APPENDIX-I (List of Primers)

Gene	**Forward Primer**	**Reverse Primer**
fgf1	ACTCTCCGGAAAAGAAACCTTG	GTGGGTTTCTGGTCCCTTCA
fgf2	ATCCGGGAGAAAAACGACCC	TTGGTCGTCTCGCTCCAAAC
fgf4	GCACCTGACCACAGGTATCC	GGCCCAGAGCAAAACATTGG
fgf8	GAGACCGACACCTTTGGGAG	TTGCCGTTACTCTTGCCGAT
fgf9	GGTCACGGACTTGGACCATT	GCTGTCTACTCCTCGGATGC
fgf10	GTGCGGAGCTACAATCACCT	TCCAATATGCTGAAGGGGCA
fgf19	GACATGGTGGGCTCCTTTGG	GTCCCTGGGCACATACCTTC
fgf20	GATTCTGGGCGGCGATACTT	TCAGCTGTATCCCAGCACATC
fgf21	GAACTCACAACACGGCGTTC	CGAGTCCAGGTTGAAGTCCC
fgfr1	CCGTCCAGCAACACCTACTT	TGAGTCCACAGACACTGTTACC
fgfr2	GGGTTCAGCAAAGGTGCAAG	TTTGGGGTCAGGTATGCGAC
fgfr3	CGCCGACATCCTCATCTACC	GCTGTCTCTTGAGGGGGAAC
pcna	TGTTCCTCTCGTTGTGGAGT	TCCCAGTGCAGTTAAGAGCC
tgfβ1	CATGGCGAACAGGAACAACAGC	AGTGGTGAGCAGCTGGAAATTG
tgfβ2	AACCACAGAGCTGCCACTTG	CTTGGTGGGATGGCATTTTCG
tgfβ3	GGGAATGGCTGATGTACGGA	TGTAGTCTCCTTCCATGCCTTC
tgfβRI	AGGGACACAAGGAAAACCAGC	GGAGTCATGTCGAACAGCCA
tgfβRII	ACTGGTGTTCGTTTCCACAGC	ATCACCACGGCAACCAAGAC
p53	CAGCCAAGTCTGTGACTTGCACG TAC	CTATGTCGAAAAGTGCTTCTGTC ATC
p21	CTGGAGACTCTCAGGGTCGAA	CCAGGACTGCAGGCTTCCT
bcl2	ATGTGTGTGGAGAGCGTCAACC	GCATCCCAGCCTCCGTTATC
bax	CCTTTTCTACTTTGCCAGCAAAC	GAGGCCGTCCCAACCAC
casp9	CCAGAGATTCGCAAACCAGAGG	GAGCACCGACATCACCAAATCC
bad	CTTTAAGAAGGGACTTCCTCGC	GTGGAGTTTCGGGATGTGGA
cytC	GAACAAAGGCATCATCTGGGG	GGCAGTGGCCAATTATTACTCA
ecadherin	GAAGACAGCCAAGGGCCTG	TCTGGTACCCCTACCCTCTTG

ncadherin	AGCCCACGGAGTTTGTAGTG	TTTGGTCCTTTTCTGAGGCCC
twist1	CGAAGCGTTCACGTCGTTAC	TAGCTGCAATTGGTCCCTCG
twist2	ATCCTGTAACCACGGCTTCG	CGCTCGTCACCAGACTATCC
snai2	AGGACTCACACCTTGCCTTG	GTAGGTGAGCCCTGAGGTTG
snai1	CGAACGCACACTGGTGAAAA	CATACGGGAGAAAGTCCGGG
mmp2	GTCTCCTGGCTCATGCCTTT	TTTCACCACTTGGCCCTCTC
mmp9	GCCTTCAAGGTGTGGAGTGA	ATCCCCGTGGTCAGCTTTTC

Wound Repair and Regeneration

ORIGINAL RESEARCH-REGENERATION SCIENCE

Inherent variations in the cellular events at the site of amputation orchestrate scar-free wound healing in the tail and scarred wound healing in the limb of lizard *Hemidactylus flaviviridis*

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ABSTRACT

Lizards are unique in having both—regeneration competent (tail) as well as non-regenerating appendages (limbs) in adults. They therefore present an appropriate model for comparing processes underlying regenerative repair and nonregenerative healing after amputation. In the current study, we use northern house gecko *Hemidactylus flaviviridis* to compare major cellular and molecular events following amputation of the limb and of the tail. Although the early response to injury in both cases comprises apoptosis, proliferation, and angiogenesis, the temporal distribution of these processes in each remained obscure. In this regard, observations were made on the anatomy and gene expression levels of key regulators of these processes during the healing phase of the tail and limb separately. It was revealed that cell proliferation markers like fibroblast growth factors were upregulated early in the healing tail, coinciding with the growing epithelium. The amputated limb, in contrast, showed weak expression of proliferation markers, limited only to fibroblasts in the later stage of healing. Additionally, apoptotic activity in the tail was limited to the very early phase of healing, as opposed to that in the limb, wherein high expression of caspase-3 was observed throughout the healing process. Early rise in VEGF-α expression reflected an early onset of angiogenesis in the tail, while it was seen to occur at a later stage in case of the limb. Moreover, the expression pattern of transforming growth factor beta members points toward a pro-fibrotic response being induced very early in the amputated limb. Collectively, these results explain why regenerating appendages are able to heal without scars and if we are to induce scar-free healing in nonregenerating limbs, what interventions can be envisaged. This is crucial to the field of regenerative medicine since it is the initial stages of repair following amputation, which decide whether the appendage will be restored or only covered with a scab.

INTRODUCTION

It has been well perceived that a quiescent tissue of a living organism almost spontaneously evokes a reparative response when challenged with an injury. Nonetheless, the healing outcome might differ based on the extent and the site of injury. In vast majority of cases, the outcome of tissue healing is limited to small scale tissue repair and formation of a scarred tissue at the site of injury.[1,2] However, even though rarely, in few instances the tissue heals by a scar free mechanism and proceeds till the lost structure is regrown. Known as scar-free wound healing, in the latter case the epithelial cells rapidly cover the wound surface which later acts as an organizer to orchestrate the events leading to the structural and functional reestablishment of the lost part.[3–5]

The scarred wound healing is achieved through a multifaceted yet overlapping sequence of event namely hemostasis, inflammation, proliferation, and remodeling at the site of injury. There are ample records to believe that these events are tightly regulated by several mediators that include, but not limited to, platelets,[6] inflammatory cells,[7] cytokines,[8] growth factors, and matrix metalloproteinases.[9] A tissue when injured would immediately relay signals to the cells at the site of injury to form a provisional matrix over the wound to curtail blood loss. Immediately following hemostasis, the newly recruited platelet cells trigger a local surge of inflammation.[10] Once the inflammation subdues the proliferation phase begins wherein the wound is rebuilt with granulation tissue which is a collection of fibroblasts, inflammatory cells, and neovasculature wrapped in a matrix of collagen and extracellular matrix. In the subsequent maturation phase, the granulation tissue undergoes substantial remodeling with attended retraction of blood vessels to form an avascular structure called scar, a dense collagen tissue, that thoroughly covers the wound.[6,11]

Not surprisingly, even the scar-free wound healing begins with a hemostasis phase, however, after an acute hike early on, the inflammatory phase is suddenly truncated, which is where the scar-free healing swerves away from that of the scarred one. This abrupt drop in the inflammation marks the beginning of proliferation that allows the epidermal cells to rapidly divide and migrate as a layer to cover the wound surface which eventually stratifies to form a multilayered apical epithelial cap (AEC). The AEC, like an embryonic organizer, sends regulatory signals to the underlying mesenchyme and the latter responds by recruiting a pool of blastemal cells which proliferate and later get respecified to recreate the lost tissue.[12] The various phases of scarred and scar-free wound healing are depicted in Figure 1.

As stated earlier, successful regeneration of the lost tissue is possible only through scar-free wound healing. Among vertebrates, fishes, urodele amphibians, and few reptiles are known to heal the cut surface of their amputated appendage in a scar-free manner prior to the regrowth of the lost structure.[15,16] However, unlike other vertebrate models of regeneration, lizards are unique since they are endowed with two different appendages with distinct wound healing pattern namely the tail and limb. The former upon amputation heals the wound surface in a scar-free manner whereas the latter follows scarred wound healing and hence remains refractive to regenerative response.

Further, wound healing, being an incredibly complex biological process with intricate molecular interaction among various cells at the site of injury, is modulated by the timed expression of a myriad of regulatory factors. Important among these factors are the members of epidermal growth factor, transforming growth factor beta (TGF-β), fibroblast growth factor (FGF), interleukin, and tumor necrosis factor-α families.[17,18] In addition, vascular endothelial growth factor (VEGF), granulocyte macrophage colony stimulating factor, platelet-derived growth factor, connective tissue growth factor are known to influence the process of wound healing.[19,20] Because the focus of the study was on three processes, members of FGF and TGF-β were targeted as there are reports of these molecules being involved in proliferation, apoptosis, and angiogenesis. Moreover, a temporal expression screening of these molecules is yet to be attempted in the appendages of lizard during the course of wound healing. Hence, it was thought pertinent to compare their expression pattern during the course of scarred as well as scar-free wound healing in the limb and the tail respectively of lizard *Hemidactylus flaviviridis.*

MATERIALS AND METHODS

Animal maintenance

Healthy adult Northern house geckos *H. flaviviridis* of either sex having average snout to vent length of 10 cm were collected from Baroda, India. They were acclimatized for a week and maintained all through the experiment in well ventilated wooden cages. The cages were housed in a room at a controlled temperature of 36 ± 2 °C and 40–70% relative humidity. The photoperiod was kept at 12 : 12 hours of light : dark. Lizards were fed with cockroach nymphs daily and water was given ad libitum. Autotomy was induced in the tail by applying mild pressure with a foot ruler on the third segment from the vent. The limb was amputated under hypothermia. The animal was placed on a precooled tile and ice pack was applied to the limb to be amputated. With a sharp sterile surgical blade, the forelimb of the lizard was amputated. The experimental protocol (MSU-Z/IAEC/15-2017) was approved by the Institutional Animal Ethics Committee (IAEC) and all the experiments were performed as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

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.[13] It has been observed through histology in our lab that following autotomy, hemostasis is achieved rapidly and a scab is formed as early as 1dpa. Subsequently, a thin layer of epithelium covers the wound surface on 2dpa. Thereafter, the epithelium continues to proliferate and stratifies into a multilayered AEC that displaces the scab on 4dpa. Hence the time points selected for tissue collection were 0dpa (representing uncut resting stage), 1dpa, 2dpa, 3dpa, and 4dpa.

On the other hand, wound healing in the amputated limb is achieved only on 9dpa in the selected housing condition. Based on a continuous morphological observation on an amputated limb and taking reference from Vitulo et al.[21] and Alibardi[22], 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 3dpa the cut end of limb shows only a scab with no epithelial layer beneath it. On 6dpa, limb displays a thick blood clot and is in granulation phase. Proper scarring is however, achieved on 9dpa. Hence, the time points selected for limb were 0dpa (representing uncut resting stage), 3dpa, 6dpa, and 9dpa. The study was composed of nine groups in total; five groups in case of tail and four groups for limb tissues. Each group consisted of six lizards for individual experiments. The tissues were processed as per the requirement described in the following sections.

Histology

Histology was performed for tail tissues at 0dpa, 2dpa, and 4dpa and at 0dpa, 3dpa, and 9dpa for limb tissues. The tissues were stored in 10% neutral buffered formalin and decalcified by EDTA, followed by dehydration and embedding into paraffin blocks. Sections were cut at a thickness of 7 μm and stained with Harris' hematoxylin & eosin (H&E). The stained samples were observed under Leica DM2500 research microscope (Leica, Wetzlar, Germany) 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 (Leica).

Immunohistochemistry

For immunolabeling, longitudinal cryosections (8–10 μm) were cut from freshly collected tissues, fixed in acetone at −20 °C for 15–20 minutes and air dried for 15 minutes.

Figure 1. Phases in wound healing. (A) Depicts the scar-free wound healing where the epidermis covers the wound and then proliferates to form the AEC as shown by the arrowhead. (B) Shows the scarred wound healing where reepithelialization starts along with fibroblast proliferation, deposition of collagen (shown by arrowheads) and ECM development to form the granulation tissue. Brown dotted line represents the hemostasis phase, red dotted line shows the inflammation phase, blue dotted line is for epidermis covering the wound and green dotted line depicts the proliferation of epidermis in scar-free wound healing while in scarred wound healing it represents proliferation of fibroblast cells and scab formation (Adapted from Murawala et al.[13] and Diegelmann and Evans[14]). [Color figure can be viewed at wileyonlinelibrary.com]

Sections were then rehydrated with phosphate buffered saline with 0.025% Tween-20 (PBST) followed by blocking with corresponding normal serum (Genei, Merck, Kenilworth, NJ; 10% in PBS with 0.5% Bovine serum albumin [PBS-BSA]) for 1 hour at room temperature (RT). Sections were then incubated with Anti-FGF2 IgG rabbit (Sigma-Aldrich, St. Louis, MO; 0.5 μg/ml), Anti-VEGF-α IgG goat (Sigma-Aldrich, 0.1 μg/ml), Anti-Cleaved caspase-3 IgG

Table 1. Primer sequence obtained from NCBI

Gene	Forward primer	Reverse primer
fgf1	ACTCTCCGGAAAAGAAACCTTG	GTGGGTTTCTGGTCCCTTCA
fgf2	ATCCGGGAGAAAAACGACCC	TTGGTCGTCTCGCTCCAAAC
fgf8	GAGACCGACACCTTTGGGAG	TTGCCGTTACTCTTGCCGAT
fgf10	GTGCGGAGCTACAATCACCT	TCCAATATGCTGAAGGGGCA
fgf20	GATTCTGGGCGGCGATACTT	TCAGCTGTATCCCAGCACATC
fgfr1	CCGTCCAGCAACACCTACTT	TGAGTCCACAGACACTGTTACC
kdr	GCTTGCTTTTTGCCGGATGA	TGCCGACCTCTGCATGTTAG
vegfα	CAGCGCATGTGAGTGTAGGT	TCACAGACAGCATCGTGAATTG
tgfβ1	CATGGCGAACAGGAACAACAGC	AGTGGTGAGCAGCTGGAAATTG
tgfβ2	AACCACAGAGCTGCCACTTG	CTTGGTGGGATGGCATTTTCG
tgfβRI	AGGGACACAAGGAAAACCAGC	GGAGTCATGTCGAACAGCCA
tgfβRII	ACTGGTGTTCGTTTCCACAGC	ATCACCACGGCAACCAAGAC
p53	CAGCCAAGTCTGTGACTTGCACGTAC	CTATGTCGAAAAGTGCTTCTGTCATC
p21	CTGGAGACTCTCAGGGTCGAA	CCAGGACTGCAGGCTTCCT
bcl2	ATGTGTGTGGAGAGCGTCAACC	GCATCCCAGCCTCCGTTATC
bax	CCTTTTCTACTTTGCCAGCAAAC	GAGGCCGTCCCAACCAC
bad	CTTTAAGAAGGGACTTCCTCGC	GTGGAGTTTCGGGATGTGGA
cytC	GAACAAAGGCATCATCTGGGG	GGCAGTGGCCAATTATTACTCA
Cl.caspase 3	AAAGATGGACCACGCTCAGG	TGACAGTCCGGTATCTCGGT
PCNA	TGTTCCTCTCGTTGTGGAGT	TCCCAGTGCAGTTAAGAGCC

goat (Sigma-Aldrich, 0.1 μg/ml). For all primary antibodies, FITC-conjugated secondary antibodies (Sigma-Aldrich, 0.1 μg/ml) were used and their expression was observed under a fluorescent microscope. The representative images were captured using a digital camera (Leica, EC3) mounted on the Leica DM2500 microscope.

BrdU labeling

Intraperitoneal injection of BrdU (Sigma-Aldrich) at a dose of 100 mg/kg body weight was given to the animals at 3dpa and tail tissue was harvested at 4dpa by inducing autotomy. In the case of the limb, BrdU was administered on 8dpa and tissue was harvested for sectioning on 9dpa. Tissues were embedded in cryo-embedding medium (Sakura Finetek, Tokyo, 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 minutes at −20 °C) and air dried for 15 minutes followed by treatment with 2 N HCl for 30–60 minutes at 37 °C. Sections were blocked using normal serum (10% in PBS-BSA) for 1 hour at RT and incubated with 1:100 dilution of Mouse Anti-BrdU (Sigma-Aldrich) in PBS. Sections were then incubated with 1 : 50 dilution of Goat Anti-Mouse IgG-FITC (Genei, Merck) 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).

Acridine orange and ethidium bromide staining

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

Western blot

Tissues were harvested from all the nine groups and homogenized in lysis buffer (50 mM tris pH 7.5, 200 mM NaCl, 10 mM $CaCl_2$ and 1% triton-X 100) containing protease inhibitor. The samples were centrifuged at 12,000 rpm for 10 minutes and protein estimation was done using Bradford method.[23] Forty micrograms of protein of each sample was loaded and separated on a 12% SDS-PAGE. These proteins were transferred onto PVDF membrane through semi-dry transfer method by applying 100 mA for 25 minutes. The primary antibodies used to probe each protein of interest were Anti-Cleaved caspase3 IgG goat (Sigma-Aldrich, 0.1 μg/ml), Anti-Fibroblast growth factor 2 IgG rabbit (Sigma-Aldrich, 0.1 μg/ml), Anti-VEGF-α IgG goat (Sigma-Aldrich, 0.1 μg/ml), Anti-GRB7 IgG mouse (DSHB, 0.5 μg/ml), Anti-PI3K IgG mouse (DSHB, 0.5 μg/ml), Anti-AKT IgG rabbit (Sigma-Aldrich, 0.1 μg/ml), Anti-PCNA IgG rabbit (Sigma-Aldrich, 0.1 μg/ml), and Anti-β-actin IgG mouse (Santa Cruz Biotechnology, Dallas, TX; 0.01 μg/ml). The blot was developed by using the ALP, BCIP-NBT system (Sigma-Aldrich).

Quantitative real-time PCR

Total RNA was isolated from the limb and tail tissue homogenates using TRIzol reagent (Applied Biosystems,

Foster City, CA). One microgram of total RNA was reverse-transcribed to cDNA using a one-step cDNA Synthesis Kit (Applied Biosystems). Primers were designed using Primer-Blast tool of NCBI, details of which are given in Table 1. Quantitative real-time PCR was performed on a Light Cycler 96 (Roche Diagnostics, Risch-Rotkreuz, Switzerland) with the following program: 3 minutes at 95 °C as initial denaturation step and 45 cycles with each cycle of 10 seconds at 95 °C, 10 seconds at 60 °C, and 10 seconds at 72 °C). Gel electrophoresis and melt curve analysis were used to confirm specific product formation. 18SrRNA was taken as endogenous control. The fold change was computed using method of Livak and Schmittgen.[24] In order to minimize variations among biological individuals, the tissue samples from six lizards were pooled and for each variable analyzed in RT-PCR three technical replicates were performed to reduce the experimental error.

Statistical analysis

All the linear variables were organized as mean ± standard error of mean. The measurements made in histology of limb was analyzed 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.

RESULTS

Histology

Lizards, as a taxonomic group, are more closely related to mammals than are the urodeles. They are also endowed with well-structured appendages to aid their terrestrial mode of life much akin to their mammalian counterparts. In the current study, the healing pattern in two different appendages, viz., tail and limb, was assessed by observing the histological status of both, postamputation and also during various stages of wound healing. Autotomy, when induced in the tail, leads to exposure of a variety of tissues like muscles and adipose along with the vertebral column (Figure 2A). However, in the amputated limbs, in addition to the soft tissues mentioned earlier, humerus bone too was vividly seen in the histological section (Figure 3A). Tail, being the fat storage organ of the lizard, exhibited thick layers of adipose tissue (Figure 2A and C), when compared to the limb (Figure 3B and C). As the bone can be seen protruding out from the limb (Figure 3A), in tail the two lateral processes protrude conspicuously (Figure 2B). After 2dpa in tail a proliferating epithelium covered the wound, as revealed in Figure 2D with an average thickness of 44.65 μm (Figure 2E and Table 2). Later at 4dpa (Figure 2F) which is the WE stage, a more thickened epidermis of 91.93 μm ($p \leq 0.001$) was observed (Figure 2G and Table 2). However, on the lateral sides of tail the thickness of epithelium was 12.52 μm (Figure 2H and Table 2). On the contrary when the amputated limb was monitored at 3dpa, no epithelial layer was visible at the wound surface (Figure 3D), instead blood clot was seen to cover the open bone surface (Figure 3E). At 9dpa, i.e., in the scarred limb tissue, a fully covered and healed structure was displayed, which differs significantly from previously described completely healed tail (4dpa). Along with epithelium, a dense connective tissue was observed over the wound surface (Figure 3F and G). On the lateral side the mean thickness of connective tissue was 193.99 μm (Figure 3H and Table 3), while immediately over the wound it was 369.15 μm which was significantly high ($p \leq 0.001$) (Figure 3I and Table 3). However, the newly formed epithelial layer was just 12 μm thick which is the normal thickness witnessed in the resting skin of limb and tail of lizard (Figure 3B and Table 3).

Early proliferation of epithelium in tail contributes to scar-free wound healing

Members of FGF family have been known since long to induce and sustain cell proliferation at the site of amputation. Hence, their expression level was studied at mRNA level during the wound healing phase of tail as well as limb in lizard (Figure 4A and B). A steady increase in the mRNA levels of *fgf1*, *fgf2*, *fgf8*, and *fgf20* was observed in regenerating tail as healing progressed from 0 dpa to 4 dpa (Figure 4A). However, when the expression levels in all the stages were compared to that of resting stage, i.e., 0dpa, a sharp increase in fold change was noticed for *fgf10* on 3dpa unlike the other fgfs screened (Figure 4A). In tandem with the increase in fgfs, *fgfr1* was also found elevated from 1dpa to 4dpa (Figure 4A). However, the limb healing stages which are 0, 3, 6, and 9dpa showed a dissimilar trend to that observed in the tail. There was an increase in transcripts of *fgf1*, *fgf2*, *fgf8*, and *fgf10* at 3dpa but a sharp decline was observed in subsequent stages (Figure 4B). Moreover, the mRNA levels of *fgf20* and *fgfr1* were significantly downregulated at 3dpa in limb as shown in Figure 4B.

Furthermore, to find the actual trend of proliferation across the stages of wound healing, *pcna* at transcript level was studied and a steady increase was seen during tail wound healing stages with a 100-fold increase at 4dpa, as compared to 0dpa (Figure 4A). The healing limb also showed a progressive increase in *pcna* level but the fold change observed at 9dpa was just 10-fold (Figure 4B). A concomitant Western blot analysis of PCNA reaffirms the above findings as elevated levels of PCNA were found in tail healing stages and limb healing stages (Figure 4C and D), nonetheless, tail tissues showed a much a higher protein level than that in the limb.

A confirmatory BrdU labeling also revealed the presence of proliferating epidermis and a pool of dividing cells underneath (Figure 4E). Quite contrary to the observations in the tail tissue at wound epithelium stage (4dpa), only faint BrdU was observed in the scarred limb epithelium (9dpa) indicating only basal levels of proliferation at a corresponding stage in the limb (Figure 4F).

Apoptosis is prominent in the scarred wound healing during the later phase

It is well perceived that immediately following injury the wound surface induces apoptosis to clear out the debris and in the subsequent phase of wound healing, regulated apoptosis facilitates a balanced proliferation and hence, transcript levels of *caspase3*, *bax*, *bad*, *cytC*, *p53*, *p21*, and *bcl2* were quantified by real time PCR in the tissues collected from tail and limb during wound healing. On amputation of tail,

Figure 2. Histology of Tail and its wound healing stages. (A) Longitudinal section of tail showing fracture plane (FP), muscles (M), adipose tissue (AT), epithelium (E), and connective tissue (CT); (B) magnified image of resting tail showing the vertebral column processes (V) projecting out; (C) magnified image showing arrangement of E, CT, and AT; (D) epidermis covering the wound surface at 2dpa and (E) the thickness of epithelium formed is 44.65 µm; (F) at 4dpa, wound epithelium formation occurs that is depicted by proliferating epidermis also called as AEC; (G) shows the magnified image of AEC by arrow which has a thickness of 91.93 µm; (H) this section clearly shows that lateral epidermis maintains its thickness at 12.62 µm whereas the epidermis moving toward the AEC has a thickness of 41.49 µm. "*" Represents the area that has been shown in the magnified image. All the sections are longitudinal in orientation and stained with H&E. Magnification for A, D, and F is 40X, B is 100X, C, G, and H is 200X and E is 400X, $n = 6$. [Color figure can be viewed at wileyonlinelibrary.com]

Figure 3. Histology of Limb and its wound healing stages. (A) The limb, which on amputation shows exposed tissues like muscles (M), connective tissues (CT), humerus bone (B), epithelium (E) and adipose tissue (AT); (B) enlarged image showing connective tissue with a thickness of 107.5 μm and epithelium having same thickness as that of tail which is 17.75 μm; (C) arrangement of tissues can be observed which is similar to that of tail except AT being thicker in tail and CT being comparatively thicker in limb; (D) displays histology of limb at 3dpa with no epithelium covering and (E) represents persistent blood clot over the bone; (F) shows the healed limb wherein connective tissue (CT) can be observed covering the wound surface; (G) depicts the chondrocytes which replace the clot over the humerus bone; (H) lateral connective tissue with no difference in thickness from that of resting limb whereas in (I) just at the wound site the thickness of connective tissue in 369.15 μm. "*" Represents the area that has been shown in the magnified image. All the sections are longitudinal in orientation and stained with H&E. Magnification for A, D, and F is 40X, C, E, H, and I are 100X, G is 200X and B is 400X, n = 6. [Color figure can be viewed at wileyonlinelibrary.com]

Table 2. Thickness of epithelium (in μm) during different stages of wound healing in tail

Tissue layers analyzed	Thickness in μm
Lateral epithelium (normal resting stage)	12.52 ± 0.91
Epithelium covering wound at 2dpa	44.65 ± 1.28[a]
Epithelium covering wound at 4dpa	91.93 ± 3.89[a,b]

Values are expressed as mean ± SEM. Thickness of epithelium at 2 and 4dpa was compared with the thickness lateral epithelium using One-way ANOVA followed by Bonferroni's multiple comparison test, represented by asterisk.
[a]$p \leq 0.001$. Wound epithelium thicknesses of 2dpa and 4dpa were also compared with each other using the same test denoted by hash.
[b]$p \leq 0.001$, $n = 6$.

within 1dpa, a twofold increase in *caspase3* was observed which remained persistent till 2dpa (Figure 5A). Nevertheless, caspase3 returned to its basal levels at 3 and 4dpa (Figure 5A). All the other apoptotic genes studied namely, *bax*, *bad*, *cytC*, *p53*, and *p21* remained downregulated all through the healing stages of the tail (Figure 5A). However, in order to gain further insight into the regulation of apoptosis, expression pattern of antiapoptotic gene *bcl2* was ascertained and it was observed that the transcript levels of *bcl2* remained subdued at the initial stages of healing tail, followed by an elevated expression at 3 and 4dpa, the observations being an exact contradiction to observed levels of *caspase3* (Figure 5A). On the other hand, in the limb healing stages, except for *bad*, all the other genes remained downregulated at 3dpa and 6dpa (Figure 5B). At 9dpa a sudden increase in these levels was noted, while in the same time frame, *bcl2* showed an opposite trend in limb to that of the tail, as higher levels were found at 3dpa and 6dpa, which subsequently declined at 9dpa (Figure 5B).

Western blot results of cleaved caspase 3 suggest the occurrence of persistent apoptosis in the tail from 0dpa to 3dpa and at the wound epithelium stage (4dpa) this level drops perhaps to allows the proliferation to outcompete apoptosis (Figure 5C). However, the Western blot image of the limb shows a steady increase in cleaved caspase 3 bands from 0dpa to 9dpa (Figure 5D). Concomitantly, cleaved caspase 3 was localized in the tail at 4dpa which did reveal a few cells undergoing apoptosis (Figure 5E and F) however, in limb a large pool of cells was positively stained for cleaved caspase 3 (Figure 5G and H). These results coincide with the Western blot and real time PCR outcomes.

Apart from the major genes involved in apoptosis, tgf-β levels were also screened as they have an important role in mediating apoptosis and wound healing. Both, *tgf-β1* and *tgf-β2* levels did not change significantly during the wound healing phase of the tail, but the scarring limb showed distinctly elevated expression of the same molecules, when compared to the resting tissue (Figure 5I). On the contrary, expression of *tgf-β3* showed an increasing trend in the tail from 1 to 4dpa (Figure 5I) while its levels were found downregulated in limb from 3dpa to 9dpa (Figure 5J). Along with the ligands, tgf-β receptors were also screened and *tgf-βRI* was found to be upregulated at 9dpa in limb (Figure 5J) and downregulated in the tail from 1dpa to 4dpa respectively (Figure 5I). *tgf-βRII* had a twofold increase in the tail from 1dpa to 4dpa (Figure 5I) but in limb no significant change was observed (Figure 5J).

In order to further validate these results, acridine orange and ethidium bromide staining was performed on fresh frozen sections of 0dpa and 4dpa tail tissue along with 0dpa and 9dpa tissues of the healing limb. Both, the tail and limb tissues at 0dpa revealed live cells emitting green signal (Figure 5K and L). By 4dpa, in tail, a proliferating epidermis characterized by green fluorescence was predominantly observed along with few pro-apoptotic cells stained yellow (Figure 5M and O). On the contrary the limb tissue on 9dpa revealed heightened apoptosis marked by orange nuclear EtBr staining at the site of injury (Figure 5N and P) which concurs with the gene expression pattern and Western blot of cleaved caspase 3.

Angiogenesis occurs in the early phase of wound healing in tail but at a later phase of granulation in the limb

Angiogenesis is regulated by molecules like vegf-α and kdr, which operate through PI3K/AKT pathway. Hence, initially at transcript level they were studied in all the stages of both, tail and limb wound healing. A constant increase in *vegf-α* and *kdr* was noted from 1 to 4dpa in tail following amputation (Figure 6A). In limb at 3dpa, a down-regulation of *vegf-α* and *kdr* was seen after which at 6dpa stage a hike in transcript level was observed which again declined at 9dpa (Figure 6B). However, the tail had a much higher transcript level of *vegf-α* at wound epithelium stage (4dpa) than the limb at scarring stage (9dpa).

A careful analysis of the Western blot images of FGF2, PI3K, AKT, and VEGFα showed an increasing trend in their protein level from 1 to 4dpa in the tail (Figure 6C). In the limb similar to the status of transcript, the protein levels of VEGFα also showed decline at 3dpa, however contradictory to transcripts the protein level was elevated even after 6dpa and remained high till 9dpa (Figure 6D). However, no significant change was observed in FGF2 levels in limb throughout the healing process (Figure 6D).

Table 3. Thickness of epithelium and connective tissue (in μm) during different stages of wound healing in limb

Tissue layers analyzed	Thickness in μm
Lateral connective tissue (normal resting stage)	107.5 ± 4.51
Connective tissue at wound site at 9dpa	369.15 ± 21.79[a]
Lateral epithelium (normal resting stage)	17.75 ± 3.87
Epithelium covering wound at 9dpa	12.78 ± 0.71

Values are expressed as mean ± SEM. Thickness of connective tissue at wound site was compared with that of the lateral side using unpaired *t* test denoted by asterisk. [a]Represents $p \leq 0.001$, $n = 6$. The epithelium thicknesses of 9dpa and lateral side were compared using unpaired *t* test which was found to be nonsignificant.

Figure 4. Cellular and molecular details of cell proliferation during wound healing stages of tail and limb. (A) Relative transcript level expression of fgfs and pcna in tail wound healing. Fold change values for time points was normalized with that of the resting stage of tail. (B) Relative transcript level expression of fgfs and pcna during limb wound healing. Fold change values for time points was normalized by those of the resting stage of limb. Error bars represent standard error of mean and asterisk represent *p*-value where * represents $p \leq 0.05$, ** stands for $p \leq 0.01$ and *** depicts $p \leq 0.001$, ($n = 6$); (C and D) depicts Western blot of PCNA for tail and limb wound healing stages respectively; (E and F) are the images for tail at 4dpa and limb at 9dpa showing BrdU incorporation wherein orange arrowhead shows the BrdU labeling in AEC and blue arrowhead represents the epithelium which is minimally representing BrdU in limb. [Color figure can be viewed at wileyonlinelibrary.com]

FGF2 and VEGF-α, being the major molecules required in the process of angiogenesis, were localized in the tail on 4dpa and in the limb on 9dpa. It was revealed that FGF2 and VEGF-α were profusely expressed in the AEC and the underlying tissue in tail at 4dpa (Figure 6E and G), while only weak signals were obtained from the 9dpa limb (Figure 6F and H).

DISCUSSION

Lizards are amniote model of regeneration having the maximum evolutionary proximity to the mammals. Hence, there are similarities between the embryonic stages of reptiles and mammals.[16] However, lizards are capable of postembryonic development, viz., regeneration of the lost tail, which is lacked by their mammalian counterparts. Interestingly, lizards can restore only their tail but no other appendage via epimorphic regeneration.[25] Nonetheless, in order to facilitate regeneration in the tail, it needs to heal without the formation of a scar and hence, is called as scar-free wound healing. On the contrary, the amputated limb heals with a scar. Herein, an attempt was made to compare the differences in the mechanisms underlying scar-free and scarred wound healing in lizard.

In scar-free wound healing, the histology of healing tail reveals a proliferating epidermis which at 2dpa was 44.65 μm thick and was measured to be of a thickness 91.93 μm during the wound epithelium stage, i.e., at 4dpa stage. It is noticeable that the epidermis is continuously proliferating at the wound site as its thickness in the normal intact skin is only 12.52 μm. Similar results were observed by McLean and Vickaryous[25] wherein the *Eublepharis* lizard shows a newly formed wound epithelium that continues to proliferate and thicken (up to 12 cell layers thick

compared to 4–7 in the original epidermis), especially at the AEC. In other regeneration models (e.g., urodeles, teleosts) the AEC has a well-documented role, as a source of morphogenetic information quite comparable to the apical ectodermal ridge of developing limb.[26,27] However, it is now accepted that under no circumstances formation of AEC happens in case of an amputated limb. Further, it has been documented that on amputation of a limb, the blood vessels rupture and the loss of blood is more as compared to that of the tail wherein the bleeding is kept at minimum due to contraction of precapillary sphincters.[28] Moreover, because of the prolonged bleeding in limb, a thick blood clot forms at the site of injury. Not surprisingly, on 3dpa it was observed that the cut surface of the limb was covered only by a scab with no epithelial lining underneath as against the autotomy surface of tail wherein the epithelial covering was observed as early as on 1dpa. However, on completion of wound closure at 9dpa, a thick scar tissue of 200 µm covers the cut surface of limb and the overlying epidermis at this point is just 17 µm thickness, same as what used to be in a

resting epithelial layer of limb, suggesting that AEC is not formed in limb and instead scarred stump is formed.

It has been well authenticated that the wound epithelium stratifies to form AEC through regulated cell division under the influence of various putative paracrine factors.[7,29] An up-regulation in fgfs was observed, in all the time points studied, in the autotomized tail till the wound epithelium formation was accomplished at 4dpa. *fgf1* and *fgf2* transcripts were significantly higher in the healing tail and so was the FGF2 protein level, than in the resting one. Alibardi[30] in one of his studies observed positive immunolocalization for FGF2 in the epidermis of lizard tail which accords with our findings wherein FGF2 was localized in the AEC and the underlying tissue, however limb showed positive staining only in the epithelium at the wound site suggesting a barrier formation between epithelium and underlying tissue which does not allow the cells to undergo proliferation. FGF2 and FGF1 are required for the epidermis to proliferate and form the AEC.[31–34] The same trend has been reported in chick limb development as well,[35] but in case of lizard limb healing, it was observed that these factors are either absent or their levels are too low to facilitate regrowth. FGF8 has a known role in the outgrowth of the limb of chick wherein it translocates from the epidermis to the underlying mesenchymal cells.[36] In lizard tail, a steady increase of FGF8 was seen from 1dpa to 4dpa suggesting that its outgrowth must be very similar to that of the limb in a developing chick embryo. On the contrary, in the lizard limb, the absence of FGF8 may lead to stalling of outgrowth as well. In order to induce expression of FGF8, FGF10 is a prerequisite and there are ample evidences suggesting that FGF10 induces FGF8 in the ectoderm and Shh in mesoderm, proposing FGF10 to be an endogenous mesenchymal factor playing a pivotal role in initial budding and outgrowth of the vertebrate limb.[35,37,38] The evidences mentioned above justifies the results in tail wound healing, wherein an increase in transcript level of *fgf10* along with *fgf8* was observed. The lack of *fgf8* and *fgf10* in lizard's limb would be playing a crucial role in the formation of the scarred stump at the amputated end. An increase in fgf8 and fgf10 leads to proliferation which was clearly seen in the western of tail healing stages wherein a constant increase in PCNA was documented. Limb healing stages did show some increase in the protein levels of PCNA though not comparable to the tail, also their appearance was at a much later stage which might be due to collagen proliferation and deposition. To support this hypothesis, BrdU was incorporated in both tail and limb during healing and the images clearly portray that BrdU gets accumulated in the AEC and the mesenchymal cells underlying it in tail while in limb BrdU is seen only in the epithelium and the collagen deposited under it. Further evidences for proliferation and apoptosis were gathered from acridine orange and ethidium bromide stained tissue sections. The tail tissue at wound epithelium stage (4dpa) were predominated by green colored proliferating cells. On the contrary, a corresponding stage in limb (9dpa) revealed largely pro-apoptotic (yellow) and apoptotic (red) cells, indicating high apoptosis ensuing during the scar formation. Apart from these FGFs mentioned above, another major one required for sustaining the epidermis is FGF20.[39] FGF20 gets triggered by Wnt/β-catenin which in turn is regulated by ROS production. In *Xenopus* tadpole tail regeneration, higher levels of FGF20 were detected in the regenerating tissue when compared to the resting tissue.[40] In this study, tail which undergoes scar-free healing showed an increase in *fgf20* but the same was downregulated in limb again implying that the limb has an alternate path of healing which in due course leads to scarring.

Scarring is normally seen in mammalian injury, and limb healing in lizard also follows similar mechanism. The major growth factor that has a known role in scarring is the TGFβ.[41,42] One of the studies performed on human fetal skin revealed that if TGFβ1 and TGFβ2 cause scarring whereas in another study done on rat skin shows if expression of TGFβ3 in increased, the scarring reduces considerably.[43,44] This leads to an inference, that TGFβ1 and TGFβ2 have a well-defined role in fibronectin deposition which was also observed during limb healing stages. In the light of the above discussion it could be presumed that for scar-less wound healing, deposition of fibronectin is avoided and hence *tgfβ3* was found to be elevated during tail wound healing stages. Not only does TGFβ have a role in fibrosis but it also plays a major role in inducing apoptosis.[45,46] Lafon and coworkers found out that TGFβ1 induces apoptosis through c-fos and c-jun genes in human ovarian cancer cells.[47] In the current study, both TGFβ induced and p53 mediated apoptosis was studied for all the stages of healing in tail as well as limb. In tail, initially a twofold increase in *caspase 3* and *p53* was observed at 1dpa and 2dpa, however, these levels drop at 3dpa and 4dpa which also can be seen in the Cleaved caspase-3 immunoblot. In order to keep

Figure 5. Expression and localization of apoptotic markers in scar-free and scarred wound healing stages. (A) Relative transcript level expression of apoptotic markers during wound healing in tail. Fold change values for time points was normalized with those of the resting stage of tail. (B) Relative transcript level expression of apoptotic markers during wound healing in the limb. Fold change values for time points was normalized with those of the resting stage of limb. Error bars represent standard error of mean and asterisk indicates *p*-value where * shows $p \leq 0.05$, ** depicts $p \leq 0.01$ and *** stands for $p \leq 0.001$. (C and D) Represent the Western blot of cleaved caspase 3 in the wound healing stages of tail and limb respectively. (E and F) Displays IHC for cleaved caspase 3 in the tail at 4dpa shown by orange arrowhead and (G and H) represent the positive immunostaining for limb at 9dpa depicted by blue arrowhead; (I and J) denote fold change values of tgf-β in tail and limb wound healing stages where error bars exemplify standard error of mean and asterisk represent *p*-value where * stands for $p \leq 0.05$, ** shows $p \leq 0.01$ and *** illustrates $p \leq 0.001$;($n = 6$), (K–P) represent acridine orange and ethidium bromide staining wherein K and L are the tail and limb post amputation while M and N are the wound healing stages for tail and limb and their higher magnified images are O and P. The arrow heads in (O) denotes the live cells which are visible due to green fluorescence whereas arrow head in (P) depicts the pro and apoptotic cells which are orange and red in color, respectively. "*" Represents the area which is depicted in the magnified image. AEC, apical epithelial cap; B, bone; CH, chondrocytes; E, epithelium; M, Muscle; SC, spinal cord). [Color figure can be viewed at wileyonlinelibrary.com]

Figure 6. Transcript levels, Western blot and Immunolocalization of angiogenesis markers. (A) Relative transcript level expression of vegf-α and kdr in tail wound healing. Fold change values for time points was normalized with those of the resting stage of tail. (B) Relative transcript level expression of vegf-α and kdr in limb wound healing. Fold change values for time points was normalized with the values of the resting stage of limb. Error bars represent standard error of mean and asterisk depicts *p* value where * highlights $p \leq 0.05$, ** marks $p \leq 0.01$ and *** stands for $p \leq 0.001$; ($n = 6$) (C and D) Western blot of VEGF-α, FGF2, PI3K, and Akt for wound healing stages of tail and limb respectively, where β-actin was taken as the loading control; (E) indicates FGF2 localization in the AEC of tail at wound epithelium stage (orange arrowhead) and (F) indicates faint localization signals of FGF2 in the limb healing stage (blue arrowhead). (G) Localization of VEGF-α in the proliferating epidermis and the underlying tissue (orange arrowhead) while it was present only in the epithelium of limb in a scattered manner (blue arrowhead). [Color figure can be viewed at wileyonlinelibrary.com]

the tail growing, proliferation has to surpass apoptosis which was observed in this study wherein *p53*, *p21*, *bad*, *bax* and even the TGFβs were downregulated. Limb healing showed higher levels of p53 and p21 only at 9dpa but a 200-fold surge was noted in bad expression at 6dpa. This is because in scarred wound healing at granulation phase it is observed that the myofibroblasts disappear, leaving behind the scar.[48] Apoptosis is a prerequisite for the entire mechanism and thus higher levels of bad at 6dpa are justified.

Granulation phase is one of the important hallmarks of scarred wound healing.[49] During this stage, angiogenesis occurs and also fibroblast keep adding collagen, which eventually leads to scarring. At 6dpa in limb, both *vegf-α* and *kdr* were seen to be high, again confirming that granulation might be starting at this point. In this phase, along with angiogenesis even deposition of collagen occurs which is induced by *tgfβ1* and *tgfβ2*. It is important to note that angiogenesis is required even for the successful realization of scar-free wound healing in tail, however the granulation phase here is highly truncated. In tail stump, the proliferating mesenchymal stem cells are maintained due to the virtue of high levels of *vegf-α* and *kdr*, which play a pivotal role in formation of blood vessels. VEGF-α was found to be localized in the AEC and more so in the underlying tissue from where actually the signals might be coming. Moreover, in case of tail, throughout all the time points, angiogenesis was observed, but it was noted in limb only at 6dpa which was confirmed at protein level by Western blot analysis. At 9dpa though, as the scar matures, all the blood vessels retract, leaving behind only the fibrous tissue. To further validate this pathway, PI3K and Akt levels were checked for all the time point as these are the key molecules required for

Figure 7. Cellular processes and the molecules involved in scar-free and scarred wound healing in *H. flaviviridis*. [Color figure can be viewed at wileyonlinelibrary.com]

signaling. Both PI3K and Akt protein levels were constantly elevated in the tail during the span of 4dpa while their levels were elevated in limb only at 6dpa again confirming granulation to be occurring at this time point. Thus, with this it makes the events in which the healing is occurring quite clear for both scar-free and scarred wound healing.

Other studies carried out in lizard tail and limb healing are focused on specific stages of regeneration like wound healing and blastema in tail and scarring in limb. For instance, a study by Vitulo et al.[21] in *Podarcis muralis* analyses the transcriptomic expression during tail and limb healing but specifically at the wound healing and blastema stages. The study did recognize wnt and fgfs for playing major roles in blastema recruitment which ultimately leads to regeneration of tail and failure of limb formation. Another study by Alibardi[22] in *Podarcis sicula* reveals that cell proliferation is reduced in limb healing as compared to tail, an observation which coincides with the present study. Nevertheless, the results presented in the current work are based on a temporal expression pattern of some of the key molecules which are required to regulate various cellular events that facilitate the successful realization of the major milestones in the process of wound healing which has not yet been carried out in any of the lizard models. Through this study it can be construed that the central events like proliferation, apoptosis, and angiogenesis do occur in both scar-free as well as in scarred wound healing, but the duration for which they last and the point at which these are patterned, differs and this difference causes the tail to follow scar free healing, while the limb undergoes the scarred wound healing process.

CONCLUSION

Lizards achieve the wound epithelium stage at 4dpa whereas the limb requires 9dpa for full wound closure. Various processes and signaling molecules play an important role in deciding the course of healing. In this study, it was observed that proliferation of epidermal cells and the mesenchymal cells is triggered as soon as there is an injury to the tail, however the limb shows proliferation only of the fibroblast cells at a later stage of healing. Various fgf genes were upregulated in order to form the proliferating epidermis which eventually forms AEC at 4dpa in tail. The proliferation of epidermal cells follows the activation of PI3K-Akt signaling pathway as their levels were found to be elevated along with fgfs. Instead of proliferation at an early stage, limb showed apoptosis to be persistent for a longer duration wherein the expression of bad and caspase 3 were seen at an early stage at 3dpa and caspase remained high till later stage along with p53 and p21 which is triggered by the p38-MAPK pathway as evident from the result. Apoptosis was apparent at 1dpa in tail but it was subsequently found to be downregulated. Apart from fgfs, tgf-β members are also a pre-requisite in wound healing and the results suggest that tgf-β1 and tgf-β2 are involved in the scarring and apoptosis as their levels were significantly high in the stages of limb wound healing. In contrast to this, tgf-β3 promotes scar-free wound healing which accorded with the results obtained from the tail wound healing stages. Apart from these two processes, angiogenesis which is the primary requirement to sustain the proliferation was also recorded in both the wound healing. In tail, angiogenesis occurred at an early stage to provide a proliferating environment for the epidermis while in limb at a very late stage this process was observed. Hence vegf-α levels were elevated throughout the process of wound healing in tail, these levels shot up only at 6dpa, which is the granulation phase in the limb which eventually leads to scarring. Thus, all these three processes depicted in Figure 7, play a major role in scar-free and scarred wound healing. Even though they all occur in both the wound healing their occurrence and duration are quite different which may be the reason which allows the tail to heal in scar-free manner and regain it completely whereas the limb undergoes scarred wound healing which ultimately forms a stump with no functionality.

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REFERENCES

1. Gurtner GC, Werner S, Barrando Y, Longaker MT. Wound repair and regeneration. *Eur Surg Res* 2012; 49: 35–43.
2. Jackson WM, Nesti LJ, Tuan RS. Mesenchymal stem cell therapy for attenuation of scar formation during wound healing. *Stem Cell Res Ther* 2012; 3: 20.
3. Ferguson MW, O'Kane S. Scar–free healing: from embryonic mechanisms to adult therapeutic intervention. *Philos Trans R Soc B Biol Sci* 2004; 359: 839–50.
4. Gurtner GC, Werner S, Barrando Y, Longaker MT. Wound repair and regeneration. *Nature* 2008; 453: 314–21.
5. Godwin JW, Rosenthal N. Scar-free wound healing and regeneration in amphibians: immunological influences on regenerative success. *Differentiation* 2014; 87: 66–75.
6. Sonnemann KJ, Bement WM. Wound repair: toward understanding and integration of single-cell and multicellular wound responses. *Annu Rev Cell Dev Biol* 2011; 27: 237–63.
7. Grose R, Werner S, Kessler D, Tuckermann J, Huggel K, Durka S et al. A role for endogenous glucocorticoids in wound repair. *EMBO Rep* 2002; 3: 575–82.
8. Gillitzer R, Goebeler M. Chemokines in cutaneous wound healing. *J Leukoc Biol* 2001; 69: 513–21.
9. Madala SK, Pesce JT, Ramalingam TR, Wilson MS, Minnicozzi S, Cheever AW et al. Matrix metalloproteinase 12-deficiency augments extracellular matrix degrading metalloproteinases and attenuates IL-13–dependent fibrosis. *J Immunol* 2010; 184: 3955–63.
10. Mutsaers SE, Bishop JE, McGrouther G, Laurent GJ. Mechanisms of tissue repair: from wound healing to fibrosis. *Int J Biochem Cell Biol* 1997; 29: 5–17.
11. Enoch S, Harding K. Wound bed preparation: the science behind the removal of barriers to healing. *Wounds* 2003; 15: 213–29.
12. Carlson BM. Tissue interactions in regeneration. In: Carlson BM, ed. *Principles of regenerative biology*, Philadelphia: Elsevier. 2011; 93–109.
13. Murawala H, Ranadive I, Patel S, Desai I, Balakrishnan S. Protein expression pattern and analysis of differentially expressed peptides during various stages of tail regeneration in *Hemidactylus flaviviridis*. *Mech Dev* 2018; 150: 1–9.
14. Diegelmann RF, Evans MC. Wound healing: an overview of acute, fibrotic and delayed healing. *Front Biosci* 2004; 9: 283–9.
15. Alibardi L. *Morphological and cellular aspects of tail and limb regeneration in lizards: a model system with implications for tissue regeneration in mammals*. Berlin: Springer Science & Business Media, 2009.
16. Lozito TP, Tuan RS. Lizard tail skeletal regeneration combines aspects of fracture healing and blastema-based regeneration. *Development* 2016; 143: 2946–57.
17. Penn JW, Grobbelaar AO, Rolfe KJ. The role of the TGF-β family in wound healing, burns and scarring: a review. *Int J Burns Trauma* 2012; 2: 18–28.
18. Makanae A, Mitogawa K, Satoh A. Cooperative inputs of Bmp and Fgf signaling induce tail regeneration in urodele amphibians. *Dev Biol* 2016; 410: 45–55.
19. Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. *Wound Repair Regen* 2008; 16: 585–601.
20. Matsumoto K, Ema M. Roles of VEGF-A signalling in development, regeneration, and tumours. *J Biochem* 2014; 156: 1–10.
21. Vitulo N, Dalla Valle L, Skobo T, Valle G, Alibardi L. Transcriptome analysis of the regenerating tail vs. the scarring limb in lizard reveals pathways leading to successful vs. unsuccessful organ regeneration in amniotes. *Dev Dyn* 2017; 246: 116–34.
22. Alibardi L. Ultrastructural features of the process of wound healing after tail and limb amputation in lizard. *Acta Zool* 2010; 91: 306–18.
23. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; 72: 248–54.
24. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *Methods* 2001; 25: 402–8.
25. McLean KE, Vickaryous MK. A novel amniote model of epimorphic regeneration: the leopard gecko, *Eublepharis macularius*. *BMC Dev Biol* 2011; 11: 50.
26. Dinsmore CE. Tail regeneration in the plethodontid salamander, *Plethodon cinereus*: induced autotomy versus surgical amputation. *J Exp Zool A Ecol Genet Physiol* 1977; 199: 163–75.
27. Poss KD, Keating MT, Nechiporuk A. Tales of regeneration in zebrafish. *Dev Dyn* 2003; 226: 202–10.
28. Delorme SL, Lungu IM, Vickaryous MK. Scar-free wound healing and regeneration following tail loss in the leopard gecko, *Eublepharis macularius*. *Anat Rec* 2012; 295: 1575–95.
29. Mescher AL. The cellular basis of limb regeneration in urodeles. *Int J Dev Biol* 2004; 40: 785–95.
30. Alibardi L. Observations on FGF immunoreactivity in the regenerating tail blastema, and in the limb and tail scars of lizard suggest that FGFs are required for regeneration. *Belg J Zool* 2012; 142: 23–28.
31. Han M, Yang X, Taylor G, Burdsal CA, Anderson RA, Muneoka K. Limb regeneration in higher vertebrates: developing a roadmap. *Anat Rec* 2005; 287: 14–24.
32. Alibardi L, Lovicu FJ. Immunolocalization of FGF1 and FGF2 in the regenerating tail of the lizard *Lampropholis guichenoti*: implications for FGFs as trophic factors in lizard tail regeneration. *Acta Histochem* 2010; 112: 459–73.
33. Yadav M, Anusree P, Desai I, Suresh B. Influence of extraneous FGF-2 and its antagonist anti-FGF-2 on the progress of tail regeneration in *Hemidactylus flaviviridis*. *Indian J Fundam Appl Life Sci* 2012; 2: 164–72.
34. Pillai A, Desai I, Balakrishnan S. Pharmacological inhibition of FGFR1 signaling attenuates the progression of tail regeneration in the northern house gecko *Hemidactylus flaviviridis*. *Int J Life Sci Biotechnol Pharma Res* 2013; 2: 263–78.
35. Ornitz DM, Itoh N. An introduction to the fibroblast growth factors. In: Simons M, ed. *Fibroblast growth factors: biology and clinical application-Fgf biology and therapeutics*, Singapore: World Scientific Publishing Co., Inc. 2016; 1–39.
36. Han MJ, An JY, Kim WS. Expression patterns of Fgf-8 during development and limb regeneration of the axolotl. *Dev Dyn* 2001; 220: 40–8.
37. Ohuchi H, Nakagawa T, Yamamoto A, Araga A, Ohata T, Ishimaru Y et al. The mesenchymal factor, FGF10, initiates and maintains the outgrowth of the chick limb bud through

interaction with FGF8, an apical ectodermal factor. *Development* 1997; 124: 2235–44.
38. Xu X, Weinstein M, Li C, Naski M, Cohen RI, Ornitz DM et al. Fibroblast growth factor receptor 2 (FGFR2)-mediated reciprocal regulation loop between FGF8 and FGF10 is essential for limb induction. *Development* 1998; 125: 753–65.
39. Whitehead GG, Makino S, Lien CL, Keating MT. fgf20 is essential for initiating zebrafish fin regeneration. *Science* 2005; 310: 1957–60.
40. Love NR, Chen Y, Ishibashi S, Kritsiligkou P, Lea R, Koh Y et al. Amputation-induced reactive oxygen species are required for successful *Xenopus* tadpole tail regeneration. *Nat Cell Biol* 2013; 15: 222–8.
41. Klass BR, Grobbelaar AO, Rolfe KJ. Transforming growth factor β1 signalling, wound healing and repair: a multifunctional cytokine with clinical implications for wound repair, a delicate balance. *Postgrad Med J* 2009; 85: 9–14.
42. Kiritsi D, Nyström A. The role of TGFβ in wound healing pathologies. *Mechanisms of ageing and development* 2017; 172: 51–58.
43. Soo C, Beanes SR, Hu FY, Zhang X, Dang C, Chang G et al. Ontogenetic transition in fetal wound transforming growth factor-β regulation correlates with collagen organization. *Am J Pathol* 2003; 163: 2459–76.
44. Larson BJ, Longaker MT, Lorenz HP. Scarless fetal wound healing: a basic science review. *Plast Reconstr Surg* 2010; 126: 1172–80.
45. Perlman R, Schiemann WP, Brooks MW, Lodish HF, Weinberg RA. TGF-β-induced apoptosis is mediated by the adapter protein Daxx that facilitates JNK activation. *Nat Cell Biol* 2001; 3: 708–14.
46. Li Y, Liu Y, Fu Y, Wei T, Le Guyader L, Gao G et al. The triggering of apoptosis in macrophages by pristine graphene through the MAPK and TGF-beta signaling pathways. *Biomaterials* 2012; 33: 402–11.
47. Lafon C, Mathieu C, Guerrin M, Pierre O, Vidal S, Valette A. Transforming growth factor beta 1-induced apoptosis in human ovarian carcinoma cells: protection by the antioxidant N-acetylcysteine and bcl-2. *Cell Growth Differ* 1996; 7: 1095–104.
48. Desmouliere A, Redard M, Darby I, Gabbiani G. Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar. *Am J Pathol* 1995; 146: 56–66.
49. Sorg H, Tilkorn DJ, Hager S, Hauser J, Mirastschijski U. Skin wound healing: an update on the current knowledge and concepts. *Eur Surg Res* 2017; 58: 81–94.

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Cyclooxygenase-2 interacts with MMP and FGF pathways to promote epimorphic regeneration in lizard *Hemidactylus flaviviridis*

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ABSTRACT
Cyclooxygenase-2 (COX-2) is an inducible enzyme known for its role in promoting inflammation, pain and cancer. It has more recently been attributed a function in epimorphic regeneration of vertebrate appendages. However, its position among the molecular regulators of regeneration remains unclear. This work was aimed at analyzing the influence of COX-2 on critical mediators of regenerative processes in the lizard *Hemidactylus flaviviridis*. It was found during the early events of regeneration that MMP and FGF genes get altered in their expression in response to administration of etoricoxib, a COX-2 inhibitor. Results herein also reflect a positive correlation between COX-2 activity and gelatinase activities in our system. These observations, for the first time, establish a definitive interaction of the COX-2 signal with the MMPs and FGFs as essential to the initiation of tail regeneration, placing it as one of the top regulators of the molecular events which characterize epimorphosis.

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Introduction

Epimorphic regeneration enables some animals to restore partially or entirely lost appendages in a manner that seems far-fetched for humans to achieve. To understand why an amputation in different organisms leads towards different fates, we must have a clear picture of the mechanism underlying regenerative and non-regenerative healing and how they are different. Although there exists a reasonable depth of understanding about the events of mammalian wound healing, the same cannot be said for epimorphosis. It must be mentioned here that while the later stages of limb regeneration share many similarities with embryonic limb development (Bryant & Gardiner, 1992), it is the initial stages which need to be dissected to gain an insight into the regenerative processes.

Tail regeneration in urodele amphibians and geckos begins with migration of epithelial cells over the amputation wound to form a wound epithelium, which later matures into a multi-layered apical epithelial cap (AEC). Factors released from the AEC towards the underlying mesenchyme are critical for formation and development of the regeneration blastema (reviewed by Stocum, 1995), which is an accumulation of multipotent progenitor cells, and is the distinguishing feature of appendage regeneration through epimorphosis. One of the critical molecules proposed by our lab as essential for wound epithelium and blastema formation is the cyclooxygenase-2 (COX-2).

The role of COX-2 as a mediator of epimorphosis has only recently emerged and studies from our lab have described its importance in a variety of cellular processes seen during wound healing and blastema formation (Suresh et al., 2009). A mechanistic understanding is however lacking and the molecular pathways acting under the influence of COX-2 have not been identified. The present work was envisaged with a view to elucidate the interactions of the COX-2 pathway during tail regeneration in lizard *Hemidactylus flaviviridis*. Etoricoxib, a pharmacological inhibitor of COX-2 enzyme, was used to study *in vivo* the effect of COX-2 inhibition on the MMP and FGF pathways in the regenerating gecko tail. Results presented herein may help explain the negative effects of COX-2 inhibition on regeneration, as reported by us earlier (Buch et al., 2017; Sharma & Suresh, 2008).

Materials and methods

Animal procurement and maintenance

Northern house geckos *H. flaviviridis* were collected from the urban habitat and used for the study.

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Protein expression pattern and analysis of differentially expressed peptides during various stages of tail regeneration in *Hemidactylus flaviviridis*

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ABSTRACT

Epimorphic regeneration is a process allowing the animal to regain its lost structure which depends on the resident pluripotent stem cells as well as de-differentiation of existing cells to form multi-potent stem cells. Many studies have been done to understand the appendage regeneration mechanism. The animal model used since decades is an urodele amphibian the axolotl. However, this ability is also seen in some members of reptiles, mainly lizards which on autotomy of tail regain the same by forming a replica of its lost tail. Lizards being closer to mammals are of greater interest and cannot be neglected. Hence, a stage specific protein profiling was undertaken in order to find the peptides playing a major role in epimorphosis. 2-DGE being basic tool for creating a protein profile was used. With advent of newer modern technology, label-free analysis which uses MS/MS was also performed. The study reports the peptides involved in apoptosis, inflammation and ECM remodelling across the stages of lizard tail regeneration for the first time. Apart from these peptides, structural protein, enzymes involved in metabolism have also been highlighted in the current study to give a bigger picture of the processes and the specific peptides required for tail regeneration.

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1. Introduction

Over the years several studies have been carried out in various models of regeneration to unravel the finer mechanisms of epimorphic regeneration (Poss et al., 2000a; Brockes et al., 2001). Initial studies focused on the morphological, histological and biochemical changes associated with regeneration (Zika, 1969; Alibardi, 2009; McLean and Vickaryous, 2011). However, with the advent of modern analytical tools, the focus of the study was lately shifted to understand the cellular and molecular mechanisms of regeneration. This led to the understanding of roles played by some of the critical signalling molecules during regeneration like FGF, Wnt, BMP, Shh and many more (Riddle et al., 1993; Poss et al., 2000b; Quint et al., 2002; Jaźwińska et al., 2007; Tal et al., 2010). Nonetheless, all the cellular and molecular processes involve a controlled expression of these signalling molecules at various combination and concentrations. It is well understood that these signals facilitate various cellular events like proliferation, apoptosis, and morphogenesis during development. Epimorphosis too involves coordination of these above mentioned cellular events which takes place in a coherent manner. However, to manage each step, it inevitably requires a large scale of protein turnover. Despite their significance in the regulation of a myriad of cellular events which are critical for the initiation and progression of epimorphosis, protein turnover during regeneration has attracted only minimum research interest. Moreover, their interaction with each other and how they modulate the stage specific physiological events during regeneration remains to be unfolded. Thus there is an immense need to identify the proteins which govern the process of epimorphic regeneration.

Proteomic studies in the field of regeneration were initiated by Slack in 1982 (Slack, 1982). He studied the protein expression pattern during few stages of axolotl limb regeneration both by 2-D gel electrophoresis and by histochemistry. Moreover, a proteomic study of axolotl regenerating limb was carried out with quantitative label-free liquid chromatography and tandem mass spectrometry by Rao in 2009 (Rao et al., 2009). They have identified 309 proteins with significant fold change in comparison to resting stage, which is involved in different biological processes. Subsequently, few other proteomics studies have also been carried out. Rao in 2014 (Rao et al., 2014) attempted a comparative analysis of fibroblastema formation in the regenerating limb of *Xenopus laevis* froglet and axolotl (2014). In 2009, King (King et al., 2009) compared the profiles of abundant proteins in larval limbs of the *Xenopus laevis* at the time of amputation (0 day post-amputation – 0dpa) and later at 3dpa, when the limb reached the blastema stage. They have observed a total of 1517 peptides, of which 1067 were identified and amongst them, 489 showed a significant change in their level

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