Materials and Methods

ANIMAL HOUSING AND MAINTENANCE

The experimental protocol (MSU-Z/IAEC/15-2017) was approved by the Institutional Animal Ethics Committee (IAEC), and all the experiments are in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

Animal procurement: Adult northern house geckos aged between 4 to 6 months of both sexes were collected from their natural habitat. The selection of animal was primarily based on lizards having intact tail and were also screened for any parasitic infestation. Other lizards were then released back to their environment which did not meet the criteria of the current study.

Housing: The acclimatization time given to all lizards was 10 days in the animal house conditions and following which all the studies were conducted. The wooden cage in which lizards were housed had the dimension of 15×10×18 inches, with one side made of glass (Figure 2.1). The other three sides had grills for cross ventilation along with one shutter for collecting and replacing the lizards as well as in giving food and water. 10 lizards were caged together and cockroach nymphs were given twice a week while purified water was given every day, ad libitum. Ambient temperature in the animal house was 35±2 ºC throughout the duration of the study and a 12-hour light-dark cycle was maintained throughout.

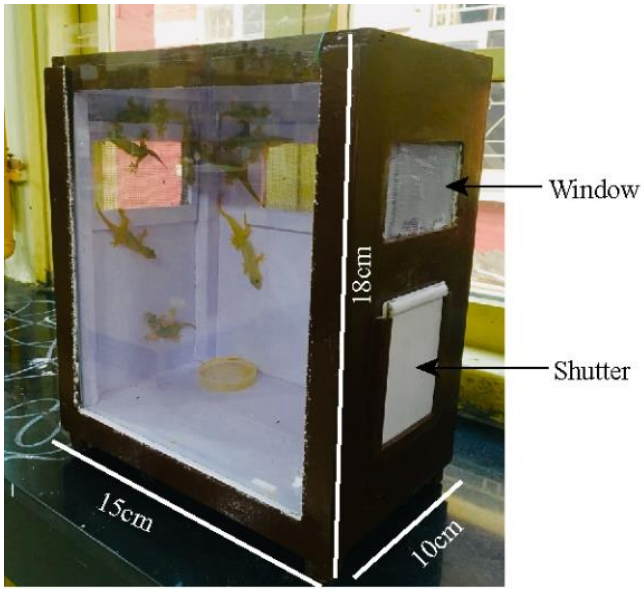

Figure 2.1: Housing of lizard in animal house

ANIMAL GROUPING AND EXPERIMENTAL DESIGN

The tissue samples were harvested in a sterile condition at the selected time interval. Time points for tissue collection were decided based on the discrete events associated with wound healing in tail and in limb. These have been characterized for the tail through the course of various studies in our lab (Buch et al., 2017; Murawala et al., 2018). Histology studies in the lab have shown that following autotomy, haemostasis is achieved rapidly and a scab is formed as early as 1 dpa. Subsequently, a thin layer of epithelium covers the wound surface on 2 dpa. Thereafter, the epithelium continues to proliferate and stratifies into a multi-layered AEC (Apical Epithelial Cap) that displaces the scab on 4 dpa. Now after 4 dpa, the cells start proliferating profusely and form blastema at 6 dpa which is characterized by a slight protrusion. Hence the time points selected for tissue collection were 0 dpa (representing uncut resting stage), 1 dpa, 2 dpa, 3 dpa, 4 dpa and 6 dpa (Figure 2.2).

On the other hand, wound healing in the amputated limb is achieved only on 9 dpa in the selected housing condition. Based on a continuous morphological observation on an amputated limb and taking reference from Alibardi, (2010) and Vitulo et al. (2016), the events occurring therein were found to be different from that of tail. Hemostasis phase in case of amputated limb is longer compared to tail. Therefore, even on 3 dpa the cut end of limb shows only a scab with no epithelial layer beneath it. On 6 dpa, limb displays a thick blood clot and is in granulation phase. Proper scarring is however, achieved on 9 dpa. The scar keeps maturing over the course of time and hence, 12 dpa was selected to see whether there are any other changes observed after 9 dpa in terms of healing. Therefore, the time points selected for limb were 0 dpa (representing uncut resting stage), 3 dpa, 6 dpa, 9 dpa and 12 dpa (Figure 2.2). The study composed of eleven groups in total; six groups in case of tail and five groups for limb tissues. Each group consisted of six lizards for individual experiments. All the experiments were performed thrice for reducing any biasness in the results obtained. The tissues were processed as per the requirement described in the following sections.

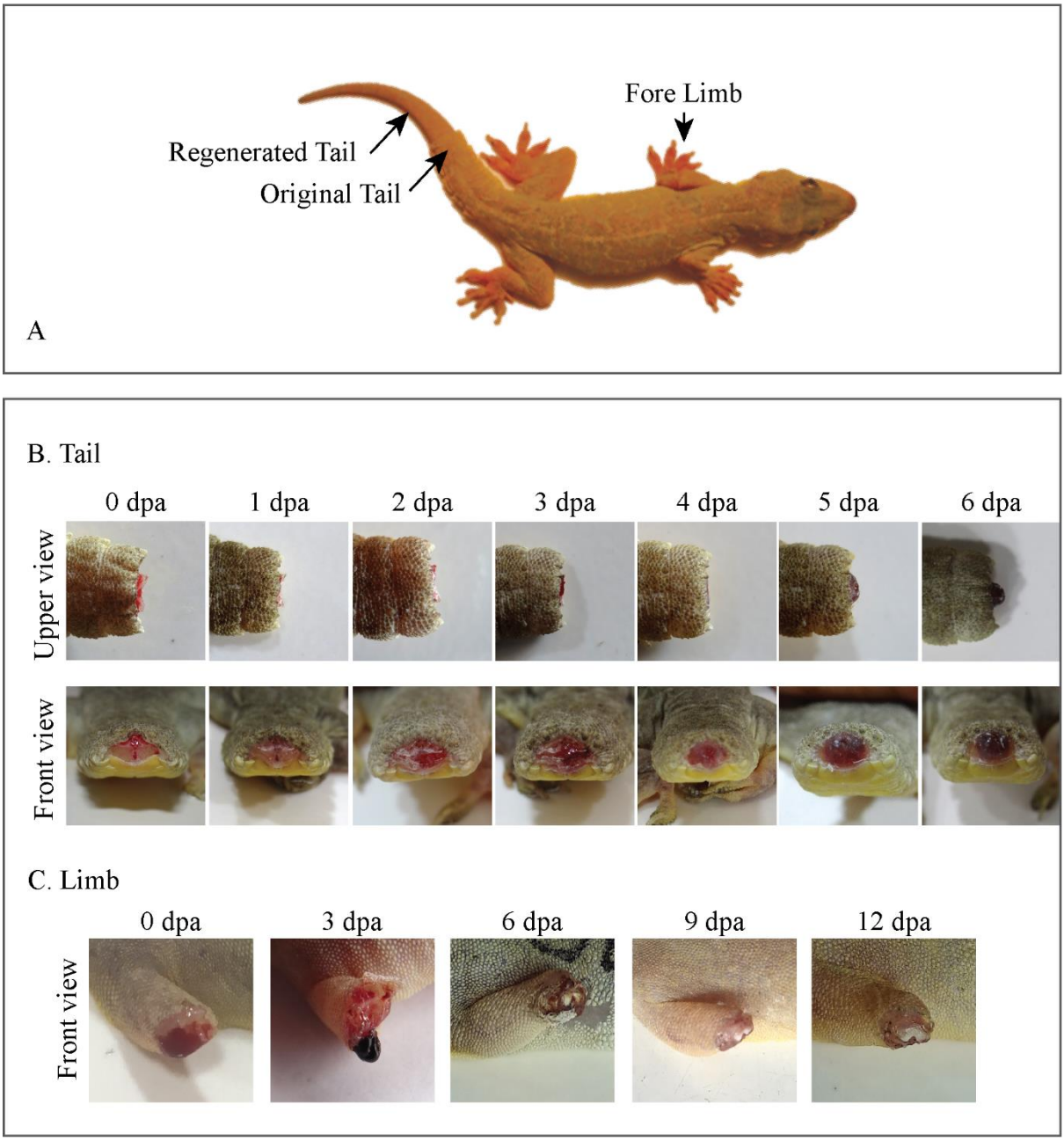

Figure 2.2: A) Morphology of the lizard; B) Tail and C) Limb wound healing stages selected for the study

BUFFERS USED FOR LYSIS OF TISSUE

- Lysis Buffer

Tris pH 7.5	50 mM
NaCl	200 mM
$CaCl_2$	10 mM
Triton-X 100	1 %
Protease inhibitor	(100X to 1X)

- RIPA Buffer

Tris pH 8	50 mM
NaCl	150 mM
Triton-X 100	1 %
Sodium deoxycholate	0.5 %
SDS	0.1 %
Protease inhibitor	(100X to 1X)

PROTEIN QUANTIFICATION

100 mg of Coomassie™ Brilliant Blue G250 (SRL) was added to 50 ml 95 % ethanol and was allowed to dissolve for 2 hours. This solution was added to 100 ml 85 % orthophosphoric acid and left overnight at room temperature. The volume was made up to 1 litre. The reagent was filtered through Whatman filter paper no. 1 and stored at 4 ºC in amber coloured bottle to protect from exposure to the light. This reagent has a characteristic brownish red colour and if there were any deviations found then the reagent was replaced. Once the reagent is made, the Bradford method (Bradford, 1976) was used for quantifying the protein samples and the readings were taken at 600 nm.

SDS-PAGE

Buffers:

1. 30 % Gel Stock

Acrylamide 29 %	29 g
Bis-acrylamide 1 %	1 g
Distilled water	100 ml

2. Tank Buffer

Tris base 125 mM	15.1 g
Glycine 1250 mM	94 g
10 % SDS	50 ml

3. 5X Sample buffer

Tris-Cl	250 mM (pH 6.6)
β-Mercaptoethanol	500 mM
SDS	10 %
Glycerol	50 %
Bromophenol blue	0.5 %

4. Resolving gel buffer (12 %; 5 ml)

30 % Gel stock	2 ml
D/W	1.6 ml
1.5 M Tris Cl (pH 8.8)	1.3 ml
10 % SDS	0.05 ml
10 % APS	0.05 ml
TEMED	0.003 ml

APS solution was prepared fresh. APS and TEMED was added just before pouring the resolving gel buffer in the PAGE assembly.

5. Stacking gel buffer (4 %; 3 ml)

30 % Gel stock	0.5 ml
D/W	2.1 ml
1 M Tris Cl (pH 6.8)	0.360 ml
10 % SDS	0.02 ml
10 % APS	0.02 ml
TEMED	0.002 ml

APS solution was prepared fresh. APS and TEMED was added just before pouring the stacking gel buffer in the PAGE assembly.

6. Fixative for gels

Methanol	50 %
Acetic acid	10 %

Protocol for SDS-PAGE

Gel casting: Resolving gel buffer was poured between PAGE plates in the casting assembly, immediately followed by D/W water. The gel was left undisturbed and allowed to fully polymerise for an hour. The excess water was removed and stacking gel buffer was poured on top of it. The comb was inserted immediately.

Sample preparation: Sample buffer (Loading buffer) at final working concentration, protein sample as per the required loading amount and water were added to prepare the final sample. Solution was heated at 80 ºC for 5 min and placed on ice till sample was loaded onto the gel.

SDS-PAGE GEL STAINING

1. Coomassie brilliant blue staining

CBB-R250	0.1 %
Methanol	40 %
Acetic acid	10 %

Procedure: The gel was kept for fixing in 40 % methanol for 30 min. Following the fixing step, the gel was shifted to a staining box containing the Coomassie stain and this set up was kept on a rocker for 2 hours. After staining the gel, destaining of the gel background was initiated. Once the bands were clearly visible and the background was cleared, image of the gel was captured for further analysis and observation.

2. Silver staining

Solutions: To be made in distilled water only

1. Fixing solution: 50 : 40 : 10 (methanol : D/W : glacial acetic acid)
2. $AgNO_3$ solution: 0.02 %
3. Developing solution: 2 ml of 0.02 % $Na_2S_2O_3$+ 2.19 % Na_2CO_3 + 125 µl formaldehyde
4. Stop solution: 1.4 % EDTA

Procedure:

- The gel was kept in fixing solution for 30 min followed by washing with 5 % methanol.
- After washing with methanol, the gel was washed with D/W twice with a time duration of 5 min
- The gel was then immersed in 0.02 % $Na_2S_2O_3$ for 3-5 min and was washed by D/W for 10 min
- The gel was kept in $AgNO_3$ solution for 20-30 min followed by addition of developing solution.
- As the bands started appearing the staining was stopped with EDTA solution.

2-DIMENSIONAL GEL ELECTROPHORESIS

Buffers:

1. Lysis Buffer (10 ml)

D/W	6.0 ml
Isopropanol	1.0 ml
Urea	3.0 g
CHAPS	0.025 g
Tween 20	0.025 g
DTT	0.15 g
Glycerol	0.5 ml

2. Sample Buffer

   Bio-Rad 2-D sample buffer (2-D starter kit rehydration buffer #1632106)

3. Equilibration Buffer 1

Urea	6 M
SDS	2 %
Tris-Cl pH 8.8	0.375 M
Glycerol	20 %
DTT	2 %

4. Equilibration Buffer 2

Urea	6 M
SDS	2 %
Tris-Cl pH 8.8	0.375 M
Glycerol	20 %
Iodoacetamide	2.5 %

5. Low melting point gel

LMP	0.05 g
D/W	9.0 ml
5X Tank buffer	1.0 ml

Protocol:

- Lysis: The tissue was collected in chilled lysis buffer and was homogenized using mortar pastel. Following homogenization step, the homogenate was allowed to rest for 2 hours at 4 °C. The sample was then centrifuged at 15000 g for 20 min at 4 °C and the supernatant was collected in a separate precooled vial.
- Precipitation: On collecting the supernatant it was precipitated by chloroform-methanol method for further use. After precipitating the protein, the precipitates were dissolved in the rehydration buffer which is also called as sample buffer. The protein was estimated by Bradford method (Bradford 1976).
- Rehydration: After preparing the protein and estimating it, 60 µg of protein was loaded on a 7 cm IPG strip (pH 3-pH 10) in the rehydration tray overnight at 18 °C. The strip was overlayed with mineral oil to prevent dehydration.
- Focusing: Once the strip rehydrates with protein being embedded in the gel, it was placed in the focusing tray and one-dimensional protein separation was done using the following programme.

Steps	Voltage	Mode	Time	Unit
1	250	Rapid	0:15	HH:MM
2	4000	Gradual	1:00	HH:MM
3	4000	Rapid	15,000	Volt hour
4	500		hold	

- After the focusing was completed the strip was removed and the mineral oil was wiped off with Kim wipes.
- Equilibration: After cleaning the IPG strip it was placed in equilibration buffer 1 for 15 min and this step was repeated twice. Similarly, after discarding equilibration buffer 1, equilibration buffer 2 was added for 15 min and the strip was washed for two more times.
- Second Dimension separation: Once the equilibration step was finished the strip was washed with 1X tank buffer and then it was placed on 12 % resolving gel above which overlay agarose was added. The gel is electrophoresed at 200 V for 30 min. On finishing the run, gel was stained with silver staining method as mentioned in the staining method.

WESTERN BLOTTING

Buffer and Chemicals

1. Semidry Transfer 5X:

Tris	240 mM
Glycine	39 mM

pH was adjusted to 9.2

2. Wet Transfer 5X:

Tris	100 mM
Glycine	960 mM

pH was adjusted to 8.3

3. Washing buffer (TBS-T)

0.1 % Triton X-100 in TBS

4. Blocking buffer (TBS-MT)

5 % skimmed milk powder in TBS-T

5. Antibody dilution buffer

BSA and Sodium azide added to TBS-T to a final concentration of 2 % and 0.02 % respectively.

6. BCIP-NBT stock solution (50X)

BCIP	9.4 mg/ml
NBT	18.75 mg/ml

Prepared in 67 % DMSO solution. Diluted to working concentration in ALP substrate buffer fresh before use.

7. ALP substrate buffer

Tris base	0.1 M
NaCl	0.1 M
$MgCl_2$	0.05 M

pH was adjusted to 9.5.

Transfer

Semidry

- 8 Whatmann filter paper soaked in 1X transfer buffer were kept ready.
- Following SDS-PAGE, the gel was transferred to the D/W for removal of buffer and then it was placed in the transfer buffer.
- After soaking the PVDF membrane first in absolute methanol it was shifted to the transfer buffer.
- Once this set was ready the sandwich of 4 filter paper, gel, PVDF membrane and 4 filter paper was made and the proteins were transferred from the gel to PVDF by applying 100 mA current for 25 min

Probing

Antibody probing: Membrane was blocked with blocking buffer at room temperature for 1 hour. It was then incubated in primary antibody at 4 °C overnight. The membrane was washed with washing buffer thrice for 10 min each time. This was followed by incubation with biotinylated secondary antibody for 30 min Membrane was washed again in washing buffer thrice same as the previous step, followed by incubation in Streptavidin-ALP conjugate for 15 min, and was washed again as in the previous steps. This was followed by colour development in ALP substrate which was BCIP-NBT.

After the development of the western blot, densitometric analysis was done by measuring the band intensity using ImageJ software and the values obtained were normalized with the band intensity of β-actin.

GELATIN ZYMOGRAPHY

Buffer:

1. Lysis buffer

Tris (pH 8)	10 mM
NaCl	150 mM
Triton X-100	1%
$CaCl_2$	10 mM

2. Sample (loading) buffer

Tris (pH 6.8)	1 M
Glycerol	10 %
SDS	2 %
BPB	0.002 %

3. Resolving gel buffer (7.5 %; 5 ml)

30 % Gel stock	1.25 ml
Tris Cl (1.5 M, pH 8.8)	1.25 ml
D/W	1.4 ml
2.5 % Gelatin	1 ml
10 % SDS	0.05 ml
10 % APS	0.05 ml
TEMED	0.005 ml

4. Stacking gel buffer (4 %; 3 ml)

30 % Gel stock	0.5 ml
D/W	2.1 ml
1 M Tris Cl (pH 6.8)	0.360 ml
10 % SDS	0.02 ml

10 % APS	0.02 ml
TEMED	0.002 ml

5. Triton wash buffer

Tris-Cl (pH 8)	20 mM
NaCl	0.15 M
$CaCl_2$	5 mM
Triton X-100	2.5 %

6. Incubation buffer

Tris-Cl (pH 7.5)	50 mM
NaCl	0.2 M
$CaCl_2$	5 mM
NaN_3	0.02 %
Brij 35	0.02 %

Protocol

Tissue collection and homogenization was done in lysis buffer containing protease inhibitor. Following homogenization and centrifugation, (11,000 g for 15 min at 4 °C) the supernatant was added to the sample buffer and 30 μg of total protein from each sample was loaded on a 7·5 % SDS-PAGE (polyacrylamide gel electrophoresis) gel, containing 0.5 % gelatin and allowed to run at 4°C at constant voltage (100 V). After electrophoresis, gels were washed with Triton wash buffer and then incubated in incubation buffer overnight at 37 °C for re-activation of the MMPs. Gels were stained with 0.5 % Coomassie brilliant blue R-250, where bands appeared as clear area against the dark blue background of stained gelatin. Relative intensities were determined using Doc-ItLs software (GeNei, Merck; www.merck.com).

RNA ISOLATION

Buffers and other reagents

1. DEPC water

0.1 % DEPC in D.W. water was prepared and autoclaved. For RNA isolation all the reagents were made in DEPC water to protect RNA from RNAse.

2. TBE Buffer (10X; 1 litre)

Tris base	108 g
Boric acid	55 g
0.5 M EDTA	40 ml

Volume made to 1 litre with DEPC water

3. RNA loading dye (5X; 1 ml)

500 mM EDTA	8 µl
40 % Formaldehyde	72 µl
Glycerol	200 µl
Formamide	30 µl
2.5 % BPB	30 µl
10X TBE	400 µl

Volume made to 1 ml with DEPC water

4. Other reagents used

- TRIzol reagent (Phenol + GITC)
- Chloroform
- 2-Propanol
- 75 % Ethanol prepared in DEPC water
- 1 % Agarose in TBE buffer
- 25 mM Ethidium bromide

All glassware and disposables were sterile and all surfaces involved were cleaned prior to the experiment. The isolation was carried out using a clean pair of gloves.

RNA isolation protocol

Isolation: Tissue was homogenized in TRIzol reagent (1 ml per 100 mg tissue), followed by centrifugation at 8,000 g for 20 min at 4 °C. The supernatant was added to 200 µl Chloroform. Tubes were allowed to stand on ice for 15 min with occasional gentle shaking. The mixture was then centrifuged at 12,000 g for 15 min at 4 °C. The organic and aqueous phases separated with a visible white interphase. The upper layer was carefully taken into a new microcentrifuge tube (strictly without contamination from the lower layers) containing 400 µl isopropanol. This

was gently mixed and refrigerated for at least 1 hour to allow precipitation. The tube was then centrifuged at 12,000 g for 15 min at 4 °C. The supernatant was discarded and the pellet, which contains RNA, was washed with 200 µl 75 % ethanol and centrifuged again. After a total of two such washes, the tube was opened and left on ice for the pellet to dry (Overdrying was avoided since it can lead to trouble with dissolution of the RNA in water). After excess ethanol had dried off, the pellet was dissolved in 30 µl DEPC water.

Quantification: RNA was quantified using one of the two methods listed below. Both the methods were used during the study at separate times.

1. UV spectrophotometry: 1 µl RNA solution obtained from the above isolation step was diluted in 1 ml DEPC water and read at 260 nm and 280 nm on a UV-Vis spectrophotometer (Toshniwal). The following formula was used to analyze the quantity of RNA: A ratio of the OD_{260} to OD_{280} was calculated to check for quality of the RNA preparation. A ratio of around 2 was accepted as indicative of good purity of the RNA solution. Further, 3 µl of the RNA solution was electrophoresed on a 1 % Agarose gel containing Ethidium bromide. Three distinct and sharp bands reflected good integrity of the RNA.

2. Qubit assay: RNA solution was diluted 1:40 and assayed on the Qubit 3.0 (Life technologies, USA) using its fluorimetric assay kit. The quality of RNA was verified by running on an Agarose gel as mentioned above.

cDNA SYNTHESIS

cDNA synthesis was performed from the isolated RNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA). The kit uses random hexamers to prime DNA polymerization reaction by the reverse transcriptase enzyme. 1 µg of total RNA was used from each sample for cDNA synthesis. The following program was set on the thermal cycler:

Temperature	**Time**
25 °C	10 min
37 °C	120 min
85 °C	5 min

The cDNA preparation was collected and stored at -20 °C till further use.

PCR

1. TBE buffer (10X; 1 litre)

Tris base	108 g
Boric acid	55 g
0.5 M EDTA	40 ml

Volume made to 1 litre with D/W water

2. DNA loading dye (6X)

Glycerol	30 %
BPB	0.25 %

3. Other reagents used

2X PCR master-mix (Sigma, USA)

Primer pairs for the respective genes

Nuclease-free water

2 % Agarose in TBE buffer

25 mM Ethidium bromide

4. Primer designing for PCR

Primers for the current study were designed using the online Primer-BLAST and BLAST tools of the NCBI. Sequences of the target mRNA in Zebrafish (*Danio rerio*) or Molly (*Poecilia spp.*) were aligned with those in Chick (*Gallus gallus*) using the BLAST tool. Regions of maximum sequence homology were identified and these regions of the mRNA were specified in the Primer-BLAST tool for designing of primers. All primers were designed in such a way that the amplicon size for each gene was below 200 bp, since real-time PCR was envisaged for gene expression studies. Primers with GC content close to 50 %, melting temperature close to 60 °C and minimal internally complementary sequences were selected and ordered. A list of the primer sequences used in this study has been appended at the end of this chapter.

PCR protocol

For each reaction, a reaction mix was prepared as follows:

Component Volume

2X master-mix	5 µl
Forward primer (5 µM)	0.5 µl
Reverse primer (5 µM)	0.5 µl
cDNA template	1 µl
Nuclease-free water	3 µl

PCRs were run on a BioRad T100 thermal cycler. The following program was used.

Temperature Time

95 °C	3 min	
95 °C	10 sec	35 cycles
60 °C	30 sec	
72 °C	30 sec	

In cases of primers not annealing well at 60 °C, annealing temperatures specific to those primers were used.

QUANTITATIVE REAL-TIME RT-PCR

For the current study, all quantitative gene expression analyses were carried out by real-time PCR using SYBR Green based master-mix (Takara Bio, Japan) on a LightCycler 96 machine (Roche Diagnostics, Switzerland).

Real-time PCR protocol

For each reaction mix, a mix with the following composition was prepared:

Component Volume

2X SYBR Green master-mix	5 µl
Forward primer (5 µM)	0.5 µl
Reverse primer (5 µM)	0.5 µl
cDNA template	1 µl
Nuclease-free water	3 µl

For every sample, each gene was assayed in triplicate. The reactions were run in 96-well plates (Genaxy, USA) sealed with clear sealing films.

The following program was used for amplification:

Temperature Time

95 °C	100 sec	
95 °C	10 sec	45cycles
60 °C	30 sec (acquisition)	
72 °C	30 sec	

This was followed by a melt-curve analysis with the following program:

Temperature Time

95°C	10 sec
65°C	60 sec
97°C	1 sec

Data analysis: Data was analyzed using the LightCycler 96 software version 1.1. Cq values were obtained for each well; normalized Cq values were calculated by subtracting the Cq values of internal control gene or reference gene (18S rRNA) from those of the target gene. Mean of these normalized Cq values were plotted.

$$\Delta Cq = Cq \text{ of target gene} - Cq \text{ of reference gene}$$

Moreover, fold change in expression was calculated by the ΔΔCq method of Livak and Schmittgen (2001). For fold change of gene expression in sample 2 as compared to that in sample 1:

$$\Delta\Delta Cq = \Delta Cq \text{ of gene in sample 2} - \Delta Cq \text{ of gene in sample 1}$$

ΔΔCq values were plotted on a graph with vertical axis following a logarithmic scale.

HISTOLOGY

Paraffin Block Preparation

Chemicals required:

1. NBF (Neutral buffered Formalin)

50 ml 37 % Formaldehyde

450 ml D/W

3.25 gm Sodium Phosphate, dibasic (Na_2HPO_4)

2 gm Sodium Phosphate, monobasic (NaH_2PO_4)

2. Ethanol
3. Xylene
4. Paraffin wax

Protocol:

Fixation: Lizard tail and limb tissues were fixed in 10 % NBF.

Paraffin Infiltration: Once fixed, tissue was processed as follows, using gentle agitation, usually on a tissue processor:

- 50 % ethanol for 1 hour.
- 70 % ethanol for 1 hour.
- First absolute ethanol for 1 hour.
- Second absolute ethanol for 1.5 hours.
- Third absolute ethanol for 1.5 hours.
- Fourth absolute ethanol for 1 hour.
- Xylene for 1 hour.
- Xylene for 1 hour.
- 25 % wax for 1 hour.
- 50 % wax for 1 hour.
- 75 % wax for 1 hour.
- 100 % wax for 1 hour.

Following these stapes the tissues were embedded in a paraffin block containing 100 % wax and were sectioned using a Microtome.

Haematoxylin & Eosin Staining: (Fischer et al., 2008)

Reagents:

1. Harri's Haematoxylin Stain (Procured from SRL)
2. Eosin Solution:

Yellow Eosin	1 g
Distilled water	80 ml
Ethanol	320 ml

Glacial Acetic Acid 2 drops

3. 0.5 % HCl.

Procedure:

1. Deparaffinize the section: the slides were flamed on the burner and placed in the xylene.
2. Hydration: Tissue sections were hydrated by passing through decreasing concentration of alcohol baths and water. (100 %, 90 %, 80 % and 70 %)
3. Sections were then stained with haematoxylin for 3-5 min followed by washing under tap water for 5 min and the in-acid alcohol (1 % HCl in 70 % alcohol) for 5 min
4. The sections were washed again in running tap water until they are blue on addition of alkaline solution (e.g., ammonia water) followed by tap water wash.
5. The sections were then stained with 1 % Eosin Y for 10 min followed by washing under the tap water for 3 min
6. After the staining was over the sections were dehydrated using increasing concentration of alcohol followed by xylene and the sections were finally mounted in DPX

The stained samples were observed under Leica DM2500 research microscope and the representative digital images were grabbed using Leica EC3 camera. The microscopic measurements of various tissues in the tissue sections were done using LAS EZ software.

IMMUNO-HISTOCHEMISTRY

Chemicals required:

1. Acetone
2. PBS
3. Blocking reagent
4. Wash buffer
5. Primary antibody
6. FITC-tagged Secondary antibody

Procedure

The tissues were cut at a thickness of 7 µm using a cryotome and were fixed on the slide by adding chilled acetone. Once the tissues were fixed, PBS was added to maintain the tissue integrity. Blocking reagent was added to the section and this setup was kept in fridge for an hour. Following the blocking stage, the sections were probed with the desired antibody overnight. Next day the sections were given three washes to completely remove the antibody following which a FITC-tagged secondary antibody was added and this set-up was kept at 4 ºC for another hour. Following the probing step, the sections were again washed with the wash buffer thrice and then were observed under the fluorescence microscope and the representative images were captured using a digital camera (Leica, EC3) mounted on the Leica DM2500 microscope.

ACRIDINE ORANGE AND ETHIDIUM BROMIDE STAINING

Cold sections (7 µm) were taken using cryotome (Reichert-Jung Cryocut E, USA) for wound epithelium stage in tail at 4 dpa and scarring in limb at 9 dpa. The sections were washed thrice with phosphate buffer of pH 7.4 (10 mM PO_4^{3-}, 137 mM NaCl and 2.7 mM KCl). Following washing, AO/EtBr stain (100 µg/ml) was added for 10 sec and the sections were immediately washed with phosphate buffer. All the images were taken under a fluorescent microscope (Leica DM2500 utilizing LAS EZ software).

BrdU LABELLING

Intraperitoneal injection of BrdU (Sigma Aldrich, USA) at a dose of 100 mg/kg body weight was given to the animals prior to the stage to be studied and on the day of collection the tail tissue was obtained by inducing autotomy. In the case of the limb, BrdU was administered similarly one day prior to the stage of interest and the next day tissue was harvested by amputating the limb. Tissues were embedded in cryo-embedding medium (Sakura Finetek, Japan) and fresh frozen sections (8-10 µm) were taken on 0.01 % poly-L-lysine coated slides. The sections were fixed in cold acetone (15-20 min at -20 ºC) and air dried for 15 min followed by treatment with 2N HCl for 30-60 min at 37 °C. Sections were blocked using bovine serum albumin (10 % in PBS-BSA) for 1 hour at RT and incubated with 1:100 dilution of Mouse Anti-BrdU (Sigma-Aldrich, USA) in PBS. The obtained sections were later incubated with 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 fluorescence microscope (Leica DM2500 utilizing LAS EZ software).

STATISTICAL ANALYSIS

All the linear variables were organized as mean ± Standard Error of Mean. The measurements made in histology of limb was analysed using Unpaired t-test. Rest of the data were subjected to One-way ANOVA followed by Bonferroni post-hoc test for multiple group comparison using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA). A 'p' value of 0.05 or less was considered as being statistically significant.