SUMMARY

Organ repair provides all living forms a chance to suit the surrounding requirements and thrive to the best of their potential. It operates in many species and at variable intensities (Suzuki and Mittler, 2012). The regenerative potential is not confined to higher taxa, neither is it equally distributed amongst all classes. Therefore, all the organisms eliciting it have a remarkable advantage over those who adjust with permanent scars post-injury.

Successful tissue regeneration and scar formation lie on the opposite sides of the wound healing spectrum. Invertebrate groups such as Cnidarians, Platyhelminthes, and Arthropods have a stunning capability to reform many organs. In contrast, more evolved organisms like birds and mammals have this potential confined only to few tissue types (Alvarado, 2000). These higher classes respond to wounds differently than the pro-regenerative ones and end up making a collagen-based, thick permanent scar.

A majority of pro-regenerative species incorporate Epimorphic route of restoration. Post-injury, the exposed tissue forms a wound epithelium, which further leads to formation of 'regenerative blastema' through de-differentiation. Eventually, blastema undergoes re-differentiation to reconstruct the structural and functional replica of the original tissue (Muneoka et al., 1986; Reddien and Alvarado, 2004; Agata et al., 2007; Kierdorf et al., 2007).

Barring a few exceptions, epimorphic capability is generally absent in amniote groups (reptiles, aves and mammals). Few lizards from Gekkonidae and Scincidae families are the only amniote members eliciting this capacity and that too in limited organ systems. For instance, post-amputation, the Northern House Gecko can regenerate only its tail, while the rest of the appendages form a thick collagenous scar. Hence, this animal-*Hemidactylus flaviviridis* has been established as an ideal model system to study the disparate regenerative potential.

A previous study from our lab (Ranadive et al., 2018) has highlighted this striking difference in wound healing as observed in lizard appendages. When autotomy was induced in lizard tail, it healed within 4-days post-autotomy (dpa) and achieved stratified wound epithelium stage. A successful signalling cross-talk between the mesenchyme underneath

and the wound epithelium is possible in this microenvironment, owing to the absence of scar between these tissues. This molecular interplay eventually triggers epimorphosis in the tail. On the contrary, limb amputation leads to a slow and scarred wound healing with remarkable collagen deposition between epithelium and mesenchyme. This collagenous scar acts as a deterrent for the molecular interaction between the epithelium and mesenchyme hence the lizard limb fails to regenerate.

Of the many processes governing wound healing, inflammation happens to be the first one taking charge and has a massive impact on the routine and outcome of wound repair. It is the first response displayed by all injured systems, which functions to remove harmful foreign entities from the wound site. It deploys all the emergency machinery to combat pathogenic influx occurring at the exposed wound site and restores homeostasis to boost healing (Filbin, 2006).

Present endeavour deals closely with inflammation as a preliminary response post-injury and the first step towards regenerative repair - wound healing. It is further governed by synchronised set of events, Inflammation, Proliferation and Remodelling. Each sub event's ratio, proportion, and time span constructs either pro-regenerative or scar making microenvironment (Murawala et al., 2012). The variation in the extent of inflammation is long suspected to be a reason behind the biased wound healing and hence the differential regeneration potential exhibited by the lizard's tail and limb.

Studies by Sharma and Suresh (2008) as well as Buch and co-workers (Buch et al., 2017; 2018) have shown that COX-2 derived PGE_2 is one of the early response inflammatory signals arriving at the wound micro-niche post-autotomy or amputation. Any attempt to impede the activity of COX-2 resulted in delayed wound healing and loss of regeneration in Northern House Gecko tail (Sharma and Suresh, 2008). Therefore, we hypothesised that the level of COX-2 induced PGE_2, a master regulator of the inflammatory mediator, could be different at the healing site of tail and limb in lizard which then leads to differential expression of other inflammatory mediators in a context specific manner resulting in either 'superhealing' followed by regeneration in tail or scarring of limb.

In order to check our hypothesis, a detailed study was devised with one primary objective, which was to study the temporal expression pattern of cyclooxygenase and ascertain its role

in the regulation of inflammatory response during wound healing in the appendages of lizard *Hemidactylus flaviviridis*. Specific aims were formulated to achieve this objective, results of which are comprehended hereby in the three chapters of this thesis. The timepoints determined for the observation were based on the pivotal hallmarks of healing observed in the tissues - haemostasis, inflammation, granulation followed by proliferation and wound epithelium formation. For tail, 0 (resting), 1, 2, 3 and 4dpa (days post-autotomy) and for limb, 0, 3, 6 and 9dpa (days post-amputation) were considered for this study, owing to the difference in repair time needed for these appendages and based on the previous studies in lab (Ranadive et al., 2018)

The first specific aim was to evaluate the roles of COX-2 driven inflammation in orchestrating differential wound healing in tail and limb, by observing the temporal status of humoral inflammatory mediators in the disparate microenvironments (Chapter 1). Enzymatic activity of COX-2, concentration of PGE_2, followed by protein (Western blot analysis) and gene expression (q-RT-PCR analysis) of various pro and antiinflammatory interleukins were evaluated here. The COX-2 activity elevated 2dpa onwards in lizard tail, while in limb it was relatively high and increased progressively at the following time points. As COX-2 belongs to the family of early response genes and is strongly induced by mitogenic and proinflammatory stimuli (Lasa et al., 2000), protein and transcript levels of COX-2 were checked in the next step. In resemblance to its hiked activity, COX-2 protein and gene levels were also found to be elevated till the 3dpa stage in tail. This suggests the participation of COX-2 in modulating early inflammation, which is reduced at 4dpa, during proliferation and epithelialisation. On the contrary, in limbs COX-2 gene expression increased from the basal level till the terminal time point of 9dpa. This suggests mRNA stabilisation in limb tissue, due to elevated proinflammatory interleukins as found in human bones, macrophages and granulosa cells by Kang and coworkers (Kang et al., 2007).

Further COX-2 activity forms PGE_2 as an early response gene product, boosted by proinflammatory cytokines, governing its transcriptional and post-transcriptional levels (Kang et al., 2007). The PGE_2 expression followed a trend of COX-2 activity in tail, while in limb, it showed significant decrease after 3dpa, until 9dpa. Interestingly, the basal level of PGE_2 in limb (0dpa) is higher than the terminal time point for tail (4dpa). This disparity could be a function of COX-2 driving multiple signalling pathways in various tissues in a context specific manner (DuBois et al., 1998; Simmons et al., 2004; Tsatsanis et al., 2006).

Also, other tissue specific inflammation curbing prostanoids might participate in causing early resolution and resultant super healing in tail, while contrasting results are observed in limb (Bos et al., 2004; Korbecki et al., 2014). Meanwhile, complete profiling of all prostanoids participating in these events would explain their roles in regulation of inflammation.

Inflammation levels stay high in the limb microenvironment owing to the elevated gene and protein levels of various proinflammatory mediators such as COX-2, PGE_2, EP2, TNF-α, iNOS, IL-6, IL-17 and IL-22, throughout its healing frame i.e., from 0dpa to 9dpa. In the tail, inflammation spiked immediately post-injury (from 0dpa to 2dpa) and resolved quickly (at 3dpa and 4dpa), allowing all the following formative processes. COX-2 and PGE_2 levels rose during the initial two days after tail amputation, but they were reduced 3dpa onwards and so did all the proinflammatory mediators. EP4 increased gradually from 0dpa to 4dpa in tail, which supports the reduced inflammatory status observed in this appendage. IL-10, a pivotal antiinflammatory mediator, was found to be increased during the healing frames of the tail. In limbs, gene and protein levels of IL-10 gradually reduced at 3, 6 and 9dpa compared to the resting stage (0dpa). Interesting functional coherence was observed amongst COX-2 derived PGE_2, EP2 and EP4, which led to increased levels of inflammation in limb (PGE_2-EP2 based) and reduced inflammatory profile in the tail (PGE_2-EP4 based).

IL-17 and IL-22 also showed a peculiarly differential behaviour in the two appendages. IL-17 showed significant reduction in gene expression traversing all the time points, for tail group, after the early inflammation (2dpa). This supports the idea that reduction of chief proinflammatory mediators cause an overall decline of inflammation at tissue level in tail and promote regeneration supportive wound healing. As opined by Veldhoen and group (2006), reduction in IL-17 expression can be a coherent effect of another regulatory mediator like IL-6, which has shown a major decline in tail. It could even be due to the specific signalling dictated by the EP receptors (Hinson et al., 1996). Nevertheless, in limb tissue, IL-17 was elevated, except at the time when scar formation and collagen deposition started at the site of healing. IL-22, in the tail tissue showed well pronounced increase in its transcripts from 1dpa till 3dpa, after which its level reduced significantly by 4dpa. This ensures its participation in early epithelialisation, as achieved in tail. IL-22 elicits a protective role, when combined with IL-17, which specifically induces anti-microbial peptides in human keratinocytes (Sabat et al., 2013).

In limb, IL-22 followed the trend of IL-17, with noticeable rise in gene expression at the time of scab formation in limb tissue, in congruence with other proinflammatory mediators like IL-6, TNF-α, iNOS and IL-17. It is thus proved that IL-22 plays its part in repairing the wound in the two appendages, in synergy with IL-17 and reconstructs the framework for scar-free healing in tail, however, it supports scar formation under the prolonged inflammatory response in limb. Discovering this novel participation of IL-17 in the regeneration model recommends further investigation, where the performance of this cardinal inflammatory mediator can be explored.

Our observations, thus clearly demonstrate the impact of early resolved or prolonged inflammation on the disparate wound healing outcomes as observed in tail and limb, respectively.

The second objective was to assess the presence and roles of cellular mediators of inflammation in the two healing microniches viz., the tail and limb. Both conventional (Total WBC count) and FACS methods were deployed to check the tissue specific and systemic blood cell profile on autotomy of tail and amputation of limb. Further, we checked the expression status of various immune cells in the immediate microenvironment of both healing tail and limb. We also checked the trend of expression and recruitment of these cells on the systemic front. We attempted the contemporary technique of FACS, to decipher various blood cells' local and systemic roles in inflammation and wound healing. Although we did not receive any conclusive directive in this venture, our total WBC count method indeed clarified the image for us. We recorded an astounding rise in the levels of leukocytes during the elevated inflammatory period in the limb. Both tissue environment and systemic levels of WBCs were found high in the case of the amputated limb, while in tail, these cell numbers increased immediately post-injury and settled close to basal level at the end of wound healing. All the cells identified in the lizard blood smear and total WBC count have been well recorded, analysed and presented in Chapter 2.

We tried to simulate the 'tail-like' pro-regenerative conditions in the otherwise scarring limb for the third objective. Since the regeneration promoting blastema formation, is confined to the tail and does not occur in the scarring limb, we tried to bring these two facets of wound healing together and simulate the 'tail-like environment' in limbs of lizards. As a pilot step, we applied 3dpa tail homogenate on the healing limb ectopically, throughout its

healing time, i.e 0 to 9dpa. We observed no significant change in the morphology or any improvement in the healing pattern of the treated limb. Further, we checked the impact of blastema homogenate on the healing limb, wherein treated limbs achieved wound healing and scarring earlier than the control ones.

We then attempted to study the molecular changes appearing in the treated appendages as compared to the control ones. We received exciting results in this preliminary analysis, wherein applying blastema homogenate on the amputated limb has reduced the healing time in the treated animals (Chapter 3). The Haematoxylin & Eosin (H&E) stained sections of control and blastema homogenate treated limbs clearly show the interrupted collagen layers in the scar tissue. The epithelium of the healed limb was also multi-layered and many cells were found embedded in the collagen layers. We also checked the status of inflammatory mediators in the tissue chunks of limbs treated with blastema homogenate and found a drastic reduction in their levels of proinflammatory ones (COX-2, TNF-α, IL-6, Il-17) as compared to control samples (Chapter 3). Meanwhile, antiinflammatory mediators namely, IL-10 and IL-22, increased gradually from 0 to 9dpa stage progressively on blastema treatment.

Overall, our study proves a cardinal role of COX-2-PGE_2 derived inflammation in steering the differential wound healing episodes in the appendages of Northern House Gecko. Both cellular and humoral mediators of inflammation contribute to either early resolution of inflammation as observed in the tail of the gecko or its persistent elevated levels as found in the limbs. Further, blastema formation and its components seem to plausibly hold the cues for scar-free regeneration as seen in the tail of the lizards. It possesses all the molecular cues of early repair, which visibly expedite the healing process in the limb. Characterising blastema and observing its components can answer our queries regarding the loss of regenerative abilities in higher vertebrates.

Meanwhile, assessing complete prostanoid profile in the different appendage microniches and evaluating their tissue-specific receptor expression can provide a wholistic idea about the effector inflammation at the healing site. The promising set of results obtained here open up new research avenues to explore wound healing mediated regenerative mechanisms. A graphical summary of the entire work is presented in the following thesis summary image.