General Considerations

A constant endeavour of the human race is to find ways to improve the quality of life. A substantial amount of money and resources is spent on the advancement of medical sciences with an aim to overcome health conditions that are difficult to treat or have so far been incurable. Numerous diseases that have eluded permanent or long-lasting solutions involve tissue degeneration. If science is able to find a way to achieve complete regeneration of damaged cells and tissue in the human body, we will be in a position to credibly manage degenerative diseases like Alzheimer's, amyotrophic lateral sclerosis, Huntington's, Parkinson's, atherosclerosis, Chronic Obstructive Pulmonary Disease, Inflammatory bowel disease, heart diseases, muscle dystrophy, osteoporosis and type 2 diabetes, to name a few. It is not hard to see from this list how widespread these have become and how far they have made human life uncertain. Since surgical intervention, organ transplants and other medication have failed to qualify as viable options here, man must look to employ one of two seemingly promising approaches: (i) Treatment by stem cells, which may help rebuild damaged tissues and (ii) Induction of resident stem cells to initiate a repair programme (Gemberling et al., 2013). Whether exogenously added stem cells effectively integrate into the target tissue and whether they are able to differentiate into the required cell types remains uncertain. An effective approach to combat tissue degenerative conditions would therefore be to stimulate the repair process by activating local or blood-derived stem cells to initiate regeneration. Of course, this would involve a sizeable investment in the form of research on the basic biology of tissue regeneration.

To fulfil our motive of understanding the biology of regeneration, we must exploit the ability of some vertebrates to regenerate a wide variety of tissues and use it to our advantage. When teleost fish and urodele amphibians are considered, they together can regenerate lost or injured portions of almost all tissue types present. It is generally observed that all tetrapods are capable of regenerating tissues at some or the other time of their lives (Han et al., 2005). For examples, mammals, which lose the potential for large scale regeneration in adulthood, can regenerate tissues effectively during the embryonic period. Anuran amphibians also generally regenerate large structures as larvae but lose the ability post-metamorphosis. Han et

al. (2005) suggest that the regenerative process in all these organisms, including mammals, must be studied in depth if we hope to bring about organ regeneration in humans. This is based on the considerations that the components to induce regeneration of the limbs are preserved in birds and mammals (Bryant et al., 2002). What needs to be known is how to activate them. This makes us wonder that if we have kept the molecular machinery for large-scale regeneration intact, what keeps it from executing the regenerative repair processes? One of the explanations offered by some takes into account the 'embryonic' status of urodeles like the Axolotl. Pedomorphic urodeles have tissues which preserve embryonic characteristics and their impressive regenerative potential can be linked to this (Alibardi, 2014). Another example in support of this theory is that of *Xenopus*, in which regeneration is possible in larvae and, after transitioning into adulthood through metamorphosis, the ability is lost (Mescher et al., 2017). However, the case of salamanders and lizards is not justified by this theory. A more convincing and consistent hypothesis is that of the evolution of the immune system, as being detrimental to the potency of regeneration. Intense nature of inflammatory reactions of the immune system has been identified as a major factor limiting large scale regeneration in amniotes (Ferguson and O'Kane, 2004; Alibardi, 2014). Fukazawa et al. (2009) demonstrated at the organismal level that lowering of immune responses restores regenerative potential in tadpole tails, at a stage of development when they cannot regenerate. The lab of Mescher has used amphibian models to show that the removal of inflammatory reactions shortly after amputation is required for healing to progress towards regeneration (Harty et al., 2003; Mescher and Neff, 2006; King et al., 2009; Mescher et al., 2017). Such observations may lead one to believe that the presence of immune cells locally in an injured tissue may prevent the onset of regeneration. Interestingly, this is not the case. It has been observed in Axolotl that removal of macrophages leads to formation of a scar at the site of amputation and results in a complete failure of regeneration (Godwin et al., 2014). This slightly complicates our understanding of the relation between inflammation and regenerative competence. The most plausible explanation for this is the one given by Okabe and Medzhitov (2014), who observed switching of macrophages from pro-inflammatory to anti-inflammatory phenotype. This, while a developed immune system is broadly known to prevent regenerative repair, it seems to be in the hands of the immune system itself to allow regeneration in regeneration-competent animals. It can therefore be concluded that the local presence immune cells is essential for regeneration to occur, however the extent and timing of the resulting inflammatory response is of prime essence. A persistent presence of pro-inflammatory signals in the local environment leads a wound towards scarring (Verrecchia

and Mauviel, 2002; Leask and Abraham, 2004), while acute inflammation followed by its timely resolution takes the wound towards a scar-free phenotype (Mescher et al., 2017), which turns out to be an absolute essentiality for regeneration to begin.

It therefore cannot be said that immune cells are either good or bad for regeneration, since their presence and controlled management of inflammation by them are both important. What is evident is that to gain a mechanistic insight into epimorphic regeneration, it is required to understand the immune modulations therein. Surprisingly, the focus of regeneration biologists on growth factors and other development-related molecular pathways has not been matched by research on components of immunity. A group of lipid mediators well-known for mediating inflammatory actions, *viz.*, the eicosanoids have been neglected in studies on regeneration (Mescher et al., 2017). Our lab had therefore initiated a study to elucidate the role of cyclooxygenases (COXs) in epimorphic regeneration in the lizard *Hemidactylus flaviviridis*. Cyclooxygenase-2 (COX-2) inhibitors were found to potently retard the initial stages of regeneration in the lizard (Sharma and Suresh, 2008). COX-2 is an inducible isoform of COX and its major product Prostaglandin E_2 (PGE_2) has been implicated in a number of human diseases involving inflammation (Higgs et al., 1974; Brodie et al., 1980; Bombardieri et al., 1981; Mnich et al., 1995; Portanova et al., 1996). Interestingly, PGE_2 has also been named as an immune-suppressant in many settings (reviewed by Kalinski, 2012). It is observed that tumor cell production of Prostaglandins may be the cause of the immunodeficient environment found in tumor tissue (Plescia et al.. 1975; Bennet et al., 1977). Thus, the outcome of COX-2-induced PGE_2 appears to be context-dependent and seemed like a target worth studying in epimorphosis.

The current work was taken up to understand the temporal activity and expression pattern of COX-2 during the very early stages of regeneration in lizard, followed by testing its influence on other important signalling molecules known to be crucial for epimorphosis. All analyses were performed on tissue regenerates from amputated lizard tail at wound epithelium and blastema stages. Activity data for COX-2 revealed a sharp increase in activity on the first day post amputation (1 dpa) as compared to that in the resting (intact) tail (0 dpa). This level was seen to be maintained till 3 dpa and reduced slightly at 4 dpa, which corresponds to completion of AEC formation in our lab conditions. It was predicted that the activity pattern reflects the pattern of gene expression of COX-2 since its levels largely control the levels of prostanoids (Albrightson et al., 1985; Bailey et al., 1985). However, since there was also a

possibility of regulation of COX-2 levels at post-transcriptional levels (Evett et al., 1993; Ristimaki et al., 1994), western blot and PCR were performed. Results from both these experiments followed the theme of activity of the enzyme. Induction was found in 1 dpa samples and heightened expression was seen up to 3 dpa, after which a slight decrease was observed. It must be mentioned that this decreased activity and expression at wound epithelium stage (4 dpa) was still significantly higher than that in resting tail (0 dpa) samples. What now remained to be seen is the region of the tail regenerates where COX-2 is present. Immunolocalisation experiments performed on regenerates at wound epithelium and blastema stages helped localise COX-2. At the wound epithelium stage, it was found expressed majorly along the original plane of amputation and also along the newly formed epithelium. These are regions of high cellular activity with the epithelial cells migrating and proliferating and the stump tissue along the amputation plane undergoing repair and apoptosis. In the blastema, COX-2 was localised to the region immediately distal to the cut nerve cord. Further, a gene expression level analysis revealed that among the four known PGE receptors EP1, EP2, EP3 and EP4, only EP2 was expressed in the regenerates. Interestingly, its expression was found even in the 0 dpa (un-amputated) tissue. Thus, the COX-2/PGE2 pathway was operationally early on in tail regeneration, its effects were mediated through the EP2 receptor and its regulation based on the induction of the COX-2 gene.

Another inducible mediator of inflammation is iNOS, and isoform of Nitric oxide synthase (NOS) enzyme. It is expressed in response to inflammatory cytokines IL-1, TNFα and IFNγ, among others (Amin et al., 1995). iNOS mediates inflammation and tissue repair (Nathan and Xie, 1994; Schmidt and Walter, 1994; Marletta, 1994). iNOS product nitric oxide has shown involvement in the process of wound healing as reflected in numerous atudies in vivo and in vitro (Baudouin and Tachon, 1996; Bruch-Gerharz et al., 1996; Romero-Graillet et al., 1996; Wang et al., 1996; Shimizu et al., 1997; Yamasaki et al., 1998). Therefore, nitric oxide levels were screened for in the early time-points of wound healing in the amputated lizard tail. Interestingly, at 1 dpa, NO levels dropped to lower levels than were present in the resting tail (0 dpa). Significantly reduced NO levels were restored partially only at the wound epithelium stage (4 dpa). A similar trend was observed for iNOS protein in the western blot. Gene expression results failed to match the trend in NO concentration and remained low and rather steady through the said time-points. Thus, the control of NO may have been through post-transcriptional changes of the iNOS enzyme. If the COX-2 and iNOS pathways shared a positive correlation in the current system, NO conc would have shown an increase at 1 dpa.

Antagonising effects of NO on COX-2 have been observed by Clancy et al. (2000) in macrophages, wherein COX-2 expression suffered a setback due to NO presence.

It was hypothesised that increase in COX-2 expression prevents NO production and thereby protects the injured tissue from its harmful effects. After all, unregulated NO production can lead to strong inflammatory reactions, tissue damage and even a cancerous phenotype (Suzuki et al., 1995; Ahn et al., 1999). Moreover, timely of NO from the local environment can prevent the formation of potentially dangerous reaction products of itself and ROS (Beckman et al., 1996; Ahn et al., 1999). It seems plausible to suppose that the decrease in NO is a requisite for normal wound healing to take place since ROS may be an integral part of the wound healing process. ROS are essential for proper wound healing (Love et al., 2013) and NO may sequester the oxides and disrupt the process. Moreover, literature has evidence of COX-2 being an inducer of oxygen radicals after injury (Katusic, 1996; O'Banion, 1999; Virdis et al., 2005). In the specific case of lizard regeneration as well, this seems possible after Sharma (2008) suggested that pharmacological inhibition of COX-2 resulted in a less oxidative environment in the tail regenerates. To test the hypothesis that COX-2 inhibition may be leading to reduced NO synthesis, iNOS localisation was carried out through immunohistochemistry. If iNOS and COX-2 are directly interacting, they are likely to be present in the same region because COX-2 products are autocrine and paracrine in nature and do not exert their effects far from the site of synthesis. iNOS immunostaining indeed revealed that in wound epithelium and blastema stages, iNOS localises to the plane of amputation and the cut nerve end respectively, similar to COX-2. To obtain further confirmation on COX-2 influence on iNOS induction, the expression of iNOS was checked in response to COX-2 inhibition by etoricoxib. PCR and western blot reflect no significant change at the expression level for iNOS. Thus, COX-2 products do not seem to regulate iNOS expression.

Interleukins

An assessment of the immune status would be incomplete without information about the interleukins and other related major cytokines. A gene expression analysis was carried out for IL1β, IL2, IL6, IL10, TNFα, IFNγ and NFκB. IL10 is a well-known anti-inflammatory cytokine, while all the others mentioned here are largely known to promote inflammation. Among these, expression of only IL6 was found in the regenerating lizard tail tissue. Gene expression of IL6 was seen in resting tail (0 dpa), which gradually decreased after 1 dpa up to wound epithelium stage (4 dpa). This is consistent with the aforementioned theory that timely

resolution of inflammation is essential for regenerative healing. It must be stated here that non-amplification of the other interleukin genes in RT-PCR from lizard tails is likely a result of non-complementarity of primers with the template. While all other genes analysed in this thesis show high conservation across vertebrate species, the interleukins show minimum overlap. Since all primers were designed from homologous regions of Zebrafish and *Gallus*, they may have failed in the case of the interleukins.

Over and above these, two other interleukins – IL17 and IL22 – were selected for this study, based on their known involvement in regenerative processes in mammals (Dudakov et al., 2012; Rao et al., 2014; Lindemans et al., 2015). Expression of these interleukins was found in the resting tail and this is the first study showing their presence in lizards and also the first to reveal their expression pattern in any model of epimorphic regeneration. While it was suspected that both IL17 and IL22 may be promoting epimorphic regeneration in the lizard, results displayed a contrasting picture. Both the interleukins were found expressed in the resting tail (0 dpa) and, like in the case of IL6, diminished after 1 dpa, not appearing even when AEC formation was complete at 4 dpa. IL17 is attributed with the stimulation of the release of pro-inflammatory cytokines including IL6 from cultured monocytes (Li et al., 2000). Also, IL22, which has been recognised for roles in tissue repair, also promotes immune system activation and its excess release is associated with pathologies (Rutz et al., 2013). A close look at the RT-PCR results (Figure 3.10) reveals a sharper decline in IL17 than in IL22. This is consistent with the observation of Rutz et al. (2013) that IL17 is more potent than IL22 in eliciting a strong inflammatory reaction.

While it became clear that the interleukins IL6, IL17 and IL22 were suppressed as a prelude to wound epithelium formation, it was thus far not known whether COX-2 has an influence this trend in their expression. To gain clarity on immune regulation of the interleukins by COX-2, etoricoxib-administered were used. Treated lizards, which had diminished activity of COX-2 as compared to the untreated ones, exhibited significantly heightened expression of all the three tested interleukins IL6, IL17 and IL22. This establishes COX-2 as an upstream effector of immune suppression, at least in the context of IL6, IL17 and IL22. Additionally, since IL22 aids in the production of IL6 (Wolk et al., 2006), COX-2 helps prevent magnification of the pro-inflammatory response. To appreciate the importance of this suppression, the potential threat posed due to IL22 in a system of rapid proliferation, such as the regenerating lizard tail, must be highlighted. IL22 can induce the expression of anti-

apoptotic factors of the Bcl-2 family (Radaeva et al., 2004; Zenewicz et al., 2007; Sonnenberg et al., 2010). Therefore, the presence of IL22 throughout the stages of regeneration in the lizard tail could possibly lead to uncontrolled division. We are thus able to appreciate that a balance between proliferation and apoptosis is necessary in a rapidly regenerating tissue in order to prevent unwarranted growth and cancer.

Induction of COX-2 gene expression and a concomitant rise in its activity on the first day post amputation can now be linked to resolution of inflammation by suppression of pro-inflammatory cytokines. However, in line with the versatility of this enzyme, it has also shown influence on cell proliferation, angiogenesis and muscle redevelopment in a previous study on lizard tail regeneration (Suresh et al., 2009). It was therefore deemed necessary to check for interactions of COX-2 signals with signalling pathways participating in epimorphosis. The following discussion provides an account of the same.

Matrix metalloproteinases

In a tissue recovering from a major injury, one of the important groups of factors coming into play is the matrix metalloproteinases (MMPs). This is because tissue remodelling is a process without which the healing of large wounds is not possible. Be it regenerative healing or otherwise, the MMPs are responsible for the dynamic changes taking place in the extracellular matrix (ECM), which directly influence the survival and proliferation of the local cells (Godwin et al., 2014). MMPs are required for regeneration of foot in *Hydra* and Sea cucumber (Leontovich et al., 2000; Quinones et al., 2002). They are also upregulated during the formation of AEC in urodeles (Ferris et al., 2010). Our lab has been reported the role of MMP2 and MMP9 in fin and tail regeneration in teleost fish and lizard (Sharma, 2008; Rajaram et al., 2016). Prior evidence of involvement of COX-2 in the regulation of MMP activity during wound epithelium formation in *H. flaviviridis* is available from thr work of Sharma (2008). The study depicted reduced activities of MMP2 and MMP9 after COX-2 inhibition as seen from zymography experiments. In the current project, it was sought to understand whether this effect was seen at the level of MMP expression or not. Moreover, gene expression analysis of MT1-MMP (MMP14) and TIMP2 was also performed. MT1-MMP is an activator of MMP2 and can also itself cleave many peptides of the ECM (Seiki, 2003; Visse and Nagase, 2003). TIMP2, on the other hand, is a negative regulator of MMP activity and is often co-expressed with the MMPs to give optimal protease action (Gardner and Ghorpade, 2003). At the wound epithelium stage, etoricoxib treatment caused a

significant decrease in MMP2 and MMP9 expression, confirming that the activity decreased observed by Sharma (2008) was due to reduced protein levels. Gene expression data reflect a drastic decrease in the transcripts of both these genes and additionally, in those of MT1-MMP. MMPs may be crucial for the very early stages of regeneration since they can break down fibrinogen (Lelongt et al., 2001) and hence clear the provisional wound matrix. The gelatinases may also play an important part in wound epithelium formation since this process is characterised by migration and proliferation of cells from the wound periphery towards the centre, and digestion of cell connections to the basement membrane is essential for the same. MMP9 is required for cell migration in wound healing in mice and its defect leads to imperfect re-epithelialisation of wounds (Kyriakides et al., 2009). Over and above the decrease in gene expression of the MMPs, etoricoxib treatment also resulted into an increase in in timp2 expression. Thus, the reduced activity of the MMPs was in fact a combinatorial effect of their own expression and an activity level inhibition by TIMP2. It can therefore be said that COX-2 signalling helps maintain a balanced ratio of MMP to TIMP for proper wound healing to take place. This is exemplified by the fact that the MMP to TIMP ratio also remains high in mammalian fetal wounds (which heal without scars), as compared to adult wounds (which form scars), because a high level of MMPs prevents accumulation of collagen (Dang et al., 2003).

The hallmark of a blastema is accumulation of progenitor cells from the stump into the distal-most region beneath the AEC. Such migration and accumulation can be made possible by removal of the rigid extracellular framework in the region. Even the release of dedifferentiating cells per se depends on modifications of the ECM (Yang and Bryant, 1994), which result from MMP action. While these are processes of the early blastema, it is found that the MMPs are required up to the late blastema stage, where they regulate the differentiation of tissues (Miyazaki et al., 1996). Results from the current study reflect decreased expression of MMP2 and MMP9 proteins due to COX-2 inhibition. Gene expression of mmp2 showed a significant decrease, while the change in mmp9 and mt1-mmp genes was negative, but not biologically significant. Similarly, timp2 also showed lesser expression than controls, however this was not significant. It is evident that the effect of COX-2 inhibition on the MMP genes was not as severe in the blastema stage as in the wound epithelium stage. Nevertheless, the imperfect formation of blastema and reduced gelatinase activity due to etoricoxib, as observed earlier by Sharma (2008), could be an outcome of MMP2 expression being negatively affected. The mid-blastema stage involves intense

proliferation of its constituent cells and since the MMPs can promote proliferation of tumor cells (Itoh et al., 1998; Bergers et al., 2000; Zhou et al., 2000), they may be helping blastema cell proliferation as well.

Another outcome of MMP-mediated degradation of the ECM is the liberation of growth factors that remain embedded in it (Houck et al., 1992). Such interaction with matrix proteoglycans is a property of the fibroblast growth factors.

Fibroblast growth factors

The fibroblast growth factors (FGFs) are among the most important targets for the study of regeneration. Members of this pathway have been well-studied in embryonic development. They play a role in the formation of AER – a structure similar to the AEC in a regenerating tail (Mahmood et al., 1995; Savage and Fallon, 1995; Vogel et al., 1996; Ohuchi et al., 1997; Xu et al., 1998). Some studies also show promotion of tissue repair by FGF1, FGF2, FGF4, FGF7 and FGF10 (Abraham and Klagsburn, 1996; Werner, 1998). The current study involved assessment of expression of FGF pathway members in COX-2 inhibited lizards. At the wound epithelium stage, etoricoxib treatment caused a significant decrease in FGF2 protein expression. This was also reflected at the level of gene expression. fgf2 transcripts were significantly low in the wound epithelium stage regenerates. While other FGF ligands were not affected negatively, the gene for FGF receptor FGFR1 suffered a drastic fall in expression due to COX-2 inhibition. FGF2 transcripts have been implicated even in mammalian wound healing as they are increased after injury (Werner et al., 1992). FGFR1 is one of the most important components of FGF signalling since a number of FGF ligands are able to exert their effects through its binding (reviewed by Thisse and Thisse, 2005). Also, Anusree et al. (2013) have demonstrated in the lizard that blockage of FGFR1 signals obstructs wound healing after amputation of the tail. The cross-talk between COX-2 product PGE_2 and FGF2/FGFR1 signalling could be through MMP2. PGE_2 mediated MMP2 activation can lead to the release of FGF2 sequestered in the ECM. However, since the gene expression of the FGF members was affected, COX-2 also must have a direct influence on their gene induction. Another possibility is that of FGF2 reduction in etoricoxib treatment animals due to overexpression of TIMP2. This speculation stems from the observation of Seo et al. (2008) that TIMP2 inhibits FGF2-dependent proliferation of endothelial cells.

The blastema stage also involves FGF signals for its own formation and maintenance. This is similar to embryonic limb development wherein the limb formation depends on FGF2, FGF4,

FGF8 and FGF10 (Niswander and Martin, 1992; Savage et al., 1993; Heikinheimo et al., 1994; Martin, 1998). FGF8 and FGF10 are associated with the regeneration blastema in *Xenopus* (Yokoyama et al., 2010). Axolotl limb blastemas also express FGF8 and FGF10 (Han et al., 2001; Christensen et al., 2002). Indeed the present analysis revealed that in etoricoxib-treated lizards, the expression of all these FGFs implicated in blastema formation were severely affected. FGF2, FGF8 and FGF10 were the ligands for which transcripts were reduced. FGF2, FGF4 and FGF8 are able to induce a limb-bud in the absence of an AER (Niswander et al., 1993; Fallon et al., 1994; Cohn et al., 1995; Vogel et al., 1996). FGF8 stimulates FGF10 production (reviewed by Thisse and Thisse, 2005). Apart from these, etoricoxib also led to a major reduction in fgf20 expression. FGF20 has been previously implicated in blastema formation in Zebrafish fins.

An interesting finding by Nomura et al. (2008) was that FGF10 induced gene expression MT1-MMP in pancreatic cancer cells. This presents the possibility of a loop wherein FGFs and MMPs activate each other.

Among the receptors, fgfr1 suffered the same fate as that in wound epithelium. In fact, the fold change in gene expression was same in magnitude in both the tested stages of regeneration. FGFR1 plays a role in blastema formation and is expressed across the entire blastema in amphibians (Cannata et al., 2001).

It must be mentioned that transcripts of FGF4, FGF12 and FGFR3 were upregulated after COX-2 inhibition at blastema stage. However, the overall effect on the FGF pathway seems negative since FGF2, FGF8 and FGF10 are well known specifically for blastema function and FGFR1 relays signals from many of the FGF ligands.

Zebrafish model of fin regeneration connects FGFs with another very important developmental signalling pathway – the Wnt/β-Catenin pathway. FGFR1 inhibition by SU5402 resulted in decreased expression of Wnt pathway members lef1 (Poss et al., 2000).

Wnt/β-Catenin pathway

The Wnt pathway is a multifunction molecular pathway that has been conserved in animals since ancient history (Clevers et al., 2014). In the context of regeneration, they appear from Cnidarians (Lengfeld et al., 2009) and flat-worms (Gurley et al., 2008; Petersen and Reddien, 2008), right up to mammalian tissues (Korinek et al., 1998; Fevr et al., 2007; van Es et al., 2012). It regulates a number of cellular processes in vertebrates. Tissue repair depends on

Wnt signals in the mammalian intestine (Clevers et al., 2014). Wnt ligands are upregulated over the first few days in mouse cutaneous wounds (Okuse et al., 2005). β-Catenin stabilised in mice was able to accelerate wound healing in mice (Kapoor et al., 2008).

Since there was no information available on the profile of Wnt ligands expressed in epimorphic regeneration of the lizard tail, first a screen was carried out to find which of the canonical Wnt ligands are expressed during wound healing in lizard tail. From a set of 12 known canonical Wnts, the expression of 10 Wnts was found in the regenerates. These are wnt1, wnt2b, wnt3a, wnt4, wnt6, wnt7a, wnt7b, wnt8a, wnt10a and wnt16. Among these, wnt4 and wnt7a had significantly greater mRNA levels at wound epithelium stage as compared to an unamputated tail. At the blastema stage, wnt1 and wnt6 were expressed at higher levels as compared to the resting tail.

The Wnt pathway has shown interaction with COX-2 signals in some systems. Inhibition of cyclooxygenase activity by Aspirin reduced signals through β-Catenin (Dilhmann et al., 2001). Goessling et al. (2009) have also identified an association of the two pathways in Zebrafish fin regeneration.

It was therefore tested whether COX-2 had an influence on the members of the Wnt/β-Catenin pathway during lizard tail regeneration. During the wound epithelium stage, COX-2 inhibition by etoricoxib resulted in a significantly decreased β-Catenin presence, as seen from its western blot. Among the Wnt ligands gene expression of wnt4 was affected negatively at wound epithelium stage. When tested at the blastema stage, the same result was obtained for β-Catenin protein levels. For the Wnt ligands, it was observed that gene expression of wnt1 and wnt16 drastically reduced due to COX-2 inhibition.

wnt4 expression change could be a likely reason for etoricoxib group animals not achieving perfect wound epithelium formation in the lizard, since it has previously been identified as important for timely wound healing and is also upregulated post-trauma (Labus et al., 1998). The most noteworthy decrease in expression during the blastema stage was that for wnt1 in etoricoxib treated animals. This can be accounted for by the known pro-proliferative actions of wnt1 (Dickinson et al., 1994). Moreover, wnt1 is also able to induce satellite cell proliferation (Otto et al., 2008), a key process contributing to blastema formation.

It is interesting to note that there were many Wnt ligands for whom transcript levels increased significantly in response to COX-2 inhibition. The most pronounced such effect was seen on

wnt7a and this was observed for both the stages. A scan through literature brought forth a paper by Ramos-Solano et al. (2015), which summarises wnt7a as being detrimental to proliferative activity. Their observations in cervical cancer cells form the first report of ectopic Wnt7a inhibiting cell proliferation and migration. Moreover, silencing wnt7a in non-tumorigenic cells led to increased proliferation (Ramos-Solano et al., 2015). A question which now arises is that if multiple canonical Wnt ligands show an increased expression in treatment group animals, how then does β-Catenin protein level decrease? This may be explained by the fact that many factors other than Wnt ligands may control β-Catenin levels during proliferation (Bielefeld et al., 2011; 2012). Additionally, studies in vitro show convergence of PGE_2 pathway and β-Catenin through GPCR association with Axin (reviewed by Evans, 2009). Also, Wnt7a is able to induce both canonical and non-canonical pathways and this depends on the receptor type involved (Ramos-Solano et al., 2015). Further work in this direction must involve testing of the Wnt receptors playing a part in mediating signals during lizard tail regeneration.

Transforming Growth Factor β

One group of factors integral to the process of wound healing and tissue repair is the Transforming Growth Factor β superfamily. Three TGFβ isoforms TGFβ1, TGFβ2 and TGFβ3 are known to have profound effects on wound healing since they directly regulate proliferation and differentiation of cells and also affect the characteristics of the ECM and of the immune response (Penn et al., 2012; Finnson et al., 2013). While TGFβ1 induces a fibrotic response after injury by stimulating production of collagen and fibronectin (Varga et al., 1987; Hocevar et al., 1999), TGFβ3 is the one ligand shown to prevent fibrosis (Occleston et al., 2011). A parallel study in our lab has revealed that among the three TGFβ ligands, TGFβ3 is not expressed in the lizard tail regenerates – an observation consistent with the one reviewed by Ud-Din et al. (2014). In the present work, the influence of COX-2 activity was assessed on TGFβ expression. COX-2 inhibition resulted in a sharp rise in the levels of TGFβ2 during wound healing in the amputated tail, thereby facilitating scarless healing. This can be responsible also for resolution of inflammation as TGFβ ligands influence the recruitment of immune cells during wound healing (Cannigia et al., 2000; Soo et al., 2000; Barrientos et al., 2008).

From this study, it becomes clear that COX-2 activity has a multitude of effects on the cellular processes taking place during wound epithelium and blastema formation in the

regenerating lizard tail. It seems to prevent inflammation reactions beyond 1 dpa, thereby allowing scar-free healing of the amputation wound. An anti-fibrotic outcome of COX-2 activity during the wound epithelium stage is also a probability since its inhibition led to an increase in TGFβ2 transcripts. However, whether this is an absolute requirement for preventing scar-formation remains debatable, since continued etoricoxib treatment did not result into terminally fibrotic tissue and regeneration progressed after cessation of etoricoxib administration. What was thus far not clear was the intracellular pathway through which COX-2 signals were relayed. As a starting point, it was hypothesised that the cAMP/PKA pathway must be involved, since only EP2 receptors had shown expression in the present in the samples. EP2 and EP4 receptors activate Gs protein and thereby stimulate cAMP production through Adenylate cyclase (Reiner et al., 1992; Ichikawa et al., 1996). Western blot was performed in wound epithelium regenerates from control and etoricoxib group animals and probed for phosphor-PI3K, phosphor-CREB and p38 MAPK, representatives of three of the pathways through which the EP group receptors transduce their signals. Etoricoxib-mediated COX-2 inhibition was found to have the most notable influence on the levels of phospho-CREB reflecting the involvement of the cAMP/PKA pathway. Literature supports a role for PGE_2/cAMP signalling in the regulation of regenerative processes. Galea and Feinstein (1999) review that while PGE_2/cAMP enhances inflammation early on after injury, it can suppress prolonged inflammation. This control of immune response could well be a result of cAMP's ability to prevent T-cell activation (Birch and Polmar, 1982), prevent IL1β and TNFα secretion (Knudsen et al., 1986; Kunkel et al., 1988) and limit the recruitment of immune cells (Ghersa et al., 1994). Additionally, the cross-talk of COX-2/PGE_2 with the Wnt/β-Catenin could also be mediated through cAMP. Activation of the Wnt/β-Catenin in Zebrafish fin regeneration by PGE_2 depends on cAMP/PKA levels and not on PI3K activation (Evan et al., 2009; Goessling et al., 2009).

In summary, this thesis highlights the interactions of COX-2-mediated PGE_2 with a view to establish its position among the regulators of epimorphosis in lizard. Elucidation of the pathways resulting in scar-less wound healing and blastema formation will take us towards our goal of understanding the regenerative process in its entirety. Moreover, an understanding of the signalling pathways interacting with PGE_2 signalling will give us valuable information about many developmental processes, more so since COX-2 is also considered as a target for cancer therapeutics.

COX-/PGE2 pathway