MATERIAL AND METHODS

ANIMAL AND MAINTENANCE
Adult Northern House Gecko, Hemidactylus flaviviridis Rüppell, 1835, (both the sexes) with normal intact tails, weighing 10±2g, collected from natural habitat were procured from local animal dealer. They were housed in well ventilated wooden cages of 45x30x60 cm with glass slider on one side for light and visibility, in the Departmental animal house (827/ac/04/CPCSEA). The lizards were subjected to 12:12 hour light-dark cycles and room temperature was maintained at 30±2°C. The relative humidity was between 30 and 70%. Further, experiments were conducted in the months of March-July and September-November. These environmental conditions are necessary to evoke optimal regeneration in lizards. All animals were screened for parasitic infestation and/or wounds and the healthy ones were acclimated for a week before the commencement of experiment. The animals were fed with cockroach nymphs twice a week and purified water was given daily, ad libitum.

DRUGS AND DOSAGE
Preparation of Drug solutions
1. SU5402: A stock solution of SU5402 was prepared in DMSO and stored at 4°C. Prior to the start of an experiment, this stock was diluted to obtain a final dilution of SU5402 in 1% DMSO, which was used for treatment.
   Dosage: 0.7mg/kg body weight

2. Etoricoxib: The drug is soluble in alkaline pH and was prepared in Tris Buffer (pH 8.8) fresh before use.
   Dosage: 50mg/kg body weight

SU5402 was purchased from Calbiochem®, EMD Biosciences, Inc., USA and Etoricoxib was gifted by the manufacturer - Sunpharma Advanced research Company Ltd., Vadodara, India. All the other chemicals were of AR grade and were procured from SRL Pvt. Ltd., Mumbai, India and Qualigens fine chemicals, Mumbai, India.

Drug Dosage and Route of Administration
For SU5402, dosage was selected based on an initial dose range study. In case of etoricoxib dosage was decided according to initial studies carried out in our laboratory (Sharma and Suresh, 2008). Animals were given in loco injections (at second intact tail segment from vent) at a maximum quantity of 0.075ml/animal.
EXPERIMENTAL PROTOCOLS

Method for induced autotomy

Autotomy was induced by exerting mild thumb pressure on the normal intact tail, two segments away from the vent. All experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) in accordance with the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), India (Form B No. ZL/IAEC/13-2010). All protocols of amputation and treatment were done under hypothermic anaesthesia (Reilly, 2001). Irrespective of the experiments, sample size per group was at least five and has been duly exacted in the respective chapters.

Treatment Schedule

For morphometric study, treatment was conducted in two series. In the first series, treatment started two days prior to amputation and was continued on every alternate day till the termination of the experiment (when control animals reached differentiation stage). In the second series, after inducing autotomy, drug treatment started at different stages of tail regeneration, viz., wound epithelium stage and blastema stage and was continued on every alternate day till the termination of the experiment, to study the stage specific effects. After amputation, the process of wound healing initiates leading to the formation of a wound epithelium, which appears as a smooth shining surface. Blastemal stage is characterized by conical aggregation of cells called blastema, a regenerate about 2-3mm in length. The blastema further grows in size and later on differentiates to replace the missing structures. For the rest of the experiments, the same treatment schedule as in the first series was followed. Regenerates were collected at defined stages and were processed further according to the experimental requirement.

Morphometric measurements of tail growth

The growth of the regenerate was measured at fixed intervals using a calibrated digital Caliper (Mitutoyo, Kawasaki, Japan). The time taken to reach defined stages of regeneration such as wound epithelium, blastema and differentiation was recorded and growth rate of regenerate was calculated.

Histological study of regenerates

Regenerates fixed in 10% formalin were washed thoroughly in running water, followed by decalcification in 10% nitric acid solution. The fixed tissue samples were then dehydrated by placing them in increasing concentrations of alcohol (50, 70 and 95%) and then in a 1:1 solution of alcohol and xylene, followed by placing them in xylene alone until the tissues became transparent. The tissues were then hot infiltrated with paraffin wax by first placing them in mixtures containing xylene and wax with successively increasing concentrations of
the wax (75:25, 50:50, 25:75 mixtures of xylene and wax respectively) and finally in molten
paraffin wax alone for 1-2 hours. Wax blocks of the infiltrated tissues were prepared and
longitudinal sections (7µm) of tail tissues were cut using a Leica microtome and placed on
glass slides coated with egg albumin.

Slides were then exposed to xylene, dried and passed through decreasing order of alcohol (95,
70 and 50%) for few minutes, washed in distilled water and stained with Harris’s
haematoxylin for 2-3 minutes. The slides were then washed and stained with eosin for 1-2
minutes, dehydrated in increasing concentration of alcohol (70 and 95% for few seconds),
dried and cleared in xylene before mounting permanently with DPX. The tissue sections were
observed under a Leica DM2500 Microscope and pictures captured using EC3 Camera
(utilizing LEICA LAS EZ (V 1.6.0) software).

**Immunofluorescence localization of FGF2, COX-2, VEGF and Caspase-3**

Briefly, regenerating tails at different stages were collected by inducing autotomy to release
the regenerate along with an intact adjacent tail segment. These were embedded in optimal
cutting temperature medium (Tissue-Tek OCT, Sakura Finetek, USA) and frozen at -20ºC
until used for cryosectioning.

For immunolabelling, longitudinal cryosections (8-10µm) were fixed in acetone at -20ºC for
15-20 minutes and air dried for 15 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 [1:200 dilution of Rabbit Anti-FGF2 (Sigma-Aldrich,
USA) in PBS-BSA; 1:50 dilution of Goat Anti-COX-2 (Biolegend, USA) in PBS-BSA; 1:100
dilution of Rat Anti-VEGF (Biolegend, USA) in PBS-BSA or 1:200 dilution of Rabbit Anti-
Caspase-3 (Sigma-Aldrich, USA) in PBS-BSA] 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
IgG-FITC, Goat Anti-Rat IgG-FITC or Rabbit Anti-Goat IgG-FITC (Genei, Merck, USA) in
PBS] 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). Same procedure was followed for negative control sections except that these were
incubated with PBS-BSA instead of the primary antibody.

**Immunohistochemical localization of MMP-2 and MMP-9**

As described before, regenerates along with an intact tail segment were collected at different
stages of regeneration, embedded in OCT and fresh frozen longitudinal cryostat sections (8-
10µm) of these were taken on clean glass slides. These were fixed in cold acetone at -20°C for 15-20 minutes, air dried for 15 minutes and rehydrated with PBST, then blocked using normal serum (10% in PBS-BSA) for 1-2 hours at RT. Sections were then incubated with appropriate primary antibody [1:200 dilution of Rabbit Anti-MMP-2 or Goat Anti-MMP-9 (Sigma-Aldrich, USA) in PBS-BSA] overnight inside a moist chamber at 4°C. Next day, sections were washed with PBST (3x5min) and incubated with corresponding ALP-conjugated secondary antibodies [(Genei, Merck, USA)1:300 dilution in PBS] for 2 hours at RT, washed and stained with substrate chromogen solution (BCIP/NBT) (Genei, Merck, USA) for 10-15 minutes. Sections were washed with PBS, mounted with a solution of PBS and glycerol (1:1) and observed using a Leica DM2500 microscope. Negative control sections were incubated with PBS-BSA instead of the primary antibody.

**Estimation of Protein content**

Protein estimation of the tissue samples was done by a BCA (Bicinchoninic acid) assay kit (Genei, Merck, USA). This method, first described by Smith *et al.*, 1985, combines the reduction of Cu$^{2+}$ to Cu$^{+}$ by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu$^{+}$) using a reagent containing BCA. The purple coloured reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. It is water soluble and exhibits a strong absorbance at 562nm that is nearly linear with increasing protein concentrations over a broad working range. The macromolecular structure of protein, the number of peptide bonds and the presence of four particular amino acids (cysteine, cystine, tryptophan and tyrosine) are reported to be responsible for colour formation with BCA (Wiechelman *et al.*, 1988). The amount of protein present in a solution can be quantified by measuring the absorption spectra and comparing with protein solutions with known concentrations.

Briefly, a set of BSA protein standards ranging from 0-2mg/ml was prepared. 20µl of each standard replicate was added to a microplate well, to which 200µl of BCA reagent was added. Same procedure was followed for unknown samples. This was allowed to incubate for 30 minutes at 37°C, cooled to room temp and absorbance was read at 562nm on a plate reader (Metertech Σ960). A standard curve of absorbance versus concentration of BSA was plotted and a regression equation was calculated in ms excel to determine the amount of protein in the unknown samples.

This assay was carried out to use equal amounts of protein for FGF2 quantification, zymographic studies and SDS-PAGE analysis.
FGF2 Quantification by ELISA

FGF2 level in the regenerates was quantified using an ELISA Kit (Quantikine, R&D Systems, USA) based on a quantitative sandwich enzyme immunoassay. In brief, a set of FGF2 standards (0-1000pg/ml) was prepared and taken in replicates onto microplate wells precoated with FGF2 specific antibody. Similarly, unknown samples with equal protein concentration were also loaded onto the microplate. Standard and unknown samples were appropriately diluted with diluent buffer, the plate covered with foil and incubated for 2 hours at room temperature. The wells were then aspirated, washed thrice with the wash buffer and blotted dry by inverting on clean tissue paper. Next, biotin-conjugated antibody solution specific for FGF2 was added and incubated for 2 hours at room temperature, followed by aspiration and washing thrice. Next, incubation with Streptavidin-HRP solution was done for 2 hours at room temperature and plate was again washed thrice. Finally, substrate-chromogen solution (TMB-H2O2) was added and plate kept in dark for 30 minutes. The reaction was stopped with the stop solution and the colour change was measured at a wavelength of 450nm using an ELISA plate reader (Metertech ∑960). FGF2 concentration in the unknown samples was determined by comparing/computing the results with the standard curve/regression equation.

SDS-PAGE (Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis) Analysis of Proteins

This method is used for electrophoretic separation of proteins through a gel matrix formed of polyacrylamide chains cross linked by N,N-methylene bisacrylamide. Since the proteins in their native or partially denatured form vary in their charge to mass ratio, SDS, an anionic detergent is used to denature proteins to their primary (linearized) structure and coat them with uniform negative charge as one SDS molecule binds to 2 amino acids. Thus, the charge to mass ratio of all the denatured proteins in the mixture becomes constant and protein molecules are separated according to their molecular weights only. The resolution and focus of the protein bands is increased by using a discontinuous gel system (Laemmli, 1970) using Tris-Glycine that stacks at a pH of 6.8 and resolves at a pH of 8.8. A standard is run along with the sample to analyze the separated proteins.

Briefly, the procedure consists of the following steps:
1. Making the polyacrylamide gel using a Gel assembling apparatus. 2. Preparing the samples by boiling in sample loading buffer containing SDS, glycerol and a reducing agent 3. Loading equal protein amounts on the gel and running the electrophoresis 4. Staining of the proteins using Coomassie Brilliant Blue-R250.
Zymography analyses
Zymography and reverse zymography are techniques used to evaluate the activities of MMPs and TIMPs in biological samples. Proteins are electrophoretically separated using a polyacrylamide gel containing a co-polymerized specific substrate under denaturing (SDS), non reducing conditions. After exchange of SDS with Triton X-100, enzymes partially renature and recover their activity. Subsequently, during incubation of gel in an appropriate activation buffer, MMPs in the gel digest the substrate. These digested areas of MMP activity are seen as clear areas against a dark background of undegraded substrate upon staining. Gelatin zymography involves using gelatin as the substrate and is mainly done for the detection of the gelatinases MMP-2 and MMP-9. In reverse zymography, an altered zymography method, an MMP is also included into the gel along with gelatin, usually MMP-2. During activation, MMP-2 digests gelatin in areas where TIMPs are absent. Thus, except for the TIMP bands where MMP activity will be inhibited, the remaining gel will appear colourless upon staining (Hawkes et al., 2001).

Gelatin Zymography
Briefly 7.5% SDS polyacrylamide gels with gelatin (5mg/ml) were prepared. Samples were loaded using a non reducing loading buffer. After electrophoresis, gels were washed with 2.5% Triton X-100 (2x30 min) and rinsed in double-distilled water (ddH₂O) followed by wash in incubation buffer (50mM Tris, 0.2M NaCl, 5mM CaCl₂, 0.02% NaN₃ and 0.02% Brij-35, pH 7.4-7.6) for 30 minutes and an 18 hour incubation with the same at 37°C. Gels were stained with Coomassie Brilliant Blue-R250 (Coomassie 0.5%w/v, Methanol 40%v/v, Acetic acid 10%v/v in ddH₂O) for 1-2 hours and then destained (45:10:45 Methanol:Acetic acid:ddH₂O). Activity of MMPs could be observed as clear bands on a dark background.

Reverse Zymography
Briefly, 15% SDS polyacrylamide gels with gelatin (2.5mg/ml) and crude MMP-2(rhMMP-2, R&D Systems, USA) were prepared. Samples were loaded with a non reducing loading buffer and electrophoresed. Gels were then processed further in the same way as described for reverse zymography. After destaining, TIMP activities were detected as blue bands on a clear background. A regular SDS-PAGE gel for the samples was also run under identical conditions to discriminate between the TIMP and the protein bands, which also get stained by Coomassie blue.

TUNEL (Terminal deoxynucleotidyl transferase biotin-dUTP Nick End Labelling)
Evaluation of Apoptosis
DNA fragmentation is a characteristic hallmark of apoptosis. TUNEL assay relies on the presence of nicks in the DNA which can be identified by Terminal deoxynucleotidyl
transferase (TdT), an enzyme that catalyzes the addition of dUTPs to the free 3’OH of cleaved DNA. The dUTPs are secondarily labelled with a marker (Gorczyka et al., 1992).

A TUNEL kit (Gen Script, USA) was used to detect the apoptotic cells in the regenerates. Briefly, tissues were excised using induced autotomy, frozen at -20°C and embedded in OCT. Longitudinal cryosections (8-10µm) were taken on clean glass slides and fixed with cold acetone for 1-2 minutes. Sections were incubated with 0.1% Triton-X100 in PBS for 5-10 minutes, rinsed in PBS (2x5min), blocked using 3% H2O2 in methanol for 10 minutes at 25°C and again rinsed with PBS twice. This was followed by incubation with TUNEL reaction mixture (Equilibration buffer, biotin-dUTP and TdT) for 45-60 minutes at 37°C. Slides were rinsed thrice for five minutes each and then sections were kept in Streptavidin-HRP solution for 30 minutes at 37°C. Sections were again rinsed, and DAB-H2O2 was added and kept for 5-10 minutes at 25°C. After washing, sections were mounted with PBS:glycerol (1:1) and analyzed with a Leica DM2500 microscope. Apoptotic nuclei were stained dark brown. Same process was done for negative control sections, except that the TUNEL reaction mixture was devoid of TdT.

**In vivo BrdU incorporation and Immunofluorescent localization**

A method commonly used to label cells in the S-phase of the cell cycle involves incorporation of the thymidine analog bromodeoxyuridine (BrdU) into replicating DNA and the subsequent immunohistochemical detection of the BrdU (Gratzner, 1982; Miller and Nowakowski, 1988). Administration of BrdU to living animals or addition of BrdU to culture medium can be used to determine relative proliferation rates, the length of the cell cycle, and the percentage of cells in the cell cycle (growth fraction) (Tang et al., 2007).

Intraperitoneal injection of BrdU (Sigma Aldrich, USA) at a dose of 100mg/kg body weight was given at different stages of regeneration and the regenerate was harvested by inducing autotomy after completion of one cell cycle. Tissues were embedded in OCT and fresh frozen sections (8-10µm) were taken on 0.01% poly-l-lysine coated slides. The sections were fixed in cold acetone (15-20 minutes at -20°C) and air dried for 15 minutes followed by treatment with 2N HCl for 30-60 minutes at 37°C. Sections were then rinsed in 0.1M borate buffer (pH 8.5) for 10 minutes (2x5 min) and then rehydrated in PBS at RT. Sections were blocked using normal serum (10% in PBS-BSA) for 1-2 hours at RT, and incubated with primary antibody [1:100 dilution of Mouse Anti-BrdU (Sigma-Aldrich, USA) in PBS] overnight inside a moist chamber at 4°C. Next day, sections were washed with PBS (3x5min) and incubated with FITC conjugated secondary antibody [1:50 dilution of Goat Anti-Mouse IgG-FITC (Genei, Merck, USA) in PBS] for 2 hours at RT, washed, mounted with PBS:glycerol (1:1) and observed under a fluorescent microscope (Leica DM2500 utilizing LAS EZ software).
Acridine Orange (AO) Staining of Nucleic acids
Acridine orange (AO) is a cell permeant, nucleic acid selective, metachromatic fluorochrome useful for cell cycle determination. It interacts with DNA and RNA by intercalation or electrostatic attractions respectively. When AO bonds with DNA and forms a complex, it has an emission maximum of 525nm (green) and when it binds with RNA, the emission maximum shifts to 650 nm and the emitted light is red (Darzynkiewicz, 1990).

Regenerates were excised using induced autotomy and immediately transferred to a cryostat microtome maintained at -20ºC. After embedding in OCT, longitudinal cryosections (8µm) of these were taken on clean glass slides. Sections were immediately fixed in ice-cold acetone for 1-2 minutes, kept in phosphate buffer (pH 6.0) for 2-3 minutes and stained with a 1:10 dilution of Acridine Orange (Sigma-Aldrich, USA) stock solution (0.1% in phosphate buffer, pH 6.0) for 1-2 minutes. The sections were then observed using a fluorescent microscope (Leica DM2500).

Statistical Analysis
Morphometric data were subjected to Shapiro-Wilk test to analyse normality of distribution followed by The Mann-Whitney U Test to compare differences between the groups. All analyses were carried out by using SPSS 12.0 for Windows (SPSS Inc, Chicago, IL). The values are expressed as mean ± SE or as mode with range in parenthesis. A ‘p’ value of 0.05 or less was considered statistically significant. Graphs were prepared using GraphPad Prism (version 3.0 for Windows, GraphPad Software, San Diego, California, USA).