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 (-20°C) 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 **Material and Methods**
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 (KH₂PO₄)
  - 2.17g Disodium hydrogen phosphate (Na₂HPO₄.7H₂O)
  - 8.71g Sodium Chloride (NaCl)
  - 800ml Double distilled water (ddH₂O)
  - Adjust pH to 7.4 and bring volume to 1L with ddH₂O
- 0.3% Hydrogen peroxide (H₂O₂) 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% H₂O₂ in PBS)

**Method:**
The frozen sections were air-dried for about 20 minutes at room temperature. Endogenous peroxidase activity was blocked with 0.3% H₂O₂ 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$_2$SO$_4$)

**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 FeCl₃ 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 (HClO₄)
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% HClO₄. 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 Cu²⁺ ions from the cupric sulphate to Cu⁺ (a temperature dependent reaction). The amount of Cu²⁺ 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)

*Material and Methods*
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 37°C 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)
Material and Methods

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

1.64ml ddH₂O
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 ddH₂O
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% AgNO₃
2.5% Na₂CO₃
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$_2$CO$_3$ 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 (-20°C). 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 -20°C 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 ddH₂O)
- Zymogram Renaturing Buffer
  - Triton X-100, 25% (v/v) in ddH₂O
- Zymogram Developing Buffer
  - 50mM Tris Base
  - Tris HCl
  - 0.2M NaCl
  - 5mM CaCl₂
  - 0.02% Brij 35
  - ddH₂O
- Coomassie blue R250 staining solution (100ml)
  - 0.25g Coomassie brilliant blue R250
  - 90ml Methanol: H₂O (1:1v/v)
  - 10ml Glacial acetic acid
- Coomassie blue R250 destaining solution (100ml)
  - Methanol: Glacial acetic acid: ddH₂O (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 ddH₂O) 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 staining solution.
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 (Na₂HPO₄)
   0.44mM Potassium dihydrogen phosphate (KH₂PO₄)
   1.3mM Calcium Chloride (CaCl₂)
   1.0mM Magnesium Sulphate (MgSO₄)
   4.2mM Sodium bicarbonate (NaHCO₃)
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 amputated 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$_2$O

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$_2$O

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.
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.