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Comparative proteomic analysis of lizards' tail and limb during wound healing phase

INTRODUCTION

There have been multiple studies carried out in various models of regeneration like hydra, planaria, zebrafish, axolotls, etc. to understand the detailed mechanism of regeneration (Hobmayer et al., 2000; Poss et al., 2000; Brockes et al., 2001; Reddien and Alvarado, 2004). Several decades back in the absence of the technological advancements, the studies mainly focused on the histological and biochemical changes associated with regeneration (Zika, 1969). Now in this modern era with all the cutting-edge technology available, most studies have shifted to unearth the cellular and molecular basis of regeneration. Nowadays, studies have been initiated to elucidate the transcriptome and proteome profiles so that analysis of expression of all the genes, peptides, and transcription factors for different phases or events during regeneration is easily possible. Amongst all the cellular and molecular studies, proteomic study is a valuable tool which unveils the final products which are the peptides that regulate all the biological processes including regeneration. 2-Dimensional gel electrophoresis (2-DGE) is one such tool which aids in identifying peptides and the method adopted in the current study is represented in the Figure 3.1. In the recent past, few attempts have been made to understand the proteomic profile in regeneration models which aims at finding peptides that regulate regeneration process (Knapp et al., 2013; Looso et al., 2013; Wu et al., 2013; Hui et al., 2014).

The model organism under study is *Hemidactylus flaviviridis,* and in our lab, earlier an attempt was made to create a comparative proteome profile by performing 2-Dimensional gel electrophoresis for various stages of tail regeneration (Murawala et al., 2018). The result from 2-DGE revealed many peptides involved in the energy metabolism; however, the peptides remained unaltered throughout the stages of regeneration depicting their constant need during the regrowth of the tissue. However, peptides involved in proliferation remained high throughout the process of regeneration. Moreover, it was observed that ECM remodelling molecules gradually increased from the wound epithelium stage to the blastema stage, allowing the proliferation of cells. Levels of peptides involved in inflammation decreased after an initial surge during regeneration. Furthermore, during the wound epithelium and blastema stages, anti-apoptotic peptides were upregulated with a concomitant reduction in the expression of pro-apoptotic peptides possibly to sustain proliferation (Murawala et al., 2018). However, the above referred study was limited to the regeneration stages (i.e. Wound Epithelium and Blastema stages) of the tail and the current study focusses on both the tail and limb wound healing patterns at finer time-points. The importance of studying both the healing pattern is explained in the following paragraph.

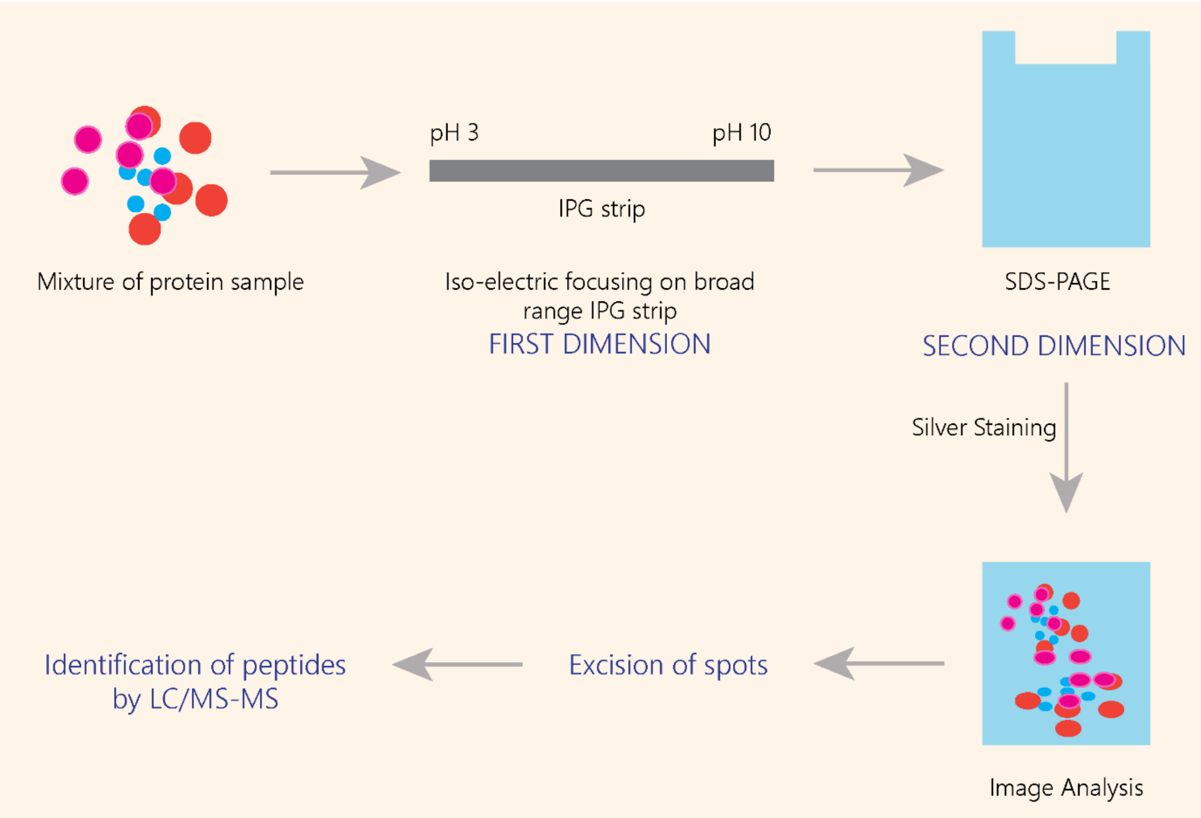


Figure 3.1: 2-Dimensional gel electrophoresis method depicted in a pictorial form

Wounding or amputation of organs or body parts in amniotes eventually leads to limited recovery of injured tissue. Most of the times, this recovery involves the formation of scarred tissue (Ferguson and O'Kane, 2004). Regeneration of a single tissue might still be possible, but regeneration of a functionally perfect novel organ is very rare (Tsonis, 2002; Ferguson and O'Kane, 2004; Carlson and Conboy, 2007; Stocum and Zupanc, 2008). An exception to this fact is the tail of a lizard which has tissue complexity very similar to the mammals yet can regenerate all the tissues on amputation. Not only does lizard regenerate all the structures in the tail, but the functionality of the tail is also restored (Bellairs and Bryant, 1985; Alibardi, 2010, 2014). However, the limbs of lizard are not endowed with this ability of regeneration and hence, discovering the mechanisms by which regeneration is elicited in one structure and not in another in the same organism is very rare opportunity (Marcucci, 1930; Barber, 1944; Alibardi, 2010). Due to this, lizards' present a unique opportunity to study the mechanism of regeneration and scarred wound healing in tail and limb, respectively. In lizard a comparison between tail and limb was studied at the transcriptome level by Vitulo and coworkers (2017), nonetheless, along with this data available, proteome data would give additional information regarding the transient molecules which will not be able to trace in a transcriptome study. Since it was mentioned that with the advanced technology being now available a need to study the comparative protein profile of both tail and limb is required, one must know how much information is available regarding other regenerating organisms in terms of proteomics study. Hence, below are a few studies listed which gives an idea about the current knowledge available in the field of proteomics, specifically in regeneration.

Axolotl limb regeneration was chosen for the first study involving 2-Dimensional gel electrophoresis. This study was initiated back in 1982 by Slack. He identified seven proteins during the early stages of regeneration, which were specific to the mesenchyme and thirteen to the epidermis. He did not find much of a difference in the gel pattern until the onset of the cytodifferentiation wherein the muscle and cartilage become substantially different and help in the blastemal mesenchyme formation. Another study was carried out by Rao and colleagues (2009) in the regenerating limb of axolotl by using label-free liquid chromatography where they could obtain around 309 proteins which were differentially expressed in different stages of regeneration when compared to the resting stage. The studies were not only directed towards the proteomics, but an attempt was made towards identifying the network-based transcription factor analysis of the regenerating axolotl limb (Jhamb et al., 2011). To investigate the potential proteome during limb regeneration of Chinese fire-bellied newt, two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) and mass spectrum (MS) was applied to examine the temporal proteome profile changes by deciding upon 11 time points (from 0 hours post amputation (hpa) to 72 hpa) after amputation. Meanwhile, several proteins were selected to validate their expression levels by western blot. The results revealed that 1476 proteins had significantly changed as compared to the control group. Gene ontology annotation and protein network analysis by Ingenuity Pathway Analysis 9.0 (IPA) software recommended that there were many peptides which were differentially expressed and were involved in 33 kinds of physiological activities including signal transduction, cell proliferation, cell differentiation, etc. Among these proteins, 407 proteins participated in cell differentiation with 212 proteins in the differentiation of skin cell, myocyte, neurocyte, chondrocyte and osteocyte (Geng et al., 2014). In 2009, King et al. compared the profiles of abundant proteins in larval limbs of the *Xenopus laevis* at the time of amputation (0 day post-amputation – 0 dpa) and later at 3 dpa when the limb reached the blastema stage. They had observed a total of 1517 peptides, of which 1067 were identified and amongst them, 489 showed a significant change in their level of expression between the two groups. Additionally, they focused on proteins which were found elevated at 3 dpa and found heightened expression of proteins belonging to the members of annexin family which are known for their immunoregulatory roles. Furthermore, they also found keratins that may facilitate cytoprotection and growth regulation, possibly at the time of wound closure. Another study performed by Rao and colleagues (2014) on the limb regeneration of axolotl revealed many differentially expressed proteins. They identified 309 unique proteins with significant fold change relative to controls (0 dpa), representing 10 biological process categories: (1) signalling, (2) Ca^{2+} binding and translocation, (3) transcription, (4) translation, (5) cytoskeleton, (6) extracellular matrix (ECM), (7) metabolism, (8) cell protection, (9) degradation and (10) cell cycle. Amongst those 309 peptides, 43 proteins belonging to the aforementioned processes exhibited exceptionally higher fold change values. Of these, the ecotropic viral integration factor 5 (EVI5), a cell cycle-related oncoprotein that prevents cells from entering the mitotic phase of the cell cycle prematurely, was of particular interest because its fold change was exceptionally high throughout blastema formation. Saxena and co-workers (2012) explored the essential proteins which are expressed during the caudal fin regeneration of zebrafish. The spectrum of changes in proteome during regeneration were analysed among controls (0 hpa) and 1, 12, 24, 48, and 72 hpa post-amputation involving quantitative differential proteomics analysis based on two-dimensional gel electrophoresis. In their study, they found around 96 proteins which were differentially expressed with approximately 1.5 fold change between the resting and the regenerating caudal fin. 35 proteins depicted differential expression amongst the 5-time points selected during the caudal fin regeneration. This does point towards the fact that there are many peptides which are expressed during multiple time points in the ongoing process of regeneration, but there are other proteins as well which are expressed transiently at a particular time point. Hence, rather than stage-specific studies, time-point based proteomic studies reveal more peptides which regulate the caudal fin regeneration. In their study the proteins identified belonged to various molecular, biological, and cellular processes.

As mentioned previously, since Murawala et al. (2018) studied the stage-specific proteome profile in *Hemidactylus flavivirdis,* there was a need to consider in between time-points ranging from resting tail tissue till the early stages of epimorphosis for finer insight into the process. Apart from the tail, understanding the mechanism behind the scarring in limb would help in enlightening the reason behind failure of regeneration. Therefore, the current study focused on identifying the peptides which are differentially expressed in the healing tail and limb in order to unravel the molecular mechanism behind scar-free and scarred wound healing respectively.

MATERIALS AND METHODS

Animal Maintenance

Healthy adult northern house geckos of either sex having average snout to vent length of 10 cm were collected from their natural habitat, screened for external infestations and the healthy ones were selected for the study. Subsequently, animals were acclimatized for a week and kept in well ventilated wooden cages housed in a room. Temperature, humidity and photoperiod of the experimental room were maintained as follows 36 ± 2 °C, 40-70 % RH and 12:12 light to dark cycle. Adequate food and water were ensured daily in addition to strict adherence to cleanliness. Autotomy of tail and amputaion of limb were performed as described in Chapter-2 . The experimental protocol (MSU-Z/IAEC/15-2017) was approved by the IAEC and all the experiments were performed as per the guidelines of 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 and blastema formation in the tail while scarring in the limb. These have been characterized for the tail through the course of various studies in our lab (Buch et al., 2018, 2018; Murawala et al., 2018; Ranadive et al., 2018). It has been observed by both histological and morphological studies that the tail healing starts with the formation of epithelial covering by 2-3 dpa and apical epithelial cap (AEC) forms by 4 dpa followed by blastema formation at 6 dpa. Since the current study focussed on healing events starting autotomy till blastema formation, the following time point was used, 0 dpa (representing uncut resting stage), 3 dpa and 4 dpa and 6 dpa.

In contrast, in the selected housing condition, the amputated limb of lizard is healed only on 9 dpa. The result of a pilot time lapse study revealed that the events occurring in the healing limb are quite different from that of the tail. Similar report by Vitulo et al., (2017) from their work conducted on a related species of lizard consolidates the current result. It was observed that haemostasis phase in case of an 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 the granulation phase. Proper scarring however, is achieved only on 9 dpa and continues till 12 dpa after which only maturation of the scar is observed. Hence, the time points selected for limb were 0 dpa (representing uncut resting stage), 6 dpa, 9 dpa, and 12 dpa.

The study composed of eight groups in total - four groups in case of the tail and four groups for limb. Each group consisted of six lizards for individual experiments. The tissues were processed as per the requirement described in the following methodology.

Tissue Processing for 2-D Gel electrophoresis

- <u>Lysis</u>: The tissue was collected in chilled lysis buffer and was homogenized using mortar pestle. Following the 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.
- <u>Precipitation</u>: On collecting the supernatant, it was precipitated by chloroform-methanol method for further use. After precipitating the protein, the precipitate was dissolved in the rehydration buffer, which is also called as sample buffer. The protein was estimated by the Bradford method (Bradford, 1976).

2-Dimensional Gel Electrophoresis

- <u>Rehydration</u>: 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 overlaid with mineral oil to prevent dehydration.

- <u>Focusing</u>: 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 program.

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 got over, 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 was electrophoresed at 200 V for 30 min. On finishing the run, gel was stained with silver staining method as mentioned in the staining method (Materials and Methods, Chapter 2).

Analysis in the PDQuest Software

- Image Acquisition

  Image acquisition for the gels was done in the Bio-Rad's densitometer. After capturing the gel images, further analysis of the gels was performed using PDQuest software.
- Image Sizing and Orientation

  In this step, the size and orientation of the image was adjusted using the cropping and rotating tools on the image menu.
- Spot Identification

  Once the images were cropped, spot identification was initiated. There is a spot detection wizard which automates the process of selecting the spots. Spots were also selected manually which were not selected by the tool. Using the Wizard, we decided various parameters to incorporate all the spots in the study. The three-dimensional Gaussian spots were created from the clarified spots. The end result was three separate images: the original unaltered scan (2-D Scan), the filtered and processed scan (Filtered image), and a synthetic image containing the Gaussian spots (Gaussian image).

- Matching and Editing

  After detecting the spots in all the gels, a MatchSet was made. A MatchSet is composed of the Raw 2-D, Filtered and Gaussian images of the gels in an experiment. In the MatchSet, the protein spots from the different gels were matched to each other and were included in a synthetic image called a MatchSet master. The master image consists of all the information about the spots in the MatchSet. Once the Master gel was prepared, the faint spot and the largest spot were selected and designated appropriately. This step is very crucial for measuring the intensity of all the other spots.

- Data Analysis

  Once all the above steps were completed, in advance settings, the sensitivity for detecting the spots was kept at 14 and size scale was set at 3. After all the spots were labelled, normalization and local regression was performed wherein fold change was set at minimum 4 for the upregulated peptides and 0.2 for the downregulated peptides.

- Scatter Plot tool

  The Scatter Plot Tool shows the relatedness of two gels (value near 1, for two gels, is considered to be highly related), replicate groups, or classes in a MatchSet. It can also be used to print scatter plots for all the gels, replicate groups and classes in a MatchSet, or a selected analysis set in a MatchSet. Scatter plots for all 3 dpa, 4 dpa, and 6 dpa were plotted against 0 dpa gel for the tail and for limb 6 dpa, 9 dpa, and 12 dpa was plotted against respective 0 dpa.

LC-MS/MS

Selected spots were excised out and sent to proteomics facility of C-CAMP (Centre for Cellular and Molecular Platforms), Bangalore for the peptide identification and further characterization. Samples were subjected to in-gel digestion followed by1D nano-LC setup connected to the mass spectrometer. For the detection of the ions, LTQ Orbitrap Discovery was used. Data were recorded and searched on the MASCOT search engine with the available database SWISS-PROT and TrEMBL of *Anolis carolinensis*. The database was searched against the *Anolis* database because of want of peptide database for *Hemidactylus sp*. Based on the data obtained, the heat map was generated using GraphPad Prism 7.0

RESULTS

2-Dimensional Gel Electrophoresis

In total 8 samples were separated on the 2D IPG strips with a range of pH3 to pH10, following which the spots were further separated on a 12 % SDS-PAGE. The samples for tail included 0, 3, 4, and 6 dpa whereas, for limb 0, 6, 9, and 12 dpa were chosen. Four representative images of the tail at all the time-points mentioned above have been depicted in Figure 3.2 (A-D) and that of the limb in Figure 3.3 (A-D). Upon obtaining the gel images, using PDQuest software firstly the total number of spots in the gel was obtained, and then the gels were superimposed with the resting stage. By superimposing the images, we obtained a number of peptides in the form of spots which were differentially expressed, and few peptides which were expressed only in certain time points. The results revealed more than 250 spots in all the selected tissues of tail and limb which have been summarised in the Table 3.1. In resting tail and in the subsequent healing stages, 296 (0 dpa), 302 (3 dpa), 325 (4 dpa) and 319 (6 dpa) spots were identified. On the other hand, in limb, resting tissue depicted 286 spots followed by 299 spots at 6 dpa, 315 at 9 dpa and 309 spots at 12 dpa.

After attaining all the spots, multiple comparisons were made between various stages of wound healing with their respective resting tissue for both the appendages studied. Venn diagrams were plotted which depicts the number of spots shared between 0 dpa and 3, 4, 6 dpa in case of the tail and 0 dpa of the limb with 6, 9, and 12 dpa. The Venn diagram showed 98 spots shared between 0 dpa and 3 dpa (Figure 3.4 A), 102 between 0 dpa and 4 dpa (Figure 3.4 B) while 106 spots were shared between 0 dpa and 6 dpa in lizards' tail (Figure 3.4C). In limb 92 spots were found shared between 0 dpa and 6 dpa (Figure 3.5 A), 103 between 0 dpa and 9 dpa (Figure 3.5 B) and 98 spots were found shared between 0 dpa and 12 dpa (Figure 3.5 C).

Spot Analysis

Upon obtaining the shared spots between different stages and the resting stage, the correlation between these spots were analysed. The correlation graph was made in the PDQuest software. The graphs generated gave 0.96, 0.94 and 0.97 correlation value for 3 dpa vs 0 dpa (Figure 3.6 A), 4 dpa vs 0 dpa (Figure 3.6 B) and 6 dpa vs 0 dpa (Figure 3.6 C) respectively in the regenerating tail (Figure 3.6). When the correlation graphs were plotted for limb 0 dpa vs. 6 dpa (Figure 3.7 A), 9 dpa (Figure 3.7 B), and 12 dpa (Figure 3.7 C), the values acquired were

0.92, 0.95, and 0.97, respectively. Since the correlation values were satisfactory, further analysis was done to check which peptides showed a differential expression across the wound healing stages of tail as well as limb. In the given Table 3.2 number of spots which were up-regulated and downregulated have been listed. A graphical representation of the data is also given in Figure 3.8.

Heat Map Analysis for Differentially Expressed Proteins

The spots obtained from 2-DGE, which depicted differential expression, were analysed further. The peptides belonged to FGF, TGF-β, VEGF-α family along with many of the peptides involved in the apoptotic pathway were identified. A heat map was made in GraphPad Prism version 7.0 to show the differential expression of proteins in the stages of wound healing of the tail and limb in *H. flaviviridis*. Analysis of the result revealed that FGFs like FGF1, FGF2, FGF8, FGF10 were mostly upregulated in the healing tail while in healing limb no change or mostly downregulation of these proteins was evident (Figure 3.9). Additionally, markers of apoptosis were seen prominently for a longer duration in the selected time points of healing limb (Figure 3.10), while in tail initial few days depict signs of apoptosis which gets resolved eventually allowing a high rate of proliferation to succeed (Figure 3.9).

Western Blot Analysis

In order to authenticate the results obtained from 2-DGE, western blot of the selected proteins was performed. FGF2 showed a gradual increase in the tail from 3 dpa till 6 dpa while in limb no significant increase was seen till 6 dpa while the levels increased marginally by 9 and 12 dpa which corresponds to the data obtained from 2-DGE (Figure 3.11). However, even though the FGF2 levels increased in limb, still in comparison to tail, the increase was not significant. Since we observed a hike in the FGF2 levels, FGFR1 and FGFR3, receptors of FGF2 were also scrutinized. The receptors were also found upregulated in the tail following amputation while in limb again no significant change was observed till 9 dpa while at 12 dpa the levels seemed to be decreasing. The values of the intensity of the bands obtained from western blot has been portrayed in Table 3.3 for healing tail and Table 3.4 for the healing limb.

Table 3.1: Total number of spots obtained from 2-Dimensional gel electrophoresis in the healing tail and limb of lizard

Sample	Number of Spots
Tail	
0 dpa	296
3 dpa	302
4 dpa	325
6 dpa	319
Limb	
0 dpa	286
6 dpa	299
9 dpa	315
12 dpa	309

Table 3.2: Number of spots upregulated and downregulated in the healing tail and limb in comparison to their respective resting tissue.

	Stages of Wound Healing		
Tail	**3 dpa**	**4 dpa**	**6 dpa**
Upregulated	46	82	85
Downregulated	52	20	21
Limb	**6 dpa**	**9 dpa**	**12 dpa**
Upregulated	32	43	37
Downregulated	60	60	61

Table 3.3: Analysis of band intensity of selected proteins extracted from the healing tail

	Protein band intensities (Mean ± SEM)			
PROTEIN	0 dpa	3 dpa	4 dpa	6 dpa
FGF2	5.98 ± 0.035	49.4 ± 5.76***	58.23 ± 0.49***	88.23 ± 0.49***
FGFR1	6 ± 0.079	19.87 ± 3.7*	25.56 ± 4.34**	25.56 ± 4.34**
FGFR3	8.27 ± 0.089	29.41 ± 2.87***	39.1 ± 3.95***	57.1 ± 2.36***

Values are expressed as Mean ± SEM, where the asterisk represents * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ (n=6)

Table 3.4: Analysis of band intensity of selected proteins extracted from the healing limb

	Protein band intensities (Mean ± SEM)			
PROTEIN	0 dpa	6 dpa	9 dpa	12 dpa
FGF2	5.89 ± 0.23	15.82 ± 0.91*	18.34 ± 1.87**	22.76 ± 1.34**
FGFR1	4.21 ± 0.34	5.19 ± 0.24	5.25 ± 0.87	6.32 ± 0.53
FGFR3	5.89 ± 0.35	9.52 ± 1.51	10.4 ± 3.85	12.41 ± 3.85

Values are expressed as Mean ± SEM, where the asterisk represents * $p \leq 0.05$, ** $p \leq 0.01$ (n=6)

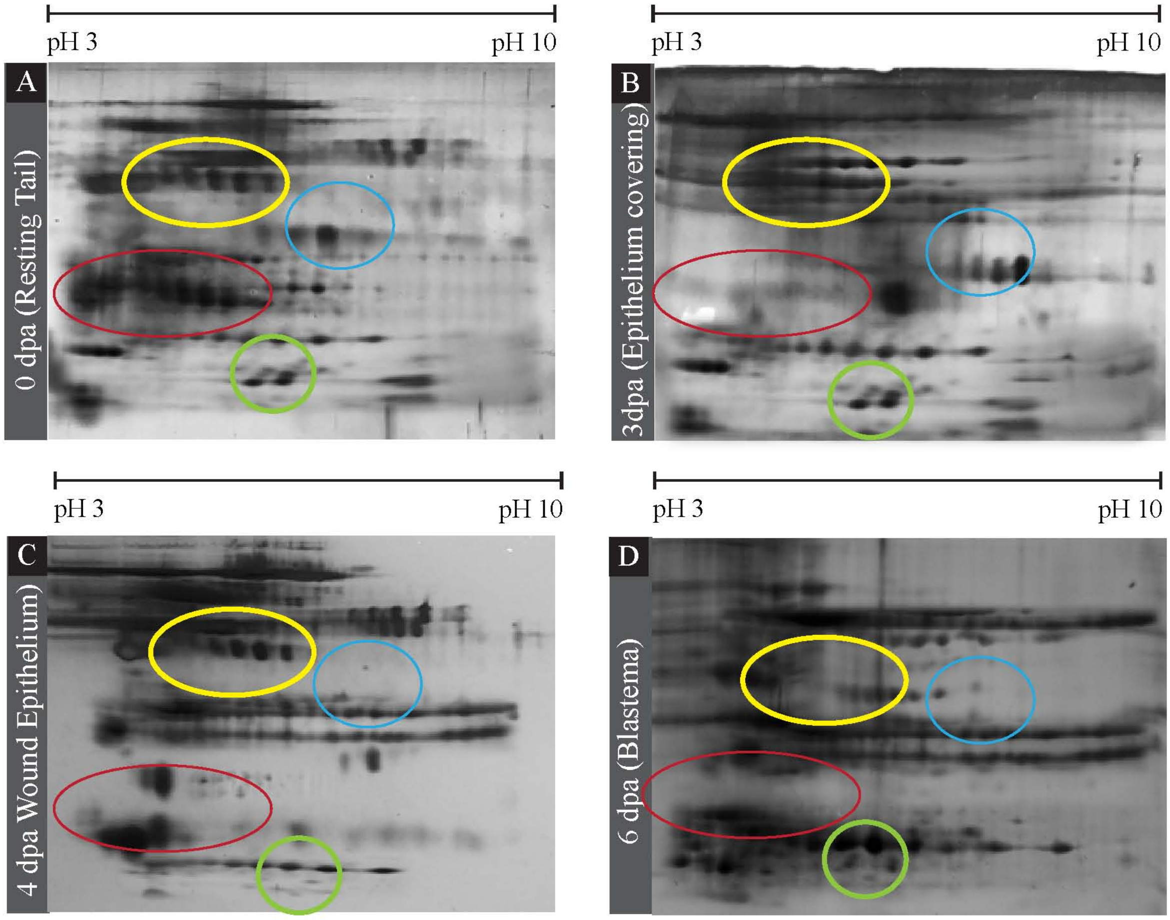


Figure 3.2: Representative images of 2-Dimensional gel electrophoresis for tail wound healing stages.

A) represents the 0 dpa while B), C) and D) depicts the 3 dpa, 4 dpa and 6 dpa. The gels were stained with silver staining protocol and the IPG strip ranges from pH3 to pH10. All the gels have been analysed in the PDQuest software. Some representative group of spots which showed differential expression have been marked with coloured rings. (n=3)

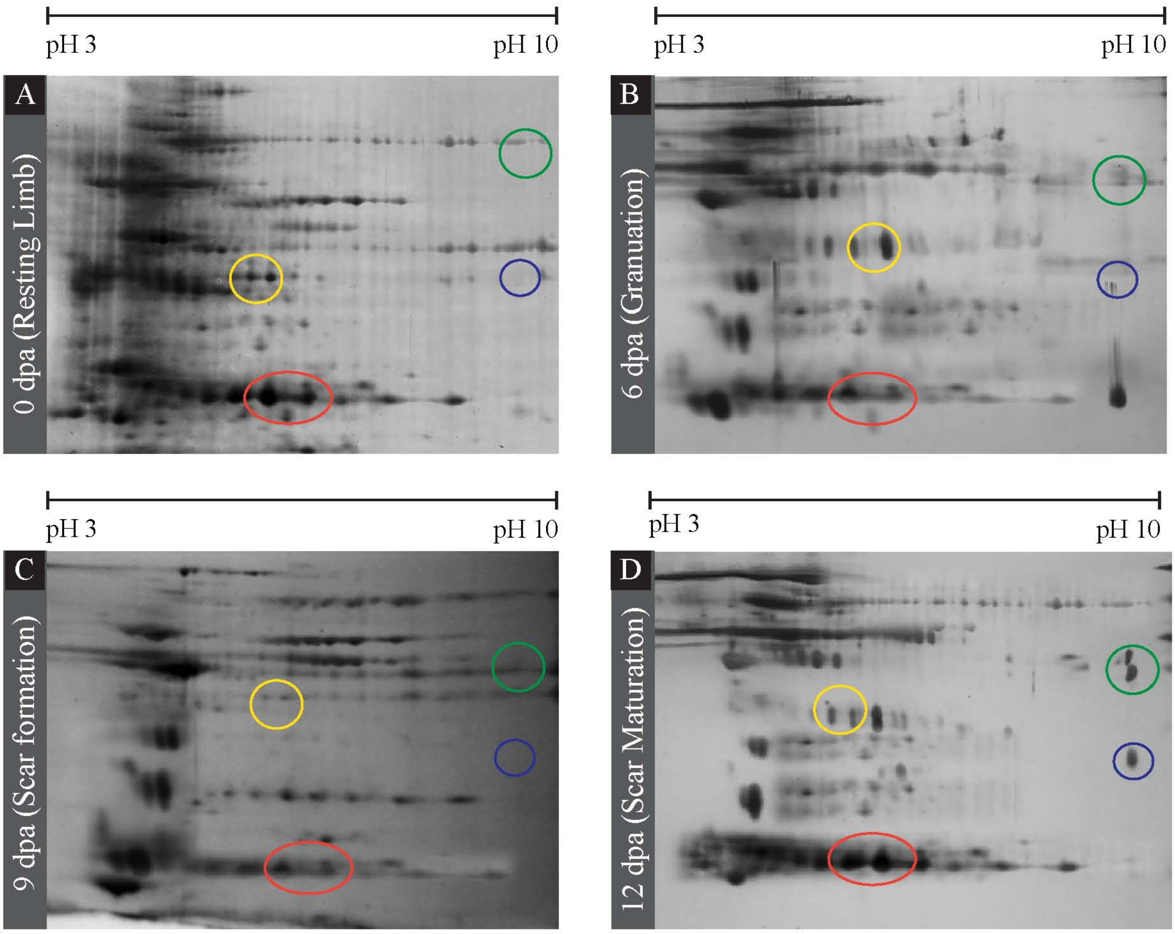


Figure 3.3: Representative images of 2-Dimensional gel electrophoresis for limb wound healing stages.

A) represents the 0 dpa while B), C) and D) depicts the 6 dpa, 9 dpa and 12 dpa. The gels were stained with silver staining protocol and the IPG strip ranges from pH3 to pH10. All the gels have been analysed in the PDQuest software. Some representative spots which showed differential expression have been marked with coloured rings. n=3

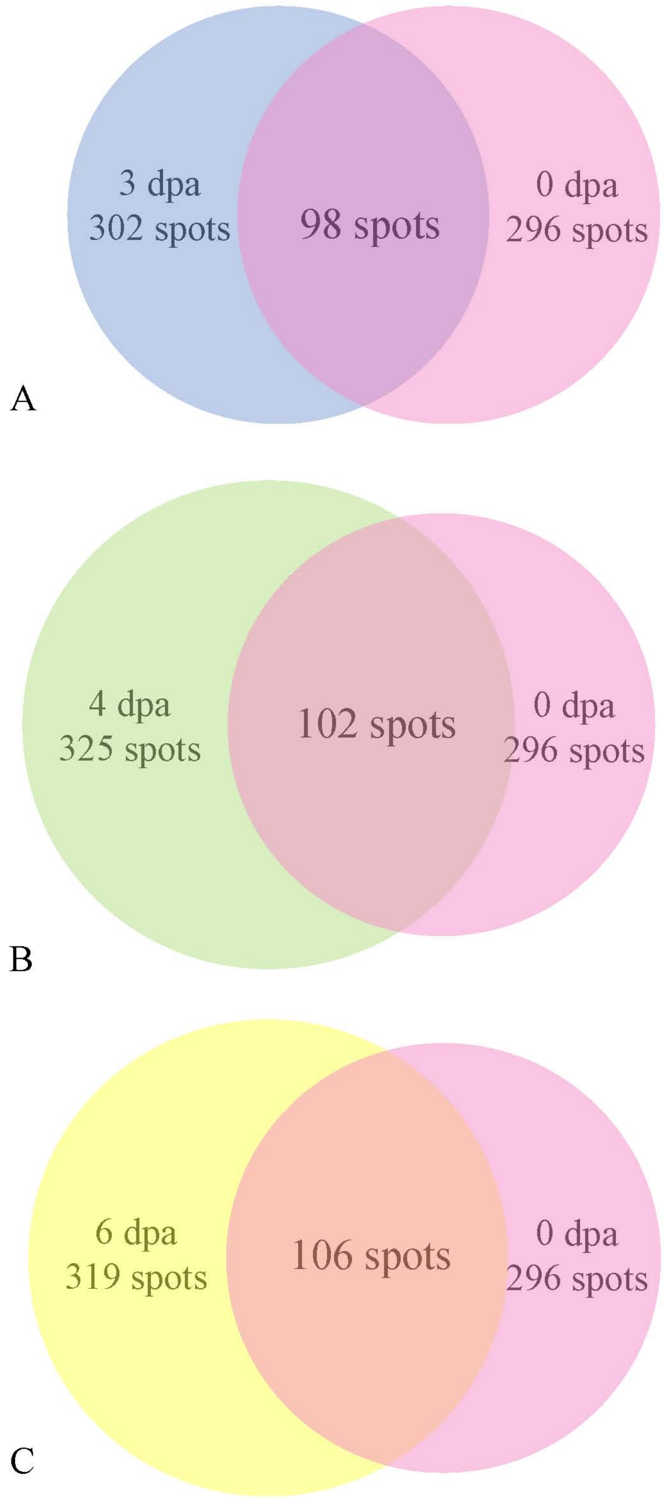
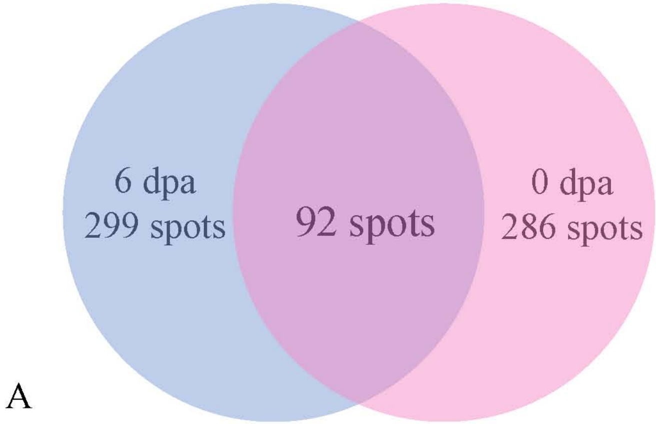
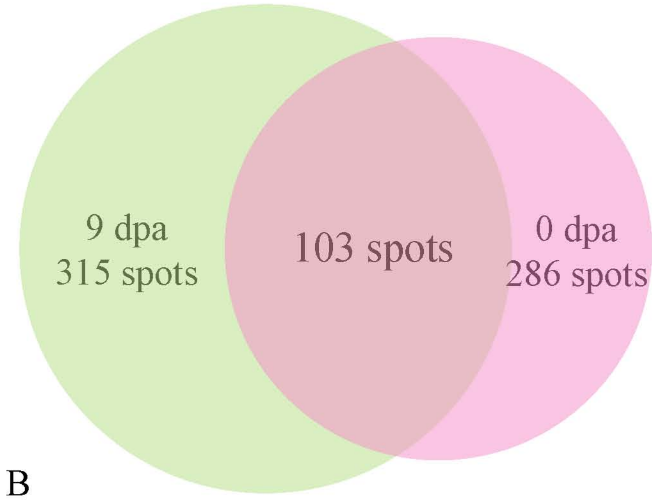


Figure 3.4: Venn diagram representing spots shared between A) 3 dpa vs 0 dpa, B) 4 dpa vs 0 dpa and C) 6 dpa vs 0 dpa.





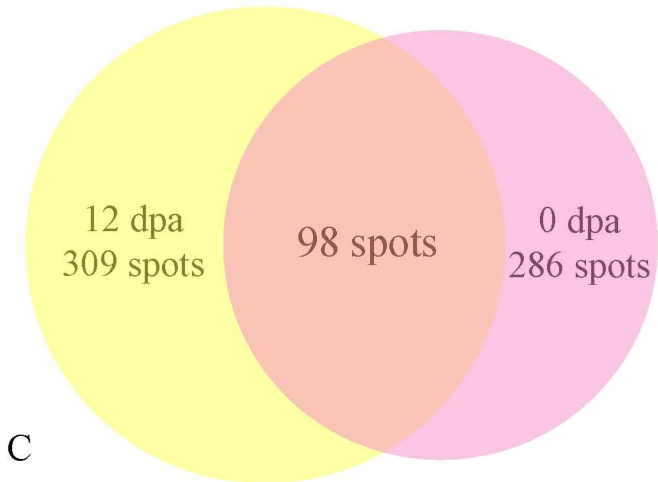


Figure 3.5: Venn diagram representing spots shared between A) 6 dpa vs 0 dpa, B) 9 dpa vs 0 dpa and C) 12 dpa vs 0 dpa in lizard tail.

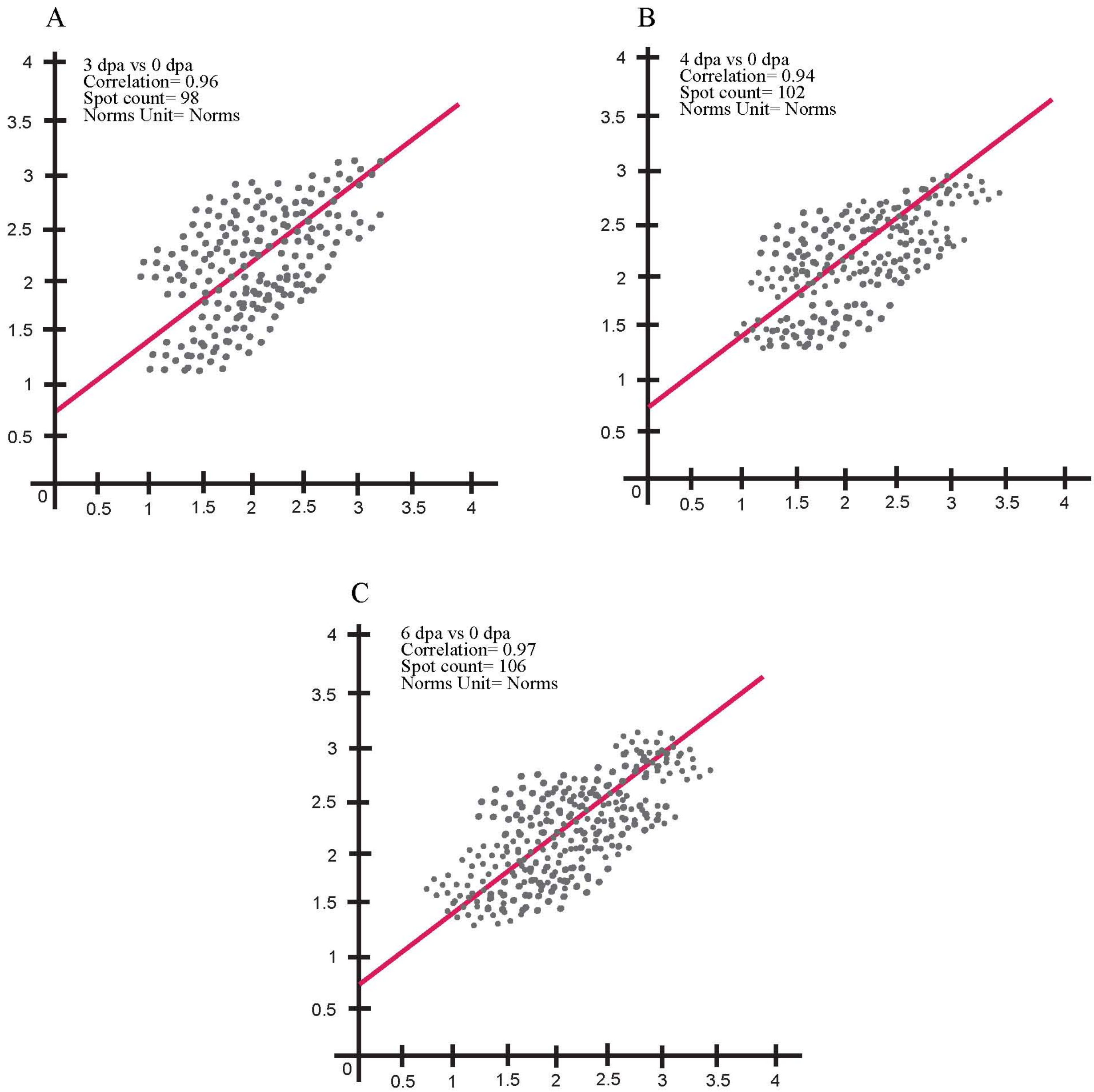


Figure 3.6: Correlation graph for the spots identified in the tail at 0 dpa with stages of wound healing during regeneration.

A) represents the 3 dpa vs 0 dpa while B) and C) depicts the 3 dpa and 4 dpa vs 0 dpa in tail. The correlation graph was plotted using the PDQuest software. n=3

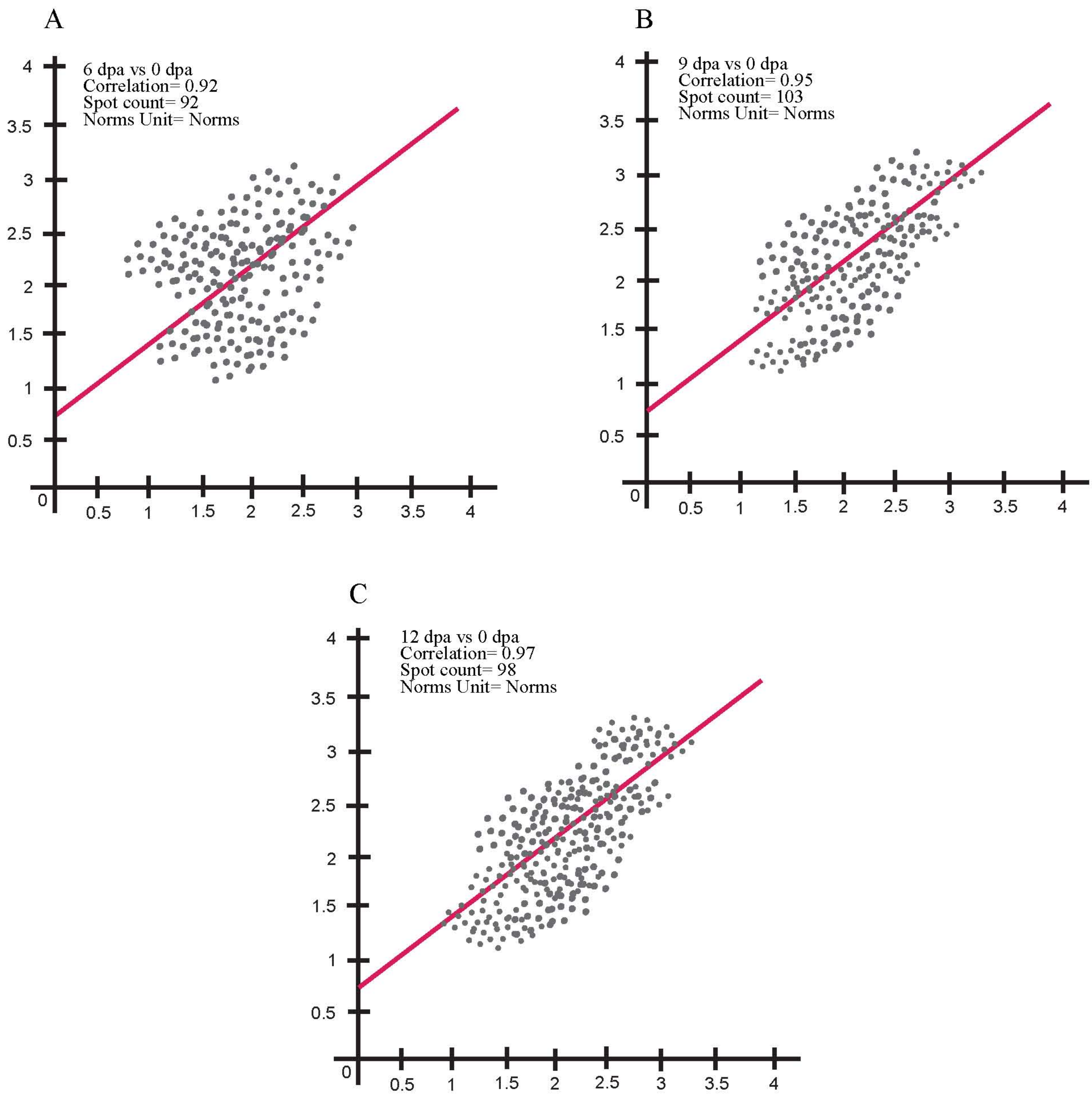
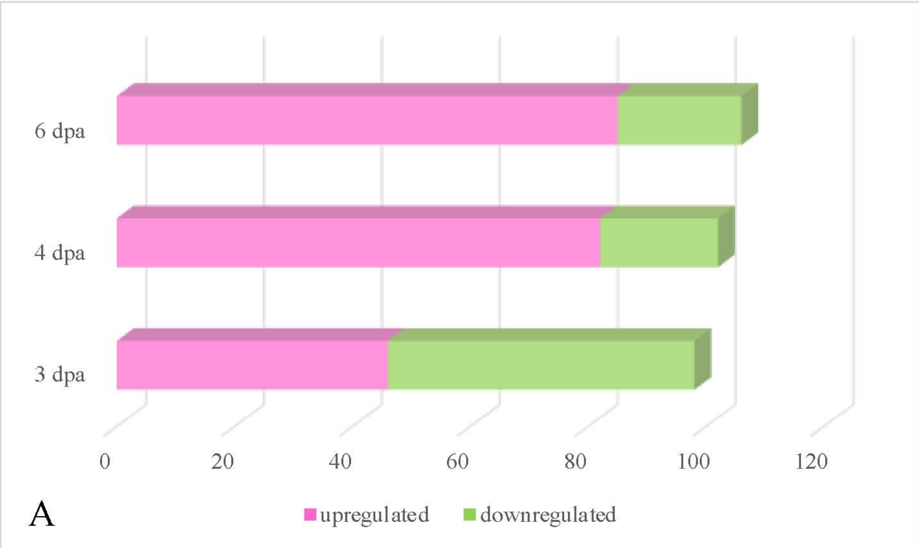


Figure 3.7: Correlation graph for the spots identified in the limb at 0 dpa with stages of wound healing.

A) represents the 6 dpa vs 0 dpa while B) and C) depicts the 9 dpa and 12 dpa vs 0 dpa in limb. The correlation graph was plotted using the PDQuest software. n=3



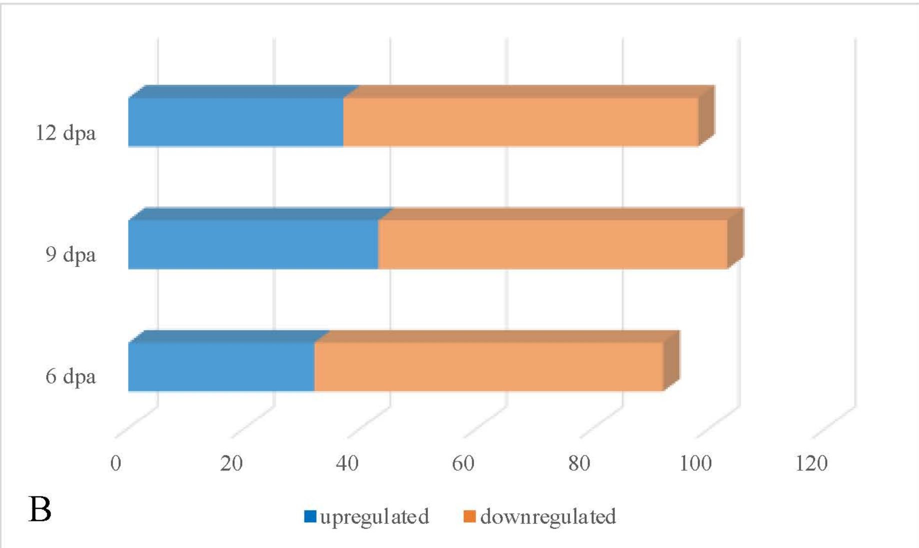


Figure 3.8: Graph A) represents the spots which were upregulated and downregulated in 3 dpa, 4 dpa and 6 dpa when compared to 0 dpa in the regenerating tail; B) represents the spots which were upregulated and downregulated in 6 dpa, 9 dpa and 12 dpa when compared to 0 dpa in the healing limb.

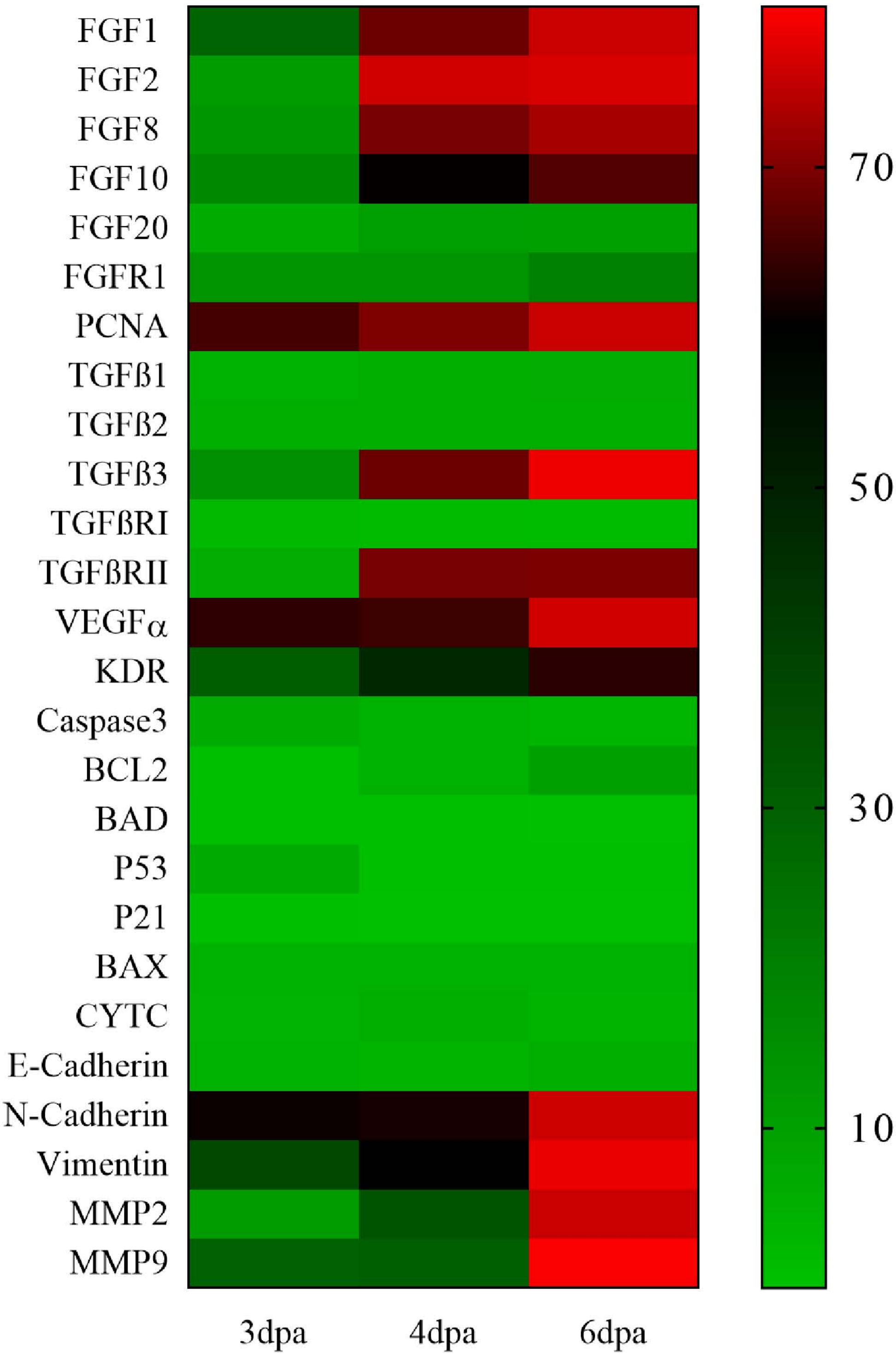


Figure 3.9: Heat Map of differential protein expression for tail and its wound healing stages. Spots obtained from 2-DGE showing differential protein expression in 3 dpa, 4 dpa and 6 dpa when compared with 0 dpa. The scale bar represnts the range of fold change value. The map was made in GraphPad Prism version 7.

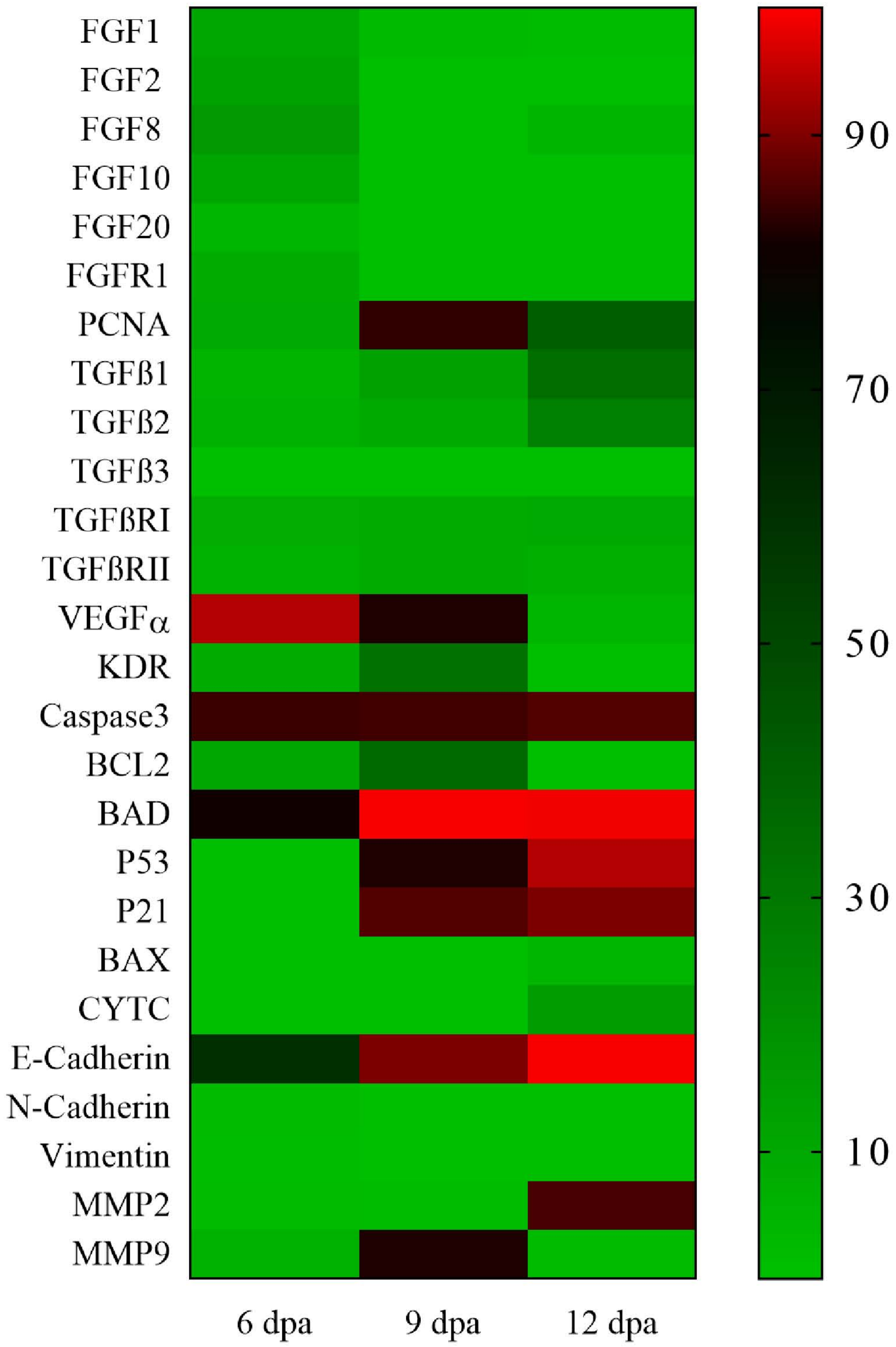


Figure 3.10: Heat Map of protein expression for limb and its wound healing stages.
Spots obtained from 2-DGE showing differential protein expression in 6 dpa, 9 dpa and 12 dpa when compared with 0 dpa. The scale bar represnts the range of fold change value. The map was made in GraphPad Prism version 7.

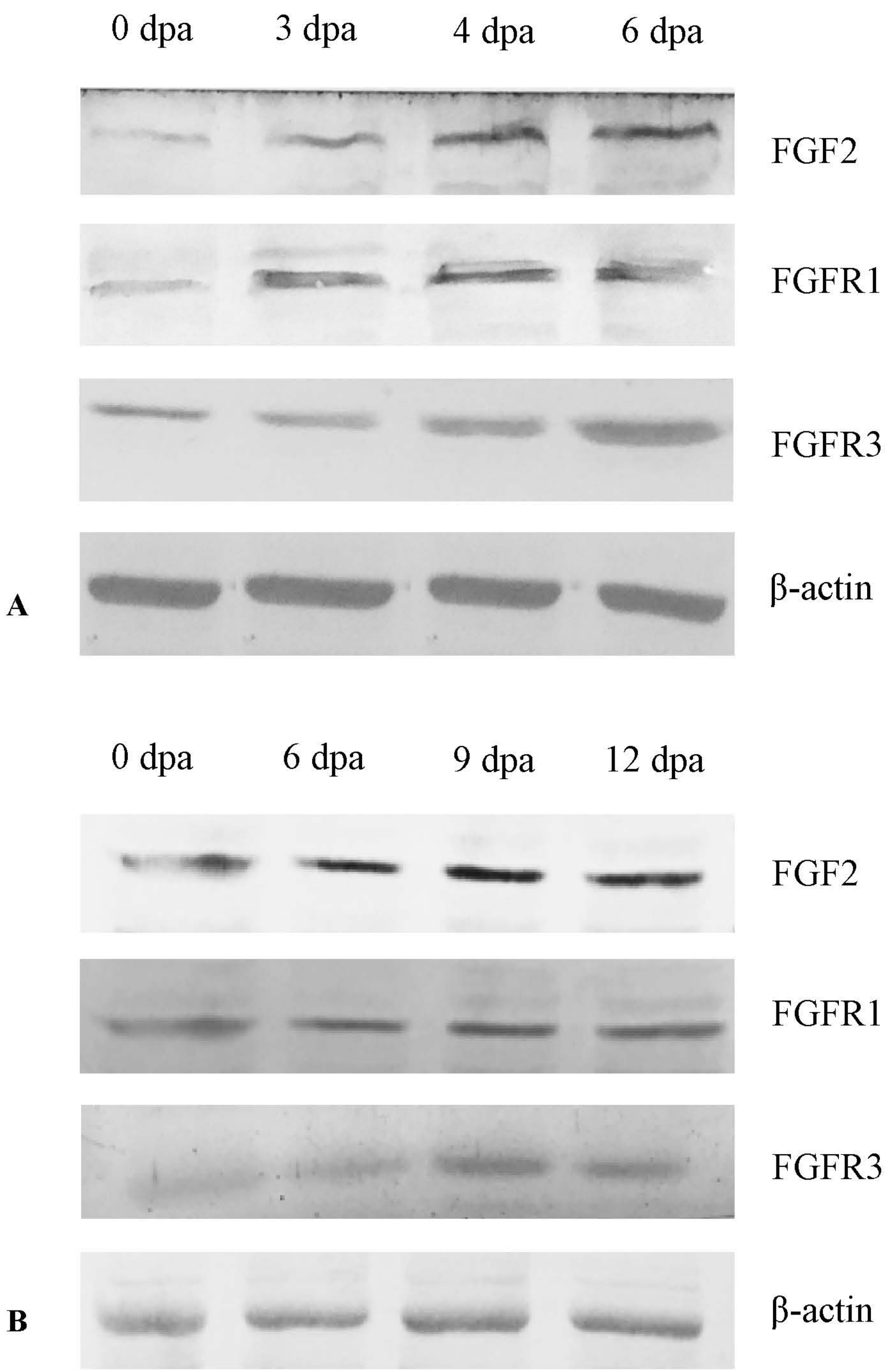


Figure 3.11: Western blot of FGF2, FGFR1 and FGFR3 in the healing tail (A) and limb (B).

DISCUSSION

The current study was aimed at identifying a series of molecules which are differentially expressed during scar-free and scarred wound healing since very few studies have been done in lizard with respect to differential regenerative response exhibited by tail and limb. Lizard is an ideal model which gives one the benefit of studying both the types of wound healing since one appendage, that is tail, follows scar-free healing while limb heals in such a way which results in scar formation. In order to understand the finer mechanism, a proteomic approach was selected to unravel the key proteins involved in the healing pattern. Proteomics provides highly useful methodologies to analyse complex protein mixtures and consequently can be used for the comparative analysis of a variety of tissues in different conditions (Ijaq et al., 2015). Here we present the first comparative proteome analysis of wound tissues at different points from tail and limb of lizard. This investigation revealed three major findings. First, we identified several known mediators involved in regeneration and wound repair. Second, the study yielded surprising results regarding new proteins with yet unknown functions in wound healing. Third, several proteins were identified that clearly distinguish a scar-free healing from a scarred healing.

Selection of the time-points was very crucial for this study as proteins identified here would make the basis of further study. In tail we selected 0 dpa which represents the resting tissue followed by 3 dpa, 4 dpa (wound epithelium characterized by AEC formation) and 6 dpa (blastema). Wound epithelium and blastema are the main hallmarks of regeneration hence it was imperative to select these time-points. For limb, granulation phase is a prominent feature of scarring therefore 6 dpa was chosen followed by 9 dpa which depicts the scarring and 12 dpa (maturation of the scar) were considered, apart from resting tissue i.e. 0 dpa. Granulation phase and scar formation are prominent stages of normal mammalian wound healing as well (Zhao et al., 2016; Frangogiannis, 2017; Belokhvostova et al., 2018).

Recently, attempts have been initiated in the reptilian group to study the differential protein expression pattern during the tail regeneration using green anoles, *Anolis carolinensis*. The tail of *A. carolinensis* at 72 hpa revealed 326 differentially expressed protein, many of them were found to be actively involved in multiple developmental and repair mechanisms. Among them, the significant genes were known for their participation in the stress response, hormonal regulation and musculoskeletal development (Eckalbar et al., 2013; Hutchins et al., 2014). In our study on performing 2-DGE with the healing tail and limb tissues using a broad range IPG strip, 296 spots in 0 dpa followed by 302, 325 and 319 spots were obtained for 3 dpa, 4 dpa and 6 dpa respectively. For limb the number of total spots marked were 286, 299, 315 and 309 in 0 dpa, 6 dpa, 9 dpa and 12 dpa. The spots obtained from 2-DGE, which were differentially expressed, were further analysed. The peptides belonged to FGF, TGF-β, VEGF-α family along with which many of the peptides involved in the apoptosis pathway were also identified. Vitulo and co-workers (2017) in their transcriptome data revealed that fgf and wnt genes were differentially expressed in the healing tail and limb. They claim that fgf and wnt are involved in proliferation and maintenance of AEC in tail while limb did not show significance difference in the expression of these molecules which may be the reason behind scarring in limb. Their mRNA data does correlate with the proteome data obtained in the current study. A heat map was plotted in GraphPad Prism version 7.0 to show the differential expression of proteins across the stages of wound healing in the tail and limb of *H. flaviviridis*. The heat map generated depicted many differentially expressed proteins of which the prominent ones belonged to FGF family, TGF-β family, peptides associated with apoptosis, angiogenesis, cell migration and ECM remodelling.

Role of FGFs in wound repair have been reported in numerous *in vivo* studies thus drawing our attention to investigate their role in wound healing of tail and limb of lizard. FGF1 and FGF2 in particular, have shown to stimulate angiogenesis in various *in vivo* and *in vitro* systems (Risau, 1990; Marwa et al., 2016). Amongst other cells, fibroblast and keratinocytes are also induced by FGFs to undergo proliferation at the wound site (Werner et al., 2007). Thus FGFs are clear candidates when it comes to contributing towards the wound healing process and this hypothesis has been supported by many studies wherein local application of FGF1, FGF2, FGF4, FGF7 or FGF10 have stimulated the process of tissue repair (Werner et al., 2007; Fumitaka et al., 2019). 2-DGE showed that FGFs were prominent by their upregulation during various time points in the healing tail but were found downregulated during the healing of limb. The FGFs identified here were, FGF1, FGF2, FGF4, FGF8, FGF10, FGF19, FGF20 and their receptors FGFR1 and FGFR3. A study by Alibardi (2012) showed positive immunolocalization for FGF2 in the epidermis of lizard tail, which does correlate with our data since during AEC formation the expression of FGF2 was heightened. In order to confirm the expression of FGF2 obtained from 2-DGE, western blot was done to quantify the protein which revealed upregulation in the healing tail tissue at specific time points. FGF2 did not show any convincing difference in the expression pattern throughout the selected time points in the healing limb. This trend was not restricted to the FGF2 levels, instead in all the FGFs analysed by 2-DGE, there was a clear upregulation of these peptides in the tail while little or no change was observed in the healing limb. There are many reports depicting role of FGFs during the chick and *Xenopus* limb development, especially during the apical ectodermal ridge (AER) formation. AER formation is very similar to the AEC formation and hence it is safe to say that FGFs would also be an integral part of the AEC formation. FGFs, in particular FGF4 and FGF8, are known to mediate the function of AER (Sun et al., 2002). FGF8 is also expressed in the urodele AEC (Han et al., 2001; Christensen et al., 2002), which accumulates FGF1 and FGF2 peptides (Mullen et al., 1996; Dungan et al., 2002; Giampaoli et al., 2003). FGF1 and FGF2 are thought to be transcribed in the nerve cell bodies and are transported to the nerve terminals present in the AEC during regeneration (Mullen et al., 1996; Dungan et al., 2002). Whitehead and co-workers (2005) made mutants in zebrafish such that they could not form blastema and this was achieved by silencing fgf20 gene. Their study was aimed at establishing the role of FGF20 in initiation of regeneration which was substantiated by failure of blastema formation in the absence of FGF20 expression. 2-DGE results in lizard depicted a higher expression of FGF20 in the healing tail whereas the limb showed meagre expression. From the results obtained via 2-DGE and the available literature supporting the data led us to carry out further investigation on FGF signalling during the wound healing stages of lizard's tail and limb which has been discussed in detail in the subsequent chapter (Chapter 4).

Of the many proteins identified, the apoptotic markers also showed a differential expression across the wound healing stages of the tail and limb. In tail though apoptotic markers like bad, bax, caspase3, cytc were identified, their levels did not show any deviation from the resting tail tissue. One mechanism by which cell number is controlled during morphogenesis is by programmed cell death (Guha et al., 2002), and apoptosis is involved in sculpting of growing tissue in a number of developmental systems including heart, limb and craniofacial patterning (Guha et al., 2002; Bastida et al., 2004), as well as nervous system pruning (Honig and Rosenberg, 2000; Bagri et al., 2003; Johnston, 2004; Mallat et al., 2005). In one study performed in *Xenopus*, early apoptosis was observed during the limb regeneration which gets resolved quickly allowing proliferation of cells (Tseng et al., 2007). Since in 2-DGE, the time-point for tissue collection in tail starts from 3 dpa, upregulation of the apoptotic markers like BAX, BAD, BCL2, cleaved Caspase 3 was not noticeable, therefore in chapter 4 even finer time-points were selected to study the trend of these markers. Apart from these molecules involved in apoptosis, transforming growth factor-β (TGF-β) were also identified. In our results from 2-DGE it was clear that TGF-β1 and TGF-β2 showed hike in the limb during the healing phases, however TGF-β3 showed a drop in the expression pattern. Tail on the contrary showed upregulation in the TGF-β3 levels. Now it is evident from a couple of recent reports that TGF-βs do have an established role during scarring and hence, it is not surprising that in the current study we found heightened expression in the limb rather than in tail (Klass et al., 2009; Kiritsi and Nyström, 2017). Hence, in chapter 4 TGF-β family was also screened during early time points in the healing tail and limb to note the trend.

A number of molecular pathways normally associated with the angiogenesis have been identified in many studies. These include the vascular endothelial growth factor (VEGF), fibroblast growth factor and platelet-derived growth factor (PDGF) pathways, as well as angiopoietin-Tie, Notch-Delta and ephrin signalling (Bicknell and Harris, 2004). VEGF-α and KDR were also identified in the 2-DGE. Both these molecules showed an increase in the protein levels from 0 dpa till 6 dpa in the tail, while in limb the levels were shown to be upregulated only at 6 dpa. Granulation phase is one of the important hallmarks of scarred wound healing (Sorg et al., 2017). During this stage, angiogenesis occurs and also fibroblast keep adding collagen, which eventually leads to scarring. This explains the hike at 6 dpa observed in limb, since by then the limb should be in the granulation phase, hence, it is but natural to find an increase in VEGF-α and KDR. Angiogenesis is also required to for continuous cell proliferation (Nishida et al., 2006; Fitzgerald et al., 2018) and hence, VEGF-α and KDR levels were maintained high in the healing tail so that the proliferating epidermis could be maintained and eventually sustain the blastema formation. In the following chapter (Chapter 4) both these molecules have been studied extensively by real-time RT-PCR and western blot analysis for scar-free as well as scarred wound healing.

Lastly amongst all the proteins identified, there were few which have a known role in inducing the process of EMT during embryogenesis. These proteins include E-Cadherin, N-Cadherin, Vimentin, MMP2 and MMP9 amongst many others. E-Cadherin is known for epithelial-epithelial cell junction, which means the cells are held together when they are expressing this protein (Markiewicz et al., 2019). Vimentin is a type III intermediate filament that is found in the mesenchymal cells of various types of tissue during the developmental stages, and it maintains cell and tissue integrity (Coulombe and Wong, 2004; Antfolk et al., 2017). N-Cadherin is a known transmembrane protein and also the major component of adherence junction. This protein is responsible for mediating cell-cell interactions and in the intracellular signalling required for organ development (Li et al., 2017). In the healing tail, N-Cadherin and Vimentin proteins were upregulated while in limb no such change was observed. Contrastingly, E-Cadherin was upregulated in the limb while the levels declined in the tail as the healing progressed. In chapter 5, molecules involved in the process of EMT were looked upon by performing real-time RT-PCR followed by western blot analysis. Key proteins were also localized at different time points in the healing tail and limb to unravel their role in EMT (Chapter 5).

By performing 2-DGE, identification of the molecules required for scar-free and scarred wound healing was achieved. However, for understanding their exact role in the wound healing required each process to be studied individually and assessment of the molecules at both transcript and protein level was a must. FGF stimulates many cellular functions, *viz*. cell proliferation, migration and protease secretion, provide the basis for matrix reorganization and angiogenesis which are important physiological functions of FGFs. Additionally, FGFs also influence cell differentiation, stimulating the process in some cell types (Robinson et al., 1995; Klint et al., 1999). Moreover, FGFs can also protect cells from undergoing apoptosis (Zheng et al., 2016; Tomida et al., 2017). Hence, next two chapters covers the wound healing pattern observed in tail and limb of lizard where processes like proliferation, apoptosis, angiogenesis and EMT were targeted keeping central focus on FGFs since they have a known role in the regulation of all these processes in one way or the other.

SUMMARY

Lizard being one of the least studied animal models of regeneration makes it difficult to explore the molecules that are involved in the process of regeneration. Hence, this study was aimed at creating a protein profile which could give some idea about the protein family which play a significant role during the tail regeneration. However, since lizard cannot regenerate its limb, this allowed us to study both the types of wound healing viz., scar-free wound healing in the tail and scarred wound healing in the limb. Hence, it was imperative to study the difference in the expression of proteins during both the types of healing. Therefore, for identifying peptides involved in scarring, limb tissues were also subjected to 2-DGE alongside tail sample. Amongst the few protein profiling studies done in regenerating tail, this study gives additional information about the limb healing pattern. More than 250 proteins were identified in all the gels of tail as well as in healing limb. Many of the selected peptides belonged to FGF family, TGF-β family, Cadherins, MMPs and even peptides involved in apoptosis and angiogenesis (summarised in Figure 3.12). Based on these findings, the further line of work was designed. FGFs and TGF-β family members were expected to show some differential expression during the healing stages since they have been reported in several regeneration studies in various animal models. Surprisingly, peptides involved in the process of EMT were also expressed in the healing tissue and hence along with the FGFs, the analysis of the expression pattern of these molecules was also carried out. Based on the result obtained, a thorough study by using various methodologies to unravel the mechanism behind healing of tail and limb was initiated which is discussed in depth in the following two chapters.

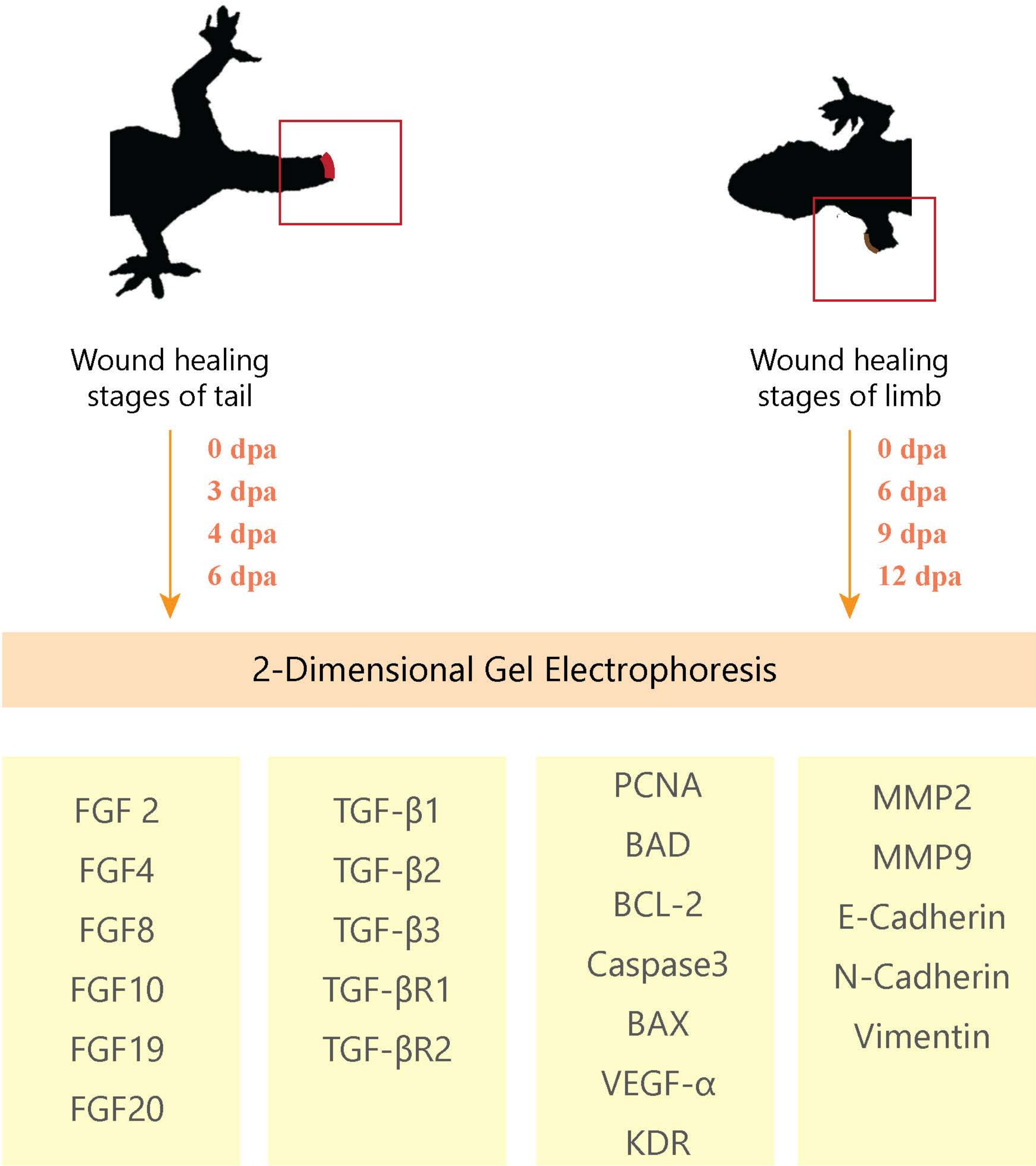


Figure 3.12: A pictorial representation:
List of peptides of significant importance that were differentially expressed during the scar-free and scarred wound healing in tail and limb respectively