Summary

The current study for the first time reports a comparative proteome profile of healing tail and limb in lizard (northern house gecko), *Hemidactylus flaviviridis*. The protein profiling was done by the aid of 2-Dimensional gel electrophoresis (2-DGE) devised in a way that not only qualitatively revealed the differentially expressed proteins but also yielded the magnitude of difference in their expression. This allowed the determination of regulatory changes during the stages of wound healing in the tail vis- à-vis the limb of *H. flaviviridis*. The sampling time points were set such that they coincide with the occurrence of major stages of regeneration and/or wound healing. Thus, while for the tail, the time points selected (0, 3, 4 and 6 dpa) were based on stages of epimorphic regeneration i.e. wound epithelium and blastema, for limb, the time points (0, 6, 9, and 12 dpa) were based on the stages observed during mammalian wound healing viz. granulation and scarring. Upon performing 2-DGE with tail samples using a broad range IPG strip, 296, 302, 325 and 319 spots were obtained differentially expressed for 0 dpa, 3 dpa, 4 dpa, and 6 dpa respectively. For limb, the number of differentially expressed spots noticeable were 286, 299, 315, and 309 at 0 dpa, 6 dpa, 9 dpa, and 12 dpa respectively. The spots acquired from 2-DGE depicting differential expression with a fold change value of more than four were identified and analysed further through LC-MS/MS. The differentially expressed peptides largely belonged to FGF, TGF-β and VEGF-α families. In addition, many peptides involved in apoptosis, angiogenesis and EMT processes were also identified (Chapter 3). This initial study made the basis of the next set of experiments in order to elucidate the healing mechanism in both the appendages of the lizard.

Nonetheless, in order to better appreciate the nuance of wound healing pattern of tail and limb it was but logical to include few more early time-points than what we selected for the study highlighted in the previous chapter. Hence, for the tail apart from the time points mentioned previously even 1 and 2 dpa were taken into consideration for further study while for limb 3 dpa group was added. Therefore, one part of the work deals with understanding the wound healing pattern by profiling the proteins differing in their expression in major stages of regeneration while the other is based on understanding the role of FGF in shaping the events of EMT during epimorphic regeneration and wound healing. While investigating the wound healing strategy used by tail and limb, first the tissue architecture of the recovering structure

was studied for both the appendages. In the scar-free wound healing, employed by the regenerating tail, histological examination of the regenerating tissue displayed a proliferating epidermis which, at 2 dpa, was 44.65 µm thick and increased its thickness to 91.93 µm by the apical epithelial cap (AEC) stage, i.e. at 4 dpa. Interestingly it was noticed that the AEC, that covered the wound surface, remained in a state of continuous proliferation as evident from the relatively thin (12.52 µm) normal intact skin lie adjacent to it. However, in case of the scarred wound healing of limb, on 3 dpa, 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 1 dpa. However, on completion of wound closure at 9 dpa, a thick scar tissue of 200 µm covered the cut surface of limb and the overlying epidermis at this point was just 17 µm thick, same as that observed in the resting epithelial layer of limb, suggesting that AEC is not formed in the limb but instead a scarred stump is formed (Chapter 4).

Based on the histology data, 2-DGE along with further investigation done with the aid of western blot and real-time RT-PCR, it was confirmed that the epidermal cells proliferate along with mesenchymal cells as soon as the tail gets injured. However, this does not hold true for limb as, instead of epidermal cells, fibroblast cells proliferate at a later stage of the healing process. In the tail, this proliferation of epidermis was brought by various FGFs like FGF2, FGF4, FGF8, FGF10, and FGF20, which leads to the formation of the apical epithelial cap at 4 dpa. It was also revealed that the proliferation of the epidermal cells triggered by the FGFs is a result of activated PI3K-Akt signalling pathway as their transcript as well as protein levels were elevated in conjunction with the FGFs. This data obtained from performing western blot and quantitative real-time RT-PCR was supported by performing BrdU incorporation wherein the tail at 4 dpa showed positive staining for the proliferating AEC, while in limb very few BrdU positive cells were visible. Western blot of PCNA also depicted the same result as mentioned above when the data was quantified, showing a significant increase of the protein in the healing tail when compared to the limb. Apart from proliferation markers, in 2-DGE, molecules associated with the apoptosis process was also identified. Therefore, when these molecules were studied at both transcript and protein level, *bad* and *caspase 3* showed an upregulation in the limb during the early phase of wound healing and remained high till the later phase along with *p53* and *p21*. These two molecules, *p53* and *p21*, were found to be triggered by the p38-MAPK pathway. In the 2-DGE results, apoptosis was not evident at 3 dpa in the healing tail, and hence, while studying the wound healing stages, finer time points were selected consequently revealing the occurrence of apoptosis at 1 dpa in the tail. However, the

levels of molecules associated with apoptosis were eventually downregulated allowing the proliferation of cells, leading to the AEC formation. In order to substantiate this claim, cleaved Caspase 3 was localized in both tail and limb, and the results depicted positive immuno-staining in the limb at 9 dpa while in the tail at 4 dpa showed lesser positive cells, hence, proving that apoptosis persists for a longer duration in the healing limb. In order to further validate these results, acridine orange and ethidium bromide staining was performed on fresh frozen sections of 0 dpa and 4 dpa tail tissue along with 0 dpa and 9 dpa tissues of the healing limb. Both the tail and limb tissues at 0 dpa revealed live cells that were emitting green fluorescence. By 4 dpa, in tail, a proliferating epidermis characterized by green fluorescence was predominantly observed along with few pro-apoptotic cells stained yellow. On the contrary the limb tissue on 9 dpa revealed heightened apoptosis marked by orange nuclear EtBr staining at the site of injury (Chapter 4).

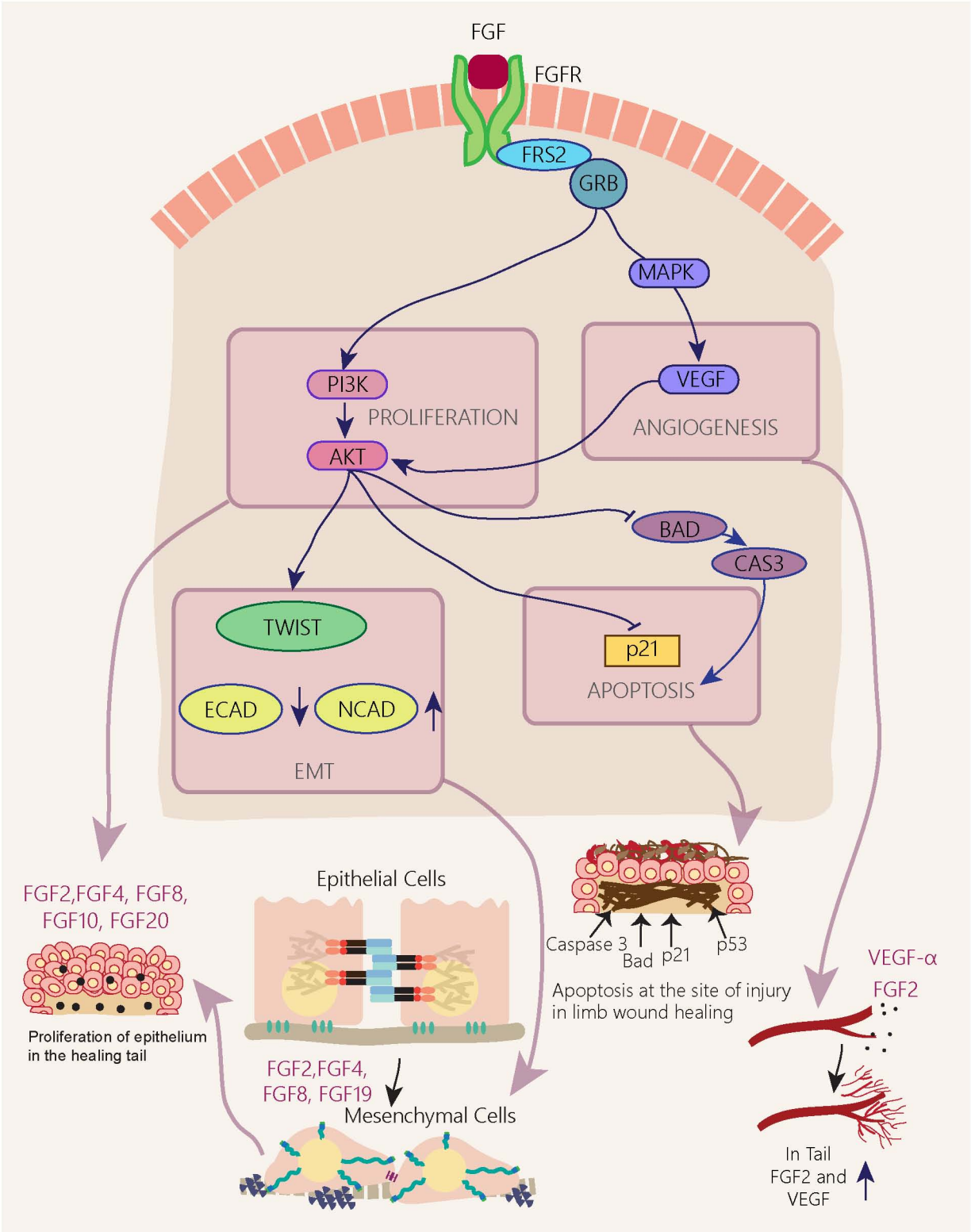
Another set of peptides identified that are differentially regulated were the TGF-β members, which are also a pre-requisite in wound healing and the results suggest that *tgf-β1* and *tgf-β2* are involved in the scarring and apoptosis as their levels were found significantly high in the stages of limb wound healing. In contrast to this, *tgf-β3* was seen to promote scar-free wound healing. These observations are in agreement with the results obtained via real-time RT-PCR during various time-points of the healing tail. Since proliferation was evident in both tail and limb though at different stages of healing, intuitively, angiogenesis may also participate in the process in order to sustain the pool of proliferating cells. Moreover, 2-DGE had revealed angiogenic markers and thus, further investigation was carried out. VEGF-α was localized in the AEC and more so in the underlying tissue from where the signals may perceivably be originating. Moreover, in case of tail, through all the time points, angiogenesis was observed, but it was noted in limb only at 6 dpa which was confirmed at protein level by western blot analysis. At 9 dpa 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 selected time points since these are the key relay molecules of the signalling pathway. Both PI3K and Akt protein levels remained elevated in the tail starting from 1 dpa, while their levels were elevated in limb only at 6 dpa again confirming the onset of granulation phase. With these results it is clear that though proliferation, apoptosis and angiogenesis occur in both healing tail and limb, their occurrence and duration are varied which may also be the reason that allows the tail to heal in a scar-free manner while the limb forms a scar tissue (Chapter 4).

Furthermore, peptides belonging to the EMT pathway were also identified to be differentially expressed in 2-DGE, however there are only few reports of involvement of this pathway in the process of regeneration. The results revealed an increase in the N-Cadherin level during the wound epithelium and blastema stage of the tail regeneration. N-Cadherin was also found to be abundantly present in the AEC and blastemal cells when localized using immunohistochemical staining followed by fluorescence microscopy. In limb, however, N-Cadherin was only scantily localized at 9 dpa. This observation can be explained by the fact that by 9 dpa, the scarring is generally initiated, and the cells, if expressing the protein now, would not be able to cross the scarred tissue and proliferate to make the blastema. It can be said that in tail, N-Cadherin expressing cells were dominant from the early phase of wound healing when compared to the limb. E-Cadherin, on the other hand, showed no change in the levels in the healing tail when compared to resting tail. While in the limb the expression of E-Cadherin kept increasing, which does indirectly indicate that the epithelial cells would not have detached from each other to undergo a transition to transform into mesenchymal cells. Vimentin was observed to be expressed only by the mesenchymal cells and healing tail showed the maximum expression of Vimentin at 6 dpa. This led us to believe that blastemal cells do have mesenchymal cells having epithelial origin present in their vast pool of proliferating cells. To reaffirm the status of EMT activation, *snail2,* and *twist1,* which inhibit the expression of E-Cadherin and initiate the process of EMT, were selected for studying their gene expression pattern during the healing of tail and limb. The results revealed an increase in the levels of the two genes in case of healing tail during different time-points whereas in the limb these levels were subdued, again suggesting that this process gets activated only in the tail and not in the limb, the reason for which is unknown (Chapter 5).

Though the study shows that the epithelial cells start expressing N-Cadherin by class-switching and get converted to mesenchymal cells, these cells still need to move towards the underlying tissue in the blastemal zone, which is possible only when the ECM is digested. MMP2 and MMP9, gelatinases required for ECM digestion, were upregulated from 4 dpa till 6 dpa in the tail. This subsequently led to a conclusion that ECM digestion occurs in the tail for tissue remodelling and for mesenchymal cell movement. Expression analysis by real-time RT-PCR, western blot and activity assay of the MMP2 and MMP9 by gelatin zymography revealed an appreciable upregulation only in the healing tail. Limb, on the contrary, showed an increase in the TIMPs – the inhibitors of MMPs, which could have been the reason that led to the reduction in expression of MMPs during the healing stages. Limb overall showed no traits which could lead to activation of the EMT pathway. *Fgf2*, *fgf4*, *fgf8*, *fgf19* were found to be elevated in the

tail, while in limb, contradictory results were obtained. FGFs have been reported to induce EMT during cancer by promoting the process of metastasis and it is possible that their expression in the healing tail leads to the activation of the EMT pathway and hence making a successful blastemal structure (Chapter 5).

Overall, from the results of the current study it could be construed that though the processes like proliferation, apoptosis and angiogenesis are found activated during the healing process of tail and limb, their initiation and extent vary decidedly so as to achieve differential outcome. Additionally, members of FGF family, with their context specific expression pattern, play an integral role in the regulation these processes thereby, tail heals by a scar-free mechanism whereas amputated limb resolves the wound with a scarred tissue. Not surprisingly, the markers of EMT is observed only in the regeneration enabled tail tissues, perhaps to supplement the pool of blastema, while the healing limb depicts no sign EMT and culminate in a scarred stump at the amputation plane. Therefore, it is logical to conclude that the crucial differences observed in the intricate orchestration of the molecular events during the healing may be amongst the many reasons why a lizard tail regenerates and a limb does not which is graphically surmised in the following diagram (Figure I).



I) A Pictorial representation of the FGF signalling pathway governing various cellular process during healing of tail and limb

Expression of FGFs during early phase of wound healing in tail leads to regeneration by accelerating proliferation, angiogenesis and promoting EMT. In limb, apoptosis persist for longer duration; expression of FGFs occurs momentarily at a later phase of limb healing; hence, eventually leading to scar formation.