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

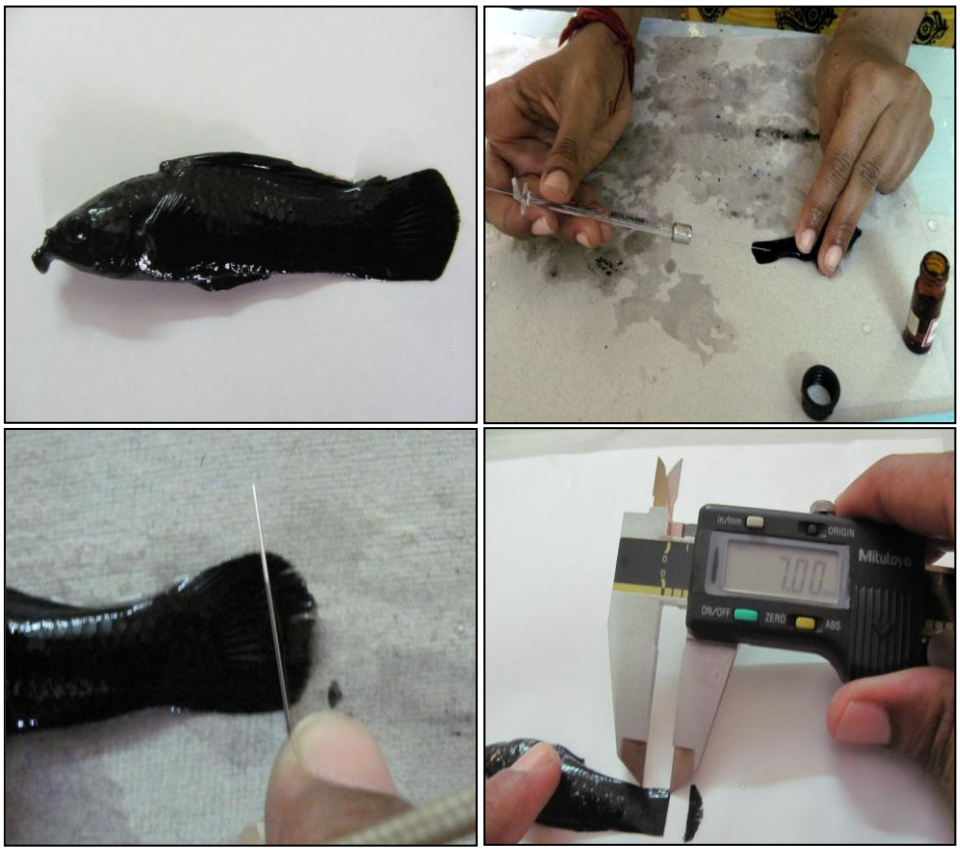
DRUG DOSAGE AND EXPERIMENTAL SETUP

A 5mM stock solution of SU5402 in 1%DMSO was prepared and stored at 4ºC. Dose was prepared freshly from the stock solution for each of the experiments. Healthy fishes of both the sexes were randomly allocated into groups named control and treated. Control fishes were injected with 1%DMSO and treated ones with 2µM/g body wt. of SU5402 at a maximum quantity of 10µl/animal in the caudal fin near to the site of amputation. The fins reaching the three consecutive stages i.e. wound healing, blastema and differentiation were collected as per requirement of each of the experiments done. The animals were acclimated a week before the beginning of any experiment. Only those animals of each group that reached a specific stage on same day were selected and further processed as per the experimental protocol.

DOSE INJECTION, CAUDAL FIN AMPUTATION AND MEASUREMENTS

The treatment in each group started a day before amputation and was continued till the animals reached differentiation stage. Fin amputations were made with disposable sterile stainless steel surgical blade (Size 11, Kehr Surgical Private Limited, India). The dose was

given using micro syringe (25µL, Hamilton Bonaduz AG, Switzerland) on the tail muscles at fixed time intervals. Tail measurements were done using a digital calliper (Mitutoyo, Japan).



A pictorial depiction of experimental protocol

IMMUNOHISTOCHEMICAL LOCALIZATION OF BASIC FIBROBLAST GROWTH FACTOR (FGF2)

FGF2 localization in the Control and Treated regenerating fins

For validating the roles of FGF2 during epimorphic regeneration of the caudal fin of *P. latipinna*, it was essential to first localize the FGF2 in the regenerating outgrowth through Immunohistochemistry (IHC).The fishes were amputated at each of the specific stages, embedded in optimal cutting temperature medium (Tissue-Tek OCT, Sakura Finetek, USA) and immediately sectioned longitudinally (9µm) by IEC make cryostat (-20ºC) on clean glass slides. The sections were then air-dried for about 15 minutes, and fixed with ice-cold acetone for about 10 minutes. The sections were again air dried at room temperature overnight and stored in a sealed slide box at -20°C for later use.

Principle:

The method involves an unlabelled primary antibody (first layer), which reacts with tissue

antigen, and a labelled secondary antibody (second layer) which reacts with the primary antibody. Secondary antibody is coupled with peroxidase. This reacts with 3, 3'-diaminobenzidine (DAB) to produce brown staining (a process known as DAB staining). Hence, the positive staining is judged by the intensity of the brown pigmentation of the specimen.

Materials:

Optimal Cutting Temperature (OCT) compound

Acetone (as fixative)

10mM phosphate buffered saline (PBS) pH 7.4

0.26g Potassium dihydrogen phosphate (KH_2PO_4)

2.17g Disodium hydrogen phosphate ($Na_2HPO_4.7H_2O$)

8.71g Sodium Chloride (NaCl)

800ml Double distilled water (ddH_2O)

Adjust pH to 7.4 and bring volume to 1L with ddH_2O

0.3% Hydrogen peroxide (H_2O_2) in PBS

Blocking buffer (10% foetal bovine serum (Genei, Merck, USA) in PBS)

Antibody dilution buffer (0.5% bovine serum albumin in PBS)

Primary antibody (rabbit anti-goat anti-FGF2 (Sigma-Aldrich, USA), dilution of 1:200)

Biotinylated secondary antibody (goat anti-rabbit (Sigma-Aldrich, USA), dilution of 1:100)

Streptavidin-Horseradish peroxidase (SAV-HRP) conjugates (dilution of 1:100)

DAB (3, 3'-Diaminobenzidine) substrate solution (freshly made just before use: 0.05% DAB - 0.015% H_2O_2 in PBS)

Method:

The frozen sections were air-dried for about 20 minutes at room temperature. Endogenous peroxidise activity was blocked with 0.3% H_2O_2 and nonspecific binding was blocked with normal goat serum. Appropriately diluted primary antibody were added to the sections and incubated in a humidified chamber overnight at 4°C. This was followed by the addition of appropriately diluted secondary antibody and incubation in a humidified chamber for 60 minutes. Later on the pre-diluted SAV-HRP conjugates were added to the slides and incubated in a humidified chamber at room temperature for 30 minutes. Freshly made DAB substrate solution was applied to reveal the colour of antibody staining. The slides were rinsed twice with PBS for 5 minutes each after each of the above mentioned steps. The tissue sections were dehydrated appropriately and the colour of the antibody staining in the tissues was observed under Leica DM2500 Microscope and pictures captured using EC3 Camera

utilizing Leica LAS EZ (V 1.6.0) software. Same protocol was followed for negative control sections except that these were incubated with PBS-BSA instead of the primary antibody.

MORPHOMETRIC STUDIES

Observing the effect of SU5402 on fin morphometry

After conformation of FGF2 localization in the regenerating fins, further experiments were aimed at finding out how the receptor inhibitor SU5402 alters the rest of the regenerative processes at each level. Therefore, many other studies were taken one by one. Meanwhile, following a dose range study a dose of 2µm/g body wt. of SU5402 had shown quite a considerable effect in reducing the FGF2 concentration as compared to the controls, however, one more dose of a still lower concentration i.e. 1µm/g body wt. was also tested for its effect. However, on comparing the results obtained we felt that the dose of 2µm/g body wt. to be more suitable in hampering the FGF2 signalling and hence, for further studies, the dose was ultimately finalized to 2µM/g body wt.

Materials:

SU5402 (Calbiochem®, EMD Biosciences, USA)

1% Dimethyl Sulfoxide (DMSO)

Method:

The animals in total were divided into 3 groups. One control and the other two as treated. The 3 groups were named as A, B and C. Group A was control, whereas B and C was treated group, where B was the low dose group and C was high dose group. Group A was injected with 1%DMSO alone; whereas group B and C received injection of 1µM/g body wt. and 2µM/g body wt. of SU5402 (in 1%DMSO) respectively at a maximum quantity of 10µl/animal.

Prior to amputation fishes were anaesthetised by immersing in water containing 0.2mg/ml Tricaine (3-aminobenzoic acid ethyl ester methanesulfonate) as per **Shao *et al*. (2009)**. The tail fins of all the animals were amputated for approximately 30% of their total length using sterile surgical knife and undisturbed regeneration of the fins was allowed. All the animals of the control group, i.e. Group A were injected with 1%DMSO. Group B was the Low Dose group and was injected 1µM/g body wt. of SU5402 and Group C or the High Dose Group received 2µM/g body wt. of SU5402. The injections were given daily in the tail muscles with a microsyringe at fixed time till the fins reached the differentiation stage. Regeneration rate

was studied by digital photographs taken every day and length variation was recorded using a calibrated digital calliper (Mitutoyo, Japan).

EXTRACTION AND ESTIMATION OF NUCLEIC ACIDS

Evaluation of the DNA and RNA content in the fin regenerates of the control and SU5402 treated fishes

Fin regenerate was harvested from the control and treated fishes at the three consecutive stages of regeneration (wound healing, blastema, differentiation) after a dosing of 1% DMSO and 2µM/g body wt. of SU5402. The fins were homogenized for 10% and extraction of nucleic acids was done by the method described by **Schneider (1957)**.

Principle:

This is a method for separation and preparation for quantitative analysis of RNA, DNA and protein in tissues. It is based on the preferential solubility of nucleic acids in hot trichloroacetic acid (TCA).

Materials:

0.25M Sucrose Solution

Trichloroacetic acid (TCA)

Ethanol

Ether

1N Potassium Hydroxide (KOH)

6N Hydrochloric acid (HCl)

Method:

10% homogenate of tissue was prepared in 0.25M sucrose at 4ºC. This was treated with 10% TCA, allowed to stand for 30 minutes at 4ºC and centrifuged at 10000 rpm for 20 minutes. Precipitate was treated with ethanol, allowed to stand at room temperature for 30 minutes and centrifuged at 10000 rpm for 15 minutes at 4ºC. This step was repeated and precipitate was treated with a 9:3 ethanol ether mixture and heated in a 60ºC water bath for 30 minutes and centrifuged at 10000 rpm for 15 minutes. Precipitate was suspended in 1N KOH and incubated in a 37ºC water bath for 20h. It was treated with 6N HCl and 5% TCA, allowed to stand for 30 minutes 4ºC and centrifuged at 10000 rpm for 20 minutes at 4ºC. The supernatant contains hydrolyzed RNA and was estimated. The precipitate was suspended in 5% TCA and heated in a 90ºC water bath with occasional stirring for 20 minutes. This was cooled to 4ºC, allowed to stand for 30 minutes at 4ºC and centrifuged at 10000 rpm for 20 minutes at 4ºC. Supernatant contains the DNA and was estimated.

ESTIMATION OF DNA

Estimation of DNA was done by the DPA (Diphenylamine) method (**Sadasivam and Manickam, 1992**).

Principle:

Under extreme acid conditions DNA is initially depurinated quantitatively followed by the dehydration of sugar to ω-hydroxylevulinylaldehyde. This aldehyde condenses in acidic medium with diphenyl amine (DPA) to produce a deep blue colored condensation product with absorption maximum at 595nm. The intensity of the blue colour measured at 595nm is directly proportional to the concentration of sugars cleaved from DNA strands during the chemical treatment with DPA.

Materials:

DNA standard (Calf thymus)

DNA samples (Regenerating caudal fins)

Saline citrate/Citrate buffer saline, pH 7.0

8.0g of Sodium Chloride (NaCl)

0.2g of Potassium Chloride (KCl)

10.51g of Citric acid (monohydrate)

Adjust pH to 7.0 with Sodium Hydroxide (NaOH)

Diphenyl amine (DPA) reagent (100ml)

3g pure DPA

100ml Glacial acetic acid

2.5ml conc. Sulphuric acid (H_2SO_4)

Method:

A set of DNA standards was prepared ranging in concentration from 100-500µg in standard saline citrate. 0.1ml of each DNA standard and sample was mixed with 0.2ml of DPA reagent (freshly made every time before use) and kept in boiling water bath for 10 minutes. Sample DNA was also dissolved in standard saline citrate. The absorbance of blue solution was read at 600nm against blank. A standard graph for DNA was plotted and this was used to determine the DNA content present in the unknown sample.

ESTIMATION OF RNA

Estimation of RNA was done by Orcinol method (**Sadasivam and Manickam, 1992**)

Principle:

Acid hydrolysis of RNA releases ribose sugar, which in presence of strong hot acid under

goes dehydration to form furfural. This furfural, in the presence of $FeCl_3$ as catalyst, reacts with orcinol and produces green coloured compound which has maximum absorbance at 665nm.

Materials:

RNA standard (Baker's yeast)

5% Perchloric acid ($HClO_4$)

Orcinol acid reagent

6% Alcohol orcinol (6g orcinol in 100ml alcohol)

Method:

A set of RNA standards ranging in concentration from 5-50µg/ml was prepared in 5% $HClO_4$. 0.1ml of each standard and sample was mixed with 0.2ml of orcinol acid reagent and 0.013ml of 6% alcohol orcinol. This was heated in a boiling water bath for 20 minutes. Absorbance was read at 660nm against the blank. A standard graph for RNA was plotted and was used to determine the RNA content of the unknown samples.

PROTEIN ESTIMATION

Determination of protein content in the fins of control and SU5402 treated

Fin regenerate at 3 specific stages (wound healing, blastema and differentiation) was harvested from the control and treated fishes after dosing as mentioned above; and 10% homogenate was made using PBS and lysis buffer (1:1); cold centrifuged at 8000 rpm for 10 minutes and the supernatant was then used for the estimation of protein content by the Bicinchoninic acid (BCA) assay kit (Genei, Merck, USA), also known as the Smith assay, after its inventor, Paul K. Smith (**Smith*, et al.*, 1985**).

Principle:

Protein assay based on Bicinchoninic acid (BCA) is a most sensitive and detergent compatible method for the colorimetric detection and quantitation of total protein. The BCA assay primarily relies on two reactions. Firstly, the peptide bonds in protein reduce Cu^{2+} ions from the cupric sulphate to Cu^{+} (a temperature dependent reaction). The amount of Cu^{2+} reduced is proportional to the amount of protein present in the solution. Next, the two molecules of bicinchoninic acid chelate with two Cu^{+} ions to form a purple-coloured product. This water-soluble complex exhibits a strong absorbance at 562nm.

Materials:

Protein standard

Bovine Serum Albumin (BSA)

BCA Stock solution

PBS

Method:

A set of BSA standard ranging in concentration from 50µg-1000µg/ml was prepared. 0.02ml of each standard as well as sample was mixed with 0.2ml of BCA working reagent. All the standards and samples were incubated at 37ºC for 30 minutes. After incubation absorbance was read at 562nm within 10 minutes. A standard curve of BSA was plotted and using the curve the protein concentration for each unknown sample was determined.

SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Expression of protein in the regenerating fins of the control and treated fishes

To determine alterations in protein expression pattern and the stage specific effect of SU5402 during regeneration, SDS-PAGE profiling of the fin regenerates for control and treated groups at all the three defined stages, *viz*., wound healing, blastema and differentiation, as well as on 15 days post amputation (dpa) was done. Fins from each group were pooled, homogenized (10% homogenate), estimated for protein by BCA assay as explained above, and 12µg of protein was loaded in each well for the SDS-PAGE procedure.

Principle:

The molecular weight of protein maybe estimated if they are subjected to electrophoresis in the presence of a detergent Sodium Dodecyl Sulfate (SDS). SDS disrupts the secondary, tertiary and quaternary structure of the protein to produce a linear polypeptide chain coated with negatively charged SDS molecules. If the proteins are denatured and put into an electric field, they will all move towards the positive pole at the same rate, with no separation by size. Hence, we need to put the proteins into a matrix that will allow different sized proteins to move at different rates. The matrix of choice is polyacrylamide, which is a polymer of acrylamide monomers. When this polymer is formed, it turns into a gel and we will use electric potential to pull the proteins through the gel so the entire process is called Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Materials:

1.0M Tris-HCl, pH 6.8 (for stacking gel)

1.5M Tris-HCl , pH 8.8 (for resolving gel)

10% SDS (Electrophoresis grade)

Resolving gel (amounts for 12.5% gels, 5ml)

1.64ml ddH_2O

2.00ml 30% (29:1) Acrylamide/Bis-Acrylamide

1.26ml 1.5M tris-HCl, pH 8.8

100µl 10% (w/v) SDS

50µl 10% (w/v) Ammonium persulphate (APS), made fresh on the day of use

2µl TEMED (N,N,N′,N′-Tetramethylethylenediamine)

Stacking gel (amounts for 5% gels, 2ml)

1.4ml ddH_2O

0.332ml 30% (29:1) Acrylamide/Bis-Acrylamide

0.252ml 1M tris-HCl, pH 6.8

20µl 10% (w/v) SDS

20µl 10% (w/v) Ammonium persulphate (APS), made fresh on the day of use

2µl TEMED (N,N,N′,N′-Tetramethylethylenediamine)

1X SDS Gel sample loading buffer

50mM tris HCl (pH6.8)

100mM dithiothreitol

2% SDS

0.1% Bromophenol blue

10% glycerol

5X Gel running buffer, pH 8.3

25mM Tris base

250mM glycine

0.2% SDS

Gel staining (Silver staining)

Fixative Solution: 30% methanol and 10% glacial acetic acid

20% $AgNO_3$

2.5% Na_2CO_3

40% formaldehyde

Method:

The glass plates were assembled properly as per instructions on the manual, and the whole clamp was adjusted on the casting stand. Then the Resolving gel was casted allowing the gel to polymerize for 45 minutes to one hour, followed by the casting of Stacking gel.

Meanwhile the samples were prepared. After the gel was polymerized completely, samples and the molecular weight markers were loaded in the wells and the gel was allowed to run at a constant current of ~100V. The run is considered completed after the tracking dye reaches the bottom. The gel was then removed from the unit, the glass plates were separated carefully and the gel was taken into a container containing fixative solution and was kept overnight. The gels were then stained with 20%$AgNO_3$ and developed using ice cold Na_2CO_3 and 40% formaldehyde. After the bands appear reaction was stopped using 7% glacial acetic acid. Gels were analyzed by Gel Doc (GeNei, Doc-ItLs software).

IMMUNOLOCALIZATION OF MMP2 AND MMP9

Gelatinases (MMP2 and MMP9) localization in the regenerating fins of the control and treated fishes 1dpa

MMP2 (gelatinase A) and MMP9 (gelatinase B) are considered to be the major modulators of the extracellular matrix reorganization occurs primarily during the wound healing period. Therefore, it was thought pertinent to observe the activity of these geltinases during this stage. The animals were grouped to 2 batches, one being control and other treated. Both the groups received injections of 1%DMSO (vehicle) and SU5402 respectively at the dose of 2µM/g body wt., with each animal receiving not more than 10µl of the dose. Dosing started a day before amputation, and the dose was injected on the day of amputation too. Later the fin having the wound epithelium was excised and longitudinal sections (9µm) were taken on clean glass slides by IEC cryostat (-20ºC). The slides were air-dried for 15 minutes, fixed with ice-cold acetone for about 10 minutes, air-dried again at room temperature overnight and then stored in a sealed slide box at -20°C for later use.

Principle: This procedure uses the techniques of targeting the antigen (MMP2/MMP9 in this case) using the right antibodies (anti-MMP2/MMP9). A primary antibody against the antigen of interest is supplemented followed by treatment with Fluorescein isothiocyanate (FITC) labelled secondary antibody that forms a complex with the former.

Materials:

Primary antibody (rabbit anti-MMP2, dilution of 1:100/ goat anti-MMP9 (Sigma-Aldrich, USA), dilution of 1:100)

FITC conjugated Secondary antibody (goat anti-rabbit, dilution of 1:500/ rabbit anti goat (Genei, Merck, USA), dilution of 1:500)

Method:

The frozen sections were fixed in acetone at -20ºC for 15-20 minutes and air dried for 20

minutes. Sections were then rehydrated with PBST (Phosphate Buffered Saline with 0.025% Tween-20) followed by blocking with corresponding normal serum [Genei, Merck, USA; 10% in PBS with 0.5% Bovine serum albumin (PBS-BSA)] for 1-2 hours at room temperature (RT). Sections were then incubated with appropriate primary antibody (rabbit anti-MMP2, dilution of 1:100/ goat anti-MMP9 (Sigma-Aldrich, USA), dilution of 1:100) overnight inside a moist chamber at 4ºC. Following day, sections were washed with PBST thrice for 5 minutes each and incubated with a corresponding FITC conjugated secondary antibody [1:50 dilution of Goat Anti-Rabbit/Rabbit Anti-Goat IgG-FITC respectively] for 2 hours at RT. Sections were then washed with PBS thrice for 5 minutes each and mounted in 1:1 mixture of PBS:glycerol and observed using a fluorescent microscope (Leica DM2500). Negative control sections were also similarly incubated but with PBS-BSA in place of the primary antibody.

GELATIN ZYMOGRAPHY

Expression of Matrix metalloproteinases MMP2 and MMP9 of the Experimental fins at Wound Healing Stage

This technique involves the electrophoresis of secreted protease enzyme through discontinuous polyacrylamide gels containing substrate. After electrophoresis removal of SDS from the gel by washing in 2.5% Triton X100 solution, followed by incubation of 18h in renaturing buffer which allows enzymes to renature and degrade the protein substrate. Staining of the gel with Coomassie Brilliant Blue allows the bands of proteolytic activity to be detected as clear bands of lysis against a blue background.

Principle:

The separation occurs in a polyacrylamide gel containing a specific substrate that is co-polymerized with the acrylamide (**Heussen and Dowdle, 1980; Fernandez-Resa *et al*., 1995**). During electrophoresis, the SDS causes the MMPs to denature and become inactive. The activation of latent MMPs during zymography is believed to involve the "cysteine switch" because the dissociation of Cys73 from the zinc molecule is caused by SDS. After electrophoresis, the gel is washed, which causes the exchange of the SDS with Triton X100, after which the enzymes partially renature and recover their activity (**Heussen and Dowdle, 1980; Woessner, 1995**). Additionally, the latent MMPs are autoactivated without cleavage (**Oliver *et al*., 1997**). Subsequently, the gel is incubated in an appropriate activation buffer. During this incubation, the concentrated, renatured MMPs in the gel will digest the substrate (**Fernandez-Resa *et al*., 1995; Hawkes *et al*., 2001**). After incubation, the gel is stained with

Coomassie blue, and the MMPs are detected as clear bands against a blue background of undegraded substrate (**Fernandez-Resa *et al*., 1995; Hawkes *et al*., 2001**). The clear bands in the gel can be quantified by densitometry (**Woessner, 1995**). The zymography is based on the following principles: (i) during electrophoresis, gelatin is retained in the gel; (ii) MMP activity is reversibly inhibited by SDS during electrophoresis; and (iii) the SDS causes the separation of MMP-TIMP complexes during electrophoresis. This enables the detection of MMPs and TIMPs independently of one another (**Hawkes *et al*., 2001**).

Materials:

Gelatin stock solution (10mg/ml in ddH_2O)

Zymogram Renaturing Buffer

Triton X-100, 25% (v/v) in ddH_2O

Zymogram Developing Buffer

50mM Tris Base

Tris HCl

0.2M NaCl

5mM $CaCl_2$

0.02% Brij 35

ddH_2O

Coomassie blue R250 staining solution (100ml)

0.25g Coomassie brilliant blue R250

90ml Methanol: H_2O (1:1v/v)

10ml Glacial acetic acid

Coomassie blue R250 destaining solution (100ml)

Methanol: Glacial acetic acid: ddH_2O (50:10:40)

Method:

12% gel was made according to standard procedures of SDS-PAGE as explained above. When preparing the running gel gelatin stock solution (10mg/ml in ddH_2O) was added to get the gelatin concentration of 0.1% (1mg/ml). Samples were applied and the gel was run with Tris-Glycine SDS Running Buffer according to the standard running conditions (~100V, constant voltage). The run is complete when the bromophenol blue tracking dye reaches the bottom of the gel. After running, the gel is incubated with zymogram renaturing buffer with gentle agitation for 30 minutes at room temperature. The gel was then incubated with zymogram developing buffer for approximately 4 hours. The gel was then stained with Coomassie blue R250 for 30 minutes, followed by destaining with an appropriate Coomassie

R250 destaining solution. Areas of protease activity appear as clear bands against a dark blue background where the protease has digested the substrate.

BROMODEOXYURIDINE INCORPORATION

Observation of Cell proliferation in the regenerating fins of the Control and Treated animals at Blastema Stage 5 days post amputation

Principle:

Bromodeoxyuridine (BrdU) is an analog of the DNA precursor thymidine. In proliferating cells the DNA has to be replicated before division can take place, which occurs during the S phase. If BrdU is injected at this stage, the cells would incorporate it into their DNA just like they would incorporate thymidine. The amount of BrdU in the DNA of the cells can be detected using specific anti-BrdU antibodies immunocytochemically. The patterns of cell proliferation, during the early steps of the regeneration process, were analyzed following injection of fish with BrdU during the Blastemal stage as this stage is considered as the most proliferative stage during fin regeneration.

Materials:

BrdU stock solution (50mg/ml BrdU prepared in Hank's solution)

Hank's solution or Hank's Buffered Salt solution (HBSS)

- 0.137M Sodium Chloride (NaCl)
- 5.4mM Potassium Chloride (KCl)
- 0.25mM Disodium hydrogen phosphate (Na_2HPO_4)
- 0.44mM Potassium dihydrogen phosphate (KH_2PO_4)
- 1.3mM Calcium Chloride ($CaCl_2$)
- 1.0mM Magnesium Sulphate ($MgSO_4$)
- 4.2mM Sodium bicarbonate ($NaHCO_3$)

Bouin's fixative

Primary antibody (mouse anti-BrdU, dilution of 1:500)

FITC tagged Secondary antibody (goat anti-mouse, dilution of 1:500)

Method:

The fishes were injected with 2µM/g body wt. of SU5402 and 1%DMSO (vehicle), serving as treated and control respectively. The treatment was started a day prior to amputation and was continued for 5 days post amputation till both the fishes reached the blastemal stage. A stock concentration of BrdU of 50mg/ml BrdU was prepared in sterile Hank's solution as per

Shao *et al*., 2009. Then the fishes were injected with 250µg/g body weight BrdU 5dpa. Six hours post amputation, the caudal fin were amputed and fixed in Bouin's fluid. Slides of the tissue sections were prepared and treated with primary mouse anti-BrdU at a dilution of 1:500 and secondary goat anti mouse antibody at a dilution of 1:500. FITC was used to visualize and photograph BrdU incorporation. Photographs were taken with the Leica DM 2500 fluorescent microscope fitted with EC3 camera. Negative control sections processed the above way but were incubated with PBS-BSA instead of the primary antibody.

HISTOLOGIC PROCESS FOR LIGHT MICROSCOPY

Staining the regenerating cells in the caudal fin during different stages of regeneration

Haematoxylin and eosin stain, a popular staining method in histology, involves application of hemalum, which is a complex formed from aluminium ions and oxidized Hematoxylin. This colors nuclei of cell blue. The nuclear staining is followed by counterstaining with an aqueous or alcoholic solution of eosin Y, which colours other eosinophilic structures in various shades of red, pink or orange.

Principle: The oxidation product of haematoxylin is haematin, and is the active ingredient in the staining solution. Haematin exhibits indicator-like properties. In acidic conditions, haematin binds to lysine residues of nuclear histones by linkage via a metallic ion mordant. To ensure saturation of chemical binding sites, the stain is applied for longer time than necessary, resulting in the overstaining of the tissues with much non-specific background colouration. This undesirable colouration is selectively removed by controlled leaching in an alcoholic acidic solution, (acid alcohol), the process being termed 'differentiation'. Differentiation is arrested by returning to an alkaline environment, whereupon the haematin takes on a blue hue, the process of "blueing-up". The haematin demonstrates cell nuclei. Full cellular detail is obtained by counterstaining with the eosin. Eosin is pink and stains proteins nonspecifically. Thus, in a typical tissue the nuclei are stained blue, whereas the cytoplasm and extracellular matrix have varying degrees of pink staining.

Materials:

Bouin's fixative
Xylene
Different grades of alcohol (70%, 80%, 95% and 100%)
Acid Ethanol (1ml concentrated HCl + 400ml 70% ethanol)
Harris haematoxylin
Eosin
Histological mounting medium: DPX (Distrene, dibutyl Phthalate, Xylene)

Method:

Fins of all the 3 stages, after appropriate dosing were fixed for 12h in Bouin's fluid, decalcified for 6h in 10% Ehylenediamine tetraacetic acid (EDTA) and embedded in paraffin after proper tissue processing. Longitudinal sections (thickness, 6µm) were cut from paraffin blocks using a microtome (Leica *RM 2155*) and collected on glass slides. Sections were dewaxed in xylene, hydrated in a descending alcohol series, and stained by a routine Hematoxylin-Eosin (HE) staining technique as explained below.

The slides containing paraffin sections of caudal fin were processed as follows:

Deparaffinizing and rehydrating sections:

3 x 3 minutes Xylene

3 x 3 minutes 100% Ethanol

1 x 3 minutes 95% Ethanol

1 x 3 minutes 80% Ethanol

1 x 5 minutes ddH_2O

The Sections were then blotted carefully to remove the excess of water before going into hematoxalin.

Hematoxalin staining:

1 x 3 minutes Hematoxalin

Rinsed in deionized water

1 x 5 minutes Tap water (to allow stain to develop)

8-12x (fast) Acid ethanol dips (to destain)

Rinsed 2 x 1 minutes Tap water

Rinsed 1 x 2 minutes ddH_2O

The Sections were again blotted carefully to remove the excess of water before going into eosin.

Eosin staining and dehydration:

1 x 30 seconds Eosin

3 x 5 minutes 95% Ethanol

3 x 5 minutes 100% Ethanol

3 x 15 minutes Xylene

After the slides were cleared properly in xylene, a drop of DPX was placed on the slide using a glass rod, taking care to leave no bubbles, and was covered gently with a cover slip. The

DPX was allowed to spread beneath the cover slip covering all tissues. It was then observed under the microscope (Leica DM2500) and photographed as described elsewhere.

STATISTICAL ANALYSIS

The data were subjected to Bartlett test for homogeneity and the significance level of the treatment groups with control group was evaluated through Student's 't' test with 95% confidence limit. The values are expressed as either Mean ± SEM or as Mode with range in parenthesis. For multiple group comparison and difference between the groups the data was subjected to One Way Analysis of Variance (ANOVA) followed by Duncan's Multiple Range Test using SPSS-PC Statistical Analysis Package (SPSS 12.0, SPSS Inc, Chicago, IL). A 'p' value of 0.05 or less was accepted as being statistically significant. Graphs are prepared by using Origin 7.0 SRO Origin Lab Corporation One Round House Plaza, Northampton MA USA.

CHAPTER 1

UNRAVELLING THE SPATIAL AND TEMPORAL EXPRESSION PATTERN OF FIBROBLAST GROWTH FACTOR2 (FGF2) IN THE REGENERATING CAUDAL FIN OF *POECILA LATIPINNA*

INTRODUCTION

Regeneration is a dynamic developmental process in which adult animals reconstruct body parts lost or damaged by injury. Repair and regeneration are universal phenomena in the biological world, but the capacity for regeneration varies considerably among species (**Yoshinari and Kawakami, 2011**). However, in most instances mammals are capable only of incomplete regeneration. In humans, for instance, the regeneration of lost parts is observed only in adult liver and infant finger tips (**Carlson, 2005; Han *et al.*, 2005**). In striking contrast, the teleost fish like zebrafish can regenerate its spinal cord tissue (**Becker *et al.*, 2004; Bareyre, 2008; Reimer *et al.*, 2008, Goldshmit *et al.*, 2012**), heart muscle (**Poss *et al.*, 2002a; Raya *et al.*, 2004; Schnabel *et al.*, 2011; Yin *et al.*, 2012**), retina (**Qin *et al.*, 2011**) as well as amputed fins (**Johnson and Weston, 1995; Stewart *et al.*, 2009; Lee *et al.*, 2010; Azevedo *et al.*, 2011; Blum and Begemann, 2012; Sing *et al.*, 2012**). In spite of these variations, we may recognize a sort of basic principle shared by many types of regeneration phenomena. For example, the detection of a wound, closure of the epithelial opening, activation of cell division and supply of new cells, cell differentiation, and morphogenesis are common elements in many types of tissue restoration processes (**Yoshinari and Kawakami, 2011**). Understanding the cellular and molecular mechanisms by which lower vertebrate model systems are able to faithfully regenerate complex organs will help illuminate potential therapies for diseases of organ damage in humans. Because of its spectacular nature and therapeutic implications, the regeneration of major appendages in non-mammalian vertebrates like teleost fish has been a subject of scientific inquiry for centuries (**Lee *et al.*, 2009**).

Teleost fish fins provide a favourable system for dissecting the regulatory niche of regeneration. Adult zebrafish regenerate their caudal fins within fourteen days after amputation (**Geraudie *et al.*, 1995; Poleo *et al.*, 2001; Poss *et al.*, 2003; Andreasen *et al.*, 2007**) through the process of epimorphic regeneration also called as “True regeneration”. The

caudal fin regeneration is the well-studied model for dissecting the molecular signalling that controls regenerative growth and angiogenesis (**Poss *et al*., 2003; Bayliss *et al*., 2006**). Comparative genomics indicate significant genetic conservation between mammals and lower vertebrates (**Stoick-Cooper *et al*., 2007a**), which beseech the question: what are the molecular differences that permit tissue regeneration in lower vertebrates, and make mammalian tissues recalcitrant to regeneration? Answers to this question will provide a path for comparative studies in vertebrates. The fins are structures comprised of bone, connective tissue, nerves, blood vessels, epidermis, and pigmentation. Experimentally useful features of teleost fish fin regeneration include the rapid and reliable nature of regenerative events, the relative simplicity of epidermal and mesenchymal structures, and the availability of genetic approaches (**Poss *et al*., 2003; Stoick-Cooper *et al*., 2007a**).

The remains of caudal fin in teleost, post-amputation, invoke a series of signalling pathways which ultimately culminating in restoration of the lost part (**Figure 1**). The central phenomenon, Morgan suggested, was the way in which an injury, however it is caused, stimulates molecular changes within the whole body of the injured organism. "It is this molecular change that, dominating the subsequent development, seems to control it, and gives us the impression of formative process at work. The formative processes are only the expression of physical, molecular structure that has been assumed by the piece" (**Morgan, 1900).**

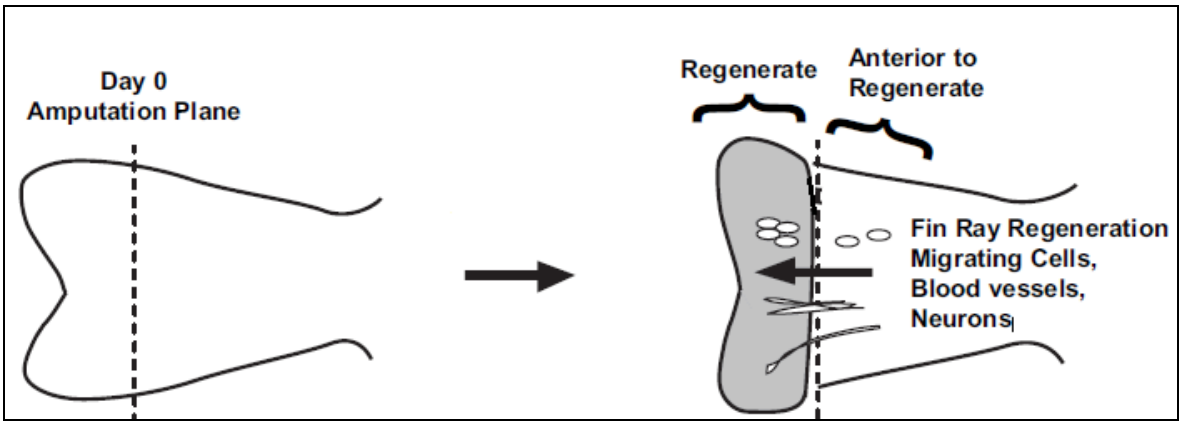


Figure 1: Processes in the teleost caudal fin after amputation (modified from **Andreasen *et al*., 2007**)

Amputation of the caudal fin causes massive trauma and disorganization, followed by rapid formation of a wound epidermis. In order to regenerate structures of correct pattern and function, intricate spatial and temporal regulation of epidermal niche signals is necessary. During the first 12 hours post amputation (hpa), epidermal cells migrate to cover the stump thereby forming the apical epidermal cap (AEC) that does not involve proliferation of cells (**Poss *et al*., 2002a)**. Within the next 12h, the wound epidermis thickens, while fibroblasts and scleroblasts (also called as lepidotrichia forming cells - LFCs) located within one or two bone segments proximal to the amputation plane lose their dense organization and show signs of distal migration thereby forming a mass of proliferating cells called blastemal cells. A highly proliferative blastema is maintained at the end of each bony ray which drives the regenerative events **(Lee *et al*., 2009)**. It is possible that the proliferating cells comprise a cellular subpopulation within the blastema and there are evidences that the mesenchymal compartmentalization is critical for regeneration, with the adjacent epidermis suspected to influence position, size, and mitotic activity of the blastema as regeneration proceeds (**Poss *et al*., 2002a; Lee *et al*., 2005;**), finally forming a faithful copy of the lost structure, the fin (**Figure 2**).

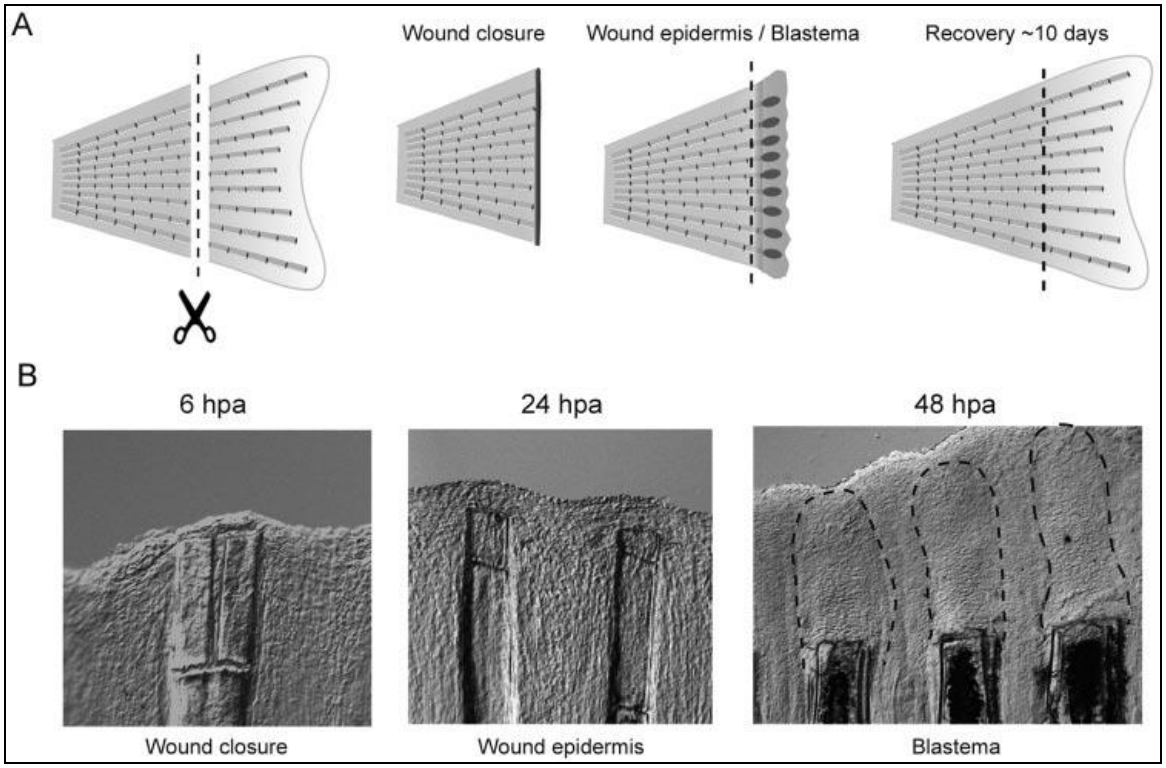


Figure 2: Processes of adult fin regeneration (from **Yoshinari and Kawakami, 2011**)
(A) Key steps in regeneration; (B) Appearance of tissues at key regeneration steps; Dotted lines in B indicate the blastema regions; hpa- hours post amputation.

Appropriate guiding factors are maintained that are continually shifted and/or re-established during the course of regeneration, to influence proliferation distally while also facilitating proximal scleroblast patterning (**Laforest *et al*., 1998)**. Recent studies have identified several developmental regulators synthesized within the regenerating epidermis, with functional data attributing positive or negative effects to these factors (**Poss *et al*., 2000a; Quint *et al*., 2002; Lee *et al*., 2005; Kawakami *et al*., 2006; Stoick-Cooper *et al*., 2007a**). However, it remains quite mysterious how such factors integrate spatially and temporally to instruct regenerative events.

The localization, expression, activation, and general effects of growth factors on tissue regeneration in amphibians are well documented (**Mesher, 1996; Geraudie and Ferretti, 1998; Gianpaoli *et al*., 2003**). Growth factors are small peptides which bind to membrane receptors to influence the various steps of the growth and development of cells through several signalling pathways (**Grounds, 1991; Chambers and McDermott, 1996; Alberts *et al*., 2002**). It has already been shown that they are capable of stimulating growth and protein secretion in many musculoskeletal cells (**Trippel *et al*., 1996**). Recent studies have also extended this analysis to regenerating tissues in lizards (**Alibardi and Loviku, 2010**). Few growth factors have been studied in wounded limb of urodeles (**Fallon *et al*., 1994; Levesque *et al*., 2007; Bell et al., 2003; Kawakami et al., 2004; Treichel et al., 2003**) and regenerating lizard tail (**Alibardi and Toni, 2005; Sharma and Suresh, 2008; Yadav *et al*., 2008; Alibardi and Loviku, 2010**).

Understanding how shape is controlled during development is one of the greatest challenges in developmental biology (**Rolland-Lagan *et al*., 2012)**. How this is achieved largely remains a mystery, but the control of growth, and therefore of shape, has been shown to involve morphogens (**Bénazet and Zeller, 2009; Lee *et al*., 2005; Schwank and Basler, 2010**). Morphogens are mobile substances that can elicit the expression of different sets of genes at different concentrations, hence generating spatial patterns of gene expression that can instruct tissue differentiation or alter tissue growth (**Schwank and Basler, 2010**). Therefore, spatial patterns of gene expression lead to the correct spatial organization of the many cell and tissue types of an organism. As morphogens can affect growth, changes in shape and growth may, in turn, affect morphogen concentration and distribution, thus altering patterning processes (**Rolland-Lagan *et al*., 2012)**. One such morphogens are fibroblast growth factors.

The Fibroblast growth factors (FGFs), among many others are the main players during the process of regeneration. The FGFs have been shown to be associated with many developmental processes including antero-posterior patterning (**Slack and Tannahill, 1992; Deimling and Drysdale, 2011**) mesoderm induction (**Slack, 1994; Bottcher and Niehrs, 2005**), angiogenesis (**Cross and Claesson-Welsh, 2001**), axon extension (**Bulow *et al*., 2004**) as well as appendage formation (**Xu *et al*., 1999**). The deregulation of the FGF pathway leads to severe pathologies including tumorigenesis and stem cell disorder as observed in the myeloproliferative syndrome (**Macdonald *et al*., 2002**).

Fibroblast growth factor (FGF) was first identified 25 years ago as a mitogenic activity in pituitary extracts (**Armelin, 1973; Gospodarowicz, 1974**). This modest observation subsequently led to the identification of a large family of proteins that affect cell proliferation, differentiation, survival, and motility (**Basilico and Moscatelli, 1992; Baird, 1994**). Recently, evidence has been accumulating that specific member of the FGF family function as key intercellular signalling molecules in embryogenesis (**Goldfarb, 1996**). Indeed, it may be no exaggeration to say that, in conjunction with the members of a small number of other signalling molecule families [including Wnt (**Parr and McMahon, 1994**), Hedgehog (shh) (**Hammerschmidt *et al*., 1997**), and bone morphogenetic protein (BMP) (**Hogan, 1996**)], FGFs are responsible for inducing and/or regulating the subsequent development of most organs in the vertebrate body (**Cotton *et al*., 2008**).

Consistent with their potential functions as intercellular signalling molecules, many of the FGFs are exported efficiently from the cells that produce them. Once released from cells, FGFs bind avidly to HSPGs such as the syndecans, glypican, and perlecan on the cell surface and in the extracellular matrix (ECM), which is thought to restrict their ability to diffuse very far from the cells that produced them (**Basilico and Moscatelli, 1992**). The binding of FGFs to heparan sulfate proteoglycans (HSPGs) (sometimes termed low-affinity fibroblast growth factor receptors FGFRs) facilitates FGF signal transduction by oligomerizing and presenting the ligands to high-affinity FGFRs **(Faham *et al*., 1996; Mason, 1994**), which are transmembrane protein tyrosine kinases.

FGFs function through a set of tyrosine kinase receptor (RTKs) known as the fibroblast growth factor receptors, for which four members have been identified in vertebrates (**Green *et al*., 1996; Pownall and Isaacs, 2010**). Upon FGF binding, the receptors are

homodimerized and autophosphorylated, leading to the activation of the kinase activity. This triggers a cascade of intracellular signals ending in the activation of target genes in the nucleus. Even though the signal transduction mechanisms by which FGFs function have been well characterized (**Eswarakumar *et al*., 2005**), identification of the targets genes is still limited.

Among all the growth factors, FGF1 and FGF2 are considered major stimulating molecules capable of replacing most of the activity of the neurotrophic factors as an inducer of organ regeneration in amphibians (**Mullen *et al*., 1996**). The presence of FGF1 and FGF2 in regenerating tail tissue of lizard has been shown by immunocytochemistry and immunoblotting (**Alibardi and Lovicu, 2010**). During the process of muscle regeneration following any injury, FGF2 is present in the extracellular space at eight hours after the injury, reaching a peak at 24 hours, with the levels slowly decreasing over a period of one week (**Anderson *et al*., 1995).**

Many studies have showed the essential roles of FGF2 *in vitro* (**Chen *et al*., 1992; Pierce *et al*., 1992; Slavin *et al*., 1992; Tsuboi *et al*., 1992; Legrand *et al*., 1993; Phillips *et al*., 1993; Gibran *et al*., 1994; Decker *et al*., 2000; Jiang *et al*., 2001**). However, it is important to recognise that regeneration *in vivo* is more complex than that *in vitro* because of the involvement of circulatory and intercellular communication (**Grounds, 1991; Chambers and McDermott, 1996)**. FGF2 was observed to enhanced muscle regeneration *in vivo* (**Menetrey *et al*., 2000**). Implantation of beads soaked in FGF2 induces extra limbs from the flank of chick embryo *in vivo* (**Cohn *et al*. 1995**). Extraneous FGF2 enhanced tail regeneration *in H. flaviviridis* (**Yadav *et al*., 2012**). Moreover, it has been shown that in urodele amphibians one of the first proteins to be formed after amputation is FGF2, which is a major regulator of the events happening during wound healing process (**Gardiner *et al*., 1995, Gardiner and Bryant, 1996**). Neutrophils are believed to be the first immune cells that are produced at the site of injury (**Martin and Leibovich, 2005; Arnold *et al*., 2007; London *et al*., 2011; Serhan *et al*., 2007**). However, an early detection of FGF2 during the initial summit of wound healing suggests that pre-existing tissue FGF2 may be important in healing rather than that synthesized *de novo* by inflammatory macrophages (**Yoshimura *et al*., 2001**). A key to understanding the precise coordination of this factor with the regenerative pathways is to determine their spatial and temporal activity during the entire regeneration event.

The present study is an attempt to evaluate the specific roles of FGF2 during epimorphic regeneration of teleost fish fin *Poecilia latipinna*. In normal tissue, basic fibroblast growth factor is present in basement membranes and in the subendothelial extracellular matrix of blood vessels. It stays membrane-bound as long as there is no signal peptide. It has been hypothesized that, during both wound healing of normal tissues and tumor development, the action of heparan sulphate degrading enzymes activates FGF 2, thus mediating the formation of new blood vessels, a process known as angiogenesis (**Gardiner and Bryant, 1996**). FGFs are known to play significant role in epimorphic regeneration as well and of all the FGFs, FGF2 is the most influential factor and very important for epimorphic regeneration (**Yamashita *et al*., 2000**). FGF2 has been localized to the WE and nerves of the regenerating amphibian limb (**Mullen *et al*., 1996**) and more recently it is shown to be localized in regenerating tissues during tail regeneration of lizard *L. guichenoti* (**Alibardi and Lovicu, 2010**).

Works by many scientists have defined the presence and significance of FGF2 during zebrafish fin regeneration (**Hata *et al*., 1998; Bouzaffour *et al*., 2009**). During teleost caudal fin regeneration, there is a network of molecular signalling that choreographs the epimorphic process (**Tal *et al*., 2010**). FGF signalling is required for initiating fin regeneration and controlling blastema formation (**Poss *et al*., 2000a; Whitehead *et al*., 2005**). Several arguments further suggest that FGF could be necessary for lepidotrichia formation (**Santos-Ruiz *et al*., 2001**). The inhibition of FGF signalling pathway stops fin outgrowth (**Poss *et al*., 2000a**) and modulation of the FGF signalling regulates the rate of fin outgrowth (**Thummel *et al*., 2006; Lee *et al*., 2005**). In addition, it has been recently shown that FGF signalling instructs position-dependent growth rate during zebrafish fin regeneration (**Lee *et al*., 2005**). In zebrafish, a cocktail of FGF ligands and FGFRs are induced during blastema formation and regenerating fin outgrowth (**Poss *et al*., 2003; Nakatani *et al*., 2007**). In particular, FGFR1 is expressed in pre-blastema mesenchymal cells during blastema formation, and maintained in subpopulations of blastemal and epidermal cells during outgrowth (**Poss *et al*., 2000a**). It has also been demonstrated that FGFR1 regulates blastemal cell proliferation during fin regeneration (**Poss *et al*., 2000a; Tawk *et al*., 2002)**. FGFs have also been shown to be required in epidermal cells for a complete epimorphic regeneration of the heart (**Lepilina *et al*., 2006**). Works by **Hata *et al*.** (**1998**) have proved that exogenous FGF2 promotes fish fin regeneration. **Lee *et al*.** (**2005**) proposed that the reduction in the amount of FGF signalling is essential to slow down and then stop regeneration. Therefore, while FGF

signalling is essential to initiate blastema formation and growth, the level of FGF expression must also be precisely regulated in order to limit the regeneration process.

To determine whether FGF2 signalling participates in *P. latipinna* caudal fin regeneration, we first determined the expression of FGF2 in the fin regenerate by immunolocalization studies of FGF2 using HRP-DAB staining procedures. The significance of FGF2 in further recruiting FGF2 in an autocrine manner was observed by blocking the FGF2 signal transduction in the treated group of fishes with a specific FGFR1 inhibitor SU5402, a drug first described as a specific inhibitor for FGFR1 phosphorylation (**Mohammadi *et al*., 1997**). The control group was injected with 1%DMSO (vehicle) alone that allowed the normal growth and differentiation of the fin so presenting the usual level of FGF2 during normal fin regeneration.

MATERIAL AND METHODS

Fish care and maintenance

Adult teleost fish, *Poecilia latipinna*, approximately 4-5 cm in length of both the sexes, were used for the experiment. They were maintained in aquaria containing constantly aerated and purified dechlorinated fresh water. The daily photoperiod was 12h of light and 12h of darkness, and the temperature was kept in the range of 26±2°C. The fishes were fed with readymade fish food (White rose fish food, Mumbai) *ad libitum*. The animals were acclimated for a week before the commencement of the experiment and the period of study was 15 days. All the experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) as per CPCSEA norms.

FGFR inhibitor treatments

A 5mM stock solution of SU5402 in 1%DMSO was prepared and was stored at 4ºC. The stock was diluted and dose was prepared freshly each time before use. Each animal received a dose of 2µM/gm body weight of SU5402 and 1% DMSO as control with the maximum quantity of 10µl/animal.

Experiment scheme and Drug dosage

The fishes were randomly allocated into two glass aquaria, with 10 fishes in each aquarium. One served as the control and the other was the treated group. The control fishes were injected with 1%DMSO, whereas the treated ones were administered 2µM/gm body weight

SU5402. The fishes were subjected to the injections a day before amputation and were continued till the fins reached the differentiation stage i.e. about 7 days post amputation (dpa). Regenerating fins were collected 1dpa (wound healing stage), 4dpa (balstema stage) and 6dpa (differentiation stage); 9µm sections were taken in IEC cryostat at -20°C and the frozen sections were then processed for HRP-DAB immunohistochemical studies.

Immunohistochemical localization of FGF2

The frozen sections were processed for the HRP-DAB staining as explained in the section **Material and Methods**. In brief, the sections were fixed using pre-cooled acetone as fixative. Endogenous peroxidase was blocked with 0.3% H_2O_2 and nonspecific binding was blocked with normal goat serum. Appropriately diluted primary antibody was added to the sections and incubated in a humidified chamber for overnight at 4°C; followed by the addition of appropriately diluted secondary antibody and incubation in a humidified chamber for 30 minutes. Later on the pre-diluted SAV-HRP conjugates were added to the slides and incubated in a humidified chamber at room temperature for 30 minutes. Freshly made DAB substrate solution was applied to reveal the colour of antibody staining. The slides were rinsed twice with PBS for 5 minutes each, after each of the above mentioned steps. The tissue sections were dehydrated appropriately and the colour of the antibody staining in the tissues was observed under microscope. Negative control sections were also incubated similarly but with PBS-BSA in place of the primary antibody to negate the possibility of nonspecific antibody binding and found no ambiguous noises in the images of negative control sections.

RESULTS

Epimorphic regeneration requires the mobilization, as well as the migration and the proliferation of progenitor cells capable of restoring the missing part. All this requires a precise coordination between proliferation and patterning, as both processes must be strictly regulated in time and space to ensure the restoration of the size and shape of the missing part. The FGF pathway is majorly involved in this process that functions through the fibroblast growth factor receptors (FGFRs). The FGF2 receptor FGFR1 is known to be highly expressed in basal epidermal cells during regenerative outgrowth. To determine requirements for FGFR activation in epidermal specification, we used a specific FGFR1 inhibitor SU5402. The experiments showed the drug to strongly inhibit the epidermal formation, blastemal

proliferation and associated regenerative events; thus proving the positive effect of FGF2 on fin regeneration.

Experimental fins examined immunohistochemically for the expression of FGF2 during the three defined stages of epimorphosis showed the presence of FGF2 at an identical locus as that of controls but at a subtle level compared to the later tissue. The fin sections at the 1dpa and 4dpa showed quite an amount of FGF2 and the FGF2 expression gradually decreased as the fin reached the differentiation stage as observed in the fin sections 7dpa. In each case, the concentration of FGF2 was more in the control fins as compared to the SU5402 treated fins; however, in the fins 1dpa the difference was negligible (**Figure 3A**).

An observation of the fin sections at wound epithelium stage revealed the presence of FGF2 in the wound and scaling epidermis. At 1 dpa, there is a thin layer of epidermis formed that covers the stump. This new epithelial covering is formed by migration of cells from the existing epidermal tissue. This epidermal tissue becomes synthetically active immediately after amputation and so is thought to release the FGF2 along with other signalling proteins to promote wound healing and further regenerative processes; and FGF2 is known to work in an autocrine order i.e. the existing stump cells release FGF2, and this further assist in synthesis and release of more FGF2 (**Yoshimura *et al*., 2001**). Thus in case of the fins treated with SU5402, the FGF2 mediated cell migration seems to be hampered to an extent, resulting in a latently formed wound epithelium as well as mediocre localization of FGF2.

The caudal fin sections at blastemal stage (4dpa) too revealed a fairly high percentage of the fins positive for FGF2 in the control fins as compared to the treated. The blastema consists of mesenchymal fibroblast cells that may be the target of FGF2. The maximum presence of FGF2 was in the growing area of the fin i.e. in the distal most blastema. It is well established that blastema formation is a main cue in epimorphic regeneration, and the further outgrowth of the fin depends on the differentiation nature of these blastemal cells. . Thus FGF2 seems to directly promote blastema growth prior to lepidotrichial formation. SU5402 was found to delay the blastema formation in the treated groups, and also reduce the expression of FGF2, as observed immunohistochemically (**Figure 3B)**.

Regenerating fins are likely to require FGF2 for their differentiation and growth. This growth and diferentiation stage is characterized by a decreased time period for cell cycles and a distal

migration of the mesenchymal cells. During this stage, the outgrowth is initiated and more blastemal cells emerge from migratory mesenchyme cells underneath the apical epidermal cap, characterized by cells exhibiting an extremely short cell cycle. At this stage of regeneration we observed a gradual decrease in FGF2 expression as the outgrowth proceeded to form its original length (7dpa). The regenerative processes seems to be somewhat independent of FGF2 as it reaches the differentiation stage, as observed by the lower levels of FGF2 in the regenerating fins even in the control fishes (**Figure 3C**). However, it is logical to infer that a modicum of FGF2 expression will be there throughout, as the teleost fish caudal fins continually grow. Considering the fact that the fin growth is isometric, it makes sense that in order to retain the proportion of body to caudal fin ratio, there must be FGF2 mediated regulation.

DISCUSSION

Teleost fin regeneration consists of several specific stages including epithelial cap formation, dedifferentiation of mesenchymal cells forming a blastema (which leads to formation of epithelium, nerves, blood vessels and many mesenchymal derivatives), and finally, outgrowth of the blastema and redifferentiation to restore the lost part of the fin (**Santamaria and Becerra, 1991**; **Johnson and Weston, 1995; Poss *et al.*, 2000b**). In order for an adult animal to replace missing structures with an exact copy of the original, it is clear that developmental programs must be redeployed. However, the dynamics of cell communication and proliferation are vastly different, as are the cell types involved. To accomplish regeneration, adult animals may invoke the proliferation of differentiated cells, the activation of reserve stem cells, the formation of new stem cells with limited capacity to self renew (progenitor cells), or a combination of these strategies (**Poss *et al.*, 2000b)**. Well-defined signalling molecules are similarly involved in regulating appendage development in a number of species from flies to humans (**Pueyo and Couso, 2005**). While there are obvious differences in size, shape and function among these nonhomologous appendages, what is more interesting from a developmental perspective is which signalling mechanisms are conserved among them; and FGF2 is one such player (**Seed *et al.*, 1988; Geetha-Loganathan *et al.*, 2008; Mariani *et al.*, 2008**), suggesting that while final structures might vary in form and function, the molecular networks used to create these appendages are conserved across multiple species and a conservation of spatial and temporal expressions of these signalling molecules exist (**Tal *et al.*, 2010**).

Upon caudal fin amputation, a remarkable series of regenerative stages are initiated that result in the complete restoration of lost part (**Tal *et al*., 2010**). This complex process, termed epimorphosis, is completed in approximately 2 weeks in adult fish (**Iovine, 2007**). Epimorphic regeneration involves the formation of a mass of undifferentiated proliferative multipotent mesenchymal cells called a blastema (**Akimenko *et al*., 2003**). In the adult model, an initial wound healing stage, characterized by nonproliferative lateral epithelial cell migration over the wound and subsequent formation of the apical epidermal cap (AEC), is initiated immediately following surgical removal of caudal fin tissue (**Nechiporuk and Keating, 2002**). Second, the wound epithelium thickens and mesenchymal tissue proximal to the amputation plane begins to disorganize. Cellular disorganization is thought to occur as a result of growth factors that originate from the mature wound epidermis and stimulate mesenchymal cells to dedifferentiate and proliferate as they migrate distally towards the area directly proximal to the AEC (**Nechiporuk and Keating, 2002)**. In the third stage, a series of blastemas form at the severed portion of each amputated fin ray. Blastema formation is the main event that distinguishes regeneration from limb development. The blastema is an accumulated mass of progenitor cells that are thought to be pluripotent or able to produce daughter cells capable of differentiating into a variety of cell types required to populate the regenerating tissue (**Tal *et al*., 2010)**

Growth factors of significant importance to wound healing are fibroblast growth factor 2 along with vascular endothelial growth factor (VEGF); both have been shown to promote angiogenesis, the formation of new blood vessels from pre-existing vessels, and to stimulate wound healing in animal models (**Pike *et al*., 2007; Liu *et al*., 2007**). As per **Poss *et al*., 2000a**, FGFR1 expressions begin to appear, approximately 18 hours post amputation. Then, FGFs synthesized in the wound epidermis bind to FGFR1 on mesenchymal cells near the amputation plane. This triggers (directly or indirectly) upregulation of *msxb* and *msxc* and new proliferative capacity in blastemal precursor cells, which organize and divide to form the blastema (**Poss *et al*., 2000a**). These signalling events may be similarly used to facilitate blastema maintenance that promotes regenerative outgrowth. Hence we hypothesize that FGF signalling contributes to fin regeneration by directing both the recruitment and proliferative ability of blastemal cells.

The abundant immunolocalization of FGF2 in the regenerating fins at initial stages suggests that FGF2 may be one of the very early players of fin regeneration. It is known that fibroblast

growth factors (FGFs) are able to induce a myriad of pleuripotent cells (**Malcolm and Reardon, 1996; Webster and Donoghue, 1996; Iseki *et al*., 1997; Burke *et al*., 1998; Greenwald *et al*., 2001; Sarkar *et al*., 2001; Tsutsumi *et al*., 2001; Ohbayashi *et al*., 2002; Ornitz and Marie, 2002; Whitehead *et al*., 2005**). Several arguments further suggest that FGF2 is necessary for lepidotrichia formation (**Santos-Ruiz *et al*., 2001**). The inhibition of FGF2 signalling pathway stops fin outgrowth (**Poss *et al*., 2000a**) and modulation of the FGF2 signalling regulates the rate of fin outgrowth (**Lee *et al*., 2005; Thummel *et al*., 2006**). Thus FGF2 is undoubtedly needed for the formation of skeletal structures and further fin formation and hence is immediately released in response to injury.

Intense immunoreactivity for FGF2 was observed in the regenerating fins of *P.latipinna* in the control group that received only the vehicle, whereas the FGF2 was comparatively scantily localized in the regenerating fins of the SU5402 treated fishes. This reduction in the concentration of FGF2 in treated animals points to a possible autocrine regulation of FGF2 recruitment by the cells in the regenerate. Further, this presence of fibroblast growth factors in the regenerating fin of *P. latipinna* indicates that FGF2 may also be one of the main candidates amongst the regenerative factors, and inhibition of this regenerative signal with pharmacological inhibitors compromise the further expression of FGF2 in the regenerating fin. Also, the latter provides opportunities to investigate a variety of other growth control problems, including: (1) How FGF2 influences initiation and growth of the fin and whether its inhibition causes fin growth retardation? (2) How FGF2 regulates the tissue remodelling and nucleic acids turnover and (3) how FGF2 influences the cellular proliferative activity during regeneration. While these questions are addressed in the subsequent chapters, as a first step in understanding these mechanisms, we conducted the current immunolocalization studies of FGF2.

Also, one of the most fascinating aspects of appendage regeneration is positional memory, the ability of the limb or fin stump to recognize and restore only those structures lost by injury (**Wills *et al*., 2008a**). Positional memory is thought to be based on a gradient of some determinant(s) existing in uninjured tissue or quickly established after amputation. Recently, work by **Lee *et al*.** (**2005**) found that the amount of FGF signalling established after amputation is graded along the proximo-distal axis, with higher amounts in more proximal tissue and lower amounts distally. Greater FGF signalling positively impacts blastemal proliferation and regenerative rate, leading to more rapid outgrowth in proximal regenerates.

Similarly, as regeneration proceeds gradually to completion, amounts of FGF signalling gradually wane. The current findings indicate that developmental signalling, including FGF2 signalling, is not inactivated after restoration of lost structures; rather, a basal level is maintained in the distal tips of intact fins. Also, studies by **Wills *et al*.** (**2008b**) prove that when the capacity to maintain this FGF signalling is experimentally blocked, tissue loss occurs, revealing an essential role in homeostatic equilibrium. So, it can be postulated that weak FGF2 presence at the distal tips of intact fins helps the fins to maintain their size as these levels of FGF signalling might oppose ongoing cell death. By contrast, an amputated fin will initiate a position dependent boost of FGF signalling for structure-restoring. Regeneration then culminates when FGF signalling decreases to an amount that no longer procures a net gain in growth vis à vis ongoing cell death.

Although this work was mainly focussed on immunodetection of FGF2 in the regenerating fins, the results showing a broad reduction in the expression of FGF2 in the SU5402 treated fins as compared to the control ones also hold great importance in showing the light to carry out further studies. Therefore, to support the hypothesis of importance of FGF2 signalling during fin regeneration in *P. latipinna*, a morphometric study was undertaken to learn how the inhibitory effect of SU5402 on FGF2 signalling via FGFR1 blockage influences the growth of the caudal fin, (**Chapter 2**) which would further assist in understanding the similarity of regulation in epimorphic regeneration amongst vertebrates.

Figure 3: Immunolocalization (in shades of brown) of FGF2 in the regenerates of control (C) and experimental (E) fish during defined stages of regeneration. CT-connective tissue; L-lepidotrichia; Arrow-wound epithelium.

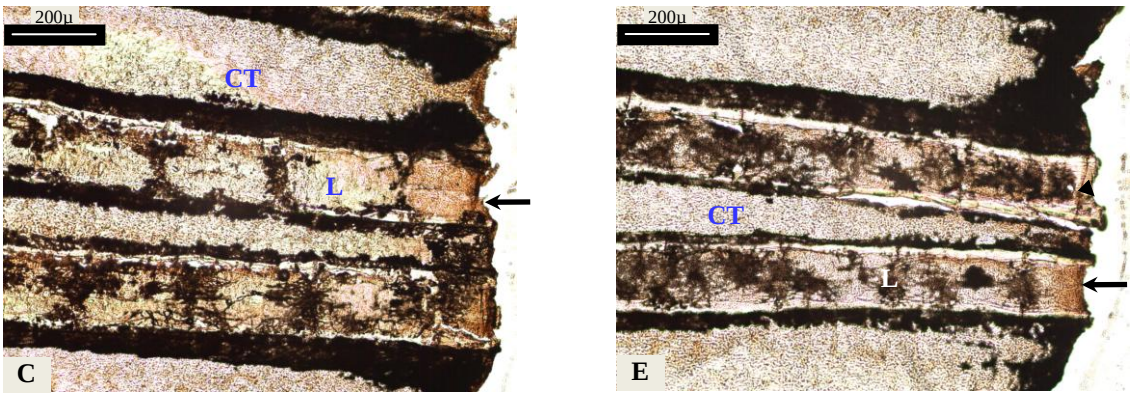


Figure 3A: Wound healing stage (1dpa)

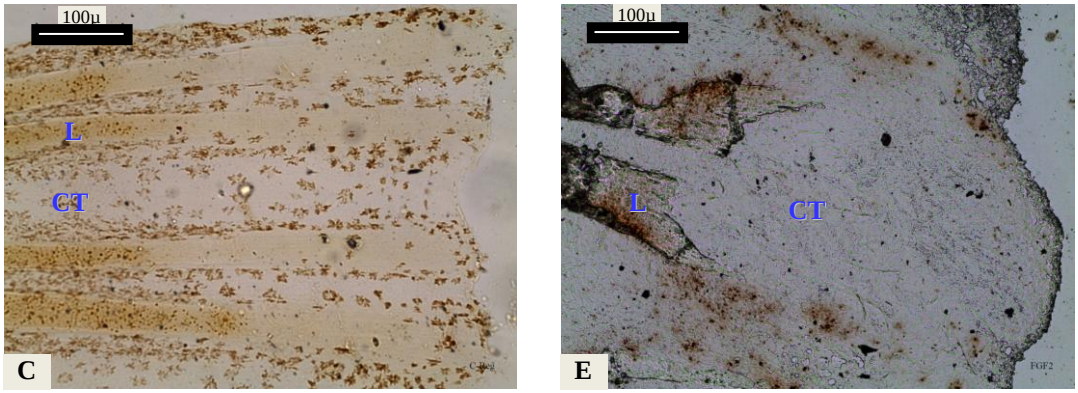


Figure 3B: Blastema stage (4dpa)

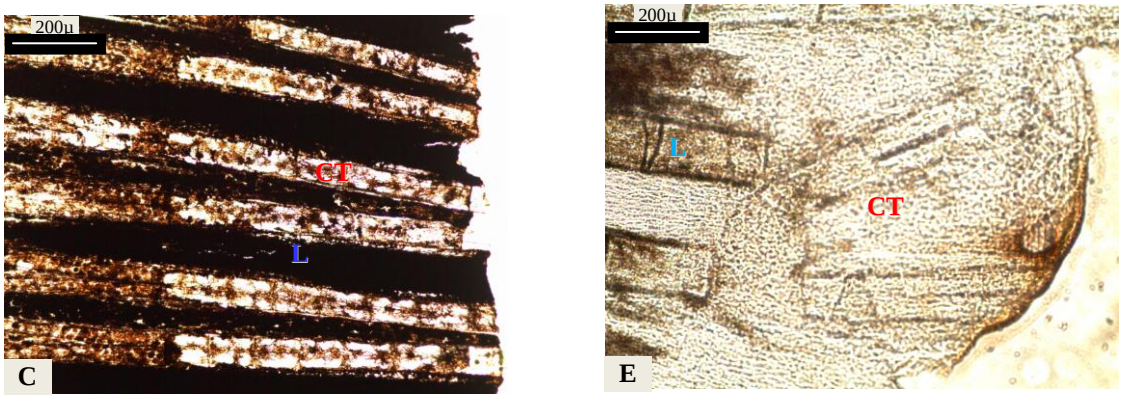


Figure 3C: Differentiation stage (7dpa)

CHAPTER 2

INVESTIGATING THE ROLE OF FGF2 SIGNALLING IN THE REGULATION OF EPIMORPHOSIS IN *POECILIA LATIPINNA*: A MORPHOMETRIC STUDY

INTRODUCTION

Both vertebrates and invertebrates possess regenerative capabilities which allow them to restore their morphology following amputations or grafts of particular parts of their bodies. Morphological regeneration is effective in adapting these populations to changing/ competitive local environment (**Mari-Beffa *et al*., 1999**). Tremendous work has been done to understand and reveal the various cellular and molecular mechanisms of this control.

Thomas Hunt Morgan is probably better known for his fundamental work on Drosophila genetics, but his research interests were first focused on regenerative capacity of various organisms (**Morgan, 1898, 1900, 1902**). Morgan chose the term "morphallaxis" (a term coined by Morgan in 1898 (**Morgan, 1898**) and later clarified by the same author in 1901) to describe the process of regeneration of Planaria in which the lost body parts are replaced by the remodelling of the remaining part and "epimorphosis" to describe the type of regeneration that, in contrast to morphallaxis, requires active cell proliferation prior to the replacement of the lost part (**Morgan, 1901**). Limb regeneration of several urodele amphibians and fin regeneration of teleost fish are examples of epimorphosis. **Morgan** (**1901, 1902**) was the first to try to understand the mechanisms underlying the ray regeneration and to investigate the process of individual ray regeneration in the context of the entire fin.

The fins are the most distinctive features of a fish, composed of bony spines protruding from the body with skin covering them and joining them together, either in a webbed fashion, as seen in most bony fish, or more similar to a flipper, as seen in sharks. These usually serve as a means for the fish to swim. Fins can also be used for gliding or crawling, as seen in the flying fish and frogfish. Fins located in different places on the fish serve different purposes, such as moving forward, turning, and keeping an upright position.

Teleost fishes have remarkable capabilities of fin, or epimorphic regeneration (**Whitehead *et al*., 2005; Stoick-Cooper, 2007a; Lee *et al*., 2010; Lehoczky *et al*., 2011; Azevedo *et al*.,**

2012). Short generation time, high fecundity, minimalism of maintenance, simplicity of the fin structure and ease of observation make the teleost fish a popular model for evaluating underlying molecular pathways in vertebrate systems. Furthermore, as the fin is non-essential for viability (especially in a laboratory setting) and has a comparatively simple structure with few cell types, it has been the focus of many studies (**Akimenko *et al.*, 2003; Poss *et al.*, 2003**). In modern bony fishes, the tail is balanced and the backbone ends where the tail begins. This type of caudal fin is called homocercal (**Figure 1**). The fin is external to the muscle mass of the body and is very flexible.

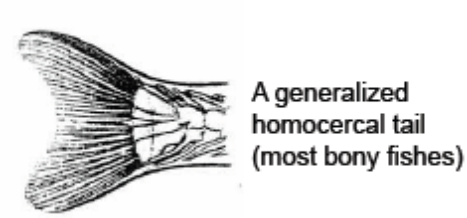


Figure 1: Homocercal fin

Although each of the five zebrafish fin types regenerates (**Johnson and Weston, 1995; Bernhardt *et al.*, 1996; Becker *et al.*, 1997**), the caudal fin is primarily used to assay regeneration. This organ is easily accessed for surgery, its injury does not compromise survival, and it possesses a relatively simple, symmetric structure with limited cell types. For instance, the caudal fin does not contain skeletal muscle, or endochondral bone formed by mineralization of cartilage. The fin skeleton instead has a dermal origin and mineralizes directly as bone. A number of segmented bony fin rays spread from the neck of the caudal fin to its posterior end.

The caudal fin is composed of multiple bony fin rays or **lepidotrichia**, most of which are bifurcated at the ends, and are called **actinotrichia** (**Montes *et al.*, 1982; Becerra *et al.*, 1983; Santamaria and Becerra, 1991; Geraudie and Singer, 1992**).

After partial ablation of the fin, the stump of each ray regenerates by an epimorphic process. The fin ray is the regenerative unit of the fin, and depends on cell-to-cell interactions to completely restore its original pattern. Restoration of the epidermis, blastema formation, cell

proliferation and differentiation, are all events that follow excision. Complete replacement of the lost fin occurs within approximately 15 days after amputation **(Mari-Beffa *et al*., 1999)**

The regeneration process in fish can be divided into three main steps (**Johnson and Weston, 1995; Poss *et al*., 2000a; Akimenko *et al*., 2003; Poss *et al*., 2003**). **Figure 2** illustrates the early events that occur during lepidotrichium regeneration, separated into four stages (A–D), at 33°C (**Goss and Stagg, 1957; Santamaria and Becerra, 1991; Johnson and Weston, 1995**).

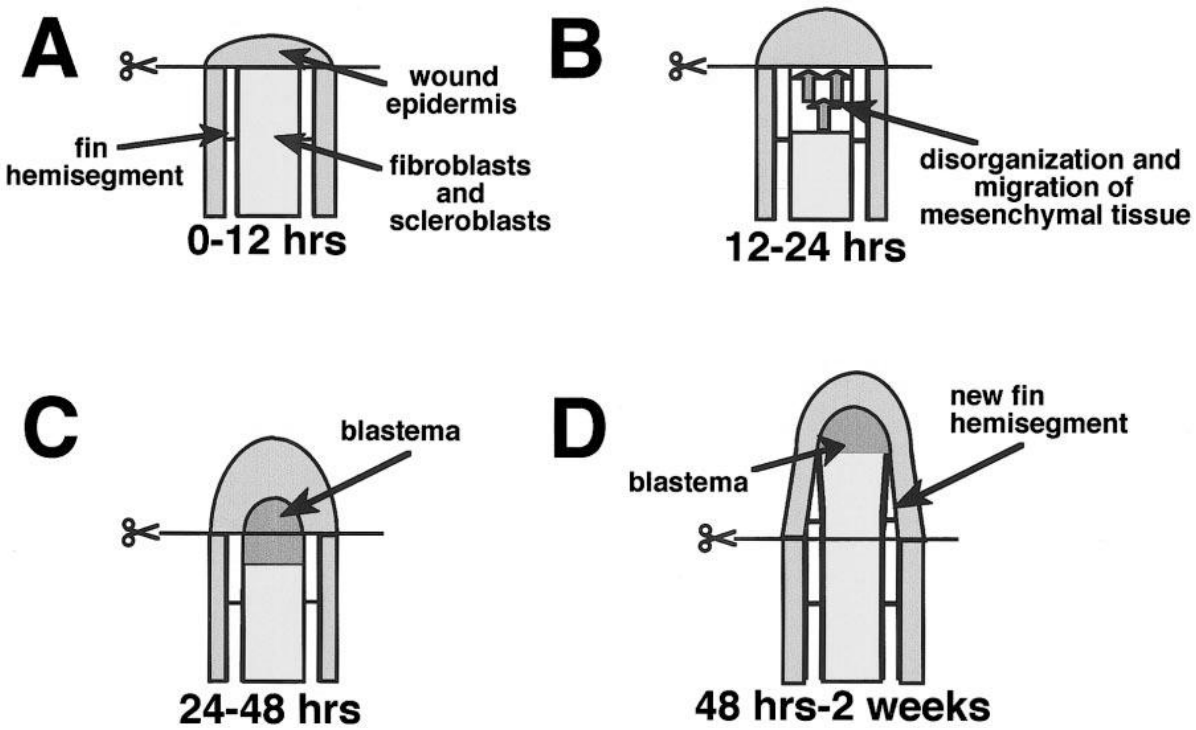


Figure 2: Early caudal fin regenerative events depicted as longitudinal sections **(**from **Poss *et al*., 2000a)**

1. Wound healing (0-24hpa): Within the 6 hours post amputation (hpa), an epithelial layer completely covers the wound, followed in the next hours by several additional layers of epidermal tissue. This forms the apical epidermal cap (AEC) in a process that does not involve cell proliferation, but migration of epithelial cells from the unamputated region (**Poleo *et al*., 2001; Nechiporuk and Keating, 2002**). The innermost cell layer (the basal epidermal layer) located against the mesenchyme, recognizable by the cuboidal shape of cells, differentiates quickly after the formation of the AEC and is the source of factors regulating epithelial-mesenchymal interactions which will control the regenerate outgrowth (**Akimenko *et al*., 1995; Laforest *et al*., 1998; Poss *et al*., 2000a; Poss *et al*., 2000b**).

2. Blastema formation (24-72hpa): Following the formation of the AEC, fibroblast-like cells located up to two segments proximally to the amputation plane start to disorganize and migrate to the distal region, at the site where the blastema will form by cell proliferation (**Poss *et al*., 2000a; Poleo *et al*., 2001; Nechiporuk and Keating, 2002).** It is still unclear whether these cells originate from the dedifferentiation of pre-existing mesenchymal cells or from a population of progenitor cells. The blastema becomes clearly visible by 2dpa. At that time, mesenchymal cell division mostly occurs in the blastema region, whereas proliferating epithelial cells are restricted to more proximal regions of the fin (**Nechiporuk and Keating, 2002**).

3. Blastema maturation and regenerative outgrowth (72hpa and later): Immediately after the blastema formation, mesenchymal cells segregate into three populations. First, a small population of slow-cycling cells is located in the distal blastema (DB) (**Santamaría *et al*., 1996; Nechiporuk and Keating, 2002**). It has been proposed that this population would constitute a pool of undifferentiated progenitor cells for the second population in the proximal blastema region (PB) which shows an intense and rapid cell cycling, twice as fast as during blastema formation (**Nechiporuk and Keating, 2002**). Finally, the most proximal part of the regenerate, the patterning zone (PZ), is mostly composed of differentiating mesenchymal cells in the core of the regenerate and scleroblasts in the periphery, adjacent to the basal epidermal layer. Cells of the PZ show little or no cell division. As regeneration continues, the blastema constantly remains distally located, driven by cell proliferation occurring in the PB, while cells of the PZ progressively differentiate into new structures which replace the amputated part of the fin. Complete regeneration is achieved maximum by 3 weeks depending on the amputation level (**Avaron *et al*., 2000**) and also varying according to species.

Understanding how shape is controlled during development is one of the greatest challenges in developmental biology (**Rolland-Lagan *et al*., 2012**). Not only do organisms develop into particular shapes according to their species, but some species are able to regenerate organs of the correct size and shape following amputation (**Yin and Poss, 2008**). How this is achieved largely remains a mystery (**Rolland-Lagan *et al*., 2012**), but it undoubtedly involves precise cell communications to restore the missing part of the injured organ **(Laforest *et al*., 1998)**. Studies showing that appendage regeneration depends on the continual presence of the wound epidermis (**Goss, 1956; Mescher, 1976**) have led to the hypothesis that a factor(s)

released from the epidermis initiates blastema formation and maintains its pleuripotent and/or proliferative properties. One class of factors might be fibroblast growth factors (FGFs), members of a large family of short polypeptides that are released extracellularly and bind with heparin to dimerize and activate specific receptor tyrosine kinases (FGFRs). FGF signalling is involved in mammalian wound healing and tumor angiogenesis (**Ortega *et al.*, 1998; Zetter, 1998**) and has numerous roles in embryonic development, including induction and/or patterning during organogenesis of the limb, tooth, brain, and heart (**Crossley *et al.*, 1996; Vogel *et al.*, 1996; Ohuchi *et al.*, 1997; Martin, 1998; Reifers *et al.*, 1998**). Evidence obtained from studies of amphibian limb regeneration supports roles for FGFs in regeneration (**Gardiner and Bryant, 1996; Mullen *et al.*, 1996**).

There are several reports that suggest the roles of FGF signalling during fin regeneration. **Poss *et al.* (2000b)** showed that an FGF receptor, FGFR1, is expressed in the fibroblast-like cells at the onset of the blastema formation, and this expression is maintained during blastema outgrowth. To investigate the role of FGF signalling, some authors used a pharmacological inhibitor of FGFR1, SU5402, and showed that incubation with SU5402 immediately following amputation prevents blastema formation without affecting wound healing. This inhibition is accompanied by an absence of cell proliferation, as shown by BrdU incorporation analysis (**Mori *et al.*, 2007**).

Here, we investigated whether FGF signalling is a necessary mediator of fin regeneration in the adult teleost fish *Poecilia latipinna*. We tested the function of FGF signalling during fin regeneration by use of a specific pharmacologic inhibitor of FGFR1. Use of this agent already showed its inhibitory effect by reducing the levels of FGF2 expressions in the regenerating caudal fins (**Chapter 1**). Therefore, next the morphometric studies were undertaken to confirm the inhibitory effect of SU5402 and essential roles of FGF2 during epimorphosis.

MATERIAL AND METHODS

Fish care and maintenance

Adult Teleost fish, *Poecilia latipinna*, approximately 4-5 cm in length of both the sexes, were used for the experiment. They were maintained in aquaria containing constantly aerated and filtrated fresh water with a daily photoperiod of 12h light and 12h darkness, and the temperature was kept in the range of 26±2°C. The fishes were fed with readymade fish food

ad libitum. The animals were acclimated for a week before the commencement of the experiment and the period of study was 15 days. All the experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

Experimental design

At the beginning of experiment the fishes were randomly allocated to three glass aquaria (n=10 per aquarium).

Group I: These fishes served as control to the treatment group (1%DMSO)
Group II: Fishes in this aquarium received low dose of SU5402 (1μM/g body wt. SU5402)
Group III: Fishes in this aquarium received high dose of SU5402 (2μM/g body wt. SU5402)

A 5mM stock solution of SU5402 in 1%DMSO was prepared and was stored at 4ºC. The 3 groups were named as A, B and C. Group A was control, whereas B and C were treated groups; where B was the low-dose group and C was high-dose group. Group A was injected with 1%DMSO alone, whereas group B and C received injection of 1μM/g body wt. and 2μM/gm body wt. of SU5402 respectively. Later, the dose was finalized to 2μM/g body wt.

Dose injection, Caudal Fin Amputation and Measurements

- The treatment in each group started a day before amputation and was continued till the animals reached differentiation stage.
- All the animals of the control group, i.e. Group A were injected with 1%DMSO, with maximum of 10μl/animal. Group B was the low dose group and was injected 1μM/g body wt. SU5402 and Group C or the high dose group received 2μM/gm body wt. of SU5402. The injections were given daily in the tail muscles at a fixed time till the fins reached the differentiation stage.
- Dosing was started a day before amputation. Fin amputations were made with a sterile surgical blade. The tail fins of all the animals were amputated for approximately 30% of their total length.
- Time taken to inject the dose as well as fin amputation for each of the fish did not exceed 30 seconds and the fishes were released immediately into the tanks with full care.

- Regeneration rate was studied by digital photographs taken every day and length variation was recorded using a digital vernier calliper.

RESULTS

Treatment of *P. latipinna* with SU5402 inhibited fin regeneration to varying degrees. Of the regenerated fins examined, the control group A showed normal growth and patterning, Group B showed slight regenerative delay and Group C showed maximum regenerative block (**Figure 7**). Normally, the adult *P. latipinna* regenerate their caudal fins within fifteen days after amputation. During the first stage of regeneration, by 12 hours post amputation (hpa), the fin formed an epidermal wound covering or cap at the site of amputation. Wound healing was clearly observed in the control group a day post amputation, whereas the treated groups took longer time to show the signs of wound healing (**Figure 7a**). Normally during 24-48hpa there is proliferation of mesenchymal cells forming a blastema and finally, from 48hpa forward, the blastema differentiates and develops structures required for fin regeneration, including blood vessels, bony rays, and connective tissue. Blastema in the control Group A could be observed clearly as a white region, almost on the third day after amputation and a clear blastema could be observed on the 4^{th} day in maximum of the fishes; Group B and C however took longer time for showing the blastemal mass (**Figure 7b**). Blastema could be observed in the low-dose group in around 4-5 days after amputation, whereas in high-dose group it could be seen at almost around 5-6 days post amputation. The Blastema then differentiated normally in the regenerating fin of the control fish and in around 7 days Post amputation, clear differentiation of regenerating tissue could be observed, whereas the differentiation process was delayed in the SU5402 treated groups (**Figure 7c)**, thereby slowing the process of regeneration. Treatment of the fins with this drug significantly arrested the growth of the fin in a dose-dependent manner. The days taken by each of the groups of experimental fishes to reach the different stages of regeneration are shown in **Table 1 and Figure 3**.

Growth is a continuous process in the teleost fin. It occurs by addition of ray segments to the end of the fin, rather than by increase in length of the established ray segments. During regeneration, fin outgrowth shows a gradient of developmental events where "Youngest" regenerative processes, such as maintenance of the blastema, are in the distal-most regenerate, and "older" regenerative processes, such as mineral deposition of the new bone, are observed in the proximal regenerate. In order to quantify the growth response, the length

of maximum outgrowth (in mm) was measured using digital vernier calliper and growth rate (in %) were calculated as depicted in **Table 2, 3 & 4**. The length of maximum outgrowth is defined as the distance from the plane of amputation to the tip of the regenerating fin. The length of maximum outgrowth (in mm) in SU5402 treated fishes was significantly lower when compared to the control animals. The control group could complete the entire regeneration within 15-17 days and the fin length reached the initial fin length recorded before amputation, whereas for the treated group it was observed that the regeneration process was not completed in 15 days, fin not reaching the initial recorded length, taking almost 18-20 days to regenerate completely, with high dose group taking still longer time than the low dose group (**Table 2**). Of the 3mm of fin amputated, on the 15^{th} dpa, almost 3mm (99%) of the amputed fin had regenerated in the control fishes, while the percentage growth of fin was about 67% (2mm) in low dose group and only about 51% (around 1.5mm) in high dose group (**Table 2 & 3; Figure 4 & 5**). The average daily percentage growth rate of the fins also differed with both the treated groups showing significantly lower growth rates as compared to the control group **(Table 4; Figure 6).** These results establish that FGF signalling is blocked with FGFR1 inhibitor SU5402 during various stages of fin regeneration resulting into a down-regulation of the regenerative outgrowth.

DISCUSSION

Maintenance of tissue morphology and function is a fundamental capability of multicellular organisms. The word regeneration covers several seemingly different phenomena such as the physiological regeneration in daily and seasonal cellular turnover; the reparative regeneration that includes epimorphic regeneration, tissue regeneration, and cellular regeneration; and the tissue hypertrophy caused by physiological demands or damage to internal organs (**Carlson, 2007**). In spite of these variations, the basic principle shared by these seems to be somewhat same (**Yoshinari and Kawakami, 2011**). Considering this preservation, it is fascinating to postulate a unifying view by which multicellular organisms organize and maintain their tissues and bodies. Despite the recent advances in mammalian stem cell biology and regenerative medicine, we still do not understand how cells are organized to form our bodies and why we have a particular size, morphology, and longevity. If a universal machinery for tissue preservation in multicellular organisms can be unveiled, it would open a way to re-form mammalian organs by enhancing our endogenous ability for tissue regeneration. A clue may come from studies of "super- healer" species such as fish (**Yoshinari and Kawakami, 2011)**

The present study deals with partial excisions and morphometric studies of the regenerative response, in order to investigate the role of FGF signalling during the caudal fin regeneration of *P. latipinna* using a specific FGFR1 inhibitor SU5402. Fin regeneration in fish is a rapid process. Each individual ray is able to regenerate on its own after ablation of a few ray segments, thus creating a replica of the lost structure. Following amputation, wound healing and proximal mesenchymal disorganization, proliferation, and migration occur in an FGF-independent manner. Then, FGFs synthesized in the wound epidermis bind to FGFR1 on mesenchymal cells near the amputation plane. In zebrafish regenerating fins, the FGFR1 is known to be expressed in the mesenchymal cells underlying the wound epidermis and also in the distal blastemal cells during outgrowth. Furthermore, treatment of FGFR inhibitor following fin amputation blocks blastema formation and prevents outgrowth (**Poss *et al*., 2000a**).

Morphometric measurements of the fin along with the regenerate taken during the period of study showed that the SU5402 treated group recorded slower rate of regeneration than the control group. Fishes injected with the vehicle (1% DMSO) regenerated their fin tissue by around 15 days, while the process of regeneration was impaired in animals injected with SU5402. At the gross level, it can be stated that SU5402 treatment inhibited fin regeneration and impaired the outgrowth phase of regeneration significantly.

It is also worthwhile to note that after complete regeneration, the regenerated fin has the same size as the original fin, suggesting that a control mechanism regulates the length of the fin during development and regeneration. Importantly, the regenerated fin is not a faithful copy of the original, because the position of the first bifurcation in the regenerated bony ray reproducibly undergoes distalization. Indeed, one to three segments intercalate between the level of amputation and the first fork (**Geraudie *et al*., 1994, 1995**). This finding is a specific trait to fins, because in regenerating vertebrate limbs the missing segments are faithfully and functionally replaced, and only exogenous retinoids can induce defects and modify segment identities in the limb (**Maden, 1983**) However, on observing with naked eyes, one cannot mark the difference and the regenerated part eventually (after pigmentation) is no different from the original fin in appearance or in function.

Signalling events between epithelial cells and adjacent mesenchyme play an important role in cell proliferation and patterning during fin regeneration as well as embryonic development

(**Akimenko *et al.*, 1995; Laforest *et al.*, 1998; Poss *et al.*, 2000a**). FGF2 via FGFR1 signalling plays a crucial role during fin regeneration is a fact well established (**Poulin *et al.*, 1993; D'Jamoos *et al.*, 1998; Poss *et al.*, 2000a; Yokoyama *et al.*, 2000**). The injury to blood vessels and nerves, which occurs as a result of amputation, is thought to be a trigger for the release of FGF2 (**Yadav, 2005**). Once this preformed FGF2 is released, it further activates the synthesis and release of more FGF2. Hence it is thought to work in an autocrine order.

As stated earlier, fish fins can fully regenerate their missing structures in 15 days after amputation. Immediately after the amputation, the epidermal cells are induced to migrate distally to cover the wound surface within a few hours and to form a thick epidermal tissue termed a 'wound epidermis' within 1dpa (**Poleo *et al.* 2001**) As per some studies, the process of Wound Healing occurs without the proliferation of cells and does not require a blood supply (**Santamaria *et al.* 1996; Poleo *et al.* 2001; Nechiporuk and Keating 2002; Santos-Ruiz *et al.* 2002; Bayliss *et al.* 2006**); however, the molecular signals that initiate this process are unknown. Consistent with the rearrangement and migration of epidermal cells, a transient downregulation of keratin expression and upregulation of actin expression in these cells have been observed (**Santos-Ruiz *et al.* 2002**). Furthermore, BrdU incorporation studies have revealed that the formation of the wound epidermis depends on cellular migration, not on cell proliferation (**Poleo *et al.*, 2001; Nechiporuk and Keating, 2002**). The wound epidermis seems to play an indispensable role in fin regeneration, because the treatment of a regenerating fin with retinoic acid, which promotes apoptosis in the wound epidermis, results in an abnormal patterning of the regenerating fin (**Ferretti and Géraudie, 1995**). The primary response of cells to a wound appears to be the rearrangement of epidermal cells and the formation of the wound epidermis.

Following the early formation of the wound epidermis, the appearance of rapidly proliferating cells designated as 'blastema' cells is a prerequisite for epimorphic regeneration to occur. Detailed analyses of cellular responses during fin regeneration have revealed that disorganization of mesenchymal cells occurs at a distance away from the wound epidermis and that these cells migrate towards the wound edge to give rise to the blastema cells (**Poleo *et al.*, 2001**). Besides covering the wound, the wound epidermis also synthesizes many secreted factors that mediate the communication between the blastema and the upper layers responsible for orchestrating the regeneration process (**Stoick-Cooper *et al.*, 2007a**) and one

of the major secreted factors is FGF (**Sousa *et al*., 2011**). It is possible that SU5402 inhibited these signals, in the treated fishes resulting in a delayed blastema formation. There are studies that have shown that fibroblast growth factor receptor 1 (FGFR1) (**Poss *et al.,* 2000b**) is well expressed in the blastemal tissue during fin regeneration. The normally released FGFs after the injury bind with these receptors and help the further outgrowth of the tissue. SU5402 treated fishes might have failed to express as abundantly as it normally does, thereby hindering the FGF2 to carry out its work of fin progression.

Once the blastema has been formed, the cells get engaged in repeated cycles of cell division, which results in the increase in length of the regenerate. Both classic and recent studies have indicated that a signal(s) released by the overlying regeneration epidermis controls or contributes to proliferation of the blastema. Previous works have found the evidence that signalling by fibroblast growth factors (FGFs) regulates blastemal proliferation during fin regeneration (**Poss *et al.,* 2000b; Tawk *et al*., 2002; Lee *et al,* 2005**). Also it has been observed that treatment of the fins with a pharmacological inhibitor of FGFRs SU5402, blocked blastemal proliferation when applied through the stages of regeneration (**Poss *et al*., 2000a**). Thus, it can be assured that FGF signalling is a prime candidate for influencing regenerative growth rate in the regenerating fish fin.

Regenerative growth then proceeds following blastema; and the definitive roles of blastema cells in the regeneration process is to supply the needed cells. During regeneration, the number of cells to be supplied depends on the amount of tissue lost. It is interesting to note that the growth is faster when a fin is amputated at a proximal position, but slower when the amputation occurs more distally (**Lee *et al*. 2005**). In other words, the amount of cells supplied by regeneration does not depend on the time required for the regeneration. Proximal amputation results in a larger blastema size and a higher mitotic index compared with the distal one. In the early 20th century, Morgan postulated the existence of 'formative factors' that regulate the growth rate at different levels (**Morgan, 1900, 1902**). An additional striking feature of appendage regeneration is the recognition and replacement of only those structures removed by amputation (**Lee *et al*., 2005**). This phenomenon, often called positional memory, has been studied most in the regenerating newt or axolotl limb. During limb regeneration, developmental regulation of regenerative growth rate is a prominent component of positional memory. For example, when a salamander is given an upper arm amputation on one limb and a digit level amputation on the other, regeneration of both limbs is completed in

approximately the same time period (**Spallanzani, 1769**). Thus, the greater amount of tissue that is amputated, the faster is the rate of regeneration. This phenomenon has been observed in many other lower vertebrate species, including teleosts goldfish, killifish and gourami, and in invertebrates such as starfish (**Morgan, 1906; Tassava and Goss, 1966**). The evolutionary persistence of position dependent growth rate suggests a fundamental role for this regulatory mechanism in the process of regeneration. In addition to the control of growth rate, it is also imperative to note that regenerative growth has to be terminated at appropriate positions in relation to the tissue morphology. Thus, a field of positional information seems to be implicated and to have an important role in the regulation of growth and termination of it during regeneration.

In the current study, we have demonstrated that fin regeneration is inhibited following SU5402 injections and the consequence is independent of the stage of regeneration. Our results are in accordance with the studies done by **Poss *et al*. (2000a)**, according to which, when a specific inhibitor of FGFR1 (SU5402) is administered immediately following fin amputation in zebrafish, blastema formation is inhibited, while administration during ongoing fin regeneration prevents further outgrowth suggesting a conserved molecular mechanism of FGF2 during fin regeneration.

To gain further insight into the importance of FGF signalling during fin regeneration, and to find out the influence of the inhibitor SU5402 on the nucleic acids and protein profile, supplementary studies were carried out and discussed in the next Chapter (**Chapter 3**).

Table 1: Days taken to reach various stages of regeneration in *P. latipinna*

Groups	Number of Days		
	WH	BL	DIFF
Control	[a]1 (1)	[a]4 (4)	[a]7(7-8)
Treated (LD)	[a]1(1-2)	[b]4(4-5) ***	[b]8(7-8) *
Treated (HD)	[b]2 (2) *	[b]5(5-6) ***	[b]9(8-9) *

WH: Wound healing stage; BL: Blastema stage; DIFF: Differentiation stage; LD: Low-dose group; HD: High-dose group. Values are expressed in Mode and Range in Parenthesis; Data prefixed with different alphabets are statistically significant within the column; * $p \leq 0.01$; *** $p \leq 0.001$; n=5.

Table 2: Progression of regenerate (in mm) in Control and Treated fishes

Day	Length of regenerate (mm)		
	Control	Treated (LD)	Treated (HD)
5	[a]0.93±0.069	[b]0.63±0.033***	[c]0.37±0.040***
10	[a]1.93±0.058	[b]1.53±0.061***	[c]1.27±0.046***
15	[a]2.97±0.029	[b]2.00±0.066***	[c]1.53±0.024***

LD: Low-dose group; HD: High-dose group. Values are expressed as Mean±SEM ; Data prefixed with different alphabets are statistically significant within the row; *** $p \leq 0.001$; n=5.

Table 3: Average Daily Percentage Growth rate of the regenerated fin

Day	Percentage growth rate		
	Control	Treated (LD)	Treated (HD)
5	[a]31.11±1.11	[b]21.11±2.34***	[c]12.22±2.22***
10	[a]64.44±2.00	[b]51.11±2.22***	[c]42.22±2.55***
15	[a]98.89±1.58	[b]66.67±3.01***	[c]51.11±3.25***

LD: Low-dose group; HD: High-dose group. Values are expressed as Mean±SEM; Data prefixed with different alphabets are statistically significant within the row; *** $p \leq 0.001$; n=5.

Table 4: Percentage growth rate of the regenerate during initial (between day 5 & 10) and final (between day 10 & 15) stages of growth phase.

Day	Percentage growth rate		
	Control	Treated (LD)	Treated (HD)
0-5	[a]31.11±2.33	[b]18.89±2.94***	[c]12.22±1.13***
5-10	[a]33.33±1.60	[b]30.00±1.33	[c]21.30±1.12
10-15	[a]34.44±2.75	[b]15.56±1.86***	[c]8.89±1.05***
Overall	[a]98.89±3.44	[b]64.44±4.01***	[c]51.11±3.79***

LD: Low-dose group; HD: High-dose group. Values are expressed as Mean±SEM; Data prefixed with different alphabets are statistically significant within the row; *** $p \leq 0.001$; n=5.

Figure 3: Days taken to reach various stages of regeneration

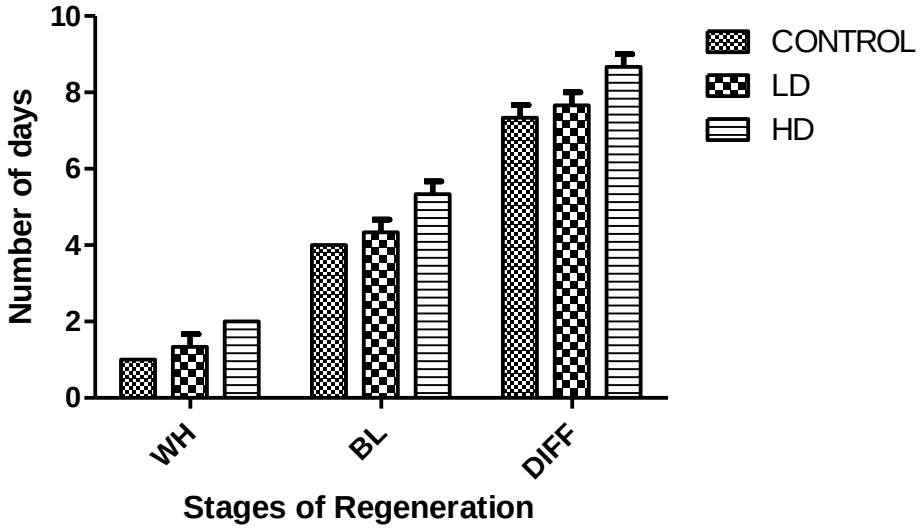


Figure 4: Progression of regeneration in mm during the process of regeneration

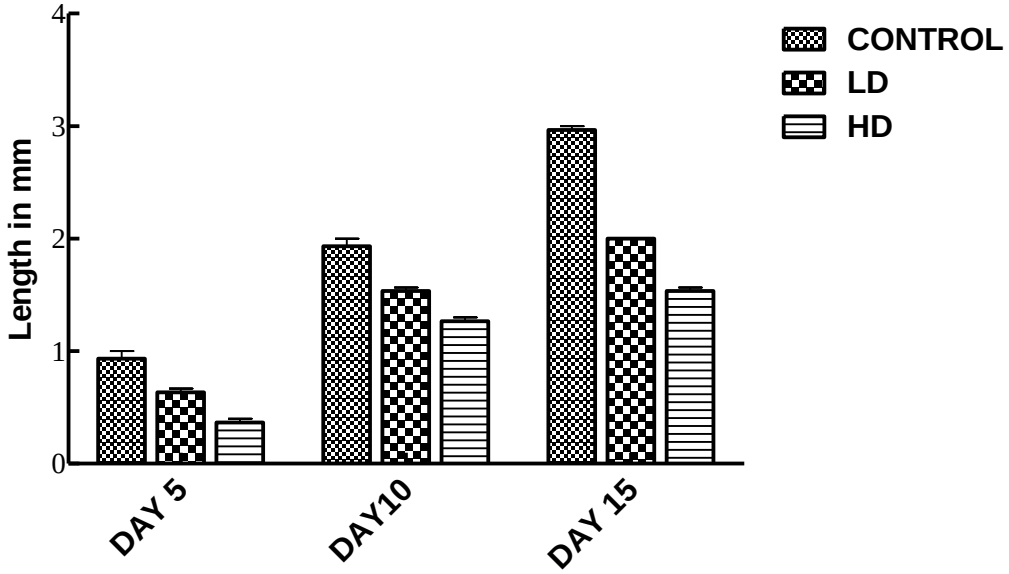


Figure 5: Percentage Growth rate during the event of regeneration

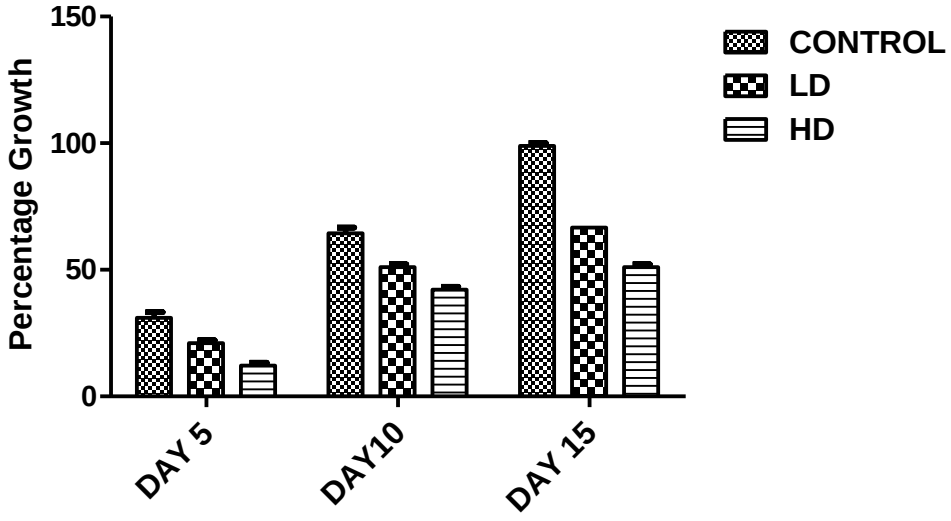


Figure 6: Daily Percentage Growth Rate

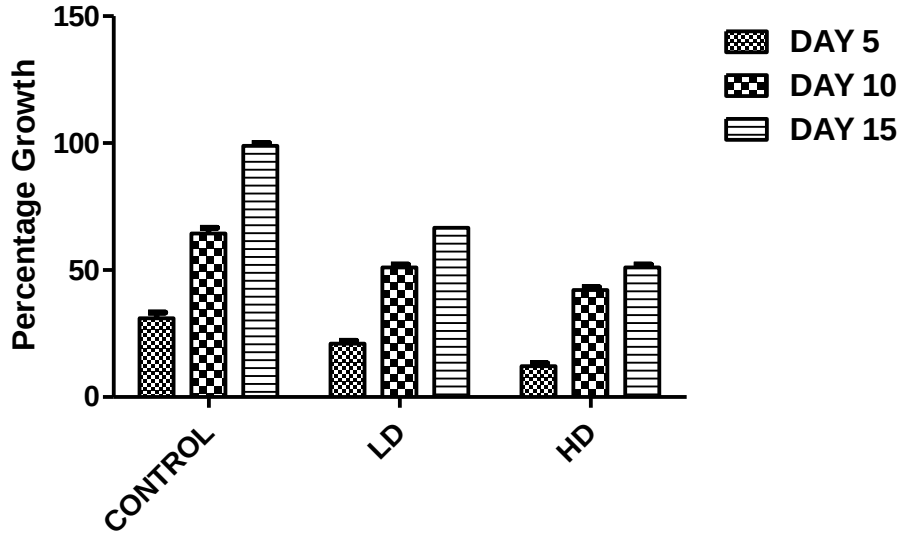


Figure 7: Morphometric analysis of regenerating tail fin treated with FGFR1 inhibitor SU5402.

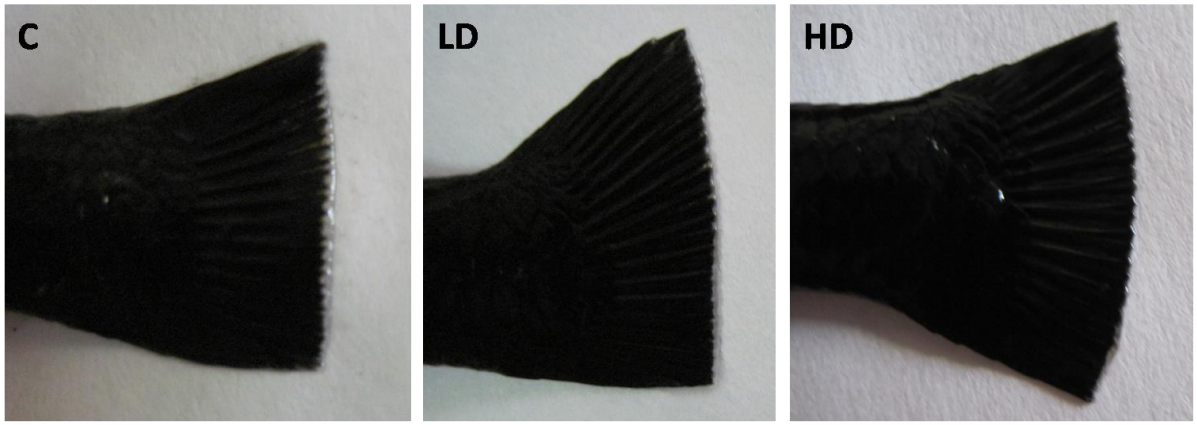


Figure 7A: Progress of regeneration in amputated fin at the **wound-epithelium** stage. **C**: Growing fin of control fish; **LD**: Growing fin of fish treated with low-dose of SU5402 (1µM/g); **HD**: Growing fin of fish treated with low-dose of SU5402 (2µM/g). Magnification: 4X.

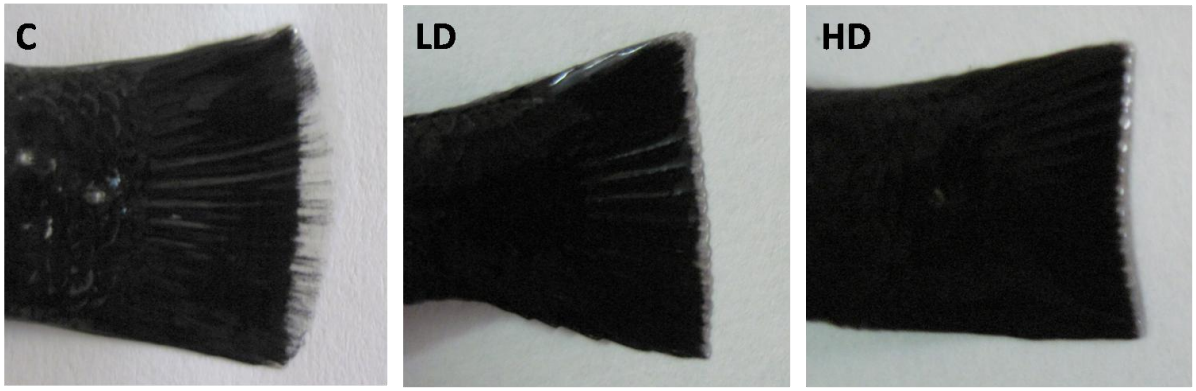


Figure 7B: Progress of regeneration in amputated fin at the **blastema** stage. **C**: Growing fin of control fish; **LD**: Growing fin of fish treated with low-dose of SU5402 (1µM/g); **HD**: Growing fin of fish treated with low-dose of SU5402 (2µM/g). Magnification: 4X.

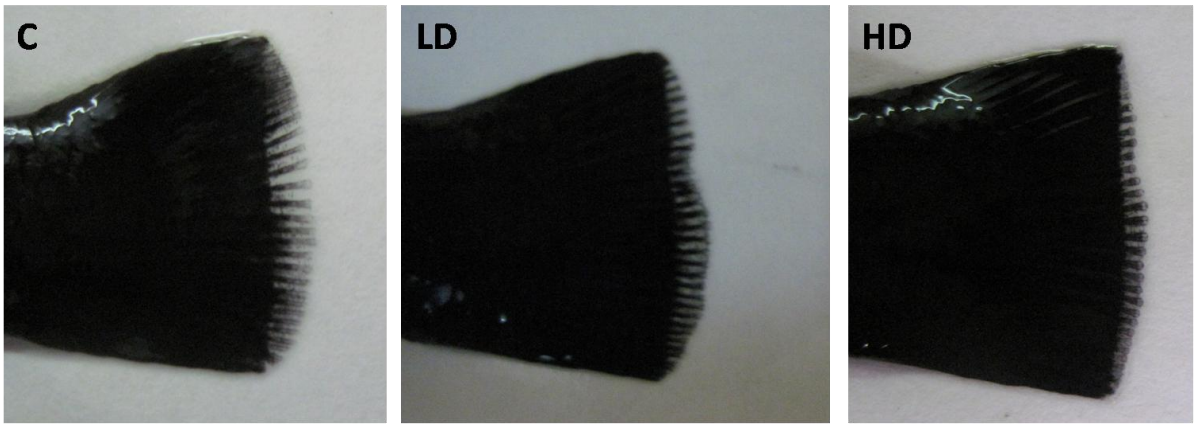


Figure 7C: Progress of regeneration in amputated fin at the **differentiation** stage. C: Growing fin of control fish; **LD**: Growing fin of fish treated with low-dose of SU5402 (1µM/g); **HD**: Growing fin of fish treated with low-dose of SU5402 (2µM/g). Magnification: 4X.

CHAPTER 3

INFLUENCE OF FGFR1 INHIBITOR SU5402 ON THE NUCLEIC ACID AND PROTEIN PROFILES OF THE REGENERATING TAIL FIN OF *POECILIA LATIPINNA*

Successful epimorphic regeneration is essential to restore the integrity and function of injured organs (**Poss *et al*., 2003**). The biomechanism of regeneration has been widely studied but poorly understood for its different extent in various animals. Understanding the basic molecular mechanism of regeneration in the wounded environment is of great significance, as it can lead to an applied possibility of making non-regenerating to a regenerating system towards therapy and healing.

When a vertebrate appendage is amputated, the transaction disrupts a number of cell substrate relations at the plane of amputation. Immediately after amputation, the deep tissues of the amputed part are directly exposed to the outside medium. After epithelial wound healing, neither a basal lamina nor the dermis intervenes between the wound epidermis and the underlying tissues. Beneath the skin, the basal lamina covering any muscle fibre and nerve axons are transacted, leaving cross sections of damaged axons and muscle fibres exposed to the fluids and clot material that collect at the amputation surface. The cut blood vessels pour out blood until constriction of the vessel wall and clotting stop the flow of blood. After the clot is formed, it attracts the neutrophils and monocytes, which are transformed into macrophages (**Broughton *et al*., 2006**). The latter are involved in phagocytosis of bacteria that may have introduced during the injury and debris of damaged and injured cells that have undergone apoptosis. These macrophages then release the growth factors which are essential for further epimorphosis. In brief, it can be said that the amputation trauma elicits a localized process similar in some respects to inflammation in the wound healing response of the vertebrates (**Mescher, 1996**); and this inflammatory response that involves activation and proliferation of satellite cells, followed by their terminal differentiation (**Tidball, 2005**) ultimately results in repair, regeneration and growth. Studies by **Campbell and Crews, (2008**) have showed that subsequent blastema formation do not occur unless the wound is healed. Wound healing phase therefore seems to be a prominent and important landmark during regeneration.

Several precise and controlled pathways as well as signalling molecules such as growth factors are involved in the wound healing and subsequent regenerative outgrowth. However, tissue repair and regeneration depends not only on the activity of such soluble factors, but also on interactions between cells and the components of the extracellular matrix (ECM). The ECM regulates the growth, proliferation, movement, and differentiation of the cells living within it. It is constantly remodelling, and its synthesis and degradation accompanies morphogenesis, regeneration, wound healing, chronic fibrotic processes, tumour invasion, and metastasis. The ECM sequesters water, providing turgor to soft tissues, and minerals that give rigidity to bone; thus it does much more than just filling the spaces around cells to maintain tissue structure (**Nechiporuk and Keating, 2002**).

A scar formation, alternatively called as apical epithelial cap (AEC) in case of vertebrate regeneration is the predominant healing process that occurs when the extracellular matrix framework is damaged by severe injury. Chronic inflammation stimulates scar formation because of local production of growth factors and cytokines that promote fibroblast proliferation and collagen synthesis, and because of accompanying damage to the ECM. Extracellular matrix components are essential for wound healing, because they provide the framework for cell migration, maintain the correct cell polarity for the re-assembly of multilayer structures and participate in the formation of new blood vessels (angiogenesis). Furthermore, cells in the ECM (fibroblasts, macrophages, and other cell types) produce growth factors, cytokines, and chemokines that are critical for regeneration and repair (**Mathew *et al*., 2006**).

Upon amputation, *Poecilia latipinna* have the capacity to completely regenerate their tail fin within 15 days by an epimorphic process; a process that occurs by the reprogramming and migration of cells that differentiate and restore a tissue to its original form. The first step of regeneration is the closure of the wound. This is a non proliferative event, involving the migration of existing epithelial cells to cover the wound (**Nechiporuk and Keating, 2002**). The epithelial cells need to break through the ECM to reach the site of amputation. The formation of wound epidermis is completed within the first 6-12 hours post amputation (hpa). Once the amputation surface is covered by a wound epithelium, the next action is characterized by the removal of many existing elements of the extracellular matrix, as the process proceeds towards dedifferentiation and blastema formation (**Hay, 1981**). The second step is blastema formation, the creation of regeneration cells that drive regeneration

(**Nechiporuk and Keating, 2002**). Shortly after the wound epidermis is formed, mesenchymal cells immediately beneath this epithelium become disorganized. In urodele amphibians, this disorganizational step requires the action of matrix metalloproteinases (**Vinarsky *et al*., 2005**). A number of cells beneath the amputation plane begin to proliferate and migrate toward the wound epidermis to form a nascent blastema (**Nechiporuk and Keating, 2002**). In the blastema, two regions can be distinguished according to their morphology and composition: the blastema proper and the blastema around the actinotrichia or actinotrichial blastemic region (**Figure 1**). Subsequently, as tissue differentiation begins within the blastema, the dynamics change towards building up an ECM that is characteristic of the stable mature regenerative outgrowth. Third, this early blastema matures to form a distal blastema consisting of slow-cycling cells that behave like stem cells even though they are derived from mesenchymal cells (**Nechiporuk and Keating, 2002**). These distal blastema cells are thought to give rise to proximal blastema cells that proliferate intensely and drive regenerative outgrowth (**Nechiporuk and Keating, 2002; Poss *et al*., 2002a**).

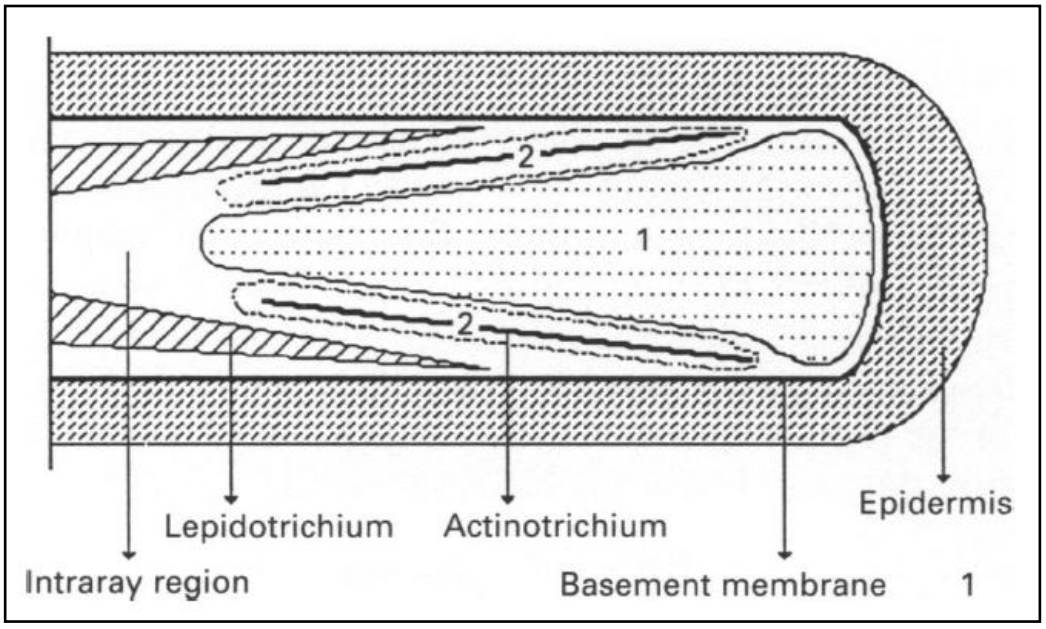


Figure 1: Schematic drawing showing the Histological analysis of the extracellular matrix of a regenerating ray (from **Santamaria *et al*., 1996**)
(1) The blastema proper and (2) blastema around the actinotrichia or actinotrichial blastemic region

Now, there are number of studies that have proved that there is an increase in the activities of a number of types of enzymes that degrade the extracellular matrix during the regressive phase of limb regeneration (**Stocum, 1995; Kato *et al*., 2003**). These enzymes include the

matrix metalloproteinases (MMPs) (e.g. collagenases, gelatinases, stromelysins), serine proteases (e.g. plasmin), and acid hydrolases (e.g. cathepsin D, acid phosphatase, carboxylic ester hydrolases) thereby altering the organization of extracellular matrix. Similarly, a sweeping change occurs in the extracellular matrix of fin too on amputation, and a series of a number of enzymes get activated for the ECM degradation and further growth of fin. Of these enzymes, the matrx metalloproteinases are considered capable of degrading most components of the extracellular matrix (**Steteler-Stevenson *et al.* 1993; Nagase and Woessner 1999**). **Figure 2** shows the pictorial representation of the events that occur during the early events of regenerative process of an adult teleost fin.

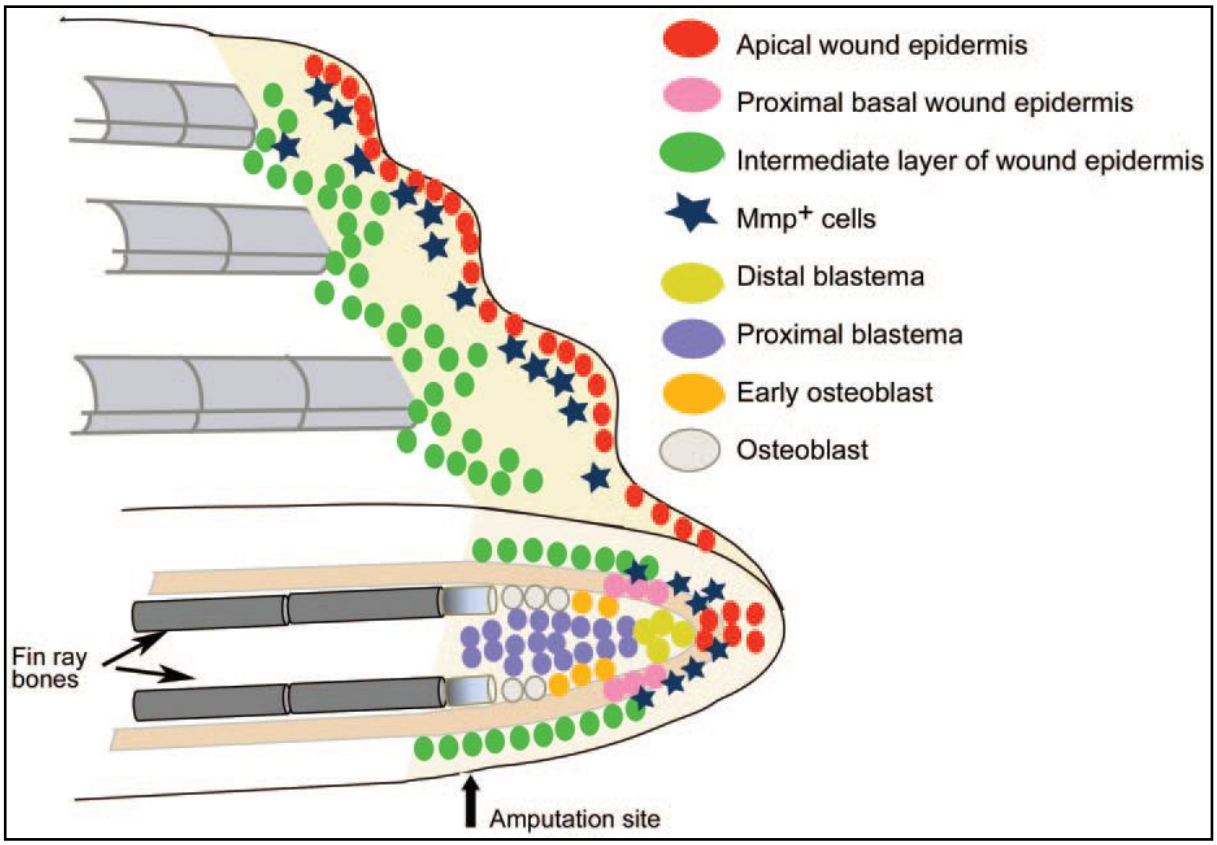


Figure 2: Cellular compartments in regenerating adult fin (from **Yoshinari and Kawakami, 2011**)

Matrix metalloproteinases were first discovered as proteases capable of digesting collagen in the tail of tadpole undergoing metamorphosis (**Gross and Lapiere, 1962; Visse and Nagase, 2003; Fujimoto *et al.*, 2006, 2007**). MMPs are a family of calcium-dependent, zinc-containing endopeptidases that are structurally and functionally related (**Bode and Maskos, 2004**); and are capable of degrading the extracellular matrix. Twenty four MMPs have been found among vertebrates (**Visse and Nagase, 2003)**. However, of lately twenty six MMPs were identified in human alone (**Verma and Hansch, 2007**). MMPs are produced and

excreted by a number of different cell types including keratinocytes, fibroblasts, phagocytes, and lymphocytes (**Brinckerhoff and Matrisian, 2002**). They are secreted in an inactive form, which is called a zymogen or a pro-MMP. These latent MMPs require an activation step before they are able to cleave extracellular matrix components (**Bode and Maskos, 2004**). They need Zn^{2+} for proper three-dimensional structure and Ca^{2+} for stability and expression of catalytic activity (**Bode *et al.*, 1999; Nagase and Woessner, 1999**). These distinct MMPs having essential roles in breaking down components of the extracellular matrix, have been classified based on their substrate specificities. MMP2 and MMP9 (also known as gelatinase A and B and) are regarded as key enzymes in degradation of the basement membrane, which consists mainly of type IV collagen (**Nagase and Woessner, 1999**).

MMP2 (Gelatinase A, 72kDa type IV collagenase) was first described and purified from highly metastatic murine tumors (**Liotta *et al.*, 1979; Salo *et al.* 1983**) and cultured human melanoma cells (**Hoyhtya *et al.*, 1990**). MMP2 is abundantly expressed in fibroblasts, endothelial and epithelial cells (**Vartio *et al.*, 1982; Salo and Oikarinen, 1985; Hipps *et al.*, 1991**). MMP9 (Gelatinase B, 92kDa type IV collagenase) was first purified from human macrophages (**Vartio *et al.*, 1982**). MMP9 expression is limited to osteoclasts, macrophages, trophoblasts, hippocampal neurocytes and migrating keratinocytes and it is controlled by growth factors, chemokines and other stimulatory signals (**Hipps *et al.*, 1991; Mohan *et al.*, 1998; Munaut *et al.*, 1999**).

The MMP activities are modulated on several levels including transcription, pro-enzyme activation, or by their endogenous inhibitors, of which the tissue inhibitors of metalloproteinases (TIMPs) are the most important. The TIMPs are also secreted proteins, but they may be located at the cell surface in association with membrane-bound MMPs (**Baker *et al.*, 2002**).

Three tissue inhibitors for MMP (TIMP-1, TIMP-2 and TIMP-3) regulate protease activity. Each TIMP inhibits various MMPs, but TIMP-1 forms complexes specifically with MMP9 (**Goldberg *et al.*, 1992**), and TIMP-2 is involved in regulation of MMP2 activity (**Itoh *et al.*, 1998; Morrison *et al.*, 2001; Zhao *et al.*, 2004**). Interestingly, TIMP-3 supports activation of MMP 2 via membrane-type MMP, as well as inhibition (**Zhao *et al.*, 2004**), and thus TIMP-3 is regarded as a major regulator of MMPs *in vivo*.

The balance between MMPs and TIMPs is largely responsible for the control of degradation of ECM proteins (**Bode *et al*., 1999**) as a deregulation of the balance between MMPs and TIMPs is a characteristic of diverse pathological conditions, such as rheumatoid and osteoarthritis, cancer progression, and acute and chronic cardiovascular diseases (**Konttinen *et al*., 1999; Tetlow *et al*., 2001; Baker *et al*., 2002**).

Previous chapter (**Chapter 2**) had proved that FGF2 signalling certainly support the epimorphosis in *P. latipinna* as evident by the delayed events throughout the regenerative process when treated with FGFR1 inhibitor SU5402. Therefore, it was logical to presume that FGF2 might play a pivotal role in the initiation and further progression of the caudal fin by acting at the first step itself i.e. ECM remodelling. There are several reports which suggest the role of FGF2 on MMPs. FGF2 is known to increase the activity of MMPs (**Palmon *et al*., 2000; Nishida *et al*., 2011**). *In vitro* studies have shown that FGF2 is one of the important regulatory factors for extracellular matrix turnover via modulation of matrix metalloproteinases and tissue inhibitors of metalloproteinases secretion from subepithelial myofibroblasts (**Yasui *et al*., 2004**). Further it was also reported that extraneous FGF2 accelerated the blastema formation in *H. flaviviridis* (**Yadav *et al*., 2012**).

Therefore, it could be presumed that FGF2 signalling must be having a decisive role in ECM remodelling of fish fin regeneration too. Roles of MMPs during fin regeneration have also been widely studies (**Bai *et al*., 2005; Andreasen *et al*., 2006)**. There are evidences that MMP activity is necessary for fin regeneration (**Bechara *et al*., 2000; Bai *et al*., 2005**). Therefore it was thought to observe the gelatinase activity in the regenerating fins of wound epithelium stage (1dpa) through gelatine zymography and immunolocalization of MMP2 and MMP9 in a case where fin regeneration was inhibited with pharmacological inhibitor, SU5402.

Furthermore, the formation of regenerating wound tissue involves not only production of new cells and ECM remoulding, but also synthesis of relatively large amounts of protein (**Dunphy and Udupa, 1955; Williamson and Fromm, 1955; Weiss and Kavanau, 1957; Fromm and Nordlie, 1959**). So logically it can be inferred that while the new tissue is being formed, nucleic acid metabolism is probably different from that observed in normal animals. There has been some indication that this may be the situation for ribonucleic acid during limb regeneration in amphibia (**Williamson and Guschlbauer, 1961**). There are several reports

indicating that specific nucleotides affect the formation of regenerating wound tissue (**Reynolds *et al*., 1958; Tremolieres and Derache, 1960**); and the problem of the formation and metabolism of both types of nucleic acid during regeneration of liver has been investigated extensively (**Nygaard and Rusch, 1955; Hecht and Potter, 1956; Jardetsky and Barnum, 1957; Bollum and Potter, 1958; Paschkis *et al*., 1959; Bollum *et al*., 1960; Fresco and Bendich, 1960**).

The earliest work on nucleic acids was in connection with exudates from regenerating wound tissue (**Meischer, 1871**). Subsequent work on such exudates still does not definitely establish whether the nucleic acids originate in the damaged cells, extraneous body tissue, leukocytes, or by synthesis in cells of the regenerating tissue (**Sherry *et al*., 1948; Schilling and Milch, 1955; Dumont, 1959**).

There are several studies which have reported that there is a series of proteomic changes that occur during the process of fin regeneration (**Bosworth *et al*., 2005; King *et al*., 2009; Encinas *et al*., 2010**). The proteins in fin are majorly associated in maintaining cellular structure and architecture. Some proteins play a recently discovered role in the complex pathways of cellular response through hormones and growth factors. These proteins, the scaffold or adapter proteins have a modular organization in which specific parts (modules) of the protein's structure recognize and bind certain structural elements in other proteins through protein-protein interactions. Furthermore, the proteins are found associated for the cytoskeleton remodelling pathway and cellular immune defense mechanism. The major proteins which were found differentially regulated during zebrafish caudal fin regeneration includes keratin and its 10 isoforms, cofilin 2, annexin a1, skeletal alpha 1 actin and structural proteins. Annexin A1 was found to be exclusively undergoing phosphorylation during regeneration (**Saxena *et al*., 2012**).Thus, obtaining differential protein expressions and the association of the various proteins during the process of regeneration might lead to a new understanding of the regeneration mechanism.

In addition it is also worthwhile to put an eye on the nucleic acid turnovers that happen during the process of regeneration. The main function of nucleic acids is to store and transmit genetic information and use that information to direct the synthesis of new protein. DNA is the permanent storage place for genetic information in the nucleus of a cell. DNA controls the synthesis of RNA. RNA transmits genetic information from DNA to the protein

synthesizers in the cell. RNA is also responsible for directing the production of the new protein by transmitting the genetic information to the protein building structures.

As to the problem of the relationship between the level of binding and the mitotic activity of the tissue, **Prodi *et al*. (1975)** reported a definite increase of binding to DNA in regenerating rat liver. Because RNA is an essential component of protein synthesis, its concentration in tissue often reflects the rate of protein synthesis. The RNA:DNA ratio provides an index of protein synthetic capacity per cell since the amount of DNA per cell is assumed not to vary with condition or with growth rate (**Bulow, 1987**). The RNA content of tissue is related to growth rate and so may also be affected by treatment with growth inhibitor. Thus, understanding the mechanisms of regeneration and repair requires knowledge of such a control of cell proliferation and signal transduction pathways, stage specific expressions of proteins and levels of nucleic acids throughout the event of regeneration and the many functions of ECM components.

Therefore, in the present study attempts were made to evaluate nucleic acids as well as protein levels in the regenerating fins of the control and SU5402 treated fishes to understand the alterations caused due to block of FGF2 signalling and performed SDSPAGE analysis to study the regulation of stage specific expression of proteins in the normal as well as the treated fish fin regenerates.

MATERIAL AND METHODS

Animals, Maintenance and drug dosage

Healthy fishes of both the sexes were selected and acclimated in glass aquariums at 26±2ºC for a week prior to the experiment. The animals were divided into two groups of control and treated and dosed according to the experiments as explained below. For the nucleic acids estimation, the animals divided into two groups, control and treated received dose of 1% DMSO and 2µM SU5402/g body weight respectively. The treatment in each group started a day prior amputation and was continued till the control animals reached the differentiation stage. The drug/vehicle was injected every day. The regenerating fins of the fishes were amputed at three defined stages of regeneration *viz*., (1) wound epithelium (1dpa), (ii) Blastema stage (4dpa) and (iii) Differentiation stage (7dpa).

Nucleic acids and Protein estimation

The fins from each group were pooled, homoginized for 10% and then further processed for estimating the nucleic acids as well as the protein contents in the tissue sample. Extraction of nucleic acids was done by the method described by **Schneider, (1957)** and the DNA and RNA levels were estimated by the DPA and Orcinol methods respectively (**Sadasivam and Manickam, 1992**). The protein estimation was done according to BCA (Bicinchoninic acid) assay kit (Genei Products, Merck, USA) as described by **Smith *et al*. (1985).**

SDS-PAGE

Expression of various proteins at each stage was identified using SDS-PAGE technique. Protein content was determined using BCA assay. Equal amount of total protein was loaded and separated by SDS-PAGE on 12.5% gels; stained with silver staining method (Details in **Material and Methods**).

Immunolocalization and detection of gelatinase activity

For the MMP2 and MMP9 immunolocalization studies, control and treated animals fins were collected. The fins reached wound epithelium were excised. Five such regenerates were used for the immunohistochemical localization of MMP2 and 9. The other five fins were pooled and used for Gelatin zymography. For immunolocalization, the fins were sectioned (9µm), fixed with pre-cooled acetone, and further processed with appropriately diluted primary antibodies, followed by FITC-labelled secondary antibodies (explained in detail in **Material and Methods**). For detection of gelatinase activity the pooled fins from each group were homoginized (10%) with PBS:Lysis buffer (1:1) and after estimating the protein content, gel (12% SDS polyacrylamide gels with gelatin concentration of 1mg/ml) was run by loading equal amount of protein in each well. After running, the gel was incubated with zymogram renaturing buffer with gentle agitation for 30 minutes at room temperature. The gel was then incubated overnight with zymogram developing buffer. The gel was then stained with Coomassie blue R250 for 30 minutes, followed by destaining with an appropriate Coomassie R250 destaining solution. Areas of protease activity appear as clear bands against a dark blue background where the protease has digested the substrate.

DETERMINATION OF MOLECULAR WEIGHT

Molecular weight of the zymographic bands were determined by using Doc-ItLs software (GeNei, Merck, USA).

SPOT DENSITOMETRY ANALYSIS

Spot densitometer was performed on all the bands using Doc-ItLs software (GeNei, Merck, USA). Densitometry was performed on the scanned images of the gel taken in charged coupled device camera (CCD) and edited in Adobe Photoshop. Similar areas were analysed for the densitometry. Auto background subtraction was performed using the same software. Using densitometric values, quantitative comparison was made in all the bands of interest and the results were expressed in arbitrary units, which was calculated by integration of the intensity of each pixel over the spot area and normalized for the gel background. In case of zymograms, the zone of clearance was quantified on the basis of area enclosed by the region in pixels together with the band intensity.

RESULTS

Transcriptional and translational profiling of regenerating caudal fin

The amount of DNA contained in regenerating tissue at three specific stages after the excision of the caudal fin is shown in Tables 2, 5 & 8. The amount of DNA (µg/100mg tissue) in the regenerating tissue appeared to increase at blastemal stage (about 4dpa) as compared to wound healing stage (1dpa) and thereafter gradually decreased by the time it reached the differentiation stage (7dpa). The amount of RNA (µg/100mg tissue) also reached its maximum at the blastemal stage. In all the cases, however, SU5402 treated group always showed a lower concentration of DNA and RNA as compared to the controls. Similar results were obtained for protein content also during all three stages (**Table 1, 4 & 7**).

Accordingly, the DNA:RNA as well as RNA:Protein ratios were also found to be lowered during the early regeneration followed by an intense increase at the blastemal stage and then a gradual decline as the cells begin to redifferentiate to form the lost structure. However, the SU5402 treatment reduced the ratios significantly at all the three stages of regeneration, (**Table 3, 6 & 9**).

A decreased amount of DNA and RNA in the tissue during the wound healing juncture are to be expected because, during healing of the wound, the cells do not undergo cell division and so can be considered as being in the lag phase; and the mitotic activity and DNA synthesis begins after this phase. After this period, a vigorous DNA synthesis starts as it is evident from the higher amounts of DNA in the regenerating fins at the blastemal stage. The values

then gradually decrease as the fin proceeds to the end of regeneration course (**Table 2, 5 & 8**).

Protein profiling by SDS-PAGE

Alterations in the protein expressions were found at all the three stages, *viz*., Wound healing, Blastema formation and Differentiation stages during regeneration. Some of the protein bands which were observed in the control group remained absent in the SU5402 treated groups. Also, the intensity of protein bands was found to be low in the treated samples as compared to the control ones.

At wound healing stage (1dpa), the protein fraction of control animals were enriched with polypeptides having molecular masses of 73.42, 59.71, 54.36, 28.61, 18.09, 13.78 and 10.71 kilo Daltons (kDa). The intensity of these bands was much less in the SU5402 treated groups as compared to the control with the exception of the band 59.71 kDa that showed the intensity somewhat greater in the treated as compared to the control ones. However, the difference in the intensity was not very high (**Table 10**).

The blastemal stage showed absence of many prominent polypeptides that were observed with great intensity in the control groups. The band of the molecular weights 116.54, 67.32 and 54.36 remained absent in the treated groups whereas they were observed with high intensity in the control groups. Some other bands with similar molecular masses were also observed (107.04, 93.24, 45.92, 13.78 kDa); nevertheless, the intensity in treated always remained low as compared to the control group (**Table 11**).

At differentiation stage, the protein fraction of control animals were enriched with polypeptides having molecular masses of 116.54, 107.04, 93.24, 73.42, 67.32, 59.71, 54.35, 45.92, 28.61, 23.71, 18.09, 13.78, 10.71 kDa. The intensity of these bands was much less in the SU5402 treated groups as compared to the control with the exception of the band 67.32 kDa (**Table 12**).

Immunolocalization of MMP2 and MMP9

The fins after the appropriate staining showed the presence of both MMP2 and MMP9. However, on comparing the regenerated fins of the control and the treated ones, the negative or inhibitory effect of SU5402 could be visualized. SU5402 had caused a significant

reduction in the amount of MMP2 and MMP9, as could be seen in Figures 4 & 5. MMP2 and MMP9 were localized abundantly in the regenerating fins of the control fins as compared to the SU5402 treated (**Figure 5**). Thus, it was valid to assume a reduction in the protease activity in the FGFR1 inhibitor treated group, which was further reaffirmed by Gelatin Zymography studies.

Gelatinase activity in the regenerating fins

Since, the interruption in fin regeneration due to SU5402 treatment was already observed in **Chapter 2**, and immunolocalization of MMPs showed the alteration of MMP2 and MMP9 expressions in the regenerating treated fins as compared to control (**Figure 5**), we conducted zymographic analysis to determine if this pharmacological FGFR1 inhibitor altered gelatinase (MMP2 and MMP9) activity in the regenerating fin. Two gelatinases were identified in the regenerating fins, a roughly 90-kDa form pre- MMP9 and a 72-kDa form pre- MMP2 (**Figure 4**). Both putative MMP2 and MMP9 activity formed two bands indicative of the physiologically inactive pre-form and the proteolytically active lower molecular weight protein. MMP2 and MMP9 activity was found elevated in regenerating fin tissue and fin tissue in the control groups at 1dpa; however, the intensity of band was found to be lower in case of the inhibitor treated group.

DISCUSSION

The fins of teleosts are appendices capable of regenerating by an epimorphic process that completely restores the original shape and size in a few days. The process of regeneration is a multifaceted one which begins with amputation and results in the complete replacement of the structures and tissues removed. Two key signals- soluble growth factors and extracellular matrix (ECM) directly influence the cell's decision to move or to stop (**Huttenlocher *et al*., 1995; Lauffenburger and Horwitz, 1996; Sander *et al*., 1998; Jimenez *et al*., 2000**). Cell migration, driven by growth factors and cytokines released concordantly into the injury site, is particularly critical during regenerative events. The signals that trigger the onset of regeneration are elicited as a direct response to the amputation; and understanding the nature of these early signalling molecules is the main objective of the present study.

After a partial amputation, the process that follows consists of: wound healing, blastema formation and cell differentiation to form a particular cell population responsible for the building of the different tissue elements. This extensive process comprises, essentially, cell

proliferation and differentiation. It is known that the interaction between the cells and the extracellular matrix (ECM) is, in part, responsible for the control of both processes (**Hay, 1981**). This interaction has been widely studied in several *in vitro* and *in vivo* models (**Trelstad, 1984**), and the teleost fin regeneration is though it has been proved that it is a good *in vivo* model for the study of the regenerative processes (**Mari-Beffa *et al*., 1999; Stoick-Cooper *et al*., 2007a,b**).

ECM is a dynamic environment that plays a crucial role in regulating cellular functions during normal and pathological remodelling processes such as embryonic development, tissue repair, inflammation, tumour invasion and metastasis. ECM macromolecules are critical for creating a conductive cellular milieu for proper proliferation and migration of different cell types. The MMPs are specifically controlled at the transcriptional level and by cell-ECM interactions. The expression and functional role of MMPs has been studied in adult zebrafish regenerating fin and have demonstrated the expression of MMP2 in the regenerating fin tissues.

Inhibition of fin regeneration by SU5402, a specific FGFR1 inhibitor was already demonstrated in **Chapter 2**. We wanted to determine whether SU5402, played any cardinal role in affecting the nucleic acids and protein levels of the regenerating caudal fins and to what extend it disrupts the activities of ECM matrix metalloproteinases MMP2 and MMP9. Thus, the present chapter deals with the alterations of the DNA-RNA-Protein levels in the pharmacological inhibitor treated group, observation of the alterations in proteins by SDS-PAGE, immunolocalization of MMP2 and MMP9 and finally running a zymogram to observe the inhibition of the protease activity in the SU5402 treated animals.

The first step in this project was to evaluate how much the inhibitor affects nucleic acid and protein levels in the experimental animals of Control (1% DMSO) and Treated (SU5402) groups. We examined the variation in total DNA, total RNA, total Protein as well as the DNA: RNA and RNA: Protein ratios during different stages of fin regeneration. Experiments dealing with mitotic counts (**Baguna, 1976**) and with cytophotometric analysis of total DNA content (**Gremigni and Miceli, 1980**) or measurements of DNA, RNA and Proteins by ^{32}P (**Martelly *et al*., 1981**) conducted on regenerating planarians have already showed the significance of DNA-RNA-Protein ratio in epimorphic regeneration. In our results, overall, the DNA: RNA: Protein content was minimal during the early stages of regeneration as

compared to the later stage and then slowly lowered by the time it reached differentiation stage. We found a radical decrease in the total DNA, RNA and Protein contents as well as the DNA: RNA and the RNA: Protein ratios of the FGFR1 inhibitor treated fins as compared to the control ones.

Decreased DNA content in the regenerating fins of SU5402 treated fishes is suggestive that these cells could not enter the new cycles, main reason probably being the insufficient availability of growth factor receptor FGFR1. Thus, there is probability of a defect in cell cycle regulation, following retardation in the rate of replication of the dividing cells, that consequently results in the low DNA content in the SU5402 treated fins. Apart from the synthesis of DNA, the proliferating blastemal cells also transcribe RNA and synthesize new proteins to meet the demands of the rapidly dividing cells. Synthesis of RNA is followed by the translation of mRNA into proteins in the regenerates. The concentration of RNA and Protein as well were lesser in the receptor inhibitor treated groups as compared to the control ones. These low rates of DNA, RNA and Protein in the treated animals, to some extent, reflect the unavailability of growth factors to the injured tissue, thereby lowering their transcriptional and translational levels. Thus, the demonstration of such changes in the DNA-RNA concentration and Protein metabolism of amputed animals as compared to normal animals points to the possibility that there also may be some change in the nucleic acid metabolism of the wounded animals. It seems quite probable that some further clue to the metabolism of the nucleic acids may be obtained from consideration of the nucleotide and protein content of the regenerating tissue. Hence, the present chapter considers an alteration in the metabolism of nucleic acids and thereby protein synthesis in regenerating fins when treated with FGFR1 inhibitor SU5402.

The synthesis and deposition of extracellular matrix (ECM) is a critical feature in the healing followed by subsequent events. Cells that synthesize the ECM proteins must meet increased production demands at the time of injury to successfully accomplish healing. Interactions among the ECM, growth factors, and cells underlie tissue generation and regeneration, including wound healing. These elements interact in an ongoing, mutually influential series of events that has been referred to as dynamic reciprocity (**Bissell *et al*., 1982**) an example of this is that during the inflammatory phase, fibronectin and other ECM protein fragments in the wound area serve as chemoattractants for monocytes, (**Clark *et al*., 1988**) which then bind to ECM proteins. This binding stimulates phagocytosis (**Brown and Goodwin, 1988**)

leading the monocytes/macrophages to further break down ECM fragments and other debris in the area (**Clark, 1993a,b**) Adherence of monocytes to ECM proteins also stimulates the expression of growth factors (**Shaw *et al*., 1990**) that can then act on cells to affect the synthesis of ECM components (e.g., proteoglycan synthesis by fibroblasts) (**Lin *et al*., 2005**). An interaction between growth factors and ECM in this dynamic reciprocity is of great significance. Degradation and remodeling of the ECM by proteases, particularly matrix metalloproteases (MMPs), is a key feature of leukocyte influx, angiogenesis, reepithelialization, and tissue remodeling. MMPs also degrade growth factors and their receptors, as well as angiogenic factors. Control of these various elements by MMPs, in part, determines whether angiogenesis will be stimulated or inhibited (**Heissig *et al*., 2003**) MMPs also play an essential role in liberating growth factors and cleaving ECM proteins to reveal regions that can activate growth factor receptors (**Mott and Werb, 2004**) Thus, MMPs act not only to degrade and remodel selected ECM components at appropriate times, but also to reveal selected bioactive ECM segments through targeted cleavage that ultimately influence cellular behaviour(**Mott and Werb, 2004**). Alterations in this complex regulatory pathway can not only directly affect the structural architecture of a tissue but can also affect cell growth, cell migration, cell-cell communication, apoptosis, and angiogenesis, since ECM also serves as a source and store of biologically active molecules which can be released and or activated by MMPs (**Mott and Werb, 2004**).

The expression and functional role of MMPs has been studied in adult zebrafish regenerating fin and have demonstrated that MMP2 mRNA transcripts were greatly expressed in the regenerating fin tissue. Fin outgrowth was significantly reduced by GM6001, a MMP inhibitor, emphasizing the magnitude of these proteinases during fin regeneration (**Bai *et al*., 2005**). There are studied which show that ECM remodelling is impaired in regenerating fins of TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) exposed adult zebrafish (**Andreasen *et al*., 2006)**. Thus, it will be not surprising then that the dynamic and complex event of fin regeneration requires a functional MMP system (**Bai *et al*., 2005**).

FGF2 is believed to increase the activity of MMPs (**Palmon *et al*., 2000; Wang *et al*., 2004; Nishida *et al*., 2011**). FGF2 enhanced the MMP2 and MMP9 production in the human bladder cancer cell lines (**Miyake *et al*., 1997**). FGF2 prompts the endothelial cells to produce MMPs thereby stimulating the endothelial cell migration, pericyte attraction and matrix deposition (**Presta *et al*., 2005**). There are also studies which showed that FGF2

released from the lens capsule by MMP2 is essential to lens epithelial cell viability and survival. Thus, it is evident that both are interdependent and work in an orchestered and highly controlled manner. Altering any of the signals via pharmaceutical inhibitors might result in unfavourable conditions for their actions.

Significance of FGF2 was already learned in the previous chapter (**Chapter 2**) by observing the delayed wound epithelium in the tyrosine kinase inhibited (SU5402) group. Thus, it was apparent to find out its role in the proteomic and proteolytic turnovers that occur during ECM remodelling and how the receptor inhibitor affects former. Therefore, the expression of proteins as well as the activity levels of the gelatinases MMP2 and 9 was evaluated in the control and SU5402 treated groups. Results depicted a high significance of FGF2 during each of the stages of regeneration. FGF2 is said to be an important candidate for ECM remodelling, as the gelatinase activity was observed to be greatly reduced in the treated tissues as compared to the control ones. The intensity of the band was much lighter in the treated as compared to the control (**Figure 4**). It is possible that the process of matrix reorganization in the animals treated with SU5402 might have been affected which consecutively resulted in delayed epimorphosis (**Chapter 2**). The immunoexpression of MMP2 and MMP9 also revealed a much lower expression in the treated fins as compared to the control (**Figure 5**). Thus, the results obtained in the previous chapter may be due to the delayed ECM remodelling due to lowered availability if FGF2 as a consequence of treatment with FGFR1 inhibitor. The less intense bands of the MMP2 and MMP9 shows that the FGF2 could not exert its property of remodelling the matrix as it does normally when treated with the receptor inhibitor, SU5402.

Also, a regulation of differential expression of proteins is required for a successful fin regrowth. The proteins expressions in the regenerating fins of the control and treated were evaluated by SDS-PAGE and the intensity of the band was measured using spot densitometry. Lower content of protein in the treated fins as compared to the control ones is showed by the absence of few of the band in the treated fins. These may be the proteins that are required for regeneration, and could not be expressed as a result of the signal inhibitor treatment; thus proving the importance of FGF2 in the expression of various essential proteins required for regeneration. Therefore, it is valid to assume that FGF2 down regulated many of the essential proteins that may have significance during caudal fin regeneration. No significant difference in the protein bands at differentiation stage between both the groups

leads to suppose that the later stage of regeneration is by and large independent of FGF2 signalling. However, minor alterations in the expression of bands in both the groups cannot be neglected. Two of the polypeptide bands having molecular weight of about 72 kDa and 92 kDa was found to have maximum intensity at 1dpa and was found absent in the later stages. This can be correlated with the zymogram studies conducted. The intensity of these two bands are as expected lower in case of the treated in compared to the control.

In summary, our data provides evidence that inhibition of FGF2-FGFR1 signalling pathways inhibits caudal fin regeneration. This inhibition may be mediated by unsuccessful interaction between the FGF2 and its receptor, thereby resulting in lower nucleic acids and protein contents and reduced level of MMPs activity and lowered protein expression in the fins. In this study, we examined the cross talk between ECM, MMPs, nucleic acids and proteins for the regrowth of the lost tissue. Interactions between the ECM and growth factors via receptors are an important affair that helps in the further responses of the fin regrowth. Thus, FGF2 signalling is unavoidable for the fin regeneration. To have a further insight in to the role of FGF2 in the maintenance of regeneration, studies on rate of cell proliferation and differentiation were conducted (**Chapter 4**).

PROTEIN CONTENT AND NUCLEIC ACID LEVELS DURING WOUND EPITHELIUM STAGE (1dpa)

Table 1: Protein content in the fin regenerates of control and SU5402 treated fish

Experimental Group	**Protein Content (mg/100mg tissue)**
Control	0.801±0.02[@]
Treated	0.712±0.012*

Table 2: Nucleic Acid levels in the fin regenerates of control and SU5402 treated fish

Experimental Group	**DNA (µg/100mg tissue)**	**RNA (µg/100mg tissue)**
Control	18.233±0.145	3.510±0.006
Treated	11.867±0.186*	2.637±0.020*

Table 3: Cellular proliferative and synthetic activities in the fin regenerates of control and SU5402 treated fish

Experimental Group	**Ratio of DNA/RNA and RNA/Protein**	
	DNA/RNA	RNA/Protein
Control	5.337±0.114	4.399±0.156
Treated	4.503±0.046*	3.769±0.081*

[@]Values are expressed as Mean ± SEM; n=5; Control: 1% DMSO; Treated: 2µM/gm body wt. SU5402; *$p<0.001$

PROTEIN CONTENT AND NUCLEIC ACID LEVELS DURING BLASTEMA STAGE (4dpa)

Table 4: Protein content in the fin regenerates of control and SU5402 treated fish

Experimental Group	**Protein Content (mg/100mg tissue)**
Control	0.900±0.012[@]
Treated	0.807±0.018*

Table 5: Nucleic Acid levels in the fin regenerates of control and SU5402 treated fish

Experimental Group	**DNA (µg/100mg tissue)**	**RNA (µg/100mg tissue)**
Control	28.167±0.167	5.033±0.033
Treated	18.667±0.441*	4.100±0.058*

Table 6: Cellular proliferative and synthetic activities in the fin regenerates of control and SU5402 treated fish

Experimental Group	**Ratio of DNA/RNA and RNA/Protein**	
	DNA/RNA	RNA/Protein
Control	5.597±0.061	5.593±0.108
Treated	4.557±0.172*	5.083±0.108*

[@]Values are expressed as Mean ± SEM; n=5; Control: 1% DMSO; Treated: 2µM/gm body wt. SU5402; *P<0.001

PROTEIN CONTENT AND NUCLEIC ACID LEVELS DURING DIFFERENTIATION STAGE (7dpa)

Table 7: Protein content in the fin regenerates of control and SU5402 treated fish

Experimental Group	**Protein Content (mg/100mg tissue)**
Control	0.860±0.012[@]
Treated	0.767±0.007*

Table 8: Nucleic Acid levels in the fin regenerates of control and SU5402 treated fish

Experimental Group	**DNA (µg/100mg tissue)**	**RNA (µg/100mg tissue)**
Control	22.333±0.167	4.590±0.038
Treated	15.333±0.333*	3.830±0.012*

Table 9: Cellular proliferative and synthetic activities in the fin regenerates of control and SU5402 treated fish

Experimental Group	**Ratio of DNA/RNA and RNA/Protein**	
	DNA/RNA	RNA/Protein
Control	4.865±0.015	5.399±0.104
Treated	4.003±0.088*	4.996±0.046*

@Values are expressed as Mean ± SEM; n=5; Control: 1% DMSO; Treated: 2µM/gm body wt. SU5402; *P<0.001

Table 10: Effect of FGFR1 inhibitor SU5402 on the protein profile on the fin regenerates of control and treated fish at Wound Epithelium stage (1dpa): blank cells indicate absence of specific protein band

LANE 1 (Control)		**LANE 2 (Treated)**	
Molecular Weight (kDa)	Optical Density (Arbitrary Unit)	Molecular Weight (kDa)	Optical Density (Arbitrary Unit)
116.54	487.13	116.54	608.13
107.04	455.68	107.04	26.36
93.24	473.85	93.24	376.24
73.42	**394.58**	**73.42**	**251.46**
67.32	611.33	67.32	111.24
59.71	839.22	59.71	921.1
54.36	**485.89**	**54.36**	**347.93**
45.92	479.72	45.92	301.79
28.61	**374.97**	**28.61**	**167.46**
23.71	490.53	23.71	295.05
18.09	**561.24**	**18.09**	**337.03**
13.78	**629.03**	**13.78**	**415.98**
10.71	**660.99**	**10.71**	**446.42**

Table 11: Effect of FGFR1 inhibitor SU5402 on the protein profile on the fin regenerates of control and treated fish at Blastema stage (4dpa): blank cells indicate absence of specific protein band

LANE 1 (Control)		**LANE 2 (Treated)**	
Molecular Weight (kDa)	Optical Density (Arbitrary Unit)	Molecular Weight (kDa)	Optical Density (Arbitrary Unit)
116.54	**289.48**	**116.54**	**-**
107.04	**142.83**	**107.04**	**114.51**
93.24	**241.12**	**93.24**	**112.2**
73.42	1.5	73.42	249.45
67.32	**110.43**	**67.32**	**-**
59.71	236.84	59.71	457.57
54.36	**104.26**	**54.36**	**-**
45.92	**160.13**	**45.92**	**104.55**
28.61	214.68	28.61	212.22
23.71	436.32	23.71	4.12
18.09	0	18.09	268.66
13.78	**331.15**	**13.78**	**326.49**
10.71	4.36	10.71	421.66

Table 12: Effect of FGFR1 inhibitor SU5402 on the protein profile of the fin regenerates of control and treated fish at Differentiation stage (7dpa): blank cells indicate absence of specific protein band

LANE 1 (Control)		**LANE 2 (Treated)**	
Molecular Wt. (kDa)	Optical Density	Molecular Wt. (kDa)	Optical Density
116.54	495.3	116.54	384.8
107.04	366.39	107.04	205.73
93.24	399.62	93.24	292.15
73.42	347.48	73.42	237.33
67.32	277.95	67.32	278.01
59.71	374.75	59.71	296.64
54.36	366.08	54.36	278.69
45.92	363.83	45.92	297.44
28.61	386.78	28.61	316.98
23.71	397.36	23.71	345.81
18.09	521.69	18.09	472.32
13.78	593	13.78	523.2
10.71	563	10.71	504.05

Figure 3: Effect of FGFR1 inhibitor SU5402 on the protein profile (SDS-PAGE ANALYSIS) of the fin regenerates of control and treated fish at three stages- WE: wound epithelium (1dpa); BL: blastema (4dpa); DF: differentiation (7dpa), MW: medium range molecular weight marker (98, 67, 44, 29, 16kDa)

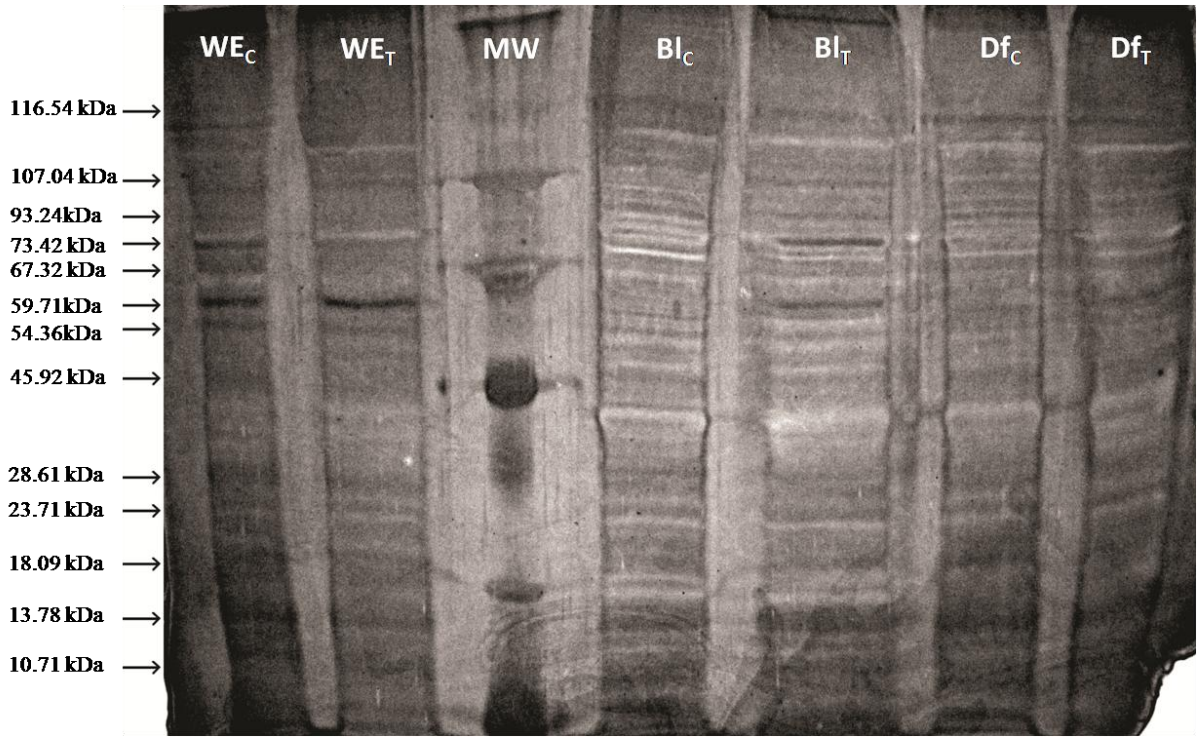


Figure 4: Effect of FGFR1 inhibitor SU5402 on MMP activity of the fin regenerates of control and treated fish at wound epithelium stage (1dpa) with molecular weight marker

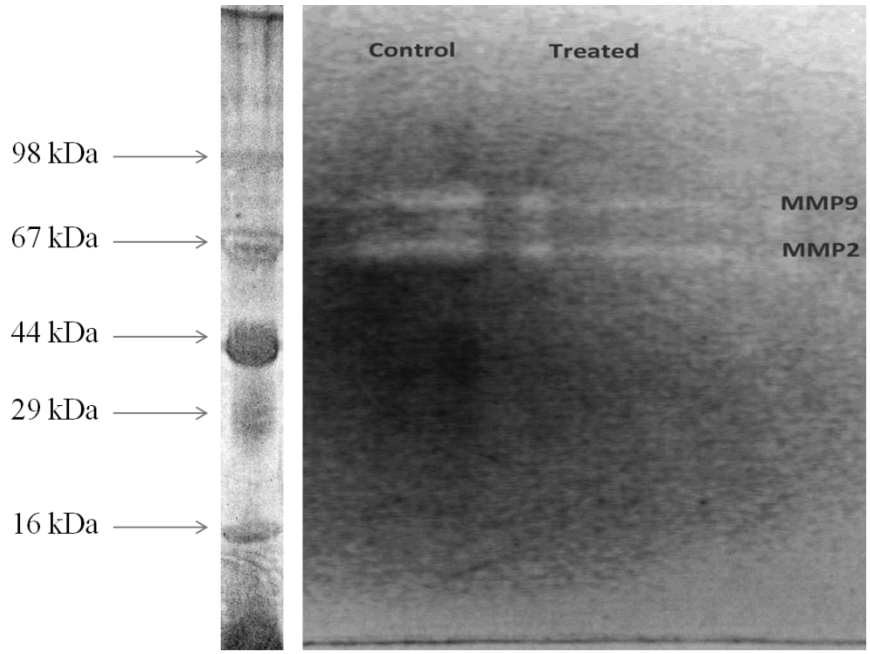
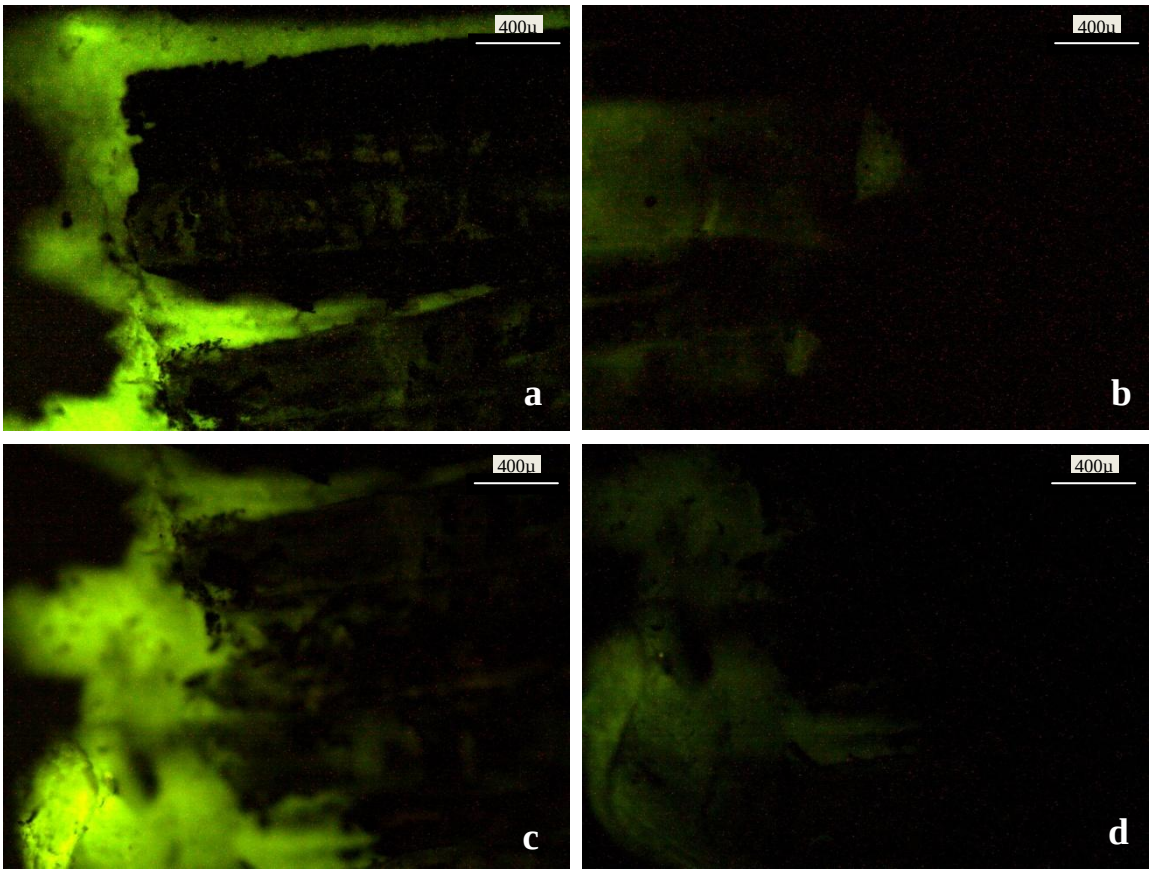


Figure 5: MMP-2 and MMP-9 localization in fin regenerates of control and SU5402 treated fish during Wound Epithelium stage(1dpa).



a: MMP9 localization in control group(1dpa); **b**: MMP9 localization in SU5402 treated group(1dpa); **c**: MMP2 localization in control group(1dpa); **d**: MMP2 localization in SU5402 treated group(1dpa).

CHAPTER 4

EVALUATING THE ROLE OF FGF2 SIGNALLING ON CELL PROLIFERATION, GROWTH AND DIFFERENTIATION DURING CAUDAL FIN REGENERATION IN *POECILA LATIPINNA*

INTRODUCTION

No animal can survive without some regenerative or self-renewal capacity. There is no doubt that regeneration, or in some case just wound healing, plays a useful part in the life and survival of animals. The skin in its normal state is continually undergoes regeneration and repair. The epidermal cells which are shed from the surface are constantly replaced from below to maintain equilibrium. The active state of regeneration normally displayed by the epidermis is probably of evolutionary significance because the skin is continually subjected to trauma and without an effective mechanism of quick repair of the outer surface, the life and survival of an individual would be precarious (**Mittal and Munshi, 1974**). Thus, the process of regeneration in the broadest sense is vegetative reproduction and the capacity for regeneration varies in different groups of animals. An inevitable fact is that all organisms posses the power to produce new cells.

The term 'wound' refers to the break in continuity of a tissue. This break may not be associated with a loss of tissue as in incised wounds or there may be varying degree of loss of substance caused by physical, chemical, microbial or immunological insult to tissue (**Johnson and Mc Minn, 1960**). There does not seem to exist a precise definition of the term 'healing' in the literature. According to many authors (**Mittal and Munshi, 1974; Phromsuthirak, 1977; Mittal *et al.*, 1978; Al-Hassan *et al.*, 1991; Ramesh *et al.*, 1993; Martin *et al.*, 1994**) a wound is said to be fully healed when it becomes fully epithelised. But it ignores the fact that many changes still continue to occur in the underlying connective tissue long after the surface cells have been restored. Regeneration, as it is well established, is the renewal of lost/removed part of the body. It is therefore resolved that, once a wound is formed the first step undergone is repair of the wound which is then followed by regeneration. The former is characteristic of all organisms, but the capability of an organism for the latter varies, being restricted to some organs in some animals (**Poss *et al.*, 2000b**).

One of the earliest signalling pathways known to be activated in response to fin injury or amputation is the FGF pathway (**Stoick-Cooper *et al*., 2007a; Whitehead *et al*., 2005**). FGF2 signalling is required prior to blastema formation to induce the proliferative response of fibroblast-like and epidermal cells in the regenerating fin of fish (**Poss *et al*., 2000b; Whitehead *et al*., 2005**). Osteoblast proliferation in the distal stump is significantly reduced after treatment with the FGF receptor 1 inhibitor SU5402 (**Knopf *et al*., 2011**).

Also, FGF2 as a crucial factor has already been established from the previous chapters; as the inhibitor treated groups failed to reach the defined stages of regeneration timely, and showed a dramatic reduction in the nucleic acids as well as protein turnovers and reduced MMP levels (**Chapter 3**). The reason understood by now was that SU5402 inhibited the expression of FGF2 thereby reducing the cellular synthetic activity at the regenerating part. Therefore, an understanding of the major cellular proliferation and differentiation that enables the FGF2 to accomplish this dynamic process of regeneration will be helpful in understanding the epimorphosis at cellular/tissue level. Histological studies of the fins of both the groups (control and treated) were carried out to identify the role of FGF2 in maintaining the tissue architecture at each defined stage.

Histological analyses of a number of small teleost fins, have shown that they are comprised of a relatively small number of cell types, including the three pigment cell types: melanocytes, xanthophores, and iridophores (**Hirata *et al*., 2005; Parichy *et al*., 2009**); osteoblasts that synthesize the bone matrix (**Akimenko *et al*., 2003; Marı´-Beffa *et al*., 1996; Poss *et al*., 2003**); dermal fibroblasts (**Marı´-Beffa *et al*., 1996; Montes *et al*., 1982**); artery and vein endothelium (**Becerra *et al*., 1983; Huang *et al*., 2009; Montes *et al*., 1982**); nerves, including the lateral line system (**Ghysen and Dambly-Chaudiere, 2007; Mari-Beffa *et al*., 1996; Martorana *et al*., 2001; Poleo *et al*., 2001; Wada *et al*., 2008**) and the intraray nerve comprised of sensory and motor nerve axons and associated glial cells **(Becerra *et al*., 1983; Montes *et al*., 1982**); skin epidermis (**Mari-Beffa *et al*., 1996; Martorana *et al*., 2001; Poleo *et al*., 2001**), and resident blood cells including macrophages, plasma cells, and Neutrophils (**Hall *et al*., 2007; Zhao *et al*., 2008**). Notably absent from the distal portion of the fin are striated muscles and cartilage (**Becerra *et al*., 1983; Marı´-Beffa *et al*., 1996; Montes *et al*., 1982**) (**Figure 1**).

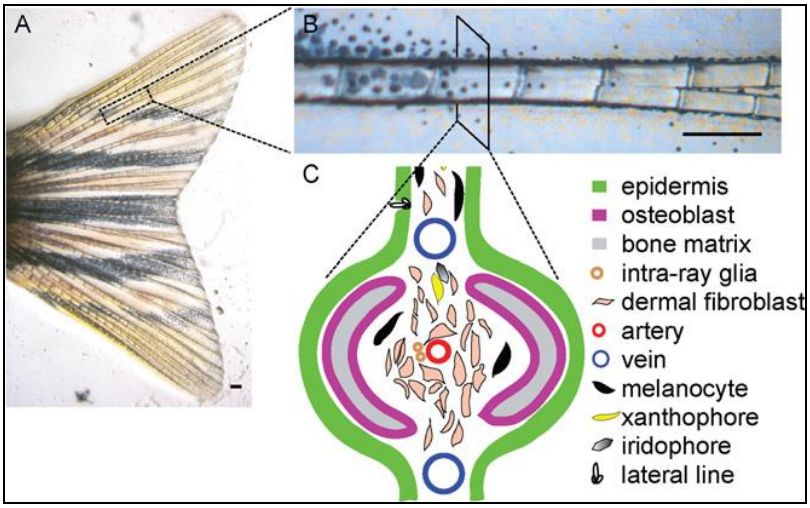


Figure 1: The anatomy and different cell types of the zebrafish caudal fin (from **Tu and Johnson, 2011**).

In order to further substantiate how FGF2 influences the fin growth an understanding cell proliferation turnover is inevitable. Decreased fin length may be the consequence of decreased cell proliferation. The latter being a very important phenomenon, was thought to be studied by using FGFR1 inhibitor SU5402 in order to explore the significance of FGF2. In this direction, BrdU-incorporation studies were undertaken.

Following an amputation or injury, the fin regenerates through a process involving successive events as mentioned earlier that are similar to those observed during the epimorphic regeneration of urodele amphibian limbs (**Tsonis, 1996**): wound healing, blastema formation, outgrowth and progressive differentiation of the blastema cells giving rise to a fin with a symmetrical pattern similar to prior amputation (**Goss and Stagg, 1957; Becerra *et al*., 1996; Johnson and Bennett, 1999; Akimenko *et al*., 2003**). During the phase of blastema formation, all blastema cells incorporate BrdU (**Santamaría *et al*., 1996; Poleo *et al*., 2001; Nechiporuk and Keating, 2002; Santos-Ruiz *et al*., 2002**). During outgrowth phase, a population of distal cells proliferates slowly, whereas the rest show an active cell proliferation rate (**Santamaría *et al*., 1996; Nechiporuk and Keating, 2002**).

Studies by **Prykhozhij and Neumann (2008)** have proved that blockage of FGF signalling with SU5402 leads to rapid loss of G1 and S-phase gene expression both in the pectoral fin buds and in the branchial arches of the zebrafish embryos. Thus, we checked if FGF2 signalling is required for proliferation of caudal tissue during regeneration. This would

provide an excellent foundation for investigating the mechanisms whereby pattern formation is integrated with proliferation.

The earlier chapters (**Chapter 1, 2 and 3**) had already proved the importance of FGF2 signalling in initiating the tail fin regeneration of *P. latipinna*. Treatment with SU5402 caused decreased the expression of FGF2 in the regenerating fins, increased the time period to attain various stages of regeneration and also altered the nucleic acids content, protein profiles and the extracellular matrix turnover. It was therefore thought to find out the alterations, if any, caused by SU5402 in the cell proliferation and subsequent differentiation to compensate the lost tail with true structural integrity. This was done by carrying out histological studies of the caudal fin of *P. latipinna* at three defined stages of regeneration as well as by evaluating the cell cycle turnover by performing BrdU-incorporation studies at the mentioned stages.

MATERIAL AND METHODS

Animals and maintenance

Adult Teleost fish, *Poecilia latipinna*, approximately 4-5 cm in length of both the sexes were maintained in aquaria containing constantly aerated and filtrated fresh water and fed daily with appropriate fish food, *ad libitum*. The animals were acclimated for a week before the commencement of the experiment and the period of study was 15 days. All the experimental protocols were approved by the IAEC in strict compliance with CPCSEA norms.

Experimental procedures

Histological analysis

The fishes were randomly divided into two groups, control and treated. The control was injected with 1%DMSO and the treated group with 2µM/g body wt. of SU5402, with each animal receiving not more than 10µl of the 1%DMSO as well as the test article. As in the earlier experiments, treatment was started a day before amputation and was continued till the control fishes reached the differentiation stage. Only those fishes that reached the specific stage on same days were selected and fins were collected for histological studies. The regenerate was excised, fixed in Bouin's fixative for 12h, decalcified with 10% EDTA for 6h and further processed for H-E staining as explained in the section **Material and Methods**.

Fin Amputation and BrdU Incorporation

The labelling of proliferating cells with BrdU was performed according to **Shao *et al*. (2009)**. The fishes were injected with 2µM/g body wt. SU5402 and 1%DMSO, serving as treated and control respectively. The treatment was started a day prior to amputation and was continued till the fishes reached the blastemal stage. A stock concentration of BrdU of 50mg/ml BrdU was prepared in sterile Hank's solution. The fishes were then injected with 250µg/g body weight BrdU at 3 defined stages of wound healing, blastema and differentiation. Frozen sections of fin tissue were taken and fixed in cold acetone followed by air drying for 15 minutes. After further treatment with 2N HCl for 30-60 minutes at 37^{o}C, sections were rinsed in borate buffer and rehydrated in PBS. Blocking was by normal serum. Sections were incubated with primary antibody (1:100 dilution of Mouse Anti-BrdU) overnight, washed in PBS, incubated with FITC conjugated secondary antibody (1:50 dilution of Goat Anti-Mouse IgG-FITC) for 2 hours and then washed and mounted with PBS:glycerol (1:1). They were observed on a fluorescence microscope (Leica DM2500; LAS EZ V1.6.0 software). The negative control sections were incubated in PBS-BSA instead of primary antibody, the rest of the protocol remaining the same. The blank photographs, however, have not been included here.

RESULTS

Epidermis formation had occurred in all the fins observed of the control group, showing regeneration right on the first day after fin excision (1dpa). A thick layer of epithelial cells could be seen that formed an apical epithelial cap (AEC). The SU5402 treated group on the other hand showed a very thin layer of epidermis covering the wound surface. No epidermal cap like structure had formed at 1dpa in this group. The epidermal layer and conjunctive tissues were all well formed and could be clearly observed in the control fishes, whereas the treated fishes showed poor formation of all these structures (**Figure 2a**).

By 5dpa, the cells of the AEC had now developed well, as could be seen in **Figure 2b,** in both the groups. The control group however, showed better growth of the epidermis basal layer and membrane as compared to the treated fins. Blastema could be localized in the interior of the conjunctive tissue of the distal extremity of the fin in regeneration. The blastema of the control showed a reduced intercellular space as compared to the treated fins. The cells of the epidermal basal layer continued to be cylindrical (**Figure 2b**) indicating that they were still in the synthesis activity of the epithelium basal membrane.

By 7dpa, the healed part of the fin had grown showing the regenerative outgrowth in both the groups. The connective tissue had been well developed by now in the control group, and the lepidotrichia seemed to be well formed by now, showing pigmentation identified by the melanocyte cells. However, each of this was observed at a much lower amount in the treated fins (**Figure 2c**). The connective tissue had not yet formed completely, leaving much of the intercellular spaces. The epidermis basal layer as well as the membrane had not shown any much improvement than at the blastemal stage letting to believe that the reason behind this may be the reduced rate of cell proliferation. Therefore, the BrdU studies were carried out parallelly.

The distribution of BrdU-labelled cells along the proximo-distal axis of the fin is shown in a series of longitudinal sections (**Figure 3**) taken at 1dpa (wound healing stage), 4dpa (blastema stage) and 7dpa (differentiation stage).

At 1dpa, a thin layer of epithelial tissue had covered the amputation wound. Surprisingly one detected no BrdU positive labels in the wound epithelium. However, initial proliferation was seen in the epidermal tissue in the stump at this point. The quantity of BrdU positive cells in fins of both control and SU5402 treated fishes were almost similar at 1 dpa **(Figure 3a)**. Very scarce BrdU labelling was seen in the control as well as in the treated fins. Observation of the fin sections at the blastema stage (5dpa) showed a vast labelling of BrdU-positive cells in both epidermal and mesenchymal cells **(Figure 3b)**. Some cells surrounding the lepidotrichia, probably scleroblasts, also showed BrdU incorporation. One can say that at this time point, we saw an explosion of labelling within the intra-ray mesenchyme compartment. The labelling could be seen right along the length of the ray extending up to about 3-4 ray segments. The concentration of the proliferating cells was found to be high in case of the control as compared to the treated sections. At differentiation stage (7dpa) again **(Figure 3c)**, the population of labelled cells, was found to be declining in both the groups. However, some BrdU-labelled cells could be observed in the distal-most part of the fin.

DISCUSSION

Following fin amputation, the injured area is repaired by rapid migration of epidermal cells over the amputation surface (**Poss *et al*., 2003; Campbell and Crews, 2008**). Subsequently, a mass of mesenchymal proliferating progenitor cells, called blastema, accumulates at the plane of amputation. The blastemal cells act like pleuripotent cells: they provide descendant

cells that build the regenerate while retaining their own undifferentiated, proliferating identity in the niche underneath the apical epidermis (**Gurley and Sánchez Alvarado, 2008**). The interruption of the contact between the wound epidermis and the blastema prevents regeneration (**Carlson, 2007; Brockes and Kumar, 2008; Campbell and Crews, 2008**). Hence, the mechanisms mediating communication between the two tissues are of central interest in the field of regenerative biology.

Epithelial–mesenchymal cell interactions play important roles during the various steps of fin regeneration and it has recently been shown that signalling by fibroblast growth factors is majorly involved in this process (**Poss *et al*., 2000**). In the current study one sought to define how early the process of lepidotrichia regeneration is initiated in the *P. latipinna* caudal fin, and identify the origin of cells that contribute to the blastema and further regenerative outgrowth and whether or not the different cellular regenerative structures appear in the absence of FGF2 signalling. In order to achieve the above goal we first examined the histology of control and SU5402-treated fin regenerates. Prior experiments had shown a significant delay in attaining various stages of regeneration (**Chapter 2**) on treatment with the mentioned drug; this delay of attaining the specific stages prompted further histological studies to unearth the reasons behind such delay. Secondly, to test if the regenerative cells underwent normal rate of DNA replication in the presence of SU5402, we examined BrdU incorporation in fins briefly treated with the inhibitory drug during regenerative outgrowth. In order to form the lost structure, a lot of cellular changes take place and the augmentation of dedifferentiated cells by cell division is one of the major mechanisms in fish caudal fin regeneration. Therefore, it was reasonable to investigate whether the inhibition of FGFR1 signalling affects the rate of cell proliferation in the regenerating tissues or not. The previous chapter (**Chapter 3**) has by now established that receptor inhibitor treatment reduced the DNA: RNA and RNA:Protein ratios in the regenerating fins of the SU5402 treated fishes. To observe the reduction in cellular proliferation and to examine as to what extent the receptor inhibitor alters the cellular activity level, we performed the BrdU incorporation studies.

During the regenerative event, in the first hours after amputation, the cells of the lateral epidermis that do not suffer any damage migrate to the amputated region of the fin in order to cover the wound in a fast way **(Bockelmann *et al*., 2010)**. In sequence, the cells of the epidermis basal layer go through a dimorphism, changing from their original cubic form to a cylindrical one, as was observed in the cells of the basal membrane. It is been postulated that

this dimorphic phase is an indication of a synthesis process, generated by the expression of the genes involved in the production of components that constitute the biochemical base of the basal membrane **(Bockelmann *et al.*, 2010)**. However, wound epidermis after amputation, failed to be induced as early as in the control fins, when FGFR activity was blocked. The SU5402 treated fins showed that the formation of wound epithelium and the beginning of a proliferative mass appeared quite later as compared to the control fins. But we could not find much of the cell proliferation when observed with the BrdU studies at 1dpa in the control fins. According to these results, it is likely that re-epithelization of the wound that ends few hours after the injury, occurs by migration of cells from the edges of the cut surfaces. Evidence for such migration studies are provided by **Santos-Ruiz *et al*. (2002**), where epidermal cells were marked with Bromodeoxyuridine and showed that the wound healing did not occur by cell proliferation but by cell migration. Molecular studies on the regeneration of zebrafish fins have shown the expression of β-catenin in the healing epidermal cells in the first hours after amputation and kept through the whole process (**Poss *et al*, 2000**). It is assumed that the expression of β-catenin works in the maintenance of the cell-cell interaction that facilitates the migration of the epidermis cells and in the maintenance of the epidermis (**Poss *et al.*, 2003**). Another gene detected in the epidermal cap, especially in the epidermis basal layer, in the last stages of regeneration, is the gene *Wnt*5. The expression of this gene seems to be strongly related to the blastema formation, leading us to suspect that the mature epidermal cap is the source of the growing factors that stimulate the formation and maintenance of the function of the blastema in regeneration, since when absent in the epidermal cap, no generation occurs, a notion shared by many (**Goss, 1991; Poss *et al.*, 2000**). The expression of genes implicated in epithelial-mesenchymal interactions, such as *msx* (**Akimenko *et al.*, 1995**), and *lef*1 (**Poss *et al.*, 2000a**), has also been reported in the basal layer of the apical epidermal cap during teleost fin regeneration.

Few hours later, cell proliferation begins at the remaining epidermis, but not at the cap covering the distal area. As observed in zebrafish caudal fin regeneration (**Santos-Ruiz *et al.*, 2002**), after 12 to 18 hours of amputation, the epidermis accumulates extra cell layers and this process of maturation also seems to happen due to cell migration and not cell proliferation. In spite of the fact that the two tissues involved in fin regeneration (epithelium and connective) begin to proliferate at different times and with different rates, they present evident relationships. Firstly, the epithelium lined the damaged connective tissue by migration of epithelial cells. Then it proliferates, probably to compensate the loss of cell

layers. Only when the distal cap is well established, the underlying connective tissue begins to proliferate (**Geraudie, 1977 and 1980**). This suggests the presence of certain factors released from the epidermal cap that could trigger proliferation in the connective tissue cells.

Following the early formation of the wound epidermis, the appearance of rapidly proliferating cells designated as blastema cells is a prerequisite for epimorphic regeneration to occur. The blastema is a crucial player in the regenerative process and is composed of a pool of proliferative cells that are responsible for the reconstitution of the lost tissue (**Sousa *et al*., 2011)**. Since no muscle cells are present in fish fin, it is thought that the blastema is preceded by only scleroblasts and fibroblasts. There are evidences that differentiated scleroblasts from the bony ray lining re-enter the cell cycle, detach from the lepidotrichia surface, migrate distally, integrate into the blastema and dedifferentiate (**Sousa *et al*., 2011)**. These findings highlight the contribution of differentiated scleroblasts to epimorphic appendage regeneration in teleost fish. Because the blastema is established beyond the amputation plane, mesenchymal migration for blastema formation has been previously proposed (**Johnson and Bennett, 1999; Poss *et al*., 2000a and 2000b**) and experimental evidence provided by **Santos-Ruiz *et al*. (2002)**. Detailed analyses of cellular responses during fin regeneration have revealed that disorganization of mesenchymal cells occurs at a distance away from the wound epidermis and that these cells migrate distally towards the wound edge to give rise to the blastema cells (**Poleo *et al*. 2001**). Such migrations occur not just to bring cells around the stump nearer to the cap but to bring cells located far from the amputation plane into the vicinity of the cap (**Santos-Ruiz *et al*., 2002)**. That the blastema is formed not by cells located at the amputation plane but by cells coming from even anterior locations has interesting morphogenetic implications. Information for patterning during amphibian limb regeneration has been shown to reside in mesenchymal cells rather than in the wound epidermis (**Stocum and Dearlove, 1972**). Each cell contains information regarding its position within the structure to which it belongs (**Wolpert, 1969, 1996**). If mesenchymal cells that migrate distally to form the blastema conserve their positional information, it would not be too daring to think of a relationship between the positional memory of these cells and the distal displacement of branches, which occurs in rays during regeneration (**Geraudie *et al*., 1993**).

But why should the blastema be formed by cells coming from a distance rather than by neighbouring cells? A precise understanding of this phenomenon undoubtedly requires

further experimentation, but the answer may be related to the kind of signal that triggers proliferation after amputation. Fibroblast growth factors are good candidates for this type of signalling, as they can stimulate both proliferation and migration in the same cell type (**Boilly *et al*., 2000**). Besides, **Poss and co-workers** (**2000b)** have demonstrated that FGF expression at the distal epidermal cap is needed for blastema formation. However, other possible sources of trophic signals should also be considered. These might be related to nerves, as innervations have been proven to be necessary for teleost fin regeneration (**Goss and Stagg, 1957; Geraudie and Singer, 1979**). The participation of other, as yet unknown signals cannot be ruled out. However since there are many evidences of FGF2 signalling during vertebrate limb development too (**Boilly *et al*., 1991; Poulin *et al*., 1993; Zenjari *et al*., 1997; Sheeba *et al*., 2012**), the effect of FGF2 signalling on cell-cycle progression in the reproving fins appears to be direct (**Prykhozhij and Neumann, 2008**). We observed a number of BrdU-labelled cells during the blastemal phase, as this time point is the definitive time of maximum mesenchymal tissue activity in the ray compartment.

Studies in zebrafish embryo have already proved the essentiality of FGF signalling for cell-cycle progression in the pectoral fin buds and in the branchial arches, since expression of G1 and S-phase cell-cycle genes in these tissues is lost after only 3 hours of inhibition of the FGF pathway. Inhibition of FGF signalling fails to affect cell-cycle progression in other organs too such as the retina and the optic tectum. The FGF signalling pathway is therefore not a global mitogenic signal in the zebrafish embryo, but instead directs proliferation in a highly tissue specific manner (**Prykhozhij and Neumann, 2008**).

Gradually these proliferating cells establish a proliferation gradient that fuels epimorphic regeneration while setting aside a small group of stem cell like, slowly dividing cells at the distal-most blastema (**Nechiporuk and Keating, 2002**), thereby forming the regenerative outgrowth called as the differentiation step. The histology sections of the control fins showed a well formed connective tissue and also showed signs of ray formation. It is reported that Lepidotrichia regeneration begins after blastema formation (**Sousa *et al*., 2011**), as patterning mechanism beginning when blastemal cells receive patterning signals from the basal layer of the epidermis; leaving the blastema and integrating into the population of scleroblasts that align at the stump to secrete lepidotrichia matrix (**Nechiporuk and Keating, 2002; Smith *et al*., 2006**). The results showed a clear visibility of lepidotrichia formation in the control fins, whereas it was quite inadequately formed in the treated fins. The possible reason would be

that the scleroblast cells never got the signal to re-enter the cell cycle. Also, the pigmentation too was overtly visible in the control fins by forming large number of melanocyte cells. However, this was poorly observed in the treated fins, leading us to believe that many or rather most of the processes post amputation, up to the complete fin regrowth, are dependent on FGF2 signalling.

On observing the BrdU incorporation results during this stage (7dpa), the BrdU labelled fewer cells, thereby proving the lower proliferation rate during this period. Observations of the sections revealed that proliferation was comparatively more in the proximal fin as compared to the distal region. One probable explanation of this could be that the adult fin reasonably must grow back the most cells lost, resulting in the need for more proliferating cells. Similarly, seeing that fin regeneration occurs proportional to its original morphology, for the whole structure to grow back at the same time, the proximal-most tissue must grow at a faster rate, needing more proliferative cells. This idea was experimentally tested by **Wills *et al*. (2008a)** in a study showing that proximal amputation results in faster regeneration rate than distal amputation. Thus, a dynamic gradient of positional information along the proximo-distal axis of the appendage is assigned, assessing region-specific instructions to the injured tissue. These instructions, known as positional memory, which could be carried out by fibroblast growth factor2, specify the amount of tissue to regenerate and a rate at which to grow (**Wills *et al*., 2008a**).

Concluding this chapter in brief, we used the hypersensitivity of SU5402, a specific inhibitor of FGFR1 inhibitor activity (**Mohammadi *et al*., 1997**), to indirectly assay the overall strength of FGFR1 signalling at different stages of caudal fin regeneration of *P. latipinna*. We found this to be an effective tool with which to uncover impairments in the FGF2 signalling pathway and the cellular alterations in tissue architecture as well as cell proliferations. Our results reinforce the observations of the previous chapters (**Chapter 1, 2 and 3**) that FGF2 has a significant role in epimorphosis of *P. latipinna* caudal fin, and indolinone tyrosine kinase inhibitor SU5402 successfully blocks the regenerative process by binding to FGFR1 and obstructs the FGF2 signalling pathway. Further, definition and manipulation of these signalling pathways may help expand regenerative capabilities in other vertebrate organisms.

Figure 2: Effect of FGFR1 inhibitor SU5402 on the histology profiles of the regenerating fin at various stages.

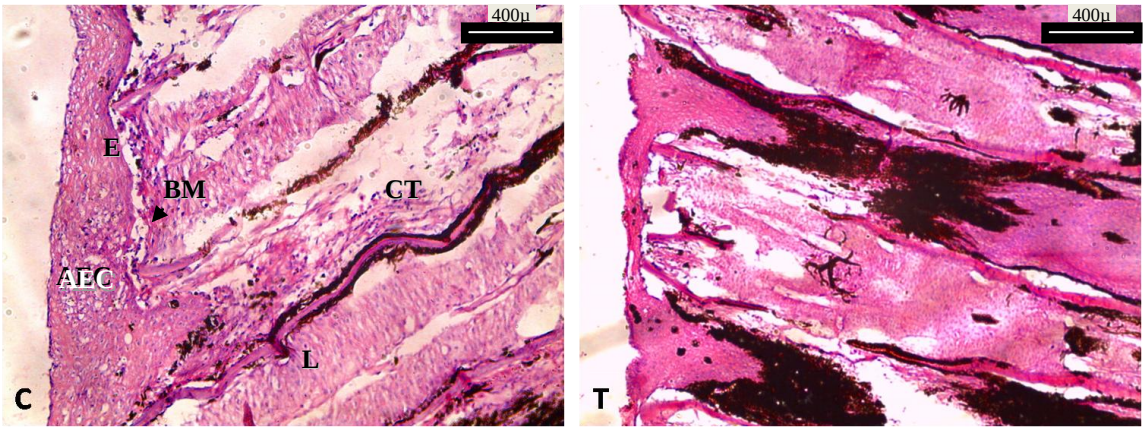


Figure 2a: Histology profiles of tail fin regenerates at Wound-epithelium stage from C: control fish injected with 1% DMSO and T: test fish injected with 2µM/g body weight of SU5402.

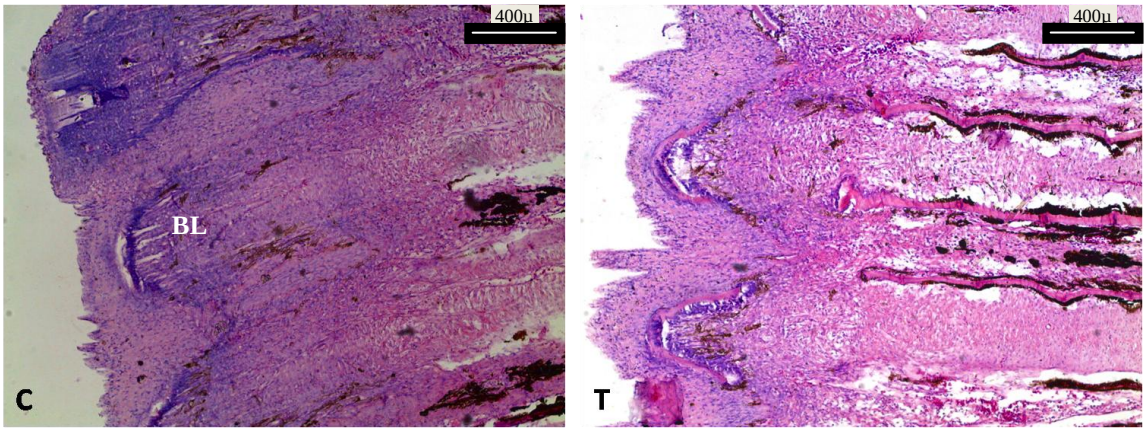


Figure 2b: Histology profiles of tail fin regenerates at Blastema stage from C: control fish injected with 1% DMSO and T: test fish injected with 2µM/g body weight of SU5402.

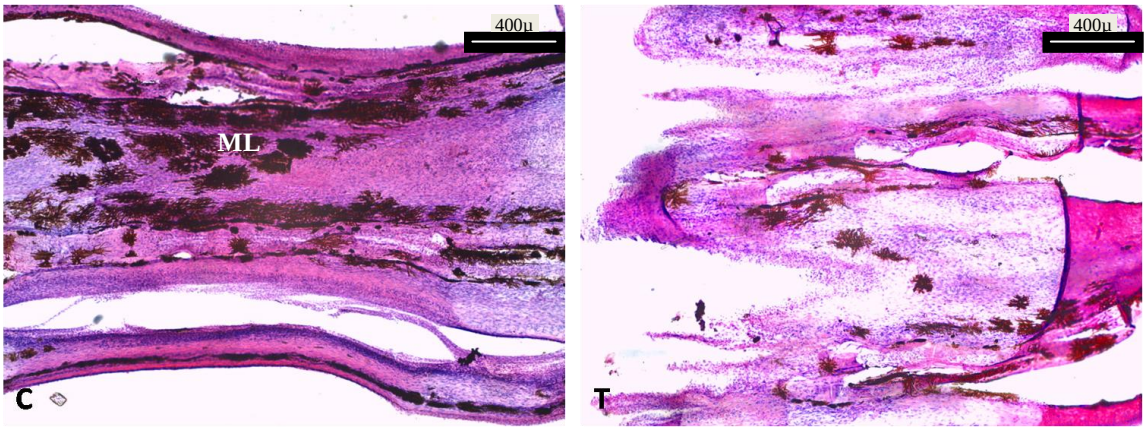


Figure 2c: Histology profiles of tail fin regenerates at Differentiation stage from C: control fish injected with 1% DMSO and T: test fish injected with 2µM/g body weight of SU5402.

E: epidermis; BM: basal membrane; CT: connective tissue; AEC: apical epithelial cap; L: lepidotrichia; BL: blastema; ML: Melanocytes.

Figure 3: BrdU localization in tail fins of control and treated fishes at various stages of regeneration.

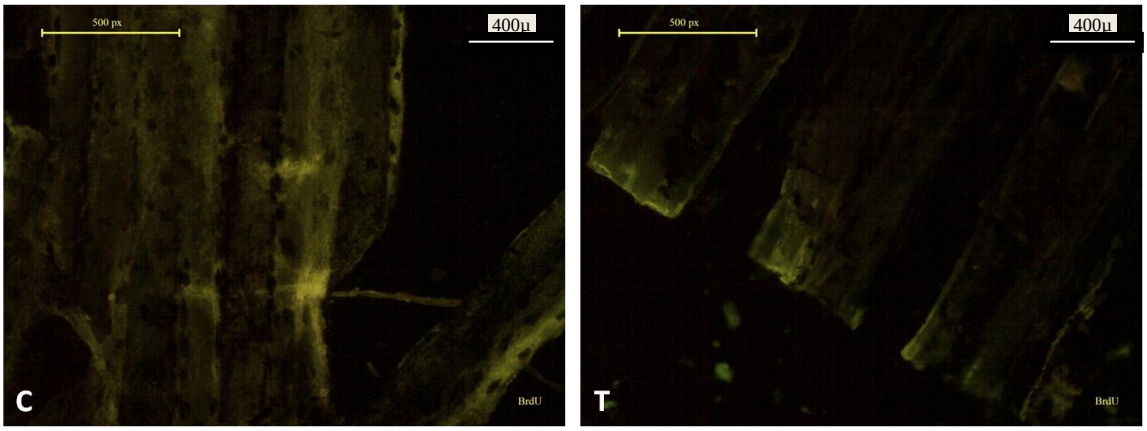


Figure 3a: BrdU localization in tail fin regenerates at Wound-epithelium stage from **C**: control fish injected with 1% DMSO and **T**: test fish injected with 2µM/g body weight of SU5402.

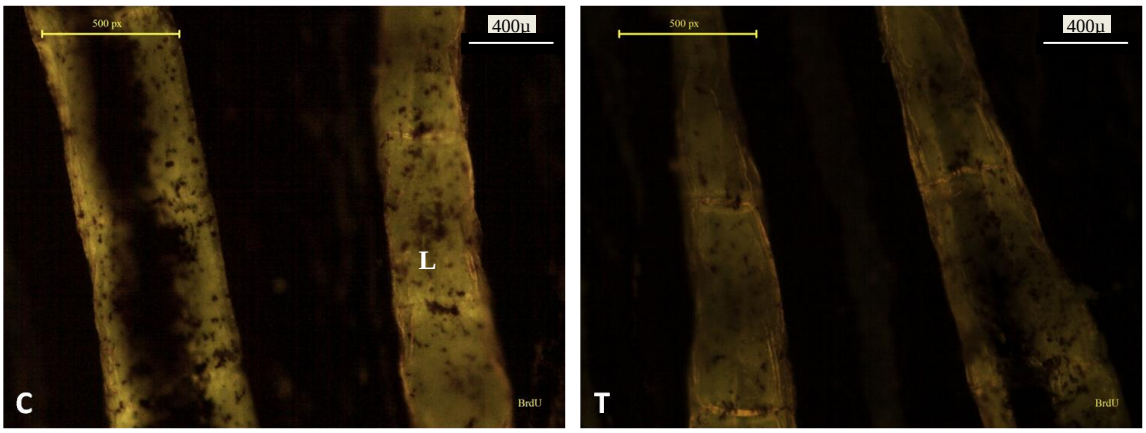


Figure 3b: BrdU localization in tail fin regenerates at Blastema stage from **C**: control fish injected with 1% DMSO and **T**: test fish injected with 2µM/g body weight of SU5402.

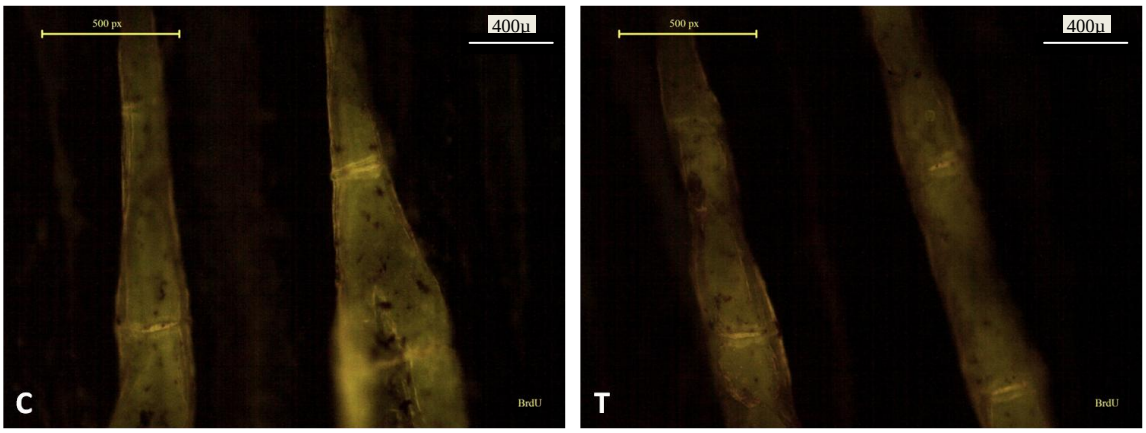


Figure 3c: BrdU localization in tail fin regenerates at Differentiation stage from **C**: control fish injected with 1% DMSO and **T**: test fish injected with 2µM/g body weight of SU5402.

L: lepidotrichia

GENERAL CONSIDERATIONS

An old Greek proverb says that when you have something precious you should guard it as we do to our body. The systematic functioning of all the organ systems in a human body is important as an injury or disease to a tissue or organ could lead to reduced quality of life or even fatality. Numerous disease conditions could be significantly improved if therapies that encourage tissue regeneration were available. The field of regenerative medicine is aimed at developing strategies to restore individual cell types, complex tissues, or structures that are lost or damaged. Most adult tissues and organs, especially in mammals, have lost their potential for further growth and differentiation. As a result, injury to a tissue or organ usually results in permanent damage (from scarring to disability). However, some non mammalian vertebrate animal models including salamanders, newts and zebrafish have retained the ability to regenerate their tissues, organs and appendages (**Brockes *et al*., 2001; Akimenko *et al*., 2003; Poss *et al*., 2003**). Since comparative genomics indicate significant genetic conservation between mammals and lower vertebrates what perplex one is that elusive molecular difference(s) that allow on one hand tissue regeneration in the non-mammalian models and on the other hand, make mammalian tissues recalcitrant to regeneration. By understanding the molecular and genetic pathways that work in harmony to accomplish regeneration in these evolutionarily lower animals, we will be in a stronger position to begin to understand why mammals fail to respond to tissue injury with a regenerative mechanism. **(Mathew *et al*., 2007)**

Injury to cells and tissues sets in motion a series of events that contain the damage and initiate the healing process called regeneration. All organisms mount a biological response to damage, but they vary widely in their ability to recover. Although, humans can regenerate an injured liver and repair limited insults to bone, muscle, digit tips and cornea, they do not regenerate the heart, spinal cord, retina or limbs. Thus, humans and other mammals are somewhat disadvantaged when compared with amphibians and teleost fish, which have a remarkable capacity to regenerate damaged organs including heart, spinal cord, retina and limbs/fins (**Akimenko *et al*., 2003; Brockes and Kumar, 2002; Poss *et al*., 2003; Poss *et al*., 2002a**). Dramatic examples of organ regeneration are that of amphibian limbs and fish fins, where intricate structures consisting of multiple cell types that are patterned into

complex tissues are faithfully restored after amputation. Elucidation of these regenerative mechanisms and an understanding of why regenerative capacity has diminished in vertebrate evolution hold the potential to revolutionize clinical medicine, with practical applications ranging from organ disease and wound treatment to possible alternatives to prosthetics for amputees (**Brockes and Kumar, 2002)**.

The promise of regenerative medicine is that therapies will be devised to promote the repair or replacement of damaged or diseased tissues and organs. This emerging field is approached from two distinct lines of work. In recent years, stem cell based models have been developed to generate a suite of differentiated cells for therapeutic applications. The use of high throughput chemical genetic screening to identify modulators of stem cell fate offers great assurance (**Ding and Schultz, 2004**). The alternative approach exploits the inherent regenerative capacity of non-mammalian models to define the molecular events that permit tissue regeneration (**Brockes and Kumar, 2005**). There are several regenerative animal models including salamanders, newts, zebrafish, hydra and flatworms that are established to evaluate tissue regeneration (**Akimenko *et al*., 2003; Bader and Oberpriller, 1978; Fujisawa, 2003; Mescher, 1996**)

The zebrafish exhibits an outstanding ability to regenerate different parts of its anatomy, including any of the paired and unpaired fins, the heart ventricle, and the spinal cord. Zebrafish is particularly useful for studies on regeneration since it has short generation times that make experiments requiring large number of animals feasible, and it has a fully sequenced and annotated genome (**Poss *et al*., 2003)**. The zebrafish caudal fin is an established model of regeneration of a complex tissue that is easy to amputate, is not required for viability, and completely regenerates in a short time frame. Regeneration of the caudal fin after experimental amputation has been appreciated for a long period of time (**Morgan, 1900; Santamarıa and Becerra, 1991**); although its other fins such as pectoral, pelvic, anal and dorsal fins also regenerate after amputation (**Kawakami *et al*., 2006; Nachtrab *et al*., 2011**). It performs such a feat by the process called epimorphic regeneration that is typically broken down into three steps. First, a wound epithelium is formed at the site of damage by migrating epithelial cells that seals the wound from the environment. Next, disorganization and dedifferentiation of tissue near the wound results in the creation of a mass of undifferentiated cells, known as the blastema. Then, proliferation of blastema cells, concomitant with patterning and differentiation, results in the regeneration of the amputated portions of the

damaged tissue (**Poss *et al*., 2002a,b**). The defining characteristic of epimorphic regeneration is the formation of the blastema at the site of amputation. A fundamental question in the field is how amputation instructs certain cells near the wound site to dedifferentiate and take part in the re-growth and subsequent reconstruction of the amputated body part.

The achievement of regeneration in caudal fin is considered to involve precise coordination of several events and a cross-talk between several signalling molecules. To understand the genetic basis of fin regeneration, several approaches have been used: mutagenesis screens (**Johnson and Weston, 1995; Gurley and Sánchez Alvarado, 2008**) candidate gene strategies (**Akimenko *et al*., 2003; Stoick-Cooper *et al*., 2007a**), suppression subtractive hybridization (**Padhi *et al*., 2004**) and microarray analysis (**Schebesta *et al*., 2006; Yin *et al*., 2008**). Progress in the last decade led to the identification of several key molecular regulators of blastema formation. Among of them there is a set of signalling molecules (**Stoick-Cooper *et al*., 2007a**). The administration of retinoic acid causes teratogenic effects and impairs fin regeneration (**White *et al*., 1994**). The ligand FGF20a is required for wound epidermis formation and for mesenchymal proliferation (**Whitehead *et al*., 2005**). Shh and BMP signalling pathways play a role in the proliferation and/or differentiation of scleroblasts that produce dermal bones (**Laforest *et al*., 1998; Quint *et al*., 2002**). The Activin-bA/TGFb pathway is required for normal wound repair and blastema proliferation (**Jazwinska *et al*., 2007**). Both canonical and noncanonical Wnts influence blastemal proliferation and patterning of the outgrowth (**Stoick-Cooper *et al*., 2007a, b**). The chemokines Sdf1a (Cxcl12a – Zebrafish Information Network) controls epithelial cell proliferation in regenerating fins (**Dufourcq and Vriz, 2006**). This long list of the signalling molecules supports the hypothesis that molecular mechanisms of organ regeneration rely on secreted factors mediating cell-cell communication.

Neurotrophic factors derived from the nerve tissue are one of such regulatory factors of regeneration. Earlier studies using amphibian model incited many to believe that the main neurotrophic factors responsible for the orchestration of regeneration could be fibroblast growth factors (FGFs), especially the prototypic FGFs, FGF1 and FGF2 (**Brockes, 1984; Mescher, 1996; Geraudie and Ferretti, 1998**). Fibroblast growth factor-2 (FGF-2) also known as basic fibroblast growth factor promotes the proliferation of a wide range of mesoderm and neuroectoderm derived cells *in vitro* (**Folkman and Klagsburn 1987; Gospodarowicz *et al*., 1986**). FGF-2 stimulates endothelial cell migration, proliferation and proteinase production *in vitro* and *in vivo* (**Pintucci *et al*., 2002**). Implantation of beads

soaked in FGF2 can induce extra limbs from the flank of chick embryo *in vivo* (**Cohn *et al.*, 1995**). Furthermore, FGF-2 stimulates *in vitro* proliferation of blastema cells from regenerating limbs of newts (**Albert *et al.*, 1987**). Isolation, ligand specificity, and reprogramming expression of FGF receptor variants have recently been revealed and are considered to play very important roles in the switching mechanism of cell proliferation and differentiation during limb regeneration of newts (**Boilly *et al.*, 1991; Poulin *et al.*, 1993**). These studies indicate that FGF2 is one of the key factors not only in ontogenesis but also during epimorphosis.

FGF2 is a member of FGF family constituting of about 23 distinct members (**Gospodarowitz *et al.*, 1974)** and is considered as a key player during epimorphic regeneration. Most FGFs (FGFs3-8, 10, 15, 17-19 and 21-23) have amino-terminal signal peptides and are readily secreted from the cells. FGFs 1 and 2 however are not secreted, but found on the cell surface and within the matrix (**Ornitz and Itoh, 2001**); and in case of any injury or wound, are released (**McNeil *et al.*, 1989; Mignatti *et al.*, 1992**). Many studies have proved the evident role of FGF2 during epimorphosis in different animal models (**Pilo and Suresh, 1994; Yadav, 2005; Sharma and Suresh 2008; Alibardi and Lovicu, 2010; Yadav *et al.*, 2012**). Fish fin regeneration is also studied to be evidently dependent on FGF2 signalling (**Hata *et al.*, 1998; Poss *et al.*, 2000a**). Therefore, it was thought pertinent to investigate the significance of FGF2 signalling in the regulation of key milestones of epimorphosis in a teleost fish - Sailfin Molly, *Poecilia latipinna* (Lesueur, 1821). The said species was selected because it was readily available with the local animal suppliers and was found easy to maintain. Moreover, our ongoing studies (**Yadav, 2005; Anusree, 2012**; **Yadav *et al.*, 2012)** have proved beyond doubt that FGF2 signalling is a quintessential modulator for the successful completion of epimorphosis in northern house gecko *Hemidactylus flaviviridis.* It was therefore, thought interesting to understand whether similar regulatory mechanisms govern the process of epimorphosis in an evolutionarily different group of organisms with regenerative ability. These results might throw some light on the evolutionary conservation or otherwise of molecular signalling in organisms belonging to different taxonomic hierarchical positions.

In order to prod further the above notion one explored the possibility to experimentally target the FGF2 signalling in the selected animal model. It is well documented that the biological activity of the FGF2 requires the presence of both heparan sulfate proteoglycans (HSPGs)

and FGF tyrosine kinase receptors (FGFRs) to transduce signals for cell proliferation (**Ornitz *et al*., 1992; Ornitz and Itoh, 2001**). FGFRs are transmembrane proteins that dimerize and undergo autophosphorylation following FGF binding (**Nugent and Iozzo, 2000**). Members of the FGF family have a high affinity for cell-surface heparan sulfate proteoglycans and heparin (**Rapraeger *et al*., 1991**). Heparan sulphate proteoglycans are complex molecules consisting of a core protein with covalently attached heparan sulfate chains. Binding to heparin sulfate is an essential part of the formation of active FGF-FGFR complexes and a prerequisite for effective intracellular signalling (**Nugent and Iozzo, 2000**). In brief it can be said that FGF2 completely depends on heparan sulphate proteoglycans (HSPG) to transduce an intracellular signal through its receptors (**Rapraeger *et al*., 1991; Yayon *et al*., 1991**) through the formation of the ternary complex HSPG-FGF2-FGFR (**Pellegrini, 2000**).

The tyrosine kinase domain of FGFR is activated upon FGF binding, resulting in the activation of a transcription factor by means of a signal transduction cascade. Blocking the FGF2 signalling pathway via inhibition of tyrosine activity of its receptor would be of great experimental value. Based on the crystallographic studies of the catalytic domain of FGFR1 with indolinones (**Mohammadi *et al*., 1997; Sun *et al*., 2000; Laird *et al*., 2000**) several classes of indolinones have emerged as inhibitors of various split kinases. SU5402 is one such indolinone that inhibits the tyrosine kinase activity of FGFR1 by interacting with its catalytic domain. SU5402 directly interacts to the catalytic domain of FGFR1 (**Simon, 2000**), and inhibits the phosphorylation activity of the receptor. The two FGFs, FGF1 and FGF2 bind with the FGFR1 with high affinity. However, there are studies which have shown that FGF1 can transduce its signals by binding to other receptors too. Therefore, an increasing number of studies have targeted the FGF2 pathway through inhibition of the tyrosine kinase activity of the fibroblast growth factor receptor 1 by use of SU5402 (**Mohammadi *et al*., 1997; Poss *et al*., 2000a; Lefevre *et al*., 2009)**.

In the current study to probe caudal fin regeneration in *P. latipinna*, an inhibitory screen was developed. The underlying assertion was that if a chemical inhibits or modulates an essential molecular target, then regeneration will be impacted. The identification of the chemical target will thus help to reveal underlying molecular pathways that permit tissue regeneration. Studies have shown that inhibition of FGFR1 with SU5402, or activation of the aryl hydrocarbon receptor (AHR) disrupted tissue regeneration (**Kawakami *et al*., 2004; Mathew *et al*., 2006; Nakatani *et al*., 2007**). Therefore, to investigate the possible regulation of

FGF2-FGFR1 signalling pathway in regenerating teleost fin, activity of the FGFR1 was blocked using the drug SU5402 and evaluated the significance of FGF2 signalling during caudal fin epimorphosis.

The first chapter (**Chapter 1**) dealt in immunolocalizing FGF2 in the regenerating caudal fins. The study focussed on the distribution of FGF2 during the key events of fin regeneration i.e. at the formation of wound epithelium, blastema and differentiation stages. The results revealed the presence of FGF2 in the regenerating fins. Intense FGF2-positive reactions were noted in the epithelial cells at 1dpa. At 5dpa much of the FGF2 could be localized in the growing area of the fin. Such FGF2-positive reactions were also seen in the fins of SU5402 treated fishes. However, the intensity was quite low in the treated fins as compared to control. The amount of FGF2 localized was found to be gradually decreased by the 7^{th} dpa in both groups. The presence of FGF2 during the initial stages is a possible testimony that FGF2 plays a crucial role in the initial stages of fin regeneration. The decreased presence of FGF2 during the later stages of regeneration (7dpa) indicates that at this point of time, the other downstream signalling mechanisms might have overshadowed the FGF2 signalling, as also opined by **Hata *et al.*, (1998**). It is known that immediately after amputation, FGF2 is released from the cell surface in a novel exocytic way independent of the classic endoplasmic reticulum-Golgi complex route and possibly binds to heparan-sulfate proteoglycan in the extracellular matrix (**Mignatti *et al.*, 1992**); and the receptor for FGF2, FGFR1 has been reported found in caudal fins, during the initial stages of regeneration (**Santos-Ruiz *et al.*, 2001**). **Poss *et al.* (2000a)** have shown FGFR1 mRNA expression in proliferative blastemal cells thus hypothesizing FGFR1 implication in the control of cell proliferation during fin regeneration. Inhibition of this signalling molecule through the kinase inhibitor SU5402 substantially altered the expression of these receptors during different stages of regeneration thereby lowering the FGF2 signals. There are several studies which have proved that FGF is necessary for lepidotrichia formation (**Santos-Ruiz *et al.*, 2001**). The inhibition of FGF signalling pathway stops fin outgrowth (**Poss *et al.*, 2000a**) and modulation of the FGF signalling regulates the rate of fin outgrowth (**Lee *et al.*, 2005; Thummel *et al.*, 2006**). Thus, this chapter concludes that FGF2 is undoubtedly needed for the initiation and further fin formation and therefore, is evidently expressed in the regenerating fins; and treatment with the inhibitor greatly reduces the FGF2 signalling thereby providing the baseline for carrying out the further studies.

After the localization and confirmation of FGF2 in the regenerating fins, the second step was to find out the significance of FGF2 in gaining back the lost part of tail fin post amputation. Therefore, a morphometric study was carried out by amputating 30% of the fin experimentally and treating with SU5402. The progression of fin regenerates of treated fish were compared with controls that received the vehicle (1%DMSO) (**Chapter 2**). Studies carried out in adult teleosts have shown that the amputation of the caudal fin leads to a succession of steps (wound healing, blastema formation and regenerative outgrowth) that restore the various tissues of the fin, including blood vessels, nerves, connective tissue, epidermis, pigment cells and lepidotrichia, the skeletal elements that support the fin structure. Lepidotrichia are elongated bony rays of dermal origin that run from proximal to distal in the caudal fin (**Akimenko *et al*., 2003**). Each lepidotrichia is composed of concave and opposed hemirays with intra-ray mesenchymal tissue (**Montes *et al*., 1982**). The caudal fin skeletal tissue is laid down by scleroblasts, skeletogenic cells equivalent to mammalian osteoblasts that secrete the lepidotrichia matrix (**Hall, 2005**). It has been hypothesised that upon amputation of the adult zebrafish caudal fin, bony ray regeneration arises from the intraray mesenchymal cells that become disorganized, change their shape, re-enter the cell cycle and migrate distally (**Poleo *et al*., 2001; Nechiporuk and Keating, 2002; Santos-Ruiz *et al*., 2002**).

In the morphometric experiments in order to decide on a correct dose, two doses of 1µM/g body wt. and 2µM/g body wt. of SU5402 were evaluated. The dosing was started a day before amputation. The results revealed a decisive role of FGF2 during the fin regeneration as the treated groups significantly lagged behind in attaining each of the stage of the epimorphic event. One day after partial amputation of the tail fin, epidermal cells migrated and completely covered the cut edge in control fishes. The requirement of FGF2 during wound healing is known (**Bikfalvi *et al*., 1997**). SU5402 treated fishes showed a delay in the formation of wound epithelium as well as the apical epidermal cap (**studied later in Chapter 4**) as was evident by a poor wound healing when observed 1dpa. All the observed control fishes could however, form a good wound epithelium by this time. Thus, it became evident that FGF2 is required for the initial healing of the wound. Meanwhile the dosing regimen was continued till the time the control fishes are expected to reach the blastema stage i.e 4dpa (personal observation by preliminary studies). A well formed blastema constituting a mass of cells could be seen at 4dpa. The blastema is enriched in differentiation and patterning signals that are known to be involved in bone tissue specification, such as FGF, BMPs and Shh

(**Laforest *et al*., 1998; Poss *et al*., 2000a,b; Quint *et al*., 2002; Lee *et al*., 2009)**, which are secreted by the basal layer of the epidermis. There is documented evidence that FGF2 is an obligatory requirement during blastemal proliferation in zebrafish caudal fin (**Hata *et al*., 1998**). The current findings were in accordance with this observation. The fishes when injected for five days with SU5402 not only showed a delay in attaining the blastemal stage, but also showed a very poor blastema formation as compared to that of controls. In the control animals the blastema began to form by 2dpa and by 4dpa one could observe a full formed blastema. Thus it could be construed that FGF2 is required for the proliferation of the blastemal cells and downregulation of FGFR1 inhibits their FGF2-induced proliferation. **Lee *et al.* (2005)** studied transgenic zebrafish that expressed a dominant negative FGF receptor, and demonstrated that FGF signalling instructs position-dependent growth rate by modulating *shh* expression in the wound epidermis and position dependent blastemal function. The blastemal proliferation ultimately leads to the regenerative outgrowth. The regeneration of the fin rays implies a significant re-growth of bone tissue. Many bone regeneration studies have been attempted in the caudal fin for better elucidating the underlying molecular mechanisms (**Smith *et al*., 2006**). Genes that specify skeletal lineages, e.g. *sox9a*, are believed to appear at few days post amputation and further help the fin to restore its original architecture (**Smith *et al*., 2006)**. In the present study it was observed that the fishes in the treated group could not reach the final stages of regeneration within 15 dpa as the control. This finding demonstrates the key role of FGFR1 activation in mediating FGF2 induced cell proliferation and growth in the regenerating fins. All the results mentioned above were of the fishes treated with the dose of 2µM/g body wt. of SU5402. The fishes of the group treated with 1µM/gm body wt. did not show significant difference in the progression of regeneration compared to the controls. Therefore the dose of 2 µM/g body wt. was opted for the rest of the studies.

Further, for successful epimorphosis, several modulators are required to act in unison. It will be interesting to understand the extent to which the injury-induced regeneration correlates with the activity of ongoing homeostatic regeneration maintained by FGF2 ligands and its signalling pathways. One of the earliest events during epimorphic regeneration is the extracellular matrix (ECM) remodelling. Matrix metalloproteinases (MMPs) are known to play the most important role during matrix degradation. These enzymes are encoded by different genes and are implicated in several normal and pathological tissue remodelling processes such as wound healing, angiogenesis and tumour invasion (**Forget *et al*., 1999**).

They are upregulated very early after amputation and are required for regeneration, and it is postulated that they play a role in matrix degradation, contributing to formation of the wound epithelium (**Call and Tsonis, 2005; Vinarsky *et al*., 2005**). The temporal expression pattern of MMPs in the regenerating newt limb suggests these enzymes to be involved in blastema formation, maintenance and growth (**Vinarsky *et al*., 2005**).

Of them, gelatinase-A (MMP2) and gelatinase-B (MMP9) are able to degrade extracellular matrix protein, including type IV collagen. Gelatinases have been linked to cell invasion and the process of metastasis (**Stetler-Stevenson, 2001**). MMPs are activated in regenerating limbs of newts and salamanders (**Yang and Bryant, 1994; Grillo *et al*., 1968; Park and Kim, 1999**) and are also activated during inflammation of wound healing and function to clear inflammatory debris in mammals (**Parks, 1999; Broughton *et al*., 2006**). In 2005, **Vinarsky *et al*.** reported that some MMPs are upregulated very early after amputation and that urodele limb regeneration can be partially inhibited by treatment with a synthetic MMP inhibitor. These findings suggest that MMPs are specifically required for regeneration, especially during initiation (wound epithelium formation/subsequent blastema formation) and furthermore, are also necessary for the successive regenerative events (**Vinarsky *et al*., 2005)**.

In order to understand the effect of FGF2 inhibition on degradative events involved in dedifferentiation, we examined the involvement of MMPs in the regenerating fins. Study was performed by immunolocalizing the MMP2 and MMP9 and performing gelatin zymography.

The regeneration of the wounded tissue not only involves in the ECM remodelling, but also involves in the synthesis of relatively large amounts of protein (**Dunphy and Udupa, 1955; Williamson and Fromm, 1955; Weiss and Kavanau, 1957; Fromm and Nordlie, 1959**). Therefore, it can be inferred that while the new tissue is being formed, nucleic acid metabolism is probably different from that observed in normal animals. Hence, attempts were also made to evaluate the transcriptional as well translational activities in the control and the treated fins by calculating the DNA:RNA and RNA:Protein levels in the regenerating fins of the control and the treated animals. Moreover, a lower level of transcriptional and translational activities was observed in the treated fins as compared to the controls. This was exemplified by the lower DNA, RNA and protein contents in the FGFR1 inhibitor treated groups as well as by the lowered DNA:RNA as RNA:protein ratios. The results indicate that

the process of DNA synthesis was inhibited in the treated animals. Decreased DNA content in the regenerating fins of SU5402 treated fishes is suggestive that these cells could not enter the new cycles, main reason probably being the insufficient availability of FGF2 signalling, thereby leading to the possibility of a defect in cell cycle regulation, following retardation in the rate of replication of the dividing cells, that ultimately results in the low DNA content in the SU5402 treated fins. Also the proliferating cells transcribe RNA and synthesize new proteins to meet the demands of the rapidly dividing cells. Since it is well known that the amount of protein is directly related with growth and proliferation, these studies hold great significance. Results exemplify lower levels of RNA as well as proteins in the receptor inhibitor treated groups as compared to the control ones. This low turnover of DNA, RNA and protein in the treated animals, to some extent, reflects the unavailability of growth factors to the injured tissue, thereby lowering their transcriptional and translational levels. Thus, the demonstration of such changes in the nucleic acids and protein content of the treated animals as compared to controls points to the possibility that there also could be some change in the nucleic acid metabolism of the wounded animals. It seems quite probable that some further clue for the regulation of regeneration by proteins may be obtained from consideration of the nucleotide and protein content of the regenerating tissue. Observing the protein bands of the control and treated confirmed the above outcome. SDS-PAGE analysis was made to understand the stage-specific expression of proteins, which was quantified using spot densitometer (**Chapter 3**). We found many polypeptide bands to be absent in the SU5402 treated group as compared to the control. These may be the proteins expressed through FGF2 signalling and essential for fin regeneration. Also, the intensities of many bands in the treated samples were lower as compared to control; a plausible reason being the downregulation of the signalling proteins. From these observations it can be deduced that impaired regeneration observed in the inhibitor treated animals could be due to the downregulation of several proteins being regulated by FGF2, pointing again towards its requirement for a proper regenerative response.

Suppression of gelatinase activity was illustrated on blocking the FGF2 signalling at wound healing stage. This was evident from the both, immunolocalization studies of MMP2 and 9 as well as the zymography results. This suppressed gelatinase activity possibly might be the reason for delayed wound epithelium formation as well as subsequent cell migration and differentiation observed in the inhibitor-treated fishes during morphometric studies.

To supplement these studies, the evaluation of the role of FG2 signalling on cell proliferation, growth and differentiation during caudal fin regeneration was done in the next chapter (**Chapter 4**).

By now it was established that FGF2 has a very putative role in the initial stages of caudal fin epimorphosis of *P. latipinna*. Therefore, studies were extended to evaluate the role of FGF2 signalling on further progression of the regenerate. Histological studies of the fins of both the groups (control and treated) were carried out to identify the role of FGF2 in maintaining the tissue architecture at each defined stage. This would also complement in our understanding of the morphometric alterations (decreased fin length) that occurred during the initial studies. In addition, dedifferentiation of cells is the most important process of the epimorphic regeneration (**Holly *et al*., 2003**). Increase in cell proliferation in the regenerate is essential so that the regenerate can step into its successive stages without any hindrance. Decreased fin length observed in the receptor inhibitor-treated fishes may be the consequence of decreased cell proliferation. The latter being a very important phenomenon, was thought to be studied by using FGFR1 inhibitor SU5402 in order to explore the significance of FGF2. In this direction, BrdU-incorporation studies were undertaken.

Regeneration of the caudal fins in this experiment too followed the same trend as the previous observations. Restoration of the lost tissue in the control started immediately after the fin amputation and by 1dpa, the epidermal cells had completely covered the cut edge. The histology of the fins of both the groups (control and treated) when observed under the microscope showed an array of differences during each of the stage of epimorphosis. In the control fins the regeneration had started in the distal region of the fin, on the inside of the connective tissue matrix adjacent to the epidermis. A well formed apical epithelial cap could be observed in the control fins as was not the case for the treated fins. By 4dpa, some blastema cells formed a row of cells, one next to the other, immediately beneath the epidermis, in strong association with the basal layer on both sides in the fin. Such cells known as lepidotrichia forming cells (LFCs) (**Bockelmann *et al*., 2010**) are responsible for the synthesis and deposition of the lepidotrichial extracellular matrix in the region turned to the basal layer, and therefore, seen between the row of scleroblasts and the basal layer of the epidermis. However such well formed blastemal cells could not be observed in the regenerates of the treated groups. It is known that the members of the sonic hedgehog signalling pathway, sonic hedgehog (*shh*), patched 1 (*ptc1*), and bone morphogenetic protein

(*bmp2*) are all expressed in the basal layer of the epithelium during fin regeneration (**Laforest *et al.*, 1998**). SU5402 is also known to downregulate the *shh* genes**,** thereby contributing to the deterrence of fin regeneration as observed in the present studies. By 7dpa, pigmented cells (melanocytes) were seen in the control fins whereas the abundance of such pigmented cells was much lower in the SU5402 treated groups. At this stage the control regenerate showed sufficient growth in the length as well as width of the lepidotrichia. This increase in the width of lepidotrichia is reported to be the action of scleroblasts. In zebrafish, by about 6dpa, scleroblasts migrate to the other side of the hemisegment of the regenerating lepidotrichia and get interposed between the epidermis and the hemisegment, maintaining the disposition of a single layer of cells involving both sides of the lepidotrichial hemisegment and start to secrete extracellular matrix to the hemisegment direction (**Bockelmann *et al.*, 2010)**. A deregulation of all the above cited processes could be reasoned for the delay in the SU5402 treated fins to restore its original structures.

Mitotic index of the regenerate was evaluated by labelling the cells with BrdU when cells are at S-phase 2of the cell cycle at all the three stages: wound healing, blastema and differentiation. The study demonstrated that FGF2 is one of the extracellular factors to exert positive regulation on cell proliferation. Minimal BrdU was localized in the initial stage of wound healing, as this is the phase that mainly depends on cell migration rather than cell proliferation. The blastemal phase is known to be composed of a mass of proliferating cells and therefore, as expected showed an abundance of BrdU labelling in the fins at this phase of regeneration. Nevertheless, the quantity of BrdU labelled cells was much less in the treated group as compared to the control group. From the results it could be construed that the blockage of FGF2 by a specific receptor inhibitor (SU5402) resulted in less number of cells entering the S-phase of the cell cycle and more number of cells remained in the quiescent state when compared to the control animals. There are reports showing that FGF2 induces cell cycle progression from G0/G1 to S phase in endothelial cells (**Zeitler *et al.*, 1997**). Therefore, it could be hypothesized that the absence/unavailability of FGF2 signal led to a decrease in the fraction of cells re-entering the cell cycle and the induction of G0/G1 cell cycle arrest thereby proving the proliferative potential of FGF2. The later stages however, also showed a lowered or decreased BrdU labelling in both the groups. Thus, the current study proved beyond doubt that FGF2 signalling is essential for the initiation and maintenance of epimorphic regeneration not only in amphibians and lizards but also for

orchestration of the restorative growth in teleosts, an evolutionarily primitive group of vertebrate.

In addition, many approaches world over have been done for dissecting molecular functions underlying fin regeneration using inhibitors/agonists. **Quint *et al.*, (2002)** used cyclopamine, an inhibitor of signalling in fin-ray bone differentiation. In addition, an inhibitor of the vascular endothelial growth factor receptor was used to knockdown angiogenesis during regeneration to determine the role of blood vessels in regeneration **(Bayliss *et al.*, 2006)**, though the fin could regenerate without direct interaction with endothelial cells and at a distance from a blood supply. In further instances, the chemical 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and inhibitors of phosphoinositide 3-kinase (PI3K) signalling were found to impair regeneration of the caudal fin in the zebrafish and medaka, respectively (**Zodrow and Tanguay, 2003; Nakatani *et al.*, 2007**). Furthermore, it was reported that the inhibitors of MMPs also negatively affect regeneration (**Bai *et al.*, 2005)**. **Yoshinari and Kawakami (2011)** successfully used an inhibitor of JUN N-terminal kinase (JNK) to demonstrate the role of JunB family proteins and their phosphorylation in regeneration (**Ishida *et al.*, 2010). Lee *et al.*, (2005)** studied transgenic zebrafish that expressed a dominant-negative FGF receptor, and demonstrated that FGF signalling instructs position dependent growth rate by modulating *shh* expression in the wound epidermis and position dependent blastemal function (**Lee *et al.*, 2009**). Using the same transgenic fish, other investigators suggested the involvement of FGF signalling in the homeostatic growth of fins and heart in response to population density (**Wills *et al.*, 2008a, b)**. In a study similar to those on FGF signalling, **Stoick-Cooper *et al.* (2007b)** used dominant-negative Tcf, a transcription factor downstream of Wnt/β-catenin signalling, and Dkk1, a Wnt antagonist, and showed the role of Wnt signalling in regeneration. Similar roles of FGF and Wnt signalling pathways have also been suggested in tail regeneration of the *Xenopus* larva (**Lin and Slack, 2008)**. **Bechara *et al.* (2000)** observed that aspirin, a non-steroidal anti-inflammatory drug like naproxen, inhibited actinotrichia formation, and they suggested that this inhibition could have been because the aspirin probably interfered with the *Shh* signalling pathway. Thus, these advances in molecular analytical methods in recent years have greatly accelerated our understanding about molecules and signalling pathways operating in regeneration. Another way to understand the molecular mechanisms underlying fin regeneration is to identify genes differentially expressed during the different steps of the regeneration process (**Akimenko *et al.*, 2003**). It is known that fin regeneration in teleosts is intimately related to the expression

of some genes (**White *et al*., 1994; Akimenko *et al*., 1995; Brulfert *et al*., 1998; Géraudie and Ferretti, 1998; Poss *et al*., 2000b; Borday *et al*., 2001**), and that the inhibition of these genes could alter the configuration of the newly formed fin (**Laforest *et al*., 1998; Poss *et al*., 2000a)**. Thus it can be said that a set of signalling molecules influences the proliferation and patterning during regenerative outgrowth (**Jazwinska *et al*., 2007; Laforest *et al*., 1998; Quint *et al*., 2002; Smith *et al*., 2006; Stoick-Cooper *et al*., 2007**); a comparison of these signals known to regulate cell proliferation and specification in different regenerating systems reveals that FGF2 signalling could be implicated in almost all of them. Therefore, an in depth understanding of the role of FGF2 during the regenerative event will be of great restorative value.

To conclude, due to the accessibility of the fin and the simplicity of its structure, the fin regenerate in fish is a very attractive system to conduct research on developmental dynamics during postembryonic period. The current study revealed that the major signalling mechanism (FGF2 signalling) in fish fin regeneration is much akin to that of other vertebrates which are endowed with the power to regenerate, despite their positions in the taxonomic hierarchy. Nevertheless, as mentioned earlier the master regulator – the FGF2 signalling, once expressed at the site of amputation (in response to injury) enter into a series of cross-talks with a whole gamut of putative factors. From the available literature it could be with reasonable conviction, postulate that the major co-regulators of vertebrate epimorphosis are the likes of BMPs, shh, Wnt and PGE_2. Therefore, currently efforts have been initiated in our lab to understand the significance of these communications (especially between the FGF2 and BMP2 as well as between FGF2 and PGE_2) in the regulation of proper regeneration in both anamniote and amniote models. The results of the current as well as the proposed future plans of research shall help us unravel the intricate interplay between various transcriptional regulators of epimorphosis and also might give further corroborative evidences for the evolutionary conservation of the mechanisms of appendage regeneration amongst vertebrates. The present study was however, a humble beginning to understand that seemingly impossible yet fascinating task of regeneration.

BIBLIOGRAPHY

Agata, K., Saito, Y. and Nakajima, E. 2007. Unifying principles of regeneration I: Epimorphosis versus morphallaxis. *Develop. Growth Differ*., **49**(2): 73-78.

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.

Akimenko, M. A., Mari-Beffa, M., Becerra J. and Geraudie, J. 2003. Old questions, new tools, and some answers to the mystery of fin regeneration. *Developmental Dynamics*, **226**:190-201.

Albert, P., Boilly, B., Courty, J. and 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. Dev*., **21**(1): 63-68.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P. *et al*. 2002. Signaling through enzyme-linked cell-surface receptors. *Molecular Biology of the Cell*, 4th Ed., Garland Science, New York.

Albertson, S., Hummel, R. P., Bresden, M. and Greenshalgh, D. G. 1993. PDGF and FGF reverse the healing impairment in protein-malnourished diabetic mice. *Surgery*, **114**(2): 368-372.

Al-Hassan, J. M., Dyson, M., Young, S. R., Thomson, S. R. and Criddle, R. S. 1991. Acceleration of wound healing responses induced by preparations from the epidermal secretions of the Arabian gulf catfish (*Arius bilineatus*, Valenciennes). *J. Wilderness Med.*, **2**(3): 153-163.

Alibardi, L and Toni, M. 2005. Wound keratins in the regenerating epidermis of lizard suggest that the wound reaction is similar in the tail and limb. *J Exp Zool*., **303**(A): 845-860.

Alibardi, L. and Lovicu, J. F. 2010. Immunolocalization of FGF1 and FGF2 in the regenerating tail of the lizard *Lampropholis guichenoti*: Implications for FGFs as trophic factors in tail regeneration. *Acta Histochem*., **112**(5): 459-473.

Allouche, M. and Bikfalvi, A. 1995.The role of fibroblast growth factor 2 in hematopoiesis. *Prog. Growth Factor Res*., **6**(1): 35-48.

Alonso, L. Palacios, J. M., Garcia, E. and Moliner, R. 2000. Characterization of Mn and Cu oxides as regenerable sorbents for hot coal gas desulfurization. *Fuel Processing Tech*., **62**: 31.

Alvarado, A. S. 2000. Regeneration in the metazoans: Why does it happen? *BioEssays*, **22**(6):578-590.

Anderson, J. E., Mitchell, C. M., McGeachie, J. K. and Grounds, M. D.1995. The time course of basic fibroblast growth factor expression in crush injured skeletal muscles of SJL/J and BALB/c mice. *Exp. Cell Res.*, **216**(2): 325-34.

Andreasen, E. A., Mathew, L. K., and Tanguay, R. L. 2006. Regenerative growth is impacted by TCDD: Gene expression analysis reveals extracellular matrix modulation. *Toxicol. Sci*., **92**: 254-269.

Andreasen, E. A., Mathew, L. K., Lohr, C. V., Hasson, R. and Tanguay, R. L. 2007. Aryl hydrocarbon receptor activation impairs extracellular matrix remodeling during zebrafish fin regeneration. *Toxicological Sciences*, **95**(1): 215-226.

Anusree, P. 2012. Role of fibroblast growth factor 2 (FGF2) in the initiation and progression of tail regeneration in northern house gecko *Hemidactylus flaviviridis*. Ph. D thesis submitted to The M. S. University of Baroda, Vadodara, India.

Anusree, P., Saradamba, A., Tailor, N., Desai, I. and Suresh, B. 2011. Caudal fin regeneration is regulated by COX-2 induced PGE_2 in teleost fish, *Poecilia latipinna. J. of Cell and Tissue Research*, **11** (2) 2795-2801.

Armelin, H. A. 1973. Pituitary extracts and steroid hormones in the control of 3T3 cell growth. *PNAS*, **70**(9): 2702-2706.

Arnold, E. N. 1984. Evolutionary aspects of tail shedding in lizards and their relatives. *Journal of Natural History*, **18**(1): 127-169.

Arnold, E. N. 1988. Caudal autotomy as a defense. In: C. Gans and R. B. Huey and A. B. Liss (Eds.) *Biology of the Reptilia*, Alan R. Liss, New York, **16**: pp. 235-273.

Arnold, L., Henry, A., Poron, F., Baba-Amer, Y., Van, R. N., Plonquet, A. *et al*. 2007. Inflammatory monocytes recruited after skeletal muscle injury switch into anti-inflammatory macrophages to support myogenesis. *J. Exp. Med*., **204**(5): 1057-1069.

Avaron, F., Smith, A. and Akimenko, M. A. 2000. Sonic hedgehog signaling in the developing and regenerating fins of zebrafish. Madame Curie Bioscience Database, Austin (TX): Landes Bioscience.

Azevedo, A. S., Grotek, B., Jacinto, A., Weidinger, G. and Saude, L. 2011. The regenerative capacity of the zebrafish caudal fin is not affected by repeated amputations. *PLoS ONE*, **6**(7): e22820.

Azevedo, A. S., Sousa, S., Jacinto, A. and Saude, L. 2012. *BMC Developmental Biology*, **12**(1): 24.

Bader, D. and Oberpriller, J. O. 1978. Repair and reorganization of minced cardiac muscle in the adult newt (*Notophthalmus viridescens*). *J. Morphol*., **155**: 349-357.

Baguna, J. 1976. Mitosis in the intact and regenerating planarian *Dugesia Mediterranean* sp. II. Mitotic studies during regeneration and a possible mechanism of blastema formation. *J. Exp. Zool.*, **195**: 65-80.

Bai, S., Thummel, R., Godwin, A. R., Nagase, H., Itoh, Y., Li, Y. *et al*. 2005. Matrix metalloproteinase expression and function during fin regeneration in zebrafish: Analysis of MT1-MMP, MMP2 and TIMP2. *Matrix Biol*., **24**(4): 247-260.

Baird, A. 1994. Fibroblast growth factors: activities and significance of non-neurotrophin neurotrophic growth factors. *Curr. Opin. Neurobiol*., **4**(1): 78-86.

Baird, A. and Klagsbrun, M. 1991. The fibroblast growth factor family. *N.Y. Acad. Sci.*, **638**: 1-513.

Baird, A., Schubert, D., Ling, N. and Guillemin, R. 1988. Receptor and heparin-binding domains of basic fibroblast growth factor. *Proc. Natl. Acad. Sci*., **85**(7): 2324-2328.

Baker, A. H., Edwards, D. R. and Murphy, G. 2002. Metalloproteinase inhibitors: biological actions and therapeutic opportunities. *J. Cell Sci*., **115**: 3719-3727.

Bareyre, F. M. 2008. Neuronal repair and replacement in spinal cord injury. *J. Neurol. Sci*., **265**(1-2): 63-72.

Basilico, C. and Moscatelli, D. 1992. The FGF family of growth factors and oncogenes. *Adv. Cancer Res*., **59**: 115-165.

Bayliss, P. E., Bellavance, K. L., Whitehead, G. G., Abrams, J. M., Aegerter, S., Robbins, H. S. *et al*. 2006. Chemical modulation of receptor signaling inhibits regenerative angiogenesis in adult zebrafish. *Nat. Chem. Biol.*, **2**(5): 265-273.

Becerra, I. J., Montes, G. S., Bexiga, S. R. R. and Junqueira, L. C. U. 1983. Structure of the tail fin in teleosts. *Cell and Tissue Research*, **230**(1): 127-137.

Becerra, J., Junqueira, L. C., Bechara, I. J. and Montes, G. S. 1996. Regeneration of fin rays in teleosts: A histochemical, radioautographic, and ultrastructural study. *Arch. Histol. Cytol.*, **59**(1): 15-35.

Bechara, I. J., Joazeiro, P. P., Marí-Beffa, M., Becerra, J. and Montes, G. S. 2000. Collagen affecting drugs impair regeneration of teleost tail fins. *J. Submicrosc. Cytol. Pathol*., **32**(2): 273-280.

Beck, C. W., Christen, B. and Slack, J. M. 2003. Molecular pathways needed for regeneration of spinal cord and muscle in a vertebrate. *Dev. Cell*, **5**(2): 429-439.

Becker, C. G., Lieberoth, B. C., Morellini, F., Feldner, J., Becker, T. and Schachner, M. 2004. L1.1 is involved in spinal cord regeneration in adult zebrafish. *J. Neurosci*., **24**(36): 7837-7842.

Becker, T., Wullimann, M. F., Becker, C. G., Bernhardt, R. R. and Schachner, M. 1997. Axonal regrowth after spinal cord transection in adult zebrafish. *J. Comp. Neurol.*, **377**(4): 577-595.

Bell, S. M., Schreiner, C. M., Waclaw, R. R., Campbell, K., Potter, S. S. and Scott, W. J. 2003. Sp8 is crucial for limb outgrowth and neuropore closure. *Proc. Natl. Acad. Sci*., **100**(21): 12195-12200.

Bely, A. E. and Nyberg, K. G. 2010. Evolution of animal regeneration: Re-emergence of a field. In: A. E. Bely and K. G. Nyberg (Eds.) *Trends in Ecology and Evolution*, **25**(3): pp. 161-170.

Bénazet, J. and Zeller, R. 2009. Vertebrate limb development: moving from classical morphogen gradients to an integrated 4-dimensional patterning system. *Cold Spring Harbor Perspect. Biol.*, **1**: a001339.

Bernhardt, R. R., Tongiorgi, E., Anzini, P. and Schachner, M. 1996. Increased expression of specific recognition molecules by retinal ganglion cells and by the optic pathway glia accompanies the successful regeneration of retinal axons in adult zebrafish. *J. Comp. Neuro.*, **376**(2): 253-264.

Bhushan, A. Itoh, N., Kato, S., Thiery, J. P., Czernichow, P., Bellusci, S. *et al*. 2001. FGF10 is essential for maintaining the proliferative capacity of epithelial progenitor cells during early pancreatic organogenesis. *Development*, **128**: 5109-5117.

Bikfalvi, A. and Han, Z. C. 1994. Angiogenic growth factors are hematopoietic growth factors and *vice versa*. *Leukemia*, **8**(3): 523-529.

Bikfalvi, A., Klein, S., Pintucci, G. and Rirkin, D. B. 1997. Biological roles of fibroblast growth factor-2. *Endocr. Rev*., **18**: 26-45.

Bissell, M. J., Hall, H. G. and Parry, G. 1982. How does the extracellular matrix direct gene expression? *J. Theor. Biol*., **99**(1): 31-68.

Blum, N. and Begemann, G. 2012. Retinoic acid signalling controls the formation, proliferation and survival of the blastema during adult zebrafish fin regeneration. *Development*, **139**(1): 107-116.

Bockelmann, P. K., Ochandio, B S. and Bechara, I. J. 2010. Histological study of the dynamics in epidermis regeneration of the carp tail fin (*Cyprinus carpio,* Linnaeus, 1758). *Braz. J. Biol.*, **70**(1): 217-223.

Bode, W. and Maskos, K. Matrix metalloproteinases. 2004. In: A. Messerschmidt, W. Bode, M. Cygler (Eds.). *Handbook of Metalloproteins*. JohnWiley & Sons, Chichester, England, pp. 130-147.

Bode, W., Fernandez-Catalan, C., Grams, F. X., Gomis-Ruth, H., Nagase, H., Tschesche, H. *et al*. 1999. Insights into MMP-TIMP interaction. *Ann. NY. Acad. Sci*., **878**: 73-91.

Bohlen, P., Baird, A., Esch, F., Ling, N. and Gospodarowicz, D. 1984. Isolation and partial molecular characterization of pituitary fibroblast growth factor. *Proc. Natl. Acad. Sci*., **81**(17): 5364-5368.

Bohn, H. 1976. Tissue interactions in the regenerating cockroach leg. In: P. A. Lawrence (Ed.) *Insect* Development, Symp. Roy. ent. Soc., **8**: pp. 170-185.

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 blastemas of axolotls and binds specifically to blastema tissues. *Dev Biol*., **145**(2): 302-310.

Boilly, B., Vercoutter-Edouart, A. S., Hondermarck, H., Nurcombe, V. and Le-Bourhis, X. 2000. FGF signals for cell proliferation and migration through different pathways. *Cytokine Growth Factor Rev*., **11**: 295-302.

Bollum, F. J. and Potter, V. R. 1958. Incorporation of thymidine into deoxyribonucleic acid by enzymes from rat tissues. *J. Biol. Chem.*, **233**(2): 478-482.

Bollum, F. J., Anderegg, J. W., Mcelya, A. M. and Potter, V. R. 1960. Nucleic acid metabolism in regenerating rat liver. VII. Effect of x-radiation on enzymes of DNA synthesis. *Cancer Res*., **20**: 138-143.

Borday, V., Thaeron, C., Avaron, F., Brulfert, A., Casane, D., Laurenti, P. *et al*. 2001. Evx1 trascription in bony fin rays segment boundaries leads to a reiterated pattern during zebrafish fin development and regeneration. *Developmental Dynamics*, **220**(2): 91-98.

Bosworth C. A. IV, Chau-Wen, C., Cole, R. B. and Bernard, B. R. 2005. Protein expression patterns in zebrafish skeletal muscle: Initial characterization and the effects of hypoxic exposureeesinitial characterization and the effects of hypoxic exposure. *Proteomics*, **5**: 1362-1371.

Bottcher, R. T. and Niehrs, C. 2005. Fibroblast growth factor signaling during early vertebrate development. *Endocrine Reviews*, **26**(1): 63-77.

Bouzaffour, M., Dufourcq, P., Lecaudey, V., Haas, P. and Vriz, S. 2009. Fgf and Sdf-1 pathways interact during zebrafish fin regeneration, **4**(6): e5824.

Brinckerhoff, C. E. and Matrisian, L. M. 2002. Matrix metalloproteinases: a tail of a frog that became a prince. *Nature Reviews Molecular Cell Biology*, **3**: 207-214.

Brockes, J. P. and Kumar, A. 2002. Plasticity and reprogramming of differentiated cells in amphibian regeneration. *Nat. Rev. Mol. Cell Biol*., **3**(8): 566-74.

Brockes, J. P. and Kumar, A. 2005. Appendage regeneration in adult vertebrates and implications for regenerative medicine. *Science*, **310**: 1919-1923.

Brockes, J. P. and Kumar, A. 2008. Comparative aspects of animal regeneration. *Annu. Rev. Cell Dev. Biol*., **24**: 525-549.

Brockes, J. P., Kumar, A., and Velloso, C. P. 2001. Regeneration as an evolutionary variable. *J. Anat*., **199**(1-2): 3-11.

Brookes, N. 1984. Neurohumoral influences and [3H]2-deoxyglucose uptake in primary astrocytes. *Fed. Proc*., **43**: 289.

Broughton, G., Janis, J. E. and Attinger, C. E. 2006. The basic science of wound healing. *Plast. Reconstr. Surg*., **117**(7): 12S-34S.

Broussonet, M. 1786. Observations sur la régénérations de quelques parties du corps des poissons. *Hist. d. l'Acad. Roy. Des. Sciences*.

Brown, E. J. and Goodwin, J. L. 1988. Fibronectin receptors of phagocytes. Characterization of the Arg-Gly-Asp binding proteins of human monocytes and polymorphonuclear leukocytes. *J. Exp. Med*., **167**: 777–93.

Brulfert, A., Monnot, M. J. and Géraudie, J. 1998. Expression of two even-skipped genes eve1 and evx2 during zebrafish fin morphogenesis and their regulation by retinoic acid. *Int. J. Dev. Biol*., **42**: 1117-1124.

Bulow, F. J. 1987. RNA-DNA ratios as indicators of growth in fish. In: R. C. Summerfelt and G. C. Hall (Eds.) *The age and growth of fish*, The Iowa State University Press, Ames, pp. 45- 64.

Bulow, H. E., Boulin, T. and Hobert, O. 2004. Differential functions of the *C. elegans* FGF receptor in axon outgrowth and maintenance of axon position. *Neuron*, **42**(3): 367-374.

Buntrock, P., Buntrock, M., Marx, I., Kranz, D., Jentzsch, K. D. and Heder, G. 1984. Stimulation of wound healing, using brain extract with fibroblast growth factor (FGF) activity III, Electron microscopy, autoradiography, and ultrastructural autoradiography of granulation tissue. *Exp. Pathol*., **26**: 247-54.

Buntrock, P., Jentzsch, K. D and Heder, G. 1982a. Stimulation of wound healing, using brain extract with fibroblast growth factor (FGF) activity I. Quantitative and biochemical studies into formation of granulation tissue. *Exp. Pathol*., **21**: 46-53.

Buntrock, P., Jentzsch, K. D. and Heder, G. 1982b. Stimulation of wound healing, using brain extract with fibroblast growth factor (FGF) activity II. Histological and morphometric examination of cells and capillaries. *Exp. Pathol*., **21**: 62-7.

Burgess, W. H. and Maciag, T. 1989. The heparin-binding (fibroblast) growth factor family of proteins. *Annu. Rev. Biochem*., **58**: 575-606.

Burke, D., Wilkes, D., Blundell, T. L. and Malcolm, S. 1998. Fibroblast growth factor receptors: lessons from the genes. *Trends Biochem. Sci*., **23**(2): 59-62.

Burrus, L. W., Zuber, M. E., Lueddecke, B. A. and Olwin, B. B. 1992 Identification of a cysteine-rich receptor for fibroblast growth factors. *Mol. Cell Biol*., **12**: 5600-5609.

Cai, S., Fu, X. and Sheng, Z. 2007. Dedifferentiation: A New Approach in Stem Cell Research. *BioScience*, **57**(8): 655-662.

Call, M. K. and Tsonis, P. A. 2005. Vertebrate limb regeneration. *Adv. Biochem. Eng. Biotechnol*., **93**: 67-81.

Campbell, L. J. and Crews, C. M. 2008. Wound epidermis formation and function in urodele amphibian limb regeneration. *Cell Mol. Life Sci*., **65**(1): 73-79.

Carlson, B. M. 1974. Factors controlling the initiation and cessation of early events in the regenerative process. In: G. V. Sherbet (Ed.) *Neoplasia and cell* differentiation, Basel, Karger, pp. 60-105.

Carlson, B. M. 2005. Some principles of regeneration in mammalian systems. *Anat. Rec. B New Anat.*, **287**(1): 4-13.

Carlson, B. M. 2007. Principles of regenerative biology. Elsevier Ltd., Academic Press, New York, pp. 68-71.

Chambers, R. L. and McDermott, J. C. 1996. Molecular basis of skeletal muscle regeneration. *Can. J. Appl. Physiol.*, **21**(3): 155-84.

Chen, W. Y., Rogers, A. A. and Lydon, M. J. 1992. Characterization of biologic properties of wound fluid collected during early stages of wound healing. *J. Invest. Dermatol*., **99**(5): 559-564.

Christen, B. and Slack, J. M. 1997. FGF-8 is associated with anteroposterior patterning and limb regeneration in *Xenopus*. *Dev. Biol*., **192**(2): 455-466.

Christensen, R. N., Weinstein, M. and Tassava, R. A. 2002. Expression of fibroblast growth factors 4, 8, and 10 in limbs, flanks, and blastemas of *Ambystoma*. *Dev. Dyn*., **223**(2): 193-203.

Clark, R. A. 1993a. Basics of cutaneous wound repair. *J. Dermatol. Surg. Oncol*., **19**: 693-706.

Clark, R. A. 1993b. Biology of dermal wound repair. *Dermatol. Clin*., **11**(4): 647-666.

Clark, R. A., Wikner, N. E., Doherty, D. E. and Norris, D. A. 1988. Cryptic chemotactic activity of fibronectin for human monocytes resides in the 120-kDa fibroblastic cell-binding fragment. *J. Biol. Chem*., **263**(24): 12115-12123.

Cohn, M. J., Izpisua-Belmonte, J. C., Abud, H., Heath, J. K. and Tickle, C. 1995. Fibroblast growth factors induce additional limb development from the flank of chick embryos. *Cell*, **80**(5): 739-746.

Cotton, L. M., O'Bryan, M. K. and Hinton, B. T. 2008. Cellular signaling by fibroblast growth factors (FGFs) and their receptors (FGFRs) in male reproduction. *Endocr. Rev*., **29**(2): 193-216.

Coughlin, S. R., Barr, J., Coussens, L. S., Fretto, L. J. and Williams L. T. 1988. Acidic and basic fibroblast growth factors stimulate tyrosine kinase activity *in vivo. J. Biol. Chem.*, **263**: 988-993.

Courtenay, W. R. and Meffe, G. K. 1989. Small fishes in strange places: a review of introduced poeciliids. In: G. K. Meffe and F. F. Snelson (Eds.) *Ecology and evolution of livebearing fishes (Poeciliidae),* Englewood Cliffs, Prentice Hall, pp. 319-332.

Cross, M. J. and Claesson-Welsh, L. 2001. FGF and VEGF function in angiogenesis: signalling pathways, biological responses and therapeutic inhibition. *Trends Pharmacol. Sci*., **22**(4): 201-207.

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

Cuevas, P. 1997. Therapeutic prospects for fibroblast growth factor treatment of brain ischemia. *Neurol. Res.*, **19**: 355-356.

Cutroneo, K. R. 2003. Gene therapy for tissue regeneration. *J. Cell. Biochem*., **88**(2): 418-425.

D'Jamoos, C. A., McMahon, G. and Tsonis, P. A. 1998. Fibroblast growth factor receptors regulate the ability for hindlimb regeneration in *Xenopus laevis*. *Wound Repair Regen.*, **6**(4): 388-397.

Davidson, J. M., Klagsbrun, M., Hill, K. E., Buckley, A., Sullivan, R., Brewer, P. S. *et al*. 1985. Accelerated wound repair, cell proliferation, and collagen accumulation are produced by a cartilage-derived growth factor. *J. Cell. Biol*., **100**: 1219-27.

Decker, L., Avellana-Adalid, V., Nait-Oumesmar, B., Durbec, P. and Baron-Van, E. A. 2000. Oligodendrocyte precursor migration and differentiation: combined effects of PSA residues, growth factors, and substrates. *Mol. Cell Neurosci*., **16**(4): 422-439.

Deimling, S. J. and Drysdale, T. A. 2011. FGF is required to regulate anterior-posterior patterning in the *Xenopus* lateral plate mesoderm. *Mech. Dev*., **128**(7-10): 327-41.

Dial, B. E. and Fitzpatrick, L. C. 1983. Lizard tail autotomy: Function and energetics of postautotomy tail movements in *Scin-cella lateralis* (Sauria: Scincidae). *Science*, **219**(4583): 391-393.

Dietrich, W. D., Alonso, O., Busto, R. and Finklestein, S. P. 1996. Post treatment with intravenous basic fibroblast growth factor reduces histopathological damage following fluid percussion brain injury in rats. *J. Neurotrauma*, **13**: 309-316.

Ding, S. and Schultz, P. G. 2004. A role for chemistry in stem cell biology. *Nature Biotechnology*, **22**: 833-840.

Dinsmore, C. E. 1992. *A history of regeneration research: Milestones in the evolution of a science*. Cambridge University Press, Cambridge.

Douglas, B. S. 1972. Conservative management of guillotine amputations of the finger in children. *Australian Paediatric Journal*, **8**(2): 86-89.

Dufourcq, P. and Vriz, S. 2006. The chemokine SDF-1 regulates blastema formation during zebrafish fin regeneration. *Development Genes and Evolution*, **216**(10): 635-639.

Dumont, A. E. 1959. Wound healing and extracellular deoxyribonucleic acid. *Ann. Surg*., **150**: 799-807.

Dunphy, J. E. and Udupa, K. N., 1955. Chemical and histochemical sequences in the normal healing of wounds. *N. Engl. J. Med*., **253**(20): 847-851.

Dvorak, P. and Hampl, A. 2005. Basic fibroblast growth factor and its receptors in human embryonic stem cells. *Folia Histochemica. Et. Cytobiologica*., **43**(4): 203-208.

Edel, M. J., Menchon, C., Menendez, S., Consiglio, A., Raya, A. and Ipzisua-Belmonte, J. C. 2010. Rem2 GTPase maintains survival of human embryonic stem cells as well as enhancing reprogramming by regulating p53 and cyclin D1. *Genes Dev*., **24**(6): 561-573.

Encinas, P., Rodriguez-Milla, M. A., Novoa, B., Estepa, A., Figueras, A. and Coll, J. 2010. Zebrafish fin immune responses during high mortality infections with viral haemorrhagic septicaemia rhabdovirus. A proteomic and transcriptomic approach. *BMC Genomics*, **11**: 518.

Ericksson, A. E., Cousens, L. S., Weaver, L. H. and Matthews, B. W. 1991. Three dimensional structure of human basic fibroblast growth factor. *Proc. Natl. Acad. Sci*., **88**(8): 3441-3445.

Eswarakumar, V. P, Lax, I. and Schlessinger, J. 2005. Cellular signaling by fibroblast growth factor receptors. *Cytokine Growth Factor Rev.*, **16**(2): 139-149.

Faham, S., Hileman, R. E., Fromm, J. R., Linhardt, R. J and Rees, D. C. 1996. Heparin structure and interactions with basic fibroblast growth factor. *Science*, **271**: 1116-1120.

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**(5155): 104-107.

Fernandez-Resa, P., Mira, E. and Quesada, A. R. 1995. Enhanced detection of casein zymography of matrix metalloproteinases. *Anal. Biochem*., **224**(1): 434-435.

Ferretti, P. and Geraudie, J. 1995. Retinoic acid-induced cell death in the wound epidermis of regenerating zebrafish fins. *Dev. Dyn*., **202**(3): 271-283.

Floss, T., Arnold, H. H. and Braun, T. 1997. A role for FGF-6 in skeletal muscle regeneration. *Genes & Development*, **11**: 2040-2051.

Folkman, J. and Klagsbrun, M. 1987. Angiogenic factors. *Science*, **235**: 442-7.

Folkman, J., Klagsbrun, M., Sasse, J., Wadzinski, M., Ingber, D. and Vlodavsky, I. 1988. A heparin-binding angiogenic protein--basic fibroblast growth factor--is stored within basement membrane. *Am. J. Pathol*., **130**(2): 393-400.

Forget, M. A., Desrosiers, R. R. and Beliveau, R. 1999. Physiological roles of matrix metalloproteinases: implications for tumor growth and metastasis. *Canadian Journal of Physiology and Pharmacology*, **77**(7): 465-480.

Fresco, J. R. and Bendich, A. 1960. The metabolic stability of rat liver deoxyribonucleic acid: A Turnover Study. *J. Biol. Chem*., **235**: 1124-1128.

Fromm, H. J. and Nordlie, R. C. 1959. Redistribution of methionine and cystine during experimental wound healing. *Biochem. Biophys. Acta*., **31**(2): 357-364.

Fu, L., John, L. M, Adams, S. H., Yu, X. X., Tomlinson, E., Renz, M. *et al.* 2004. Fibroblast growth factor 19 increases metabolic rate and reverses dietary and leptin deficient diabetes. Endocrinology, **145**: 2594-2603.

Fujimoto, K., Nakajima, K. and Yaoita, Y. 2006. One of the duplicated matrix metalloproteinase-9 genes is expressed in regressing tail during anuran metamorphosis. *Dev. Growth Differ*., **48**(4): 223-241.

Fujimoto, K., Nakajima, K. and Yaoita, Y. 2007. Expression of matrix metalloproteinase genes in regressing or remodeling organs during amphibian metamorphosis. *Dev. Growth Differ*., **49**(2): 131-143.

Fujisawa, T. 2003. Hydra Regeneration and Epitheliopeptides. *Developmental Dynamics*, **226**: 182-189.

Galzie, Z., Kinsella, A. R. and Smith, J. A. 1997. Fibroblast growth factors and their receptors. *Biochem. Cell Biol*., **75**: 669-685.

Gardiner, D. M. and Bryant, S. V. 1996. Molecular mechanisms in the control of limb regeneration: the role of homeobox genes. *International Journal of Developmental Biology*, **40**(4): 797-805.

Gardiner, D. M., Blumberg, B., Komine, Y. and Bryant, S. V. 1995. Regulation of HoxA expression in developing and regenerating axolotl limbs. *Development*, **121**(6): 1731-1741.

Geetha-Loganathan, P., Nimmagadda, S. and Scaal, M. 2008. *Wnt* signaling in limb organogenesis. *Organogenesis*, **4**(2): 109-115.

Geraudie, J. 1977. Initiation of the actinotrichial development of the early fin bud of the fish Salmo. *J. Morphol*., **151**: 353-362.

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

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

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

Géraudie, J. and Singer, M. 1992. The fish fin regeneration. In: H.W. Sauer (Ed.) *Keys to regeneration*: *Monographs in Developmental Biology*, **23**: pp. 62-72.

Geraudie, J. and Singer, M., 1985. Necessity of an adequate nerve supply for regeneration of the amputated pectoral fin in the teleost *Fundulus*. *J. Exp. Zool*., **234**(3): 367-374.

Geraudie, 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*., **268**(1): 12-22.

Geraudie, 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. *Int. J. Dev. Biol*., **39**(2): 373-381.

Géraudie, J., Monnot, M. J., Ridet, A., Thorogood, P. and Ferretti, P. 1993. Is exogenous retinoic acid necessary to alter positional information during regeneration of the fin in zebrafish? *Prog. Clin. Biol. Res*., **383**(B): 803-814.

Ghosh, A. and Greenberg, M. E. 1995. Distinct roles for bFGF and NT-3 in the regulation of cortical neurogenesis. *Neuron*, **15**: 89-103.

Ghysen, A. and Dambly-Chaudiere, C. 2007. The lateral line microcosmos. *Genes and development*, **21**: 2118-2130.

Gianpaoli, S., Bucci, S., Ragghianti, M., Mancino, G., Zhang, F. and Ferretti, P. 2003. Expression of FGF2 in the limb blastema of two salamandridae correlates with their regenerative capability. *Proc. Biol. Sci.*, **270**(1530): 2197-2205.

Gibran, N. S., Isik, F. F., Heimbach, D. M. and Gordon, D. 1994. Basic fibroblast growth factor in the early human burn wound. *J. Surg. Res*., **56**(3): 226-234.

Givol, D. and Yayon, A. 1992. Complexity of FGF receptors: genetic basis for structural diversity and functional specificity. *FASEB J*., **6**: 3362-3369.

Goldberg, G. I., Strongin, A., Collier, I. E., Genrich, L. T. and Marmer, B. L. 1992. Interaction of 92-kDa type IV collagenase with the tissue inhibitor of metalloproteinases prevents dimerization, complex formation with interstitial collagenase, and activation of the proenzyme with stromelysin. *J. Biol. Chem*., **267**(7): 4583-4591.

Goldfarb, M. 1996. Functions of fibroblast growth factors in vertebrate development. *Cytokine Growth Factor Rev.*, **7**: 311-325.

Goldshmit, Y., Sztal, T. E., Jusuf, P. R., Hall, T. E., Nguyen-Chi, M. and Currie, P. D. 2012. FGF-dependent glial cell bridges facilitate spinal cord regeneration in zebrafish. *The Journal of Neuroscience*, **32**(22): 7477-7492.

Gospodarowitz, D. 1990. Fibroblast growth factor. Chemical structure and biologic function. *Clin. Orthop*., **257**: 231-48.

Gospodarowitz, D. 1974. Localisation of a fibroblast growth factor and its effect alone and with hydrocortisone on 3T3 cell growth. *Nature*, **249**: 123-127.

Goss, R. and Stagg, M., 1957. The regeneration of fins and fin rays in *Fundulus heteroclitus*. *Journal of Experimental Zoology*, **136**(3): 487-507.

Goss, R. J. 1956. Regenerative inhibition following limb amputation and immediate insertion into the body cavity. *Anat. Rec*., **126**: 15-27.

Goss, R. J. 1969. *Principles of Regeneration.* Academic Press, New York.

Goss, R. J. 1991. The natural history (and mystery) of regeneration. In: C. E. Dinsmore (Ed.) *A history of regeneration research*, Cambridge University Press, Cambridge, pp. 7-23.

Grass, S., Arnold, H. H. and Braun, T. 1996. Alterations in somite patterning of myf-5 deficient mice: A possible role for FGF-4 and FGF-6. *Development*, **12**: 141-150.

Green, P. J., Walsh, F. S. and Doherty, P. 1996. Promiscuity of fibroblast growth factor receptors. *BioEssays*, **18**(8): 639-646.

Greenwald, J. A., Mehrara, B. J., Spector, J. A., Warren, S. M., Fagenholz, P. J., Smith, L. P. *et al*. 2001. *In vivo* modulation of FGF biological activity alters cranial suture fate. *Am. J. Pathol*., **158**(2): 441-452.

Gremigni, V. and Miceli, C. 1980. Cytophotometric evidence for cell 'transdifferentiation' in planarian regeneration. *Wilhelm Roux's Archives*, **188**: 107-113.

Grillo, H. C., Lapiere, C. M., Dresden, M. H. and Gross, J. 1968. Collagenolytic activity in regenerating forelimbs of the adult newt (*Triturus viridescens*). *Dev. Biol*., **17**(5): 571-583.

Gross, J. and Lapiere, C. M. 1962. Collagenolytic activity in amphibian tissues: a tissue culture assay. *Proc. Natl. Acad. Sci.*, **48**(6): 1014-1022.

Grounds, M. D. 1991. Towards understanding skeletal muscle regeneration. *Path. Res. Pract.*, **187**(1): 1-22.

Guimond, S. E., Turnbull, J. E. 1999. Fibroblast growth factor receptor signalling is dictated by specific heparan sulphate saccharides. *Curr. Biol*., **9**(22): 1343-6.

Guimond, S., Turner, K., Kita, M., Ford-Perriss, M. and Turnbull, J. 2001. Dynamic mechanism during development. *Biochem. Soc. Trans*., **29**: 177-81.

Gurley, K. A. and Sánchez-Alvarado, A. 2008. Stem cells in animal models of regeneration. In: *StemBook*.

Halaban, R., Fan, B., Ahn, J., Funasaka, Y., Gitay-Goren, H. and Neufeld, G. 1992. Growth factors, receptor kinase and protein tyrosine phosphatase in normal and malignant melanocytes. *J. Immunopathol*., **12**(3): 154-161.

Hall, B. K. 2005. Bones and Cartilage: Developmental and evolutionary skeletal biology. Elsevier Academic Press, San Diego, CA, **1**: 760pp.

Hall, C., Flores, M. V., Storm, T., Crosier, K. and Crosier P. 2007. The zebrafish lysozyme C promoter drives myeloid-specific expression in transgenic fish. *BMC developmental biology*, **7**: 42.

Hammerschmidt, M., Brook, A. and McMahon, A. P. 1997. The world according to hedgehog. *Trends Genet.*, **13**: 14-21.

Han, M. J., An, J. Y. and Kim, W. S. 2001. Expression patterns of FGF-8 during development and limb regeneration of the axolotl. *Dev. Dyn*., **220**(1): 40-48.

Han, M., Yang, X., Taylor, G., Burdsal, C. A., Anderson, R. A. and Muneoka, K. 2005. Limb regeneration in higher vertebrates: Developing a roadmap. *Anat. Rec. B. New. Anat.*, **287**(1): 14-24.

Harty, M, Neff, A. W., King, M. W. and Mescher, A. L. 2003. Regeneration or Scarring: An Immunologic Perspective. *Developmental Dynamics*, **226**(2): 268-279.

Hata, J., Oshima, S., Takeshita, A. and Yamashita, S. 1998. Blastemal growth promoted by fibroblast growth factor 2 in fish fin regeneration. *J. Mar. Biotechnol*., **6**: 91-94.

Hawkes, S. P., Li, H. and Taniguchi, G. T. 2001. Zymography and reverse zymography for detecting MMPs, and TIMPs. In: I. Clark (Ed.) *Matrix Metalloproteinases Protocols*. Humana Press, Totowa, NJ, **151**: pp. 399-410.

Hay, E. D. 1981. *Cell Biology of Extracellular Matrix*, Plenum Press, New York.

Hayek, A., Culler, F. L., Beattie, G. M., Lopez, A. D., Cuevas, P. and Baird, A. 1987. An *in vivo* model for study of the angiogenic effects of basic fibroblast growth factor. *Biochem. Biophys. Res. Commun*., **147**: 876-80.

Hecht, L, I. and Potter, V. R. 1956. Nucleic acid metabolism in regenerating rat liver Part I. The rate of deoxyribonucleic acid synthesis *in vivo*. *Cancer Research*, **16**(10): 988-933.

Heissig, B., Hattori, K., Friedrich, M., Rafii, S. and Werb, Z. 2003. Angiogenesis: vascular remodeling of the extracellular matrix involves metalloproteinases. *Curr. Opin. Hematol*., **10**: 136-41.

Heussen, C. and Dowdle, E. B. 1980. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. *Anal. Biochem.*, **102**(1): 196-202.

Hinchliffe, J. R. 2002. Developmental basis of limb evolution. *Int. J. Dev. Biol.*, **46**: 835-45.

Hipps, D. S, Hembry, R. M., Docherty, A. J., Reynolds, J. J. and Murphy, G. 1991. Purification and characterization of human 72-kDa gelatinase (type IV collagenase). Use of immunolocalisation to demonstrate the non-coordinate regulation of the 72-kDa and 95-kDa gelatinases by human fibroblasts. *Biol. Chem. Hoppe-Seyler*, **372**: 287-296.

Hirata, M., Nakamura, K. I. and Kondo, S. 2005. Pigment cell distributions in different tissues of the zebrafish, with special reference to the striped pigment pattern. *Dev. Dyn*., **234**: 293-300.

Hobmayer, B., Rentzsch, F., Kuhn, K., Happel, C. M., Von-Laue, C. C., Snyder, P. *et al*. 2000. WNT signalling molecules act in axis formation in the diploblastic metazoan *Hydra*. *Nature,* **407**(6801): 186-189.

Hogan, B. L. 1996. Bone morphogenetic proteins: Multifunctional regulators of vertebrate development. *Genes and Dev.*, **10**: 1580-1594.

Holly, L. D., Nye, J. A., Cameron, E., Chernoff, A. G. and Stocum, D. L. 2003. Regeneration of the urodele limb: A Review. *Developmental dynamics*, **226**: 280-293.

Höyhtyä, M., Hujanen, E., Turpeenniemi-Hujanen, T., Thorgeirsson, U., Liotta, L. A. and Tryggvason, K. 1990. Modulation of type-IV collagenase activity and invasive behavior of metastatic human melanoma (A2058) cells *in vitro* by monoclonal antibodies to type-IV collagenase. *Int. J. Cancer*, **46**: 282-286.

Huang, H. Y., Dai, E. S., Liu, J. T., Tu, C. T., Yang, T. C. and Tsai, H. J. 2009. The embryonic expression patterns and the knockdown phenotypes of zebrafish ADP-ribosylation factor-like 6 interacting protein gene. *Dev. Dyn*., **238**(1): 232-240.

Huttenlocher, A., Sandborg, R. R. and Horwitz, A. F. 1995. Adhesion in cell migration. *Curr. Opin. Cell. Biol*., **7**(5): 697-706.

Illingworth, C. M. 1974. Trapped finger and amputated tips in children. *J. Paediatr. Surg.*, **9**(6): 853-859.

Ingham, P. W. and Mcmahon, A. P. 2001. Hedgehog signaling in animal development: paradigms and principles. *Genes Dev*., **15**: 3059-3087.

Iovine, M. K. 2007. Conserved mechanisms regulate outgrowth in zebrafish fins. *Nature Chemical Biology*, **10**(3): 613-618.

Iseki, S., Wilkie, A. O. M., Heath, J. K., Ishimaru, T., Eto, K. and Morriss-Kay, G. M. 1997. *Fgfr2* and *osteopontin* domains in the developing skull vault are mutually exclusive and can be altered by locally applied FGF2. *Development*, **124**(17): 3375-3384.

Ishida, T., Nakajima, T., Kudo, A., and Kawakami, A. 2010. Phosphorylation of Junb family proteins by the Jun N-terminal kinase supports tissue regeneration in zebrafish. *Dev. Biol*., **340**(2): 468-479.

Itoh, T., Tanioka, M., Yoshida, H., Nishimoto, H. and Itohara, S. 1998. Reduced angiogenesis and tumour progression in gelatinase A-deficient mice. *Cancer Res.*, **58**: 1048-1051.

Jardetsky, C. and Barnum, C. P. 1957. Metabolic activity and intracellular distribution of nucleic acid phosphorus in regenerating liver. *Arch. Biochem*., **67**(2): 350-365.

Jazwinska, A., Badakov, R. and Keating, M. T. 2007. Activin-betaA signaling is required for zebrafish fin regeneration. *Curr. Biol*., **17**(16): 1390-1395.

Jiang, F., Frederick, T. J. and Wood, T. L. 2001. IGF-I synergizes with FGF-2 to stimulate oligodendrocyte progenitor entry into the cell cycle. *Dev. Biol*., **232**(2): 414-423.

Jianwu, F., Siluo, H., Huisheng, L., Crepin, M., Tao, X. U. and Jianfeng, L. I. U. 2003. Role of FGF-2/FGFR signaling pathway in cancer and its signification in breast cancer. *Chinese Science Bulletin*, **48**(15): 1539-1547.

Jimenez, C., Portela, R. A., Mellado, M., Rodríguez-Frade, J. M., Collard, J., Serrano, A. *et al*. 2000. Role of the PI3K regulatory subunit in the control of actin organization and cell migration. *J. Cell Biol*., **151**(2): 249-261.

Johnson, F. R. and McMinn, R. M. H. 1960. Biological Reviews: The cytology of wound healing of body surfaces in mammals, **35**(3): 364-410.

Johnson, S. and Weston, J., 1995. Temperature-sensitive mutations that cause stage-specific defects in zebrafish fin regeneration. *Genetics,* **141**(4): 1583-1595.

Johnson, S. L. and Bennett, P. 1999. Growth control in the ontogenetic and regenerating zebrafish fin. *Methods Cell Biol*., **59**: 301-311.

Kato, T., Miyazaki, K., Shimizu-Nishikawa, K., Koshiba, K., Obara, M., Mishima, H.K. *et al*., 2003. Unique expression patterns of matrix metalloproteinases in regenerating newt limbs. *Dev. Dyn*., **226**(2): 366-376.

Kawakami, Y., Esteban, C. R., Matsui, T., Rodriguez-Leon, J., Kato, S. and Belmonte, J. C. 2004. Sp8 and Sp9, two closely related buttonhead-like transcription factors, regulate Fgf8 expression and limb outgrowth in vertebrate embryos. *Development*, **131**(19): 4763-4774.

Kawakami, Y., Esteban, C. R., Raya, M., Kawakami, H., Marti, M., Dubova, I. *et al*. 2006. *Genes Dev.*, **20**(23): 3232-3237.

Kemp, N. E. and Park J. H. 1970. Regeneration of lepidotrichia and actinotrichia in the tailfin of the teleost *Tilapia mossambica*. *Devl. Biol*., **22**(2): 321-342.

Kharitonenkov, A., Shiyanova, T. L., Koester, A., Ford, A. M., Micanovic, R., Galbreath, E. J. *et al*. 2005. FGF-21 as a novel metabolic regulator**.** *J. Clin. Invest.*, **115**: 1627-1635.

King M. W., Neff A.W. and Mescher A. L. 2009. Proteomics analysis of regenerating amphibian limbs: Changes during the onset of regeneration. *Int. J. Dev. Biol.*, **53**, 955-969.

Knopf, F., Hammond, C., Chekuru, A., Kurth, T., Hans, S., Weber, C. W. *et al*. 2011. Bone regenerates via dedifferentiation of osteoblasts in the zebrafish fin. *Developmental Cell*, **20**(5): 713-724.

Konishi, M., Micami, T., Yamasaki, M., Miyake, A., and Itoh, N. 1999. Fibroblast growth factor-16 is a growth factor for embryonic brown adipocytes. *J. Biol. Chem.*, **255**: 12119-12122.

Konttinen, Y. T., Ainola, M., Valleala, H., Ma, J., Ida, H., Mandelin, J. *et al*. 1999. Analysis of 16 different matrix metalloproteinases (MMP-1 to MMP-20) in the synovial membrane: different profiles in trauma and rheumatoid arthritis. *Ann. Rheum. Dis*., **58**(11): 691-697.

Ku, P. T. and D'Amore, P. A. 1995. Regulation of basic fibroblast growth factor (bFGF) gene and protein expression following its release from sublethally injured endothelial cells. *J. Cell Biochem.*, **58**: 328-343.

Kuhn, H. G., Winkler, J., Kempermann, G., Thal, L. J. and Gage, F. H. 1997. Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult rat brain. *J. Neurosci.*, **17**: 5820-5829.

Laforest, L., Brown, C. W., Poleo, G. Géraudie, J, Tada, M., Ekker, M. *et al*. 1998. Involvement of the sonic hedgehog, patched 1 and bmp2 genes in patterning of the Zebrafish dermal fin rays. *Development*, **251**(21): 4175-4184.

Laird, A. D., Vajkoczy, P., Shawver, L. K., Thurnher, A., Liang, C., Mohammadi, M. *et al*. 2000. SU6668 is a potent antiangiogenic and antitumor agent that induces regression of established tumors. *Cancer Res*., **60**: 4152-4160.

Lamont, F. R., Tomlinson, D. C., Cooper, P. A., Shnyder, S. D., Chester, J. D. and Knowles, M. A. 2011. Small molecule FGF receptor inhibitors block FGFR-dependent urothelial carcinoma growth *in vitro* and *in vivo*. *Brit. J. Cancer*, **104**(1): 75-82.

Lauffenburger, D. A. and Horwitz, A. F. 1996. Cell migration: a physically integrated molecular process. *Cell*, **84**(3): 359-369.

Lee, P. L., Johnson, D. E., Cousens, L. S., Fried, V. A. and Williams, L. T. 1989. Purification and complementary DNA cloning of a receptor for basic fibroblast growth factor. *Science*, **245**: 57-60.

Lee, Y., Grill, S., Sanchez, A., Murphy-Ryan, M. and Poss, K. D. 2005. FGF signalling instructs position-dependent growth rate during zebrafish fin regeneration. *Development*, **132** (23): 5173-5183.

Lee, Y., Hami, D., Val, S. D., Kagermeier-Schenk, B., Wills, A. A., Black, B. L. *et al*. 2009. Maintenance of blastemal proliferation by functionally diverse epidermis in regenerating zebrafish fins. *Developmental Biology*, **331**(2): 270-280.

Lee, Y., Nachtrab, G., Klinsawat, P. W., Hami, D. and Poss, K. D. 2010. Ras controls melanocyte expansion during zebrafish fin stripe regeneration. *Dis. Model. Mech*., **3**(7-8): 496-503.

Lefevre, G., Babchia, N., Calipel, A., Mouriaux, F., Faussat, A. M., Mrzyk, S. and Mascarelli, F. 2009. Activation of the FGF2/FGFR1 Autocrine Loop for Cell Proliferation and Survival in Uveal Melanoma Cells. *Investigative Ophthalmology and Visual Science*, **50**(3): 1047-1057.

Legrand, E. K., Burke, J. F., Costa, D. E. and Kiorpes, T. C. 1993. Dose response effects of PDGF-BB, PDGF-AA, EGF and bFGF on granulation tissue in a guinea pig partial thickness skin excision model. *Growth Factors*, **8**(4): 307-314.

Lehoczky, J. A., Robertb, B. and Tabina, C. J. 2011. Mouse digit tip regeneration is by fate-restricted progenitor cells. *PNAS*, **108**(51): 20609-20614.

Lenhoff, S. G. and Lenhoff, H. M. 1986. Hydra and the Birth of Experimental Biology, 1744*: Abraham Trembley's Memoirs Concerning the Natural History of a Type of Freshwater Polyp with Arms Shaped Like Horns*, Boxwood Press, Pacific Grove, **17**: 241-242.

Lepilina, A., Coon, A. N., Kikuchi, K., Holdway, J. E., Roberts, R. W., Burns, C. G. *et al*. 2006. A dynamic epicardial injury response supports progenitor cell activity during Zebrafish heart regeneration. *Cell*, **127**(3): 607-619.

Levesque, M., Gatien, S., Finnson, K., Desmeules, S., Villiard, E., Pilote, M. *et al*. 2007. Transforming Growth Factor: b signaling is essential for limb regeneration in axolotls. *PLoS ONE*, 2(11): e1227.

Lin, F., Ren, X. D., Doris, G. and Clark, R. A. 2005. Three-dimensional migration of human adult dermal fibroblasts from collagen lattices into fibrin/fibronectin gels requires syndecan-4 proteoglycan. *J. Invest. Dermatol*., **124**: 906-13.

Lin, G. and Slack, J. M. 2008. Requirement for Wnt and FGF signaling in *Xenopus* tadpole\ tail regeneration. *Dev. Biol*., **316**(2): 323-335.

Lin, X., Buff, E. M., Perrimon, N. and Michelson, A. M. 1999. Heparan sulfate proteoglycans are essential for FGF receptor signaling during *Drosophila* embryonic development. *Development*, **126**(17): 3715-3723.

Liotta, L. A., Abe, S., Robey, P. G. and Martin, G. R. 1979. Preferential digestion of basement membrane collagen by an enzyme derived from a metastatic murine tumor. *Proc. Natl. Acad. Sci*., **76**: 2268-2272.

Liozner, L. D. 1974. Organ regeneration: a study of developmental biology in mammals. *Consultants Bureau*, New York: 1-15.

Liu, Y., Cai, S., Shu, X. Z., Shelby, J. and Prestwich, G. D. 2007. Release of basic fibroblast growth factor from a crosslinked glycosaminoglycan hydrogel promotes wound healing. *Wound Rep. Reg*., **15**(2): 245-251.

Logan, A. and Berry, M. 1993 Transforming growth factor-b1 and basic fibroblast growth factor in the injured CNS. *Trends Pharmacol. Sci*., **14**(9): 337-342.

Logan, A., Frautschy, A. S. and Baird, A. 1991. Basic fibroblast growth factor and central nervous system injury. *Ann. NY. Acad. Sci.*, **638**: 474-476.

London, A., Itskovich, E., Benhar, I., Kalchenko, V., Mack, M., Jung, S. *et al*. 2011. Neuroprotection and progenitor cell renewal in the injured adult murine retina requires healing monocyte-derived macrophages. *J. Exp. Med*., **208**(1): 23-39.

Loof, S. 2008. Contracting Muscles for Regeneration: Myogenic dedifferentiation in limb progenitor cell proliferation. Ph.D Thesis submitted to Karolinska Institute, Stockholm, Sweden.

Louis, D. S., Palmer, A. K., and Burney, R. E. 1980. Open treatment of digital tip injuries. *Journal of the American Medical Association*, **244**(7): 697-698.

Macdonald, D., Reiter, A. and Cross, N. C. 2002. The 8p11 myeloproliferative syndrome: a distinct clinical entity caused by constitutive activation of FGFR1. *Acta Haematol*., **107**(2): 101-107.

Maden, M. 1983. Vitamin A and the control of pattern in regenerating limbs. *Prog. Clin. Biol. Res*., **110**(A): 445-454.

Malcolm, S. and Reardon, W. 1996. Fibroblast growth factor receptor-2 mutations and craniosynostosis. *Ann. NY. Acad. Sci*., **785**: 164-170.

Mariani, F. V., Ahn, C. P. and Martin, G. R. 2008. Genetic evidence that FGFs have an instructive role in limb proximal-distal patterning. *Nature*, **453**(7193): 401-405.

Mari-Beffa, M., Palmqvist, P., Marin-Giron, F, Montes, G. S. and Becerra, J. 1999. Morphometric study of the regeneration of individual rays in teleost tail fins. *J. Anat*., **195**(3): 393-405.

Mari-Beffa, M., Santamaria, J. A., Fernandez-Llebrez, P. and Becerra, J. 1996. Histochemically defined cell states during tail fin regeneration in telost fishes. *Differentiation*, **60**(3): 139-149.

Martelly, I., Rey, C. and Le-moigne, A. 1981. Planarian regeneration: DNA metabolism in adults. *Int. J. Inv. Reprod.*, **4**: 107-121.

Martin, G. F., Ghooray, G. T., Wang, X. M., Xu, X. M. and Zou, X. C. 1994. Models of spinal cord regeneration. In: F. J. Seil (Ed.) *Progress in brain research: neural regeneration*. Elsevier, Amsterdam, **103**: 175-201.

Martin, G. R. 1998. The roles of FGFs in the early development of vertebrate? limbs. *Genes Dev.*, **12**: 1571-1586.

Martin, P. and Leibovich, S. J. 2005. Inflammatory cells during wound repair: the good, the bad and the ugly. *Trends cell. Biol*., **15**(11): 599-607.

Martinez, D. E. 1998. Mortality patterns suggest lack of senescence in hydra. *Exp. Gerontol.*, **33**(3): 217-225.

Martorana, M. L., Tawk, M., Lapointe, T., Barre, N., Imboden, M., Joulie, C. 2001. Zebrafish keratin 8 is expressed at high levels in the epidermis of regenerating caudal fin. *The International Journal of Developmental Biology*, **45**: 449-452.

Masaki, H. and Ide, H. 2007. Regenerative potency of mouse limbs. *Development, growth and differentiation*, **49**: 89-98.

Mason, I. 1994. The ins and outs of fibroblast growth factors. *Cell*, **78**: 547-552.

Mathew, L. K., Andreasen, E. A. and Tanguay, R. L. 2006. Aryl hydrocarbon receptor activation inhibits regenerative growth. *Mol. Pharmacol*., **69**(1): 257-265.

Mathew, L. K., Sengupta, S., Kawakami, A., Andreasen, E. A., Lohr, C. V., Loynes, C. A. *et al*. 2007. Unraveling tissue regeneration pathways using chemical genetics. *The Journal of Biological Chemistry*, **282**(48): 35202-35210.

McAvoy, J. M., Chamberlain, G. C., De Longh, R. V., Richardson, N. A. and Lovicu, F. J. 1991. The role of fibroblast growth factor in eye lens development. *Ann. NY. Acad. Sci*., **638**: 256-274.

McLean, K. E. and Vickaryous, M. K. 2011. A novel amniote model of epimorphic regeneration: the leopard gecko, *Eublepharis macularius*. *BMC Developmental Biology*, **11**: 50.

McNeil, P. L., Muthukrishan, L., Warder, E. and D'Amore, P. A. 1989. Growth factors are released by mechanically wounded endothelial cells. *J. Cell Biol*., **109**: 811-822.

Meffe, G. K. and Snelson, F. F. 1989. An ecological overview of poecilid fishes. In: G. K. Meffe and F. F. Snelson (Eds.) *Ecology and evolution of livebearing fishes (Poeciliidae)*. Englewood Cliff, Prentice Hall, NJ, pp. 13-31.

Meinhardt, H. 2002. The radial-symmetric hydra and the evolution of the bilateral body plan: an old body became a young brain. *Bioessays*, **24**(2): 185-191.

Menetrey, J., Kasemkijwattana, C., Day, C. S., Bosch, P.,Vogt, M., Fu, F. H. *et al*. 2000. Growth factors improve muscle healing *in vivo*. *J. Bone Joint. Surg. Br*., **82**(1): 131-137.

Mescher, A. L. 1976. Effects on adult newt limb regeneration of partial and complete skin flaps over the amputation surface. *J. Exp. Zool*., **195**(1): 117-128.

Mescher, A. L. 1996. The cellular basis of limb regeneration in urodeles. *Int. J. Dev. Biol*., **40**: 785-795.

Miescher, F. 1871. Ueber die chemische zusammensetsung der eiterzellen. *Hoppe- Seyler's Medicinische-Chemische Untersuchunge*, **4**: 441-460.

Mignatti, P., Morimoto, T. and Rifkin, D. B. 1992. Basic fibroblast growth factor, a protein devoid of secretory signal sequence, is released by cells via a pathway independent of the endoplasmic reticulum-Golgi complex. *J. Cell. Physiol*., **151**: 81-93.

Mittal, A. K. and Munshi, J. S. D. 1974. On the regeneration and repair of superficial wounds in the skin of *Rita rita* (Ham) (Bagridae, Pisces). *Acta Anat*., **88**: 424-442.

Mittal, C. K., Arnold, W. P. and Murad, F. 1978. Characterization of protein inhibitors of guanylate cyclase activation from rat heart and bovine lung. *J. Biol. Chem*., **253**(4): 1266-1271.

Miyake, H., Yoshimura, K., Hara I., Eto, H., Arakawa, S. and Kamidono, S. 1997. Basic fibroblast growth factor regulates matrix metalloproteinases production and invasiveness in human bladder cancer cell lines. *J. Urol*., **157**(6): 2351-2355.

Mocchetti, I. and Wrathall, J. R. 1995. Neurotrophic factors in central nervous system trauma. *J. Neurotrauma*, **12**: 853-870.

Mohammadi, M., McMahon, G., Sun, L., Tang, C., Hirth, P., Yeh, B. K. *et al*., 1997. Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. *Science*, **276**(5314): 955-960.

Mohan, R., Rinehart, W. B., Bargagna-Mohan, P. and Fini, M. E. 1998. Gelatinase B/lacZ transgenic mice, a model for mapping gelatinase B expression during development and injury-related tissue remodeling. *J. Biol. Chem*., **273**: 25903-25914.

Montes, G. S., Becerra, J., Toledo, O. M. S., Gordilho, M. A. and Junqueira, L. C. U. 1982. Fine structure and histochemistry of the tail fin ray in teleosts. *Histochemistry*, **75**(3): 363-376.

Montesano, R., Vassalli, J. D., Baird, A., Guillemin, R. and Orci, L. 1986. Basic fibroblast growth factor induces angiogenesis *in vitro*. Proc. Natl. Acad. Sci., U S A, **83**: 7297-301.

Morgan, 1900. Regeneration: Old and new interpretations. *Biological lectures*: 185-208.

Morgan, T. H. 1898. Experimental studies of the regeneration of Planaria Maculata. *Arch. Entw. Mech. Org*., **7**: 364-397.

Morgan, T. H. 1898. Experimental studies of the regeneration of Planaria *Maculata*. *Arch. Entw. Mech. Org*., **7**: 364-397.

Morgan, T. H. 1900. Regeneration in teleosts. *Arch. Entw. Mech. Org.*, **10**: 120-131.

Morgan, T. H. 1901. Regeneration. New York: The Macmillan Co.

Morgan, T. H. 1902. Further experiments on the regeneration of the tail fins of fishes. *Arch. Entw. Mech. Org.*, **14**: 539-561.

Morgan, T. H. 1906. The physiology of regeneration. *J. Exp. Zool.*, **4**: 457-500.

Mori, H., Shukunami, C., Furuyama, A., Notsu, H., Nishizaki, Y. and Hiraki, Y. 2007. Immobilization of Bioactive Fibroblast Growth Factor 2 into Cubic Proteinous Microcrystals (*Bombyx mori* Cypovirus Polyhedra) that are insoluble in a physiological cellular environment. *The Journal of Biological Chemistry*, **282**(23): 17289-17296.

Morrison, C. J., Butler, G. S., Bigg, H. F., Roberts, C. R., Soloway, P. D. and Overall, C. M. 2001. Cellular activation on MMP-2 (gelatinase A) by MT2-MMP occurs via a TIMP-2 independent pathway. *J. Biol. Chem.*, **276**: 47402-47410.

Mott, J. D. and Werb, Z. 2004. Regulation of matrix biology by matrix metalloproteinases. *Curr. Opin. Cell Biol*., **16**(5): 558-564.

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.

Munaut, C., Salonurmi, T., Kintusaari, S., Reponen, P., Morita, T., Foidart, J. M. *et al*. 1999. Murine matrix metalloproteinase 9 gene. 5'-upstream region contains cis-acting elements for expression in osteoclasts and migrating keratinocytes in transgenic mice. *J. Biol. Chem*., **274**: 5588-5596.

Nachtrab, G., Czerwinski, M. and Poss, K. D. 2011. Sexually Dimorphic Fin Regeneration in Zebrafish Controlled by Androgen/GSK3 Signaling. *Curr. Biol*., **21**(22): 1912-7.

Nagase, H. and Woessner, J. F. Jr. 1999. Matrix metalloproteinases. *J. Biol. Chem*., **274**: 21491-21494.

Nakatani, Y., Kawakami, A. and Kudo, A. 2007. Cellular and molecular processes of regeneration, with special emphasis on fish fins. *Dev. Growth Differ*., **49**(2): 145-154.

Nechiporuk, A. and Keating, M. T. 2002. A proliferation gradient between proximal and msxb-expressing distal blastema directs zebrafish fin regeneration. *Development*, **129**(1): 2607- 2617.

Newth, D. R. 1958. New (and better?) parts for old. In: M. L. Johnson, M. Abercrombie and G. E. Fogg (Eds.) *New biology*. Penguin Books, London, **26**: pp. 47-62.

Nishida, T., Kubota, S., Aoyama, E., Janune, D., Maeda, A. and Takigawa, M. 2011. Effect of CCN2 on FGF-2 induced proliferation and MMP9 and MMP13 productions by chondrocytes. *Endocrinology*, **151**(11): 4232-4241.

Nugent, M. A. and Iozzo, R. V. 2000. Fibroblast growth factor-2. *Int J Biochem Cell Biol*., **32**: 115-120.

Nygaard, O. and Rusch, H. P. 1955. Incorporation of radioactive phosphate into nucleic acids of regenerating rat liver. *Cancer Res*., **15**(4): 240-245.

Ohbayashi, N., Shibayama, M., Kurotaki, Y., Imanishi, M., Fujimori, T., Itoh, N. *et al*. 2002. FGF18 is required for normal cell proliferation and differentiation during osteogenesis and chondrogenesis. *Genes Dev*., **16**(7): 870-879.

Ohuchi, H., Hori, Y., Yamasaki, M., Harada, H., Sekine, K., Kato, S. *et al*. 2000. FGF10 acts as a major ligand for FGF receptor 2 IIIb in mouse multiorgan development. *Biochem. Biophys. Res. Commun*., **277**: 643-649.

Okada, T. S. 1996. A brief history of regeneration research- For admiring Professor Niazi's discovery of the effect of vitamin A on regeneration. *J. Biosci.*, **21**(3): 261-271.

Oliver, G. W., Leferson, J. D., Stetler-Stevenson, W. G. and Kleiner, D. E. 1997. Quantitative reverse zymography: analysis of picogram amounts of metalloproteinase inhibitors using gelatinase A and B reverse zymograms. *Anal. Biochem*., **244**(1): 161-166.

Ornitz, D. M. and Itoh, N. 2001. Fibroblast growth factors. *Genome Biol*., **2**(3): 1-12.

Ornitz, D. M. and Marie, P. J. 2002. FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease. *Genes Dev*., **16**: 1446-1465.

Ornitz, D. M., Xu, J., Colvin, J. S., McEwen, D. G., MacArthur, C. A., Coulier, F. *et al*. 1996. Receptor specificity of the fibroblast growth factor family. *J. Biol. Chem*., **271**(25): 15292-15297.

Ornitz, D. M., Yayon, A., Flanagan, J. G., Svahn, C. M., Levi, E. and Leder, P. 1992. Heparin is required for cell-free binding of basic fibroblast growth factor to a soluble receptor and for mitogenesis in whole cells. *Mol. Cell Biol*., **12**(1): 240-247.

Ortega, S., Ittmann, M., Tsang, S. H., Ehrlich, M. and Basilico, C. 1998. Neuronal defects and delayed wound healing in mice lacking fibroblast growth factor 2. *Proc. Natl. Acad. Sci*., **95**: 5672-5677.

Padhi, B. K., Joly, L., Tellis, P., Smith, A., Nanjappa, P., Chevrette, M. *et al*. 2004. Screen for genes differentially expressed during regeneration of the zebrafish caudal fin. *Dev. Dyn*. **231**(3): 527-541.

Palmon, A., Ross, H., Edel, J., Sax, B., Savion, N., Grosskop, A. *et al*. 2000. Inverse dose and time dependent effect of bFGF on the gene expression of collagen type I and MMP-1 by periodontal ligament cells in culture. *J. Periodontol*., **71**(6): 974-980.

Parenti, L. R. and Rauchenberger, M. 1989. Systematic overview of the poeciliines. In: G. K. Meffe and F. F. Snelson (Eds.) *Ecology and evolution of livebearing fishes (Poeciliidae)*. Englewood Cliffs, Prentice Hall NJ, pp. 3-12.

Parichy, D. M., Mellgren, E. M., Rawls, J. F., Lopes, S. S., Kelsh, R. N., and Johnson, S.L. 2000. Mutational analysis of endothelin receptor b1 (rose) during neural crest and pigment pattern development in the zebrafish *Danio rerio*. *Dev. Biol*., **227**: 294-306.

Park, I. S. and Kim, W. S. 1999. Modulation of gelatinase activity correlates with the dedifferentiation profile of regenerating salamander limbs. *Mol. Cells*, **9**(2): 119-126.

Parks, W. C. 1999. Matrix metalloproteinases in repair. *Wound Repair Regen*., **7**(6): 423-432.

Parr, B. A. and McMahon, A. P. 1994. Wnt genes and vertebrate development. *Curr. Opin. Genet. Dev.*, **4**: 523-528.

Paschkis, K. E., Goddard, J., Cantarow, A. and Adibi, S. 1959. Stimulation of growth by partial hepatectomy. *Proc. Soc. Exp. Biol. Med*., **101**(1): 184-186.

Pazmany, C. C. 2003. *In Vitro* Analysis of FGF-23 Induced Gene Expression. Ph.D thesis Submitted to the Faculty of the Worcester Polytechnic Institute, Worcestershire, England.

Pellegrini, L., Burke, D. F., Von-Delft, F., Mulloy, B. and Blundell, T. L. 2000. Crystal structure of fibroblast growth factor receptor ectodomain bound to ligand and heparin. *Nature*., **407**(6807): 1029-34.

Petrovicky, I. 1988. Aquarium fish of the world. The Hamlyn Publishing Group Limited, London, 499pp.

Pettmann, B., Labourdette, G., Weibel, M. and Sensenbrenner, M. 1986. The brain fibroblast growth factor (FGF) is localized in neurons. *Neurosci. Lett.*, **68**: 175-180.

Phillips, L. G., Abdullah, K. M., Geldner, P. D., Dobbins, S., Ko, F., Linares, H. A. *et al*. 1993. Application of basic fibroblast growth factor may reverse diabetic wound healing impairment. *Ann. Plast. Surg*., **31**(4): 331-334.

Phromsuthirak, P. 1977. Electron microscopy of wound healing in the skin of *Gasterosteus aculeatus*. *J. Fish Biol*., **11**(2): 193-206.

Pierce, G. F., Tarpley, J. E., Yanagihara, D., Mustoe, T. A., Fox, G. M. and Thomson, A. 1992. Platelet derived growth factor (BB homodimer), transforming growth factor-β1 and basic fibrobast growth factor in dermal wound healing. Neovessel and matrix formation and cessation of repair. *Am. J. Pathol.*, **140**(6): 1375-1388.

Pike, D. B., Cai, S., Pomraning, K. R., Firpo, M. A., Fisher, R. J., Shu, X. Z. *et al*. 2007. Heparin-regulated release of growth factors *in vitro* and angiogenic response *in vivo* to implanted hyaluronan hydrogels containing VEGF and bFGF. *Biomaterials*, **27**: 5242-5251.

Pilo, B. and Suresh, B. 1994. Effect of EGF on progress of tail regeneration in the gekkonid lizard, *Hemidactylus flaviviridis*. *J. Anim. Morphol. Physiol*., **41**: 61-66.

Pintucci, G., Moscatelli, D., Saponara, F., Biernacki, P. R., Baumann, F. G., Bizekis, C. *et al*. 2002. Lack of ERK activation and cell migration in FGF-2 deficient endothelial cells. *FASEB J*., **16**(6): 598-600.

Plotnikov, A. N., Schlessinger, J., Hubbard, S. R. and Mohammadi, M. 1999. Structural basis for FGF receptor dimerization and activation. *Cell*, **98**(5): 641-50.

Poleo, G., Brown, C. W., Laforest, L. and Akimenko, M. A. 2001. Cell proliferation and movement during early fin regeneration in zebrafish. *Dev. Dyn.*, **221**(4): 380-390.

Poss, K. D., Keating, M. T. and Nechiporuk, A. 2003. Tales of regeneration in zebrafish. *Developmental Dynamics*, **226**(2): 202-210.

Poss, K. D., Wilson, L. G. and Keating, M. T. 2002a. Heart regeneration in zebrafish. *Science*, **298**(5601): 2188-2190.

Poss, K. D., Nechiporuk, A., Hillam, A. M., Johnson, S. L. and Keating, M. T. 2002b. Mps1 defines a proximal blastemal proliferative compartment essential for zebrafish fin regeneration. *Development*, **129**(22): 5141-5149.

Poss, K. D., Shen, J., Nechiporuk, A. McMahon, G., Thisse, B. Thisse, C. *et al*. 2000a. Roles for FGF signaling during zebrafish fin regeneration. *Dev. Biol*., **222**(2): 347-358.

Poss, K. D., Shen, J. and Keating, M. T. 2000b. Induction of *lef1* during zebrafish fin regeneration. *Dev. Dyn*., **219**(2): 282-286.

Poulin, M. L., Patrie, K. M., Botelho, M. J., Tassava, R. A. and Chiu, I. M. 1993. Heterogeneity in the expression of fibroblast growth factor receptors during limb regeneration in newts (*Notophthalmus viridescens*). *Development*, **119**(2): 353-361.

Powers, C. J., McLeskey, S. W. and Wellstein, A. 2000. Fibroblast growth factors, their receptors and signaling. *Endocr. Relat. Cancer*, **7**(3): 165-197.

Pownall, M. E. and Isaacs, H. V. 2010. FGF signalling in vertebrate development. San Rafael, Morgan & Claypool Life Sciences.

Presta, M., Dell'Era, P., Mitola, S., Moroni, E., Ronca, R. and Rusnati, M. 2005. Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. *Cytokine Growth Factor Rev*., **16**(2): 159-178.

Prodi, G., Rocchi, P. and Grilli, S. 1975. The effect of polycyclic hydrocarbons on the synthesis of DNA in lymphoid organs, bone marrow and regenerating rat liver. *Experientia*, **31**(3): 358-360.

Prykhozhij, S. V. and Neumann, C. J. 2008. Distinct roles of Shh and FGF signalling in regulating cell proliferation during zebrafish pectoral fin development. *BMC Developmental Biology*, **8**: 91-101.

Pueyo, J. I. and Couso, J. P. 2005. Parallels between the proximal-distal development of vertebrate and arthropod appendages: homology without an ancestor? *Curr. Opin. Genet. Dev*., **15**(4): 439-446.

Qin, Z., Kidd, A. R., Thomas, J. L., Poss, K. D., Hyde, D. R., Raymond, P. A. *et al*. 2011. FGF signalling regulates rod photoreceptor cell maintenance and regeneration in Zebrafish. *Exp. Eye Res*., **93**(5): 726-734.

Quarto, N., Bouche, G., Bugler, B., Chailleux, C., Prats, A. C., Prats, H. *et al*. 1993. The basic fibroblast growth factor isoforms: endogenous and exogenous behaviour. In: T. Moody (Ed.) *Growth Factors, Peptides and Receptors*. Plenum Press, New York, pp. 147-158.

Quarto, N., Talarico, D., Florkiewicz, R. and Rifkin D. B. 1991. Selective expression of high molecular weight basic fibroblast growth factor confers a unique phenotype to NIH3T3 cells. *Cell Regul.*, **2**: 699-708.

Quint, E., Smith, A., Avaron, F., Laforest, L., Miles, J., Gaffield, W. *et al*. 2002. Bone patterning is altered in the regenerating zebrafish caudal fin after ectopic expression of sonic hedgehog and bmp2b or exposure to cyclopamine. *Proc. Natl. Acad. Sci*., **99**(13): 8713-8718.

Ramesh, M., Sivakumari, K., Kamagaraj, M. K. and Manavalaramanujam, K. 1993. Toxicity of dye effluent in lactatedehydrogenase activity in *Labeo Rohita*. *J. Environ. Protect*., **13**: 124-127.

Rapraeger, A. C., Krufka, A. and Olwin, B. B. 1991. Requirement of heparan sulfate for bFGFmediated fibroblast growth and myoblast differentiation. *Science*, **252**: 1705-1708.

Ray, J., Peterson, D. A., Schinstine, M. and Gage, F. H. 1993. Proliferation, differentiation, and long-term culture of primary hippocampal neurons. *Proc. Natl. Acad. Sci.*, **90**: 3602-3606.

Raya, A., Consiglio, A., Kawakami, Y., Rodriguez- Esteban, C. and Izpisua-Belmonte, J. C. 2004. The zebrafish as a model of heart regeneration. *Cloning Stem Cells*, **6**(4): 345-351.

Reaumur, R. A. F. 1712. Sur les diverses reproductions quises font dans Ecrevisse, les omars, les Crabes, etc et entrautres sur celles de leurs Jambes et de leurs Ecailles. *Mem. Acad. Roy. Sci*.: 223-245.

Reifers, F., Böhli, H., Walsh, E. C., Crossley, P. H., Stainier, D. Y. R. and Brand, M. 1998. *Fgf8* is mutated in zebrafish *acerebellar(ace)* mutants and is required for maintenance of midbrain-hindbrain boundary development and somitogenesis. *Development*, **125**: 2381-2395.

Reimer, M. M., Sorensen, I., Kuscha, V., Frank, R. E., Liu, C., Becker, C. G. *et al*. 2008. Motor Neuron Regeneration in Adult Zebrafish. *The Journal of Neuroscience*, **28**(34): 8510-8516.

Renko, M., Quarto, N., Morimoto, T. and Rifkin D. B. 1990. Nuclear and cytoplasmic localization of basic fibroblast growth factor species. *J. Cell. Physiol.*, **144**: 108-114.

Reynolds, B. L., Codington, J. B. and Buxton, R. W. 1958. Wound healing: a study of the response of injured tissues to the coenzyme adenosine 5-monophosphate. *Surgery*, **44**(1): 33.

Rifkin, D. B. and Moscatelli, D. 1989. Recent developments in the cell biology of basic fibroblast growth factor. *J. Cell Biol*., **109**: 1-6.

Riley, B. B., Savage, M. P., Simandl, B. K., Olwin, B. B. and Fallon, J. F. 1993. Retroviral expression of FGF-2 (bFGF) affects patterning in chick limb bud. *Development*, **118**(1): 95-104.

Robins, R. 2003. Biological profile of the sailfin molly. Florida Museum of Natural History website.

Robinson, M. L., MacMillan-Crow, L. A., Thompson, J. A. and Overbeek, P. A. 1995. Expression of a truncated FGF receptor results in defective lens development in transgenic mice. *Development*, **121**: 3959-3967.

Roghani, M. J., Mansukhani, A., Dell'Era P, Bellosta, P., Basilico, C., Rifkin, D. B. *et al*. 1994. Heparin increases the affinity of fibroblast growth factor for its receptor but is not required for binding. *J. Biol. Chem.*, **269**: 3976-3984.

Rolland-Lagan, A. G., Paquette, M., Tweedle, V. and Akimenko, M. A. 2012. Morphogen based simulation model of ray growth and joint patterning during fin development and regeneration. *Development*, **139**(6): 1188-1197.

Sadasivam, S. and Manickam,A. 1992. *Biochemical Methods for Agricultural Sciences*. Wiley Eastern Limited, NewDelhi.

Sakaue, H., Konishi, M., Ogawa, W., Asaki, T., Mori, T., Yamasaki, M. *et al*. 2002. Requirement of fibroblast growth factor 10 in development of white adipose tissue. *Genes Dev.*, **16**(8): 908-912.

Salo, T. and Oikarinen, J. 1985. Regulation of type IV collagen degrading enzyme by cortisol during human skin fibroblasts growth. *Biochem. Biophys. Res. Commun*., **130**: 588-595.

Salo, T., Liotta, L. A. and Tryggvason, K. 1983. Purification and characterization of a murine basement membrane collagen-degrading enzyme secreted be metastatic tumor cells. *J. Biol. Chem*., **258**: 3058-3063.

Sander, E. E., Van-Delft, S., Ten-Klooster, J. P., Reid, T., Van Der Kammen, R. A., Michiels, F. *et al*. 1998. Matrix-dependent Tiam1/Rac signalling in epithelial cells promotes either cell cell adhesion or cell migration and is regulated by phosphatidylinositol 3-kinase. *J. Cell Biol*., **143**(5): 1385-1398.

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

Santamaria, J. A., Mari-Beffa, M. and Becerra, J. 1992. Interactions of the lepidotrichial matrix components during tail fin regeneration in teleosts. *Differentiation*., **49**: 143-150.

Santamaría, J. A., Marí-Beffa, M., Santos-Ruiz, L. and Becerra, J. 1996. Incorporation of bromodeoxyuridine in regenerating fin tissue of the goldfish *Carassius auratus*. *The J. of Exp. Zool*., **275**(4): 300-307.

Santos-Ruiz, L., Santamaría, J. A., and Becerra, J. 2001. Differential expression of FGF receptors during zebrafish fin regeneration. *Int. J. Dev. Biol*., **45**(S1): S131-S132.

Santos-Ruiz, L., Santamaria, J. A., Ruiz-Sanchez, J. and Becerra, J. 2002. Cell proliferation during blastema formation in the regenerating teleost fin. *Dev. Dyn.*, **223**(2)**:** 262-272.

Sarkar, S., Petiot, A., Copp, A., Ferreti, P. and Thorogood, P. 2001. FGF2 promotes skeletogenic differentiation of cranial neural crest cells. *Development*, **128**(11): 2143-2152.

Saxena, S., Singh, S. K., Meena-Lakshmi, M. G., Meghah, V., Bhatti, B., Brahmendra-Swamy C. V. *et al.* 2012. Proteomic analysis of zebrafish caudal fin regeneration. *Mol Cell Proteomics,* **11**(6): 1.

Schaffer, C. J. and Nanney, L. B. 1996. Cell biology of wound healing. *Int. Rev. Cytol*., **169**: 151-181.

Schebesta, M., Lien, C. L., Engel, F. B. and Keating, M. T. 2006. Transcriptional profiling of caudal fin regeneration in zebrafish. *Scientific World Journal*, **6**(1): 38-54.

Schilling, J. and Milch, L. E. 1955. Fractional analysis of experimental wound fluid. *Proc. Sot. Exptl. Biol. Med*., **89**(2): 189-192.

Schlessinger, J., Plotnikov, A. N., Ibrahimi, O. A., Eliseenkova, A. V., Yeh, B. K., Yayon, A. *et al*. 2000. Crystal structure of a ternary FGF-FGFR- heparin complex reveals a dual role for heparin in FGFR binding and dimerization. *Mol. Cell*., **6**(3): 743-50.

Schnabel, K., Wu, C. C., Kurth, T. and Weidinger, G. 2011. Regeneration of cryoinjury induced necrotic heart lesions in zebrafish is associated with epicardial activation and cardiomyocyte proliferation. *PLoS ONE*, **6**(4): e18503.

Schnapp, E., Kragl, M., Rubin, L., and Tanaka, E. M. 2005. Hedgehog signaling controls dorsoventral patterning, blastema cell proliferation and cartilage induction during axolotl tail regeneration. *Development*, **132**(14): 3243-3253.

Schneider, W.C. 1957. *Methods in Enzymology*. Acad. Press, NewYork.

Schwank, G. and Basler, K. 2010. Regulation of organ growth by morphogen gradients. *Cold Spring Harbor Perspect. Biol.*, **2**: a001669.

Schwartz, S. M. and Liaw, L. 1993. Growth control and morphogenesis in the development and pathology of arteries. *J. Cardiovasc. Pharmacol*., **21**(1):S31-S49.

Seed, J., Olwin, B. B. and Hauschka, S. D. 1988. Fibroblast growth factor levels in the whole embryo and limb bud during chick development. *Dev. Biol*., **128**(1): 50-57.

Serhan, C. N., Brain, S. D., Buckley, C. D., Gilroy, D. W., Haslett, C. O'Neill, L. A. J. *et al*. 2007. Resolution of inflammation: state of the art, definations and terms. *FASEB J*., **21**(2): 325-332.

Shao, J., Qian, X., Zhang, C. and Xu, Z. 2009. Fin regeneration from tail segment with musculature, endoskeleton, and scales. *J. Exp. Zool. B. Mol. Dev. Evol.*, **312**(7): 762-769.

Sharma, P. 2007. Studies on cellular and molecular mechanisms of lizard tail regeneration: Role of PGE_2 in recruitment, proliferation and differentiation of blastemal cells *in vivo*. Ph.D thesis submitted to The Maharaja Sayajirao University of Baroda, Vadodara.

Sharma, P. and Suresh, B. 2008. Influence of COX-2-Induced PGE2 on the initiation and progression of tail regeneration in Northern House Gecko, *Hemidactylus flaviviridis*. *Folia Biologica (Praha)*, **54**: 193-201.

Shaw, R. J., Doherty, D. E., Ritter, A. G., Benedict, S. H. and Clark, R. A. 1990. Adherence dependent increase in human monocyte PDGF(B) mRNA is associated with increases in c- fos, c-jun, and EGR2 mRNA. *J. Cell Biol*., **111**(1): 2139-48.

Sheeba, C. J., Andrade, R. P. and Palmeirim, I. 2012. Joint interpretation of AER/FGF and ZPA/SHH over time and space underlies hairy expression in the chick limb. *Biology open*: 1-9.

Sherry, S., Tillet, W. S. and Christensen, L. R. 1948. Presence and significance of desoxyribose nucleoprotein in the purulent pleural exudates of patients. *Proc. Sot. Exptl. Biol. Med*., **66**(1): 179-184.

Simon, M. A. 2000. Receptor tyrosine kinases: specific outcomes from general signals. *Cell*, **103**(1): 13-15.

Singer, A. J. and Clark, R. A. F. 1999. Cutaneous wound healing. *N. Engl. J. Med*., **341**(10): 738-746.

Singer, M., Weckesser, E. C., Geraudie, J., Maier, C. E., and Singer, J. 1987. Open finger tip healing and replacement after distal amputation in Rhesus monkey with comparison to limb regeneration in lower vertebrates. *Anatomy and Embryology*, **177**(1): 29-36.

Singh, S. P., Holdway, J. E. and Poss, K. D. 2012. Regeneration of amputed Zebrafish fin rays from *de novo* osteoblasts. *Dev. Cell*., **22**(4): 879-886.

Slack, J. 1994. Role of fibroblast growth factors as inducing agents in early embryonic development. *Mol. Reprod. Dev*., **39**(1): 124-125.

Slack, J. M. and Tannahill, D. 1992. Mechanism of anteroposterior axis specification in vertebrates: Lessons from the amphibians. *Development*, **114**(2): 285-302.

Slack, J. M., Darlington, B. G. Heath, J. K. and Godsave, S. F. 1987. Mesoderm induction in early *Xenopus* embryos by heparin-binding growth factors. *Nature*, **326**: 197-200.

Slavin, J., Hunt, J. A., Nash, J. R., Williams, D. F. and Kingsnorth, A. N. 1992. Recombinant basic fibroblast growth factor in red blood cell ghosts accelerates incisional wound healing. *Br. J. Surg*., **79**(9): 918-921.

Sleeman, M., Fraser, J., McDonald, M., Yuan, S., White, D., Gradison, P. *et al*. 2001. Identification of a new fibroblast growth factor receptor, FGFR5. *Gene*, **271**(2): 171-82.

Smith, A., Avaron, F., Guay, D., Padhi, B. K. and Akimenko, M. A. 2006. Inhibition of BMP signaling during zebrafish fin regeneration disrupts fin growth and scleroblasts differentiation and function. *Dev. Biol*., **299**(2): 438-454.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M.D. *et al*., 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.*, **150**(1): 76-85.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mllia, A. K., Gartner, F. H., Provenzano, M. D. *et al.* 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.*, **150**(1): 76-85.

Sorensen, V., Nilsen, T. and Wie˛dłocha, A. 2006. Functional diversity of FGF-2 isoforms by intracellular sorting. *BioEssays*, **28**: 504-514.

Sousa, S., Afonso, N., Bensimon-Brito, A., Fonseca, M., Simoes, M., Leon, J. *et al*. 2011. Differentiated skeletal cells contribute to blastema formation during zebrafish fin regeneration. *Development*, **138**(18): 3897-3905.

Spallanzani, L. 1769. An essay on animal reproductions. London, T. Becket: 86pp.

Spivak-Kroizman, T., Lemmon, M. A., Dikic, I., Ladbury, J. E., Pinchasi, D., Huang, J. *et al.* 1994. Heparin-induced oligomerization of FGF molecules is responsible for FGF receptor dimerization, activation, and cell proliferation. *Cell*, **79**(6): 1015-1024.

Stauber, D. J., Digabriele, A. D. and Hendrickson, W. A. 2000. Structural interactions of fibroblast growth factor receptor with its ligands. *Proc. Natl. Acad. Sci.*, **97**: 49-54.

Stetler-Stevenson, W. G., Liotta, L. A. and Kleiner, D. E. Jr. 1993. Extracellular matrix 6: role of matrix metalloproteinases in tumor invasion and metastasis. *FASEB J.*, **7**(15): 1434-1441.

Stewart, S., Tsuna, Z. and Belmontea, J. C. I. 2009. A histone demethylase is necessary for regeneration in zebrafish. *PNAS*, **106**(47): 19889-19894.

Stocum, D. L and Dearlove, G. E. 1972. Epidermal-mesodermal interaction during morphogenesis of the limb regeneration blastema in larval salamanders. *J. exp. Zool*., **181**: 49-62.

Stocum, D. L. 1995. Wound Repair, Regeneration and Artificial Tissues, Springer, Berlin.

Stocum, D. L. 2004. Tissue restoration through regenerative biology and medicine. Berlin:Springer-Verlag.

Stoick-Cooper, C. L., Moon, R. T. and Weidinger, G., 2007a. Advances in signaling in vertebrate regeneration as a prelude to regenerative medicine. *Genes Dev*., **21**(11): 1292-1315.

Stoick-Cooper, C. L., Weidinger, G., Riehle, K. J., Hubbert, C., Major, M. B., Fausto, N. *et al*. 2007b. Distinct Wnt signaling pathways have opposing roles in appendage regeneration. *Development*, **134**(3): 479-489.

Sun, L., Tran, N., Liang, C., Hubbard, S., Tang, F., Lipson, K. *et al*. 2000. Identification of substituted 3-[(4,5,6, 7-tetrahydro 1H indol-2- yl) methylene]-1,3-dihydroindol-2-ones as growth factor receptor inhibitors for VEGF-R2 (Flk-1/ KDR), FGF-R1, and PDGF-R beta tyrosine kinases. *J. Med. Chem*., **43**(14): 2655-2663.

Suzuki, M., Yakushiji, N., Nakada, Y., Satoh, A., Ide, H. and Tamura, K. 2006. Limb regeneration in *Xenopus laevis* froglet. *Sci. World J.*, **6**(1): 26-37.

Szebenyi G. and Fallon, J. F. 1999. Fibroblast growth factors as multifunctional signalling factors. *Int. Rev. Cytol*., **185**: 45-106.

Tal, T. L., Franzosa, J. A. and Tanguay, R. L. 2010. Molecular signaling networks that choreograph epimorphic fin regeneration in zebrafish - a mini-review. *Gerontology*, **56**(2): 231-240.

Tassava, R. A. and Goss, R. J. 1966. Regeneration rate and amputation level in fish fins and lizard tails. *Growth*, **30**(1): 9-21.

Tawk, M., Tuil, D., Torrente, Y., Vriz, S. and Paulin, D. 2002. High-efficiency gene transfer into adult fish: a new tool to study fin regeneration. *Genesis*, **32**(1): 27-31.

Taylor, G. P., Anderson, R., Reginelli, A. D. and Muneoka, K. 1994. FGF-2 induces regeneration of the chick limb bud. *Dev. Biol*., **163**(1): 282-284.

Temple, S. and Qian, X. 1995. bFGF, neurotrophins, and the control or cortical neurogenesis. *Neuron*, **15**: 249-252.

Tetlow, L. C., Adlam, D. J. and Woolley, D. E. 2001. Matrix metalloproteinase and proinflammatory cytokine production by chondrocytes of human osteoarthritic cartilage: associations with degenerative changes. *Arthritis Rheum*., **44**(3): 585-594.

Thorndyke, M. C., Chen, W. C., Beesley, P. W. and Patruno, M. 2001. Molecular approach to echinoderm regeneration. *Micro. Res. Tech*., **55**(6): 474-485.

Thorndyke, M. C., Patruno, M., Chen, W. C. and Beesley, P. W. 2001. Stem cells and regeneration in invertebrate Deuterostomes. *Symp. Soc. Exp. Biol*.**53**: 107-120.

Thummel, R., Bai, S., Sarras, M. P., Jr. Song, P., McDermott, J., Brewer, J. *et al*. 2006. Inhibition of zebrafish fin regeneration using *in vivo* electroporation of morpholinos against *fgfr1* and *msxb*. *Dev. Dyn.*, **235**(2): 336-346.

Tidball, J. G. 2005. Inflammatory processes in muscle injury and repair. *Am. J. Physiol. Regul. Integr. Comp. Physiol*., **288**(2): R345-53.

Tomlinson, E., Fu, L., John, L., Hultgren, B., Huang, X, Renz, M. *et al.* 2002. Transgenic mice expressing human fibroblast growth factor-19 display increased metabolic rate and decreased adiposity. *Endocrinology*, **143**: 1741-1747.

Treichel, D., Schock, F., Jackle, H., Gruss, P. and Mansouri, A. 2003. mBtd is required to maintain signaling during murine limb development. *Genes. Dev*., **17**(21): 2630-2635.

Trelstad, R. L. 1984. *The Role of Extracellular Matrix in Development.* Alan R. Liss, New York.

Tremolieres, J. and Derache, J. 1960. In: H. B. Stoner and C. J. Threlfall (Eds.). *The biochemical response to* injury, Springfield, Ill, pp. 23.

Tretter, Y. P., Hertel, M. Munz, B., Bruggencate, G. T., Werner, S. and Alzheimer, C. *et al*. 2000. Induction of activin A is essential for the neuroprotective action of basic fibroblast growth factor *in vivo*. *Nat. Med.*, **6**: 812-815.

Trippel, S. B, Coutts, R. D., Einhorn, T., Mundy, G. R. and Rosenfeld, R. G. 1996. Growth factors as therapeutic agents. *J. Bone Joint Surg.*, **78A**(8): 1272-1286.

Tsonis, P. A. 1996. Limb Regeneration. Cambridge Univ. Press, Cambridge, UK.

Tsonis, P. A. 2000. Regeneration in vertebrates. *Dev. Biol*., **221**: 73-284.

Tsuboi, R., Shi, C. M., Rifkin, D. B. and Ogawa, H. 1992. A wound healing model using healing impaired diabetic mice. *J. Dermatol*., **19**(11): 673-675.

Tsutsumi, S., Shimazu, A., Miyazaki, K., Pan, H., Koike, C., Yoshida, E. *et al*. 2001. Retention of multilineage differentiation potential of mesenchymal cells during proliferation in response to FGF. *Biochem. Biophys. Res. Commun.*, **288**(2): 413-419.

Tu, S. and Johnson, S. L. 2011. Fate restriction in the growing and regenerating Zebrafish fin. *Dev. Cell*, **20**(5): 725-32.

Turner, N. and Grose, R. 2010. Fibroblast growth factor signalling: From development to cancer. *Nature Reviews Cancer*, **10**(2): 116-129.

Unsicker, K., Engels, S., Hamm, C., Ludecke, G., Meier, C., Renzing, J. *et al*. 1992. Molecular control of neural plasticity by the multifunctional growth factor families of the FGFs and TGF-b. *Anat. Anz*., **174**(5): 405-407.

Vaccarino, F. M., Schwartz, M. L., Raballo, R., Nilsen, J., Rhee, J., Zhou, M. *et al*. 1999. Changes in cerebral cortex size are governed by fibroblast growth factor during embryogenesis. *Nat. Neurosci.*, **2**: 246-253.

Vartio, T., Hovi, T and Vaheri, A. 1982. Human macrophages synthesize and secrete a major 95,000-dalton gelatin binding protein distinct from fibronectin. *J. Biol. Chem*., **257**: 8862-8866.

Venkataraman, G., Shriver, Z., Davis, J. C. and Sasisekharan, R. 1999. Fibroblast growth factors 1 and 2 are distinct in oligomerization in the presence of heparin like glycosaminoglycans. *Proc. Natl. Acad. Sci.*, **96**: 1892-1897.

Verma, R. P. and Hansch, C. 2007. Matrix Metalloproteinases (MMPs): Chemical-biological functions and (Q)SARs. *Bioorg. Med. Chem*., **15**: 2223-2268.

Vicario-Abejon, C., Johe, K. K., Hazel, T. G., Collazo, D. and McKay, R. D. 1995. Functions of basic fibroblast growth factor and neurotrophins in the differentiation of hippocampal neurons. *Neuron*, **15**: 105-114.

Vinarsky, V., Atkinson, D. L., Stevenson, T. J., Keating, M. T. and Odelberg, S. J. 2005. Normal newt limb regeneration requires matrix metalloproteinase function. *Dev. Biol*., **279**(1): 86-98.

Visse, R. and Nagase, H. 2003. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function and biochemistry. *Circ. Res*., **92**(8): 827-839.

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

Wada, H., Hamaguchi, S. and Sakaizumi, M. 2008. Development of diverse lateral line patterns on the teleost caudal fin. *Dev Dyn.*, **237**: 2889-2902.

Wahl, S. M., Wong, H. and Francis, N. M. 1989. Role of growth factors in inflammation and repair. *J. Cell. Biochem.*, **40**: 193-9.

Walicke, P. A., Feige, J. J. and Baird, A. 1989. Characterization of the neuronal receptor for basic fibroblast growth factor and comparison to receptors on mesenchymal cells. *J. Biol. Chem.*, **264**: 4120-4126.

Wallace, H. 1981. Vertebrate limb regeneration. John Wiley and Sons, New York.

Wanaka, A., Johnson, E. M. Jr. and Milbrandt, J. 1990. Localization of FGF receptor mRNA in the adult rat central nervous system. *Neuron*, **5**: 267-281.

Wang, X, Manner, P. A., Horner, A., Shum, L., Tuan, R. S. and Nuckolls, G. H. 2004. Regulation of MMP-13 expression by RUNX2 and FGF2 in Osteoarthritic cartilage. *Osteoarthritis Cartilage*, **12**(12): 963-973.

Webster, M. K. and Donoghue, D. J. 1996. Constitutive activation of fibroblast growth factor receptor 3 by the transmembrane domain point mutation found in achondroplasia. *EMBO. J.*, **15**(3): 520-527.

Weiss, P. and Kavanau, J. L. 1957. A model of growth and growth control in mathematical terms. *J. Gen. Physiol*., **41**(1): 1-47.

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

Whitehead, G. G, Makino, S., Lien, C. L. and Keating, M. T. 2005. fgf20 is essential for initiating zebrafish fin regeneration. *Science*, **310**(5756): 1957-1960.

Williamson M. B. and Guschlbauer W. 1961. Changes in the Concentration of Ribonucleic Acid during Wound Tissue Regeneration. *Nature*, **192**, 454-455.

Williamson, M. B. and Fromm, H. J. 1955. The incorporation of sulfur amino acids into protein of regenerating wound tissue. *J. Biol. Chem*., **212**(2): 705-712.

Wills, A. A, Kidd, A. R., Lepilina, A. and Poss, K. D. 2008. Fgfs control homeostatic regeneration in adult zebrafish fins. *Development*, **135**(18): 3063-3070.

Wills, A. A., Holdway, J. E., Major, R. J. and Poss, K. D. 2008. Regulated addition of new myocardial and epicardial cells fosters homeostatic cardiac growth and maintenance in adult zebrafish. *Development*, **135**: 183-192.

Wirth, D. P., Johnson, C. A., Horvath, J. S. and Macgregor, J. D. 1992. *Journal of Scientific Exploration*, **6**(4): 375-390.

Woessner, J. F. 1995. Quantification of matrix metalloproteinases in tissue samples. *Methods Enzymol*., **248**: 510-528.

Wolpert, L. 1969. Positional information and the spatial pattern of cellular differentiation. *J. Theor. Biol*., **25**: 1-47.

Wolpert, L. 1996. One hundred years of positional information. *Trends Genet*., **12**: 359-364.

Xu, X., Weinstein, M., Li, C. and Deng, C. 1999. Fibroblast growth factor receptors (FGFRs) and their roles in limb development. *Cell Tissue Res*., **296**(1): 33-43.

Yadav, M. 2005. Role of Neural peptides and Growth Factors on Epimorphic regeneration: influence of FGF2 and EGF on lizard tail regeneration. Ph. D thesis submitted to The M. S. University of Baroda, Vadodara, India.

Yadav, M., Anusree, P., Desai, I. and Suresh, B. 2012. Influence of extraneous FGF-2 and its antagonist anti-FGF-2 on the progress of tail regeneration in *Hemidactylus flaviviridis*. *Ind. J. Fundam. Appl. Life Sci*., **2**(2): 164-172.

Yadav, M., Sharma, P., Desai, I., Pilo, B. and Suresh, B. 2008. Influence of administration of FGF-2 and antiFGF-2 on the nucleic acids and protein profiles of the regenerating tail of northern house gecko *Hemidactylus flaviviridis*. *Cell Tissue Res.*, **8**(3): 1557-1563.

Yamagushi, T. P. and J. Rossant. 1995. Fibroblast growth factors in mammalian development. *Curr. Opin. Genet. Dev.*, **5**: 485-491.

Yamashita, T., Yoshioka, M. and Itoh, N. 2000. Identification of a novel fibroblast growth factor, FGF-23, preferentially expressed in the ventrolateral thalamic nucleus of the brain. *Biochemical and Biophysical Research Communications*, **277**(2): 494-498.

Yang, E. V. and Bryant, S. V. 1994. Developmental regulation of a matrix metalloproteinase during regeneration of axolotl appendages. *Dev. Biol*., **166**(2): 696-703.

Yasui, H., Andoh, S., Bamba, S., Inatomi, O., Ishida, H. and Fujiyama, Y. 2004. Role of\ fibroblast growth factor-2 in the expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in human intestinal myofibroblasts. *Digestion*, **69**(1): 34-44.

Yayon, A., Aviezer, D., Safran, M., Gross, J. L., Heldman, Y., Catilly, S. *et al*.1993. Isolation of peptides that inhibit binding of basic fibroblast growth factor to its receptor from a random phage epitope library. *Proc. Natl. Acad. Sci*., **90**(22): 10643-10647.

Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P. and Ornitz, D. M. 1991. Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell*, **64**(4): 841-848.

Yin, V. P. and Poss, K. D. 2008. New regulators of vertebrate appendage regeneration. *Curr. Opin. Genet. Dev.*, **18**: 381-386.

Yin, V. P., Lepilina, A., Smith, A. and Poss, K. D. 2012. Regulation of zebrafish heart regeneration by miR-133. *Dev. Biol*., **365**(2): 319-327.

Yin, V. P., Thomson, J. M., Thummel, R., Hyde, D. R., Hammond, S. M. and Poss, K. D. 2008. Fgf-dependent depletion of microRNA-133 promotes appendage regeneration in Zebrafish. *Genes and Development*, **22**: 728-733.

Yokoyama, H., Ide, H. and Tamura, K. 2001. FGF-10 stimulates limb regeneration ability in *Xenopus laevis*. *Dev. Biol*., **233**(1): 72-79.

Yokoyama, H., Yonei-Tamura, S., Endo, T., Izpisua-Belmonte, J. C., Tamura, K. and Ide, H. 2000. Mesenchyme with FGF-10 expression is responsible for regenerative capacity in *Xenopus* limb buds. *Dev. Biol.*, **219**(1): 18-29.

Yoshimura, S., Takagi, Y., Harada, J., Teramoto, T., Thomas, S. S., Waeber, C. *et al*., 2001. FGF2 regulation of neurogenesis in adult hippocampus after brain injury. *PNAS*, **98**(10): 5874-5879.

Yoshinari, N. and Kawakami., A. 2011. Mature and juvenile tissue models of regeneration in small fish species. *Biol. Bull.*, **221**(1): 62-78.

Yu, P. J., Ferrari, G., Galloway, A. C., Mignatti, P. and Pintucci, G. 2007. Basic fibroblast growth factor (FGF-2): The high molecular weight forms come of age. *J. Cell. Biochem.*, **100**: 1100-1108.

Zauner, H., Begemann, G., Mari-Beffa, M. and Meyer, A. 2003. Differential regulation of *msx* genes in the development of the gonopodium, an intromittent organ, and of the "sword," a sexually selected trait of swordtail fishes (*Xiphophorus*). *Evolution and Development*, **5**: 466-477.

Zenjari, C., Boilly, B., Hondermarck, H. and Boilly-Marer, Y. 1997. Nerve-blastema interactions induce fibroblast growth factor-1 release during limb regeneration in Pleurodeles waltl. *Dev. Growth Differ*., **39**(1): 15-22.

Zeitler, H., Ko, Y., Glodny, B., Totzke, G., Appenheimer, M., Sachinidis, A. *et al*., 1997. Cell-cycle arrest in G0/G1 phase of growth factor induced endothelial cell proliferation by various calcium channel blockers. *Cancer Detect. Prev*., **21**(4): 332-339.

Zetter, B. R. 1998. Angiogenesis and tumor metastasis. *Annu. Rev. Med*., **49**: 407-424.

Zhang, J. D., Cousens, L. S., Barr, P. J. and Sprang, S. R. 1991. Three-dimensional structure of human basic fibroblast growth factor, a structural homolog of interleukin 1 beta. *Proc Natl Acad Sci*., **88**(8): 3446-50.

Zhao, H., Bernardo, M. M., Osenkowski, P., Sohail, A., Pei, D., Nagase, H. *et al*. 2004. Differential inhibition of membrane type 3 (MT3)-matrix metalloproteinase (MMP) and MT1-MMP by tissue inhibitor of metalloproteinase (TIMP)-2 and TIMP-3 regulates pro MMP-2 activation. *J. Biol. Chem*., **279**: 8592-8601.

Zhao, X., Findly, R. C. and Dickerson, H. W. 2008. Cutaneous antibody-secreting cells and B cells in a teleost fish. *Developmental and Comparative Immunology*, **32**: 500-508.

Zhu, X., Komiya, H., Chirino, A., Faham, S., Fox, G. M., Arakawa, T. *et al*. 1991. Three-dimensional structure of acidic and basic fibroblast growth factors. *Science*, **251**(4989): 90-93.