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; Mari’-Beffa *et al.*, 1996; Poss *et al.*, 2003); dermal fibroblasts (Mari’-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; Mari’-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°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.

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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 Wnt5. 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 lef1 (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 factor 2, 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
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; Santamaria 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 (Schebestia 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 (Cxc112a – 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 \textit{in vivo} (Cohn \textit{et al.}, 1995). Furthermore, FGF-2 stimulates \textit{in vitro} proliferation of blastema cells from regenerating limbs of newts (Albert \textit{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 \textit{et al.}, 1991; Poulin \textit{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 (Gospodarowicz \textit{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 \textit{et al.}, 1989; Mignatti \textit{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 \textit{et al.}, 2012). Fish fin regeneration is also studied to be evidently dependent on FGF2 signalling (Hata \textit{et al.}, 1998; Poss \textit{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, \textit{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 \textit{et al.}, 2012) have proved beyond doubt that FGF2 signalling is a quintessential modulator for the successful completion of epimorphosis in northern house gecko \textit{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 General Considerations
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 7th 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).

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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

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(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

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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.