5

The role of Cyclooxygenase-2 in Blastema formation

INTRODUCTION

It is clear from the previous chapters that proper healing of an amputation wound without formation of scars is absolutely inevitable for regeneration of the lizard tail. As essential as this is, it must be noted that scar-free wound healing is not itself sufficient for successful epimorphosis. As per the widely accepted definition of epimorphic regeneration, its very essence lies in the formation of the regeneration blastema. Thus, it seems that the main purpose of the Apical Epithelial Cap (AEC) formed as a result of healing in regeneration-competent animals is to allow or promote the formation of a blastema, ensuring that the severed organ is faithfully restored. From broad observations on various models across the animal kingdom, it is known that only some wounds heal in a way that they progress towards blastema formation (McCusker et al., 2015). Mammals, for instance, are not able to produce a blastema in their healing wounds and these end up as scars. But adult urodeles heal cutaneous wounds in a way that restores original skin structure and do not form scars (Seifert et al., 2012). As part of the very early response after injury, even axolotls show the presence of fibrotic tissue at the wound site, but this is reorganised to give back normal skin architecture (Neufeld and Day, 1996; Endo et al., 2004). Amputation wounds in urodele amphibians and some other vertebrates always result in the formation of a regeneration blastema at the tip of the stump. As the wound is healed, the AEC, in collaboration with nerves, directs the formation of the blastema (McCusker et al, 2015). This structure is most crucial to the success of epimorphic regeneration. The blastema, in structure and function, shares commonalities with the limb-bud of a developing embryo. The role of an AEC in regeneration in adults is reminiscent of the Apical Ectodermal Ridge (AER) of the limb-bud in that it induces the development of the blastema in the underlying mesenchyme by secreting the necessary factors such as FGF8 (Gilbert, 2014).

In view of the fact that appendage regeneration will not occur in the absence of a blastema, we must understand the mechanism behind its formation and subsequent expansion, if we are to induce large-scale regeneration in the clinical setting. Fortunately, regeneration biologists have, over the years, obtained valuable lead on the origin of the blastemal cells in various

vertebrate (and invertebrate) models of regeneration. In urodele amphibians, dedifferentiation of mature cells of the mesenchymal tissue marks the onset of blastema formation (Stocum, 1999). Of course, the role of reserve stem cells must not be undermined here and some tissues like muscles depend on these for their revival (reviewed by Santos-Ruiz et al., 2002). In the case of lizards, Gilbert et al. (2015) have provided evidence for reserve stem and progenitor cells residing in the original tail tissue. The likelihood of a combination of resident stem cell and dedifferentiating mature tissues as the source of blastemal cells in lizards remains high. Immediately following dedifferentiation, the newly formed cells begin to proliferate (Chalkley, 1954). Since this small mass of progenitor cells is entrusted with the responsibility of building an entirely new appendage, which is most often a complex of multiple tissue types, it relies on extensive proliferation, subsequent differentiation and patterning. In order to always give rise to the correct structures, the blastemal cells are under the precise regulation of a number of mitogenic and differentiation factors (Santos-Ruiz et al., 2002). The wound epidermis, which is itself induced by signals from the nerves, matures into the AEC and begins to secrete a multitude of factors into the underlying mesenchyme to regulate blastema formation and maturation. These factors induce members of the FGF, Wnt and BMP pathways, among others (reviewed by McCusker et al., 2015). This dependence of blastema function on signals from the AEC is highlighted by the observation of Stocum and Dearlove (1972) that in absence of the AEC, blastemas are unable to form correctly patterned limbs. Early work by Thornton (1968) had shown that the AEC directs regenerative outgrowth from the blastema.

In the current investigation, it is of interest to know whether the COX-2 pathway engages in cross-talk with any of the known regulators of blastema formation. A definitive involvement of COX-2 at the blastema stage of regeneration in *Hemidactylus flaviviridis* is known from the work of Sharma and Suresh (2008). Activity inhibition of the enzyme led to blockage of blastema formation. More reasons to expect such a role of COX-2 can be found in literature. PGE_2 acts on stem cells to promote their expansion. In a mouse model, exogenously added PGE_2 analogue $dmPGE_2$ was able to induce regeneration of hematopoietic stem and progenitor cells (Porter et al., 2013). Since the blastema also comprises of progenitor and multipotent cells, it is likely that they respond to PGE_2 signals. However, it remains to be ascertained whether COX-2 activity directly controls blastemal cell behaviour or does so by interacting with other major pathways. Since molecular signals almost never function independently, the latter was a more plausible hypothesis. Therefore, the current set of

experiments dealt with inhibition of COX-2 activity during the blastema stage in regenerating lizard tail and analysing its effects on the expression of major signalling molecules.

Matrix metalloproteinases

An essential requirement of dedifferentiation and accumulation of prospective blastema cells is the removal of the rigid extracellular framework. As noted earlier in this chapter, an amputation wound in regeneration-competent systems also responds with fibrosis, however, the fibrotic structure is quickly replaced due to tissue remodelling (Neufeld and Day, 1996; Endo et al., 2004). Such reorganisation of tissue must be an outcome of proteases like the matrix metalloproteinases (MMPs). Moreover, the release of dedifferentiating cells at the distal end of the stump also depends on modifications of the extracellular matrix (ECM) (Yang and Bryant, 1994), which directly result from the action of MMPs. In cutaneous wounds, MMP expression is dominant after covering of the wound and is associated with the removal of the provisional wound matrix (Gipson et al., 1988). In systems of epimorphosis like the amphibians and lizards, expression of the MMPs is induced to orchestrate regenerative repair (Yang et al., 1999; Kato et al., 2003; Vinarsky et al., 2005; Santosh et al., 2011). Their activity allows epithelial cell migration for wound covering, while also being responsible for dedifferentiation of local tissues and induction of progenitor states in cells. Dedifferentiation and recruitment of cells are processes related to the early blastema, but there is evidence for the involvement of MMP activity in the late blastema as well. (Miyazaki et al., 1996) have shown that these processes regulate differentiation of tissues through their matrix degrading activity. Therefore, MMP expression must be critically maintained throughout the major stages of regeneration. Moreover, since the blastema stage is characterised by intense proliferation of the progenitor cells, studies showing the involvement of MMP2, MMP9 and MT1-MMP in expansion of tumours (Itoh et al., 1998; Bergers et al., 2000; Zhou et al., 2000) offers support to their role as pro-mitotic signals for epimorphic regeneration.

Induction of MMP2 activity by COX-2 induced-PGE_2 is known in human endothelial cell culture (Jana et al., 2016). Additionally, and relevantly, inhibition of COX-2 by etoricoxib was found to negatively affect the activity of gelatinases MMP2 and MMP9 at the blastema stage during regeneration in *H. flaviviridis* (Sharma, 2008). It was thus tested whether this effect was exerted at the level of expression and/or regulation by the TIMPs.

Fibroblast growth factors

Among the best studied molecular signals involved in limb development and regeneration are members of the fibroblast growth factor (FGF) pathway. During early embryogenesis in vertebrates, the outgrowth of the limbs is under the influence of FGF2, FGF4, FGF8 and FGF10 after limb-bud formation (Niswander and Martin, 1992; Savage et al., 1993; Heikinheimo et al., 1994; Martin, 1998). Many roles of these FGFs are also known in systems of epimorphic regeneration. For example, FGF2 promotes regeneration of mesenchymal tissues in urodeles (Ferretti et al., 2001). FGF2 can compensate completely for the loss of expression regeneration-specific genes, which occurs in limb stumps deprived of nerve supply (Mullen et al., 1996). In *Xenopus*, FGF2 is a proven requirement for blastema formation. It also helps maintain proliferation of the blastema cells (Cannata et al., 2001). FGF8 and FGF10 are also important for their roles in regeneration. Both these ligands are expressed in the regeneration blastema and are associated with the success of regeneration, as seen in a study on *Xenopus* (Yokoyama et al., 2000). The blastemas in Axolotl limbs also express FGF8 and FGF10 (Christensen et al., 2001; 2002; Han et al., 2001). The FGFs also exert mitogenic effects in a variety of systems. FGF1 in the blastema of Axolotl has been found to regulate cell proliferation (Zenjari et al., 1996). FGF2 is required for proliferation of human embryonic stem cells (hESCs) and is even an obligate supplement in the culture medium of these cells (Thomson, 1998; Xu et al., 2005; Vallier et al., 2005; Levenstein et al., 2006; Coutu and Galipeau, 2011). Over and above this, FGFs have an important function of maintaining cells in a multipotent state and sustain a pool of progenitor cells (Coutu and Galipeau, 2011). This effect may have direct implications in recruitment of blastema cells in epimorphosis.

The major FGF receptors FGFR1 and R2, which convey signals from the FGF ligands, have been found expressed in the regeneration blastema (Poulin et al., 1993).

Wnt/β-Catenin pathway

One of the most versatile signalling pathways functioning in epimoprhic regeneration is the Wnt/β-Catenin pathway. Through regulation of β-Catenin stability and otherwise, the various Wnt ligands are able to exert a wide range of effects on cell-behaviour including cell-proliferation, migration and differentiation. During dermal wound repair in mice, levels of β-Catenin are found increased in fibroblasts, influencing their function (Cheon et al., 2002). The pathway regulates organogenesis in developing embryos as well. Wnt6 expression was closely associated with tissues of the developing heart in *Xenopus* (Lavery et al., 2008).

Another Wnt expressed in developing embryos is the Wnt10a and it has been implicated in epimoprhosis since it was found expressed in the regenerating tail of Axolotl (Caubit et al., 1997b). It may be responsible for the control of growth and patterning of the regenerating tail. Inhibition studies in systems of regeneration and cancer have proved handy in broadly identifying the function of the Wnt/β-Catenin pathway, although some of these have done little to identify the specific isoforms involved. Abrogation of signalling by this pathway using Axin or Dkk1 resulted in reduced or no regeneration in *Xenopus* (Yokoyama et al., 2007). Conversely, a constitutively stabilised β-Catenin could induce a regenerative phenotype in *Xenopus* limb buds during stages when regeneration does not occur (Yokoyama et al., 2007). The hypothesis that COX-2 may promote blastema formation via regulation of the Wnt/β-Catenin pathway is supported by the work of Dihlmann et al. (2001). They found down-regulation of the cell cycle gene Cyclin D1 in response to COX inhibition by Indomethacin. Cyclin D1 is a target gene under the influence of β-Catenin.

In view of close association of the above-stated signalling pathways with processes related to blastema formation, maintenance and differentiation, they were evaluated in lizards treated with etoricoxib for COX-2 inhibition. Regenerates were collected from control and treatment group animals at mid-blastema stage and processed for gene expression and protein expression experiments.

MATERIAL AND METHODS

Animals were divided into two experimental groups, *viz.*, control and etoricoxib-treated. Distal-most segment from the regenerating tail of each animal was collected at the mid-blastema stage and processed for RNA or protein isolation or for immunohistochemistry.

Protein expression of MMP2, MMP9, β-Catenin and FGF2 was tested using western blot. Anti-MMP2 IgG Rabbit (Sigma Aldrich, USA) and Anti-MMP9 IgG Goat (Sigma Aldrich, USA), Anti-β-Catenin IgG Mouse (SantaCruz Biotechnology, USA), Anti-FGF2 IgG Rabbit (Sigma Aldrich, USA) and Anti-β-Actin (SantaCruz Biotechnology, USA) were used at 1 µg/ml, 0.1 µg/ml, 0.2 µg/ml, 0.1 µg/ml and 0.01 µg/ml respectively respectively. The ALP-BCIP/NBT system was employed for colour development.

Immunohistochemistry for β-Catenin was performed using Anti-β-Catenin at 3 µg/ml, as per the protocol described earlier (Chapter 2).

Relative gene expression in control and treatment samples was analysed using real-time qPCR. Reaction system and primer sequences are detailed in Chapter 2: Material and Methods. Data is represented here as mean of normalised Cq values and also as mean fold change in gene expression in treatment groups as compared to control (calculated by the method of Livak and Schmittgen, 2001).

RESULTS

Matrix metalloproteinases

MMP protein expression

Regenerates at the mid-blastema stage were collected and analysed for changes in protein expression of MMP2 and MMP9. Protein from the distal-most regenerating segments of control and etoricoxib treated animals was isolated and 30 µg was used in western blot. There is a visible decrease in the expression of MMP2 in the treatment group samples as compared to control (Figure 5.1). MMP9 also showed a reduction in its expression but this was not as visibly significant as that seen for MMP2.

Gene expression of MMPs

The effect of etoricoxib on the expression of mmp2, mmp9 and mt1-mmp (mmp14) was assessed at the blastema stage. Real time qPCR was reported as mean Cq values of the stated genes normalised to those of 18S rRNA in the respective samples. These are plotted in Figure 5.2. Fold change in expression relative to the respective control samples is shown in Table 5.1. A fold change of less than 0.5 or greater than 2 was considered as significant to our study. mmp2 expression was significantly reduced in treatment group blastemas (0.477 fold expression as compared to control, $p<0.05$). mmp9 and mmp14 did show a slight reduction in expression after etoricoxib treatment (0.655 fold and 0.609 fold respectively as compared to controls); however, these changes were not accepted as biologically significant. timp2, from which the regulator of the MMPs is produced, also displayed an unchanged level of expression in treatment group blastemas (0.749 fold with respect to control).

Fibroblast growth factors

Protein expression of FGF2

Western blot of FGF2 was carried out and results revealed that, just as was seen in wound epidermis samples (Chapter 5.1), there was a reduction in its expression in COX-2 inhibited animals at the blastema stage (Figure 3).

Gene expression of FGFs

A concomitant decrease in the expression of fgf2 gene, correlating with the protein expression, was seen in the blastemas of treatment group animals. Its expression was found to be 0.40 fold as compared to the control ($p<0.01$) (Figure 5.3, Table 5.2). Other fgf ligands were tested and a significantly reduced expression of fgf8 (0.42 fold, $p<0.05$), fgf10 (0.131 fold, $p<0.01$) and fgf20 (0.16 fold, $p<0.001$) was observed. Over and above these, there were also fgf ligands which showed greater expression in the treatment group samples. fgf4 and fgf12 were upregulated at 2.55 and 2.96 fold respectively ($p<0.05$). Among the receptors of the FGFs, the gene for FGFR1 was affected most significantly. fgfr1 expression was only 0.062 fold as compared to its control ($p<0.01$). fgfr3 was upregulated (3.5 fold) in response to etoricoxib treatment ($p<0.01$) and fgfr2 showed no change in expression.

Wnt/β-Catenin pathway

β-Catenin protein levels

The levels of β-Catenin protein in the blastema tissue were analysed in the experimental groups using western blot to evaluate signalling through the canonical Wnt pathway. Results shown in Figure 5.1 demonstrate a visible decrease in expression of β-Catenin due to etoricoxib treatment.

β-Catenin localisation

There was no prior information available on the distribution of active β-Catenin in lizard regenerates at blastema stage and therefore immunohistochemical localisation was carried out. Freshly cut frozen sections of 10 µm thickness were incubated with Anti-β-Catenin (0.6 µg/ml) overnight. Colour was developed by the ALP-BCIP/NBT system. Figure 5.4 is a composite image of micrographs of a longitudinal section of the regeneration blastema. Strong blue staining is visible in the region of the cut spinal cord and relatively faint signals are seen along the epithelial covering.

Wnt ligand expression profile

Since it was thus far not known which Wnt ligands were expressed during the stages of regeneration in lizard, all the canonical Wnt ligands were first screened for their expression at the blastema stage. Expression of wnt1, wnt2b, wnt3a, wnt4, wnt6 wnt7a, wnt7b, wnt8, wnt10a and wnt16 was found (Figure 5.5, Table 5.3). Results revealed a significant dominance of wnt1 and wnt6 gene expression at this stage. These were expressed at 725 and 10.7 fold respectively as compared to the resting tail tissue ($p<0.001$).

Gene expression of Wnt ligands

The effect of COX-2 inhibition was analysed on the expression of the wnt ligands at the blastema stage. Normalised mean Cq values shown in Figure 5.6 reflect a statistically significant reduction in the expression of wnt1 and wnt16 in response to etoricoxib treatment. Fold change reduction for these two genes was 0.006 and 0.032 respectively as compared to control ($p<0.001$) (Table 5.4). Interestingly, an appreciable increase was observed in the case of wnt2b, wnt3a, wnt7a, wnt7b, wnt8a and wnt10a expression at this stage in the treatment groups ($p<0.05$, $p<0.05$, $p<0.001$, $p<0.05$, $p<0.05$ and $p<0.001$ respectively).

DISCUSSION

Epimorphic regeneration is certainly dependent on the quality of healing which takes place at the site of amputation. However, despite pro-regenerative healing being a necessity for subsequent regeneration, it is not capable of inducing regenerative outgrowth, patterning and restoration of the complete organ by itself (McCusker et al., 2015). The distinguishing feature of epimorphosis is the event following wound healing. In presence of appropriate signals from the nerve (Singer, 1974) and from the fully formed apical epithelial cap (AEC), a pool of cells accumulates beneath the AEC and takes charge of replacement of the lost organ. This aggregation of cells – the blastema – is an indispensable structure in the regeneration of appendages in vertebrates. Whether or not an organism is able to regenerate lost appendages is directly dependent on its ability to stimulate local tissues to produce a population of these cells which hold the capability of activating their cell cycle program (Tsonis, 1996; Brockes, 1997; Stocum, 1999; Santos-Ruiz et al., 2002). The salamanders, which are masters of regeneration among the tetrapods, induce cells from connective tissue of the stump after amputation to lose their tissue-specific identities and become part of the blastemal pool. This is achieved as a result of complex chemical cross-talk between the nerves in the limb stump and the newly formed AEC (Gardiner et al., 1986; Muneoka et al., 1986; Endo et al., 2004; Hirata et al., 2010; Nacu et al., 2013). Over and above recruitment of cells for the blastema, the AEC has further implications in regeneration. While the early blastema is directly dependent for its own formation on the AEC and the nerves, its transition into the mid-blastema is also brought about by the AEC. The mid-blastema stage is characterised by rapid proliferation of the constituent cells to form an enlarged conical structure over the stump. Results of mitotic indices calculated by Mescher (1976) and Globus et al. (1980) have

provided evidence that the AEC is responsible for the proliferative activity of the blastema. The progenitor cells are known to originate via two mechanisms. In urodeles, it has been demonstrated that many of the local tissues contribute cells which adopt multipotent states. In addition, some tissues maintain a pool of stem cells for the purpose of tissue repair, which also participate in the regeneration blastema (Tweedell, 2010). A pertinent question that arises is whether the blastemal cells only respond to instructions from the AEC and nerves to build the missing structures or they are able to govern some cellular processes themselves too. A possibility of the latter is logically much higher, more so due to the following observations on stem cells. Mesenchymal stem cells (MSCs) are very well known to secrete factors which can alter behaviour of the surrounding and also of their own (Falanga et al., 2007; Garg et al., 2014). They are capable, through the release of numerous factors, of directing cell proliferation, differentiation, recruitment of progenitor cells and modulation of the extracellular matrix during tissue repair (Badiavas and Falanga, 2003; Rustad et al., 2012; Garg et al., 2014; Duscher et al., 2016). Indeed, an investigation of the genes expressed during the blastema stage in Zebrafish fin regeneration revealed the presence of FGF, BMP and Wnt pathway members (Katogi et al., 2004).

One of the important small molecules released by MSCs is PGE_2, by means of which these cells control the healing process and are able to reduce scarring (Hu et al., 2014). PGE_2 has caught the attention of our lab since it is a potentially crucial promoter of blastema formation and proliferation. It is reported to help in expansion of multipotent progenitor cell populations of murine embryonic stem cells (ESCs) (North et al., 2007). The constant production of PGE_2 by COX-2 in ESCs helps also in their survival and protects them from apoptosis. This is mediated through an EP2 dependent mechanism (Liou et al., 2007). Similar results are also seen for Hematopoietic stem cells (HSCs), wherein PGE_2-mediated survival and proliferation was seen along with reduced active Caspase-3 (Hoggatt et al., 2009). Hypothesising that COX-2 activity can control the microenvironment in the blastema tissue, the influence of COX-2 inhibition was evaluated on the regulation of three major groups of signalling molecules implicated in epimorphic regeneration, *viz.*, MMPs, FGFs and Wnts.

Matrix metalloproteinases

A prerequisite for blastema formation is the clearing of the rigid framework of extracellular proteins that surround the cells. This is brought about by major modifications to the proteins which constitute the structure. Matrix degrading enzymes have the primary role of effecting the remodelling of the ECM during tissue repair. As reported in Chapter 4, they are crucial

during regenerative wound repair since it involves migration of epithelial cells through digestion of the basement membrane. At the blastema stage too, their functioning ensures that the cellular events take place unhindered, as already mentioned earlier in this chapter. Wound healing models in mice have been used to show the importance of MMP9 for the processes of cell migration and tissue remodelling (Kyriakides et al., 2009). As an eventuality of matrix degradation through MMPs, one outcome which must not be overlooked is the resulting release of regulatory factors, which otherwise remain sequestered. As a means of quick response to tissue injury, there are pro-repair proteins embedded in the ECM, which initiate the healing response promptly. Growth factors like FGF2 and VEGF are bound in this manner and, after protease action on the matrix, are released, becoming free to exert their cellular effects (Houck et al., 1992). Moreover, the TIMPs, which act to regulate the activity of the MMPs, are known for more than just MMP inhibition. TIMP1 and TIMP2 are both potent inducers of mitosis in a range of cell types including keratinocytes, fibroblasts, myocytes, chonrodcytes and more (Gasson et al., 1985; Bertaux et al., 1991; Hayakawa et al., 1992; 1994; Nemeth and Goolsby, 1993; Yamashita et al., 1996; Wang et al., 2002; Stevenson et al., 2006). TIMP1 is also able to show this effect in tumorigenic cell lines (Luparello et al., 1999). Since many of the features of blastemal cells overlap with those of cancer cells, one can speculate the TIMPs to have proliferation inducing consequences in regeneration (Stevenson et al., 2006).

The current work was undertaken to study whether the MMPs can be regulated by COX-2 signalling at the blastema stage during regeneration in lizard. Etoricoxib treatment resulted in reduced expression of MMP2 and MMP9 proteins, just as it did during the wound epidermis stage. Corresponding transcripts were also low in expression as compared to control; however, biologically significant reduction was seen only for MMP2 transcripts. mmp9 and mmp14 genes did not show a significant change. COX-2 therefore may induce the MMP genes downstream but this effect was more pronounced at the wound epidermis stage than at the blastema stage. mmp2 was faithfully regulated by COX-2 products and must have implications on blastemal cell recruitment, migration as well as proliferation. The expression of timp2 was also not significantly different in etoricoxib group than in control. The overall effect on the MMP genes was therefore less severe than that seen at the wound epidermis stage. Taking a cue from the expression pattern, it was less likely that TIMP2-mediated inhibition of MMP increased due to COX-2 inhibition at the blastema stage. Nevertheless, negative regulation of the mmp2 gene was observed, accounting for the reduced MMP2

activity observed earlier in our lab by Sharma (2008). Moreover, the functions of TIMP2 are not very straight forward to be understood. Apart from its inhibitory and mitogenic functions, it has also been seen as an activator of MMP2 protein by interaction with MT1-MMP (Strongin et al., 1995; Sternlicht and Werb, 2001).

There are reports available on an interaction between the COX-2 and MMP pathways. MMP2 activity is involved in angiogenesis in endometriosis and this is mediated through COX-2-induced PGE_2 (Jana et al., 2016). Another study in endothelial cells by Finetti et al. (2008) established that COX-2 activity induces MMP2 for mediating angiogenic signals.

Fibroblast growth factors

The MMP enzymes have been linked to the FGF pathway in cancer (Nomura et al., 2008). The role of FGFs is crucial for limb development. They are responsible for sustained proliferation in the progress zone of the limb bud (Thisse and Thisse, 2005). Martin (1998) has reviewed the part played by FGFs in initiation of the limb bud.

It was therefore tested whether the role of COX-2 in blastema formation and proliferation is dependent on the FGF pathway. Results obtained for the FGF ligands were different from those in wound epidermis stage. Inhibition of COX-2 by etoricoxib led to a drastic decrease in the gene expression of FGF2, FGF8, FGF10 and FGF20. A scan through literature reveals definitive roles of precisely these isoforms in regeneration blastemas as well as in developing limbs.

Cannata et al. (2001) have described a perfect correlation between FGF2 expression in the limb mesenchyme of *Xenopus* and its ability to form a functional blastema. It also helps sustain proliferation in the blastema. It is also associated with proliferation of undifferentiated neural progenitors and fades with their differentiation (Ferretti et al., 2001). An ectopic limb bud can be induced in absence of the AER by the addition of FGF2, FGF4 and FGF8 (Niswander et al., 1993; Fallon et al., 1994; Cohn et al., 1995; Vogel et al., 1996). Bud formation in the developing limb begins with FGF8 acting to stimulate FGF10 production (reviewed by Thisse and Thisse, 2005). FGF8 is also a proven player in successful regeneration of the *Xenopus* limb (Christen and Slack, 1997). A role for FGF10 was discovered in cancer cells, increasing their migration and invasiveness through FGFR3 (Nomura et al., 2008). Another FGF member, which has been linked with induction of proliferation of cancer cells, is FGF20 (Whitehead et al., 2005). FGF20 was also significantly altered negatively in the current analysis in lizard. That COX-2 is an upstream regulator of

FGF20 may be an important finding since the latter has been implicated in blastema formation in Zebrafish fin regeneration as well. It expression was apparent in the caudal regeneration blastema and it was found in the mesenchyme (Whitehead et al., 2005).

The genes for FGF receptors FGFR1, R2 and R3 responded in a way similar to that seen during the wound epidermis stage (Chapter 4). Whereas fgfr1 dropped significantly in its expression due to COX-2 inhibition, fgfr2 remained unchanged. fgfr3, on the contrary, displayed a heightened expression in the treatment group blastemas. While the reasons for such a trend are hard to explain, the consistent reduction in fgfr1 expression make one speculate that it is closely related to COX-2 signalling as a downstream effector. What is striking is that the fold change in expression of fgfr1 in response to etoricoxib is exactly the same at both wound epidermis and blastema stages (0.06) fold as compared to control. It is therefore hypothesised that fgfr1 gene faces direct regulation by COX-2 products without cross-interference from other regulators. Amphibian blastemas show presence of FGFR1 and R2. FGFR1 is expressed across the entire blastema, while FGFR2 is localised mainly to its core (Cannata et al., 2001). All blastemal cells must therefore be able to respond to FGF signals emanating from the AEC, the nerve and from the blastema itself.

An example of cross-talk between molecular signals is found in the case of hESCs, wherein FGF2 affects the signalling through the Wnt pathway to maintain the cells in a pleuripotent state (Ding et al., 2010). This, and an indispensable role of Wnt signals in development, was a reason to take up a study of the Wnt/β-Catenin in blastema formation in lizard.

Wnt/β-Catenin pathway

The Wnt signalling pathway interacts with FGF members to initiate limb development (Kawakami et al., 2001; Ng et al., 2002; Yang, 2003; Mercader, 2007). The limb bud shows presence of Wnt2b as an activator of FGF10 expression (Mercader et al., 2006). Thus it can be speculated that this pathway is at the forefront of regulation of blastemal cell behaviour during epimorphosis. A β-Catenin immunoblot in the present study reflects dependence of β-Catenin stabilisation on COX-2 activity in the lizard blastema. This may relate with the implication of the Wnt/β-Catenin pathway in the proliferation of cells of the regeneration blastema in Zebrafish fins (Wehner et al., 2014). Canonical Wnt ligands can be believed to play a part herein. A role for Wnt10a has been identified in newt regeneration. It is expressed in the blastema and is essential for the subsequent differentiation of these cells into the new

tissues (Caubit et a., 1997b). Cawthorn et al. (2012) have also demonstrated that Wnt6 and Wnt10a promote formation of osteoblasts.

A localisation experiment carried out in the present investigation revealed strong presence of active β-Catenin at the core of the blastema. This may represent high cell-synthetic or proliferative activity in that region. Expression of β-Catenin in specifically this region underscores it importance in formation of blastema since McLean and Vickaryous (2011 from Gilbert et al., 2015) have suggested that the proliferation of cells near the end of the original spinal cord mark the onset of blastema stage. Indeed, this region in lizards seems to be an active zone of sorts, since COX-2 and iNOS were also localised to the same region. It may reflect autocrine and paracrine signalling by COX-2 in this region. This group of cells are also among the first to differentiate, so as to form the ependymal tube, as seen from β-Catenin immunostaining of the late blastema (data not shown).

As part of the current work, it was required to first assess which Wnt ligands were specifically expressed at the blastema stage in lizards. Ten canonical wnt ligands were found expressed (wnt1, wnt2b, wnt3a, wnt4, wnt6, wnt7a, wnt7b, wnt8a, wnt10a and wnt16), of which wnt1 and wnt6 expression was highest at blastema stage as compared to resting tail and wound epidermis stage. It is reasonable to infer from this result that wnt1 and wnt6 are responsible for blastema formation and/or maintenance in the lizard. Literature lends credence to this notion. Wnt6 has been found to be a potent regulator of MSC cell fate (Cawthorn et al., 2012). Also, it has been associated with epithelial-mesenchymal transition in *Xenopus* embryos (Lavery et al., 2008). Supplement of spinal cord tissue with Wnt1 by Dickinson et al. (1994) resulted in a greater proportion of proliferating cells therein.

To test the effect of COX-2 inhibition on the Wnt ligands, all the above-mentioned wnt genes were analysed for changes in expression at blastema stage after etoricoxib treatment. wnt1 and wnt16 were negatively affected in treatment group tissue. wnt1 exhibited an extreme fall in expression due to COX-2 inhibition. The decrease was greater in magnitude than any other gene analysed at this stage in any pathway. This may have had severe repercussions on the normal function of the blastema in view of the pro-proliferative actions of wnt1 (Dickinson et al., 1994). Much worth a mention here is also an ability of wnt1 to induce satellite cell proliferation (Otto et al., 2008). As discussed in the introduction of this chapter, satellite cells are resident stem cells that could be contributors to the pool of blastemal cells. An observation that is most difficult to explain is the change in expression of wnt2b, wnt3a,

wnt7a, wnt7b, wnt 8a and wnt10a. These were significantly higher in expression than the controls, in spite of β-Catenin protein taking a dip. Nevertheless, a convincing conclusion is that abrogation of COX-2 activity causes dysregulation of the Wnt pathway components, even if its nuances are not understood completely. Moreover, wnt1 is certainly a mediator of COX-2-induced PGE_2 in functions of the lizard blastema.

The Wnt and COX-2 pathway have been known collaborators in some systems. HSC regeneration by Wnt is mediated by PGE_2, which increases cell survival and proliferation (Goessling et al., 2009). Survival of naive umbilical cord blood T cells, which depended on Wnt signalling, was also promoted by PGE_2 (Li et al., 2014).

In conclusion, COX-2 has far reaching downstream effects in the regeneration blastema of lizard *H. flaviviridis*. A mechanism is hypothesised from the obtained data. COX-2 induced PGE_2 acts on MMPs to change the organisation of the ECM, leading to release of factors like FGF2. Additionally, expression of FGF2 is also promoted. Another crucial protein induced by COX-2 is the FGFR1, which relays signals from most of the FGFs. It may also be responsible for maintenance of FGF8 and FGF10 expression, since interfering with COX-2 activity results in a fall in the expression of these ligands. These may be under the influence of Wnt1 rather than directly under COX-2, as the induction of FGF8 and FGF10 occurs under the influence of Wnt/β-Catenin in the developing limb bud. Together, the stated molecular factors concert the recruitment and subsequent proliferation of blastemal cells under the influence of COX-2.

Table 5.1: Relative gene expression data for MMP genes in control and etoricoxib-treated animals at blastema stage.

	Mean normalized Cq values		Fold change in Etoricoxib group regenerates
	Control	**Etoricoxib**	
mmp2	25.56 ± 0.27	26.68 ± 0.27 *	0.48 ± 0.09 **
mmp9	22.13 ± 0.52	22.75 ± 0.13	0.66 ± 0.06 *
mmp14	25.85 ± 0.26	26.65 ± 0.34	0.61 ± 0.15
timp2	22.44 ± 0.38	22.95 ± 0.39	0.75± 0.18

Values are represented as mean±SEM. N=6. *p<0.05, **p<0.01, ***p<0.001 as compared to control. Fold change gene expression was calculated as relative to control.

Table 5.2: Relative gene expression data for MMP genes in control and etoricoxib-treated animals at blastema stage.

	Mean normalized Cq values		Fold change in Etoricoxib group regenerates
	Control	**Etoricoxib**	
fgf2	26.02 ± 0.35	27.35 ± 0.14 **	0.40 ± 0.04 **
fgf4	24.29 ± 0.18	23.01 ± 0.43 *	2.55 ± 0.75 *
fgf8	25.01 ± 0.04	26.24 ± 0.36 *	0.44 ± 0.11 *
fgf10	27.60 ± 0.33	30.86 ± 1.40 **	0.16 ± 0.12 **
fgf12	25.16 ± 0.12	23.63 ± 0.32 ***	2.96 ± 0.65 *
fgf20	23.86 ± 0.16	26.44 ± 0.30 *	0.17 ± 0.04 ***
fgf21	24.73 ± 0.15	23.78 ± 0.19	1.94 ± 0.25 *
fgf22	23.65 ± 0.25	23.43 ± 0.15	1.17 ± 0.12
fgfr1	24.43 ± 0.45	28.43 ± 1.01 ***	0.06 ± 0.05 **
fgfr2	24.90 ± 0.34	25.05 ± 0.94	1.10 ± 0.63
fgfr3	25.35 ± 0.08	23.56 ± 0.22 ***	3.50 ± 0.52 **

Values are represented as mean±SEM. N=6. *p<0.05, **p<0.01, ***p<0.001 as compared to control. Fold change gene expression was calculated as relative to control.

Table 5.3: Relative gene expression data for Wnt genes in resting (0 dpa) versus blastema stage.

	Mean normalized Cq values		**Fold change in Wound healing compared to resting**
	Resting	**Blastema**	
wnt1	26.18 ± 0.08	16.68 ± 0.49 ***	725.75 ± 0.03 ***
wnt2b	25.16 ± 0.01	23.88 ± 0.41	2.43 ± 0.23
wnt3a	26.0 ± 0.13	25.82 ± 0.61	1.13 ± 1.37
wnt4	30.24 ± 0.37	30.53 ± 0.84	0.82 ± 0.02
wnt6	30.78 ± 1.65	27.36 ± 0.61 **	10.70 ± 2.82 ***
wnt7a	31.78 ± 0.08	30.24 ± 0.82	2.90 ± 0.47
wnt7b	25.96 ± 0.26	24.53 ± 0.95	2.69 ± 0.80
wnt8a	26.14 ± 0.10	24.19 ± 0.83	3.86 ± 0.44
wnt10a	25.95 ± 0.27	23.16 ± 0.43	6.92 ± 0.25
wnt16	29.66 ± 0.81	27.64 ± 0.62	4.07 ± 0.11

Values are represented as mean±SEM. N=6. *p<0.05, **p<0.01, ***p<0.001 as compared to control. Fold change gene expression was calculated as relative to control.

Table 5.4: Relative gene expression data for Wnt genes in control and etoricoxib treated animals at blastema stage.

	Mean normalized Cq values		**Fold change in Etoricoxib group regenerates**
	Control	**Etoricoxib**	
wnt1	16.68 ± 0.49	24.03 ± 0.71 ***	0.01 ± 0.01 ***
wnt2b	23.88 ± 0.41	19.79 ± 0.68 ***	16.95 ± 10.25 *
wnt3a	25.82 ± 0.61	20.80 ± 0.25 ***	32.52 ± 5.95 *
wnt4	30.53 ± 0.84	31.73 ± 0.42	0.43 ± 0.15
wnt6	27.36 ± 0.61	29.54 ± 0.66	0.22 ± 0.12
wnt7a	30.24 ± 0.82	19.25 ± 0.38 ***	2043.2 ± 636.7 ***
wnt7b	24.53 ± 0.95	20.93 ± 0.34 **	12.13 ± 3.03 *
wnt8a	24.19 ± 0.83	21.19 ± 0.44 *	8.00 ± 2.65 *
wnt10a	23.16 ± 0.43	20.55 ± 0.04 *	6.12 ± 0.19 ***
wnt16	27.64 ± 0.62	32.62 ± 0.97 ***	0.03 ± 0.03 ***

Values are represented as mean±SEM. N=6. *p<0.05, **p<0.01, ***p<0.001 as compared to control. Fold change gene expression was calculated as relative to control.

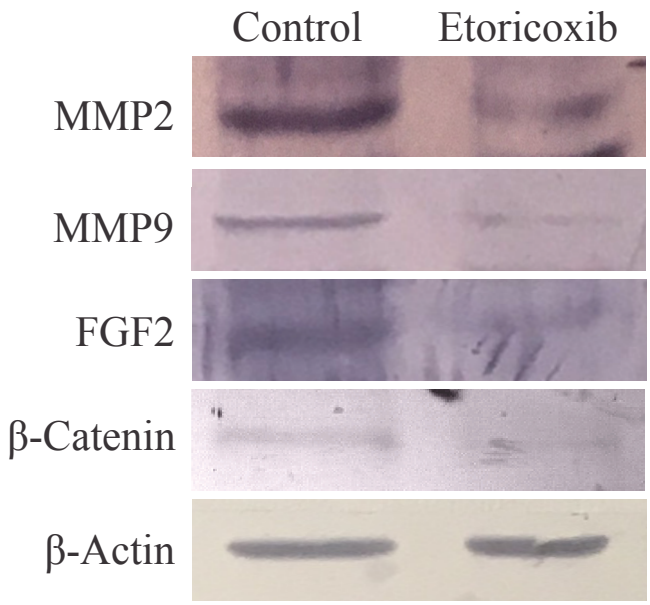

Figure 5.1: Western blot showing expression of various proteins in control and etoricoxib group animals at blastema stage. β-Actin was used as internal loading control.

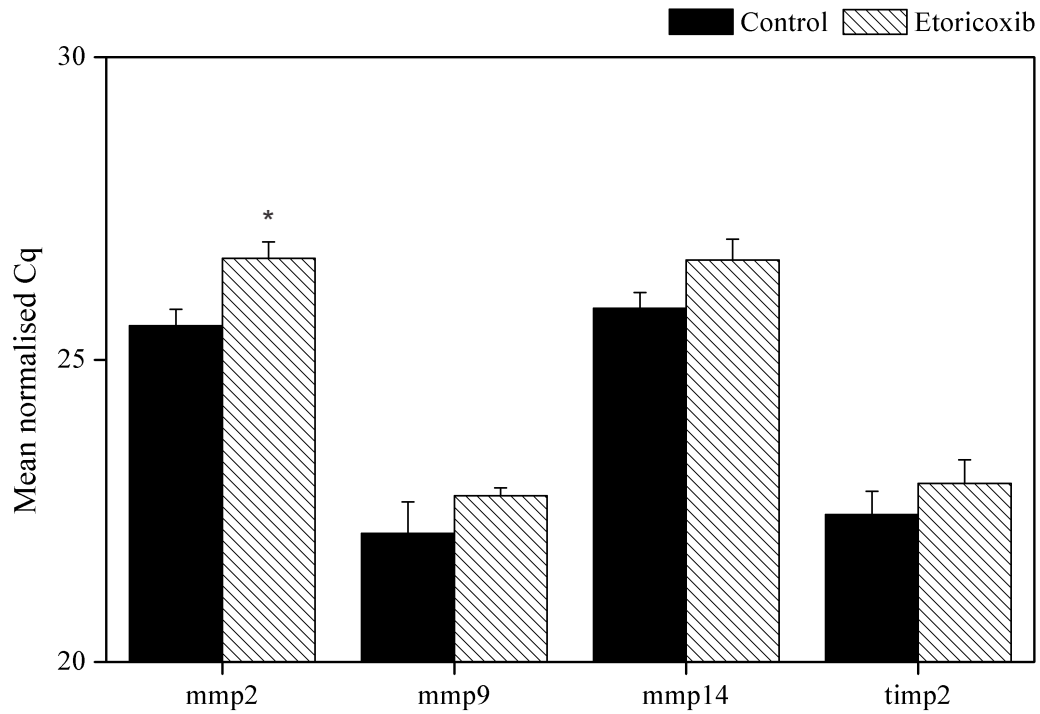

Figure 5.2A: Mean normalised Cq values for MMP genes in control and etoricoxib group animals at blastema stage. Cq value is inversely related to gene expression levels.

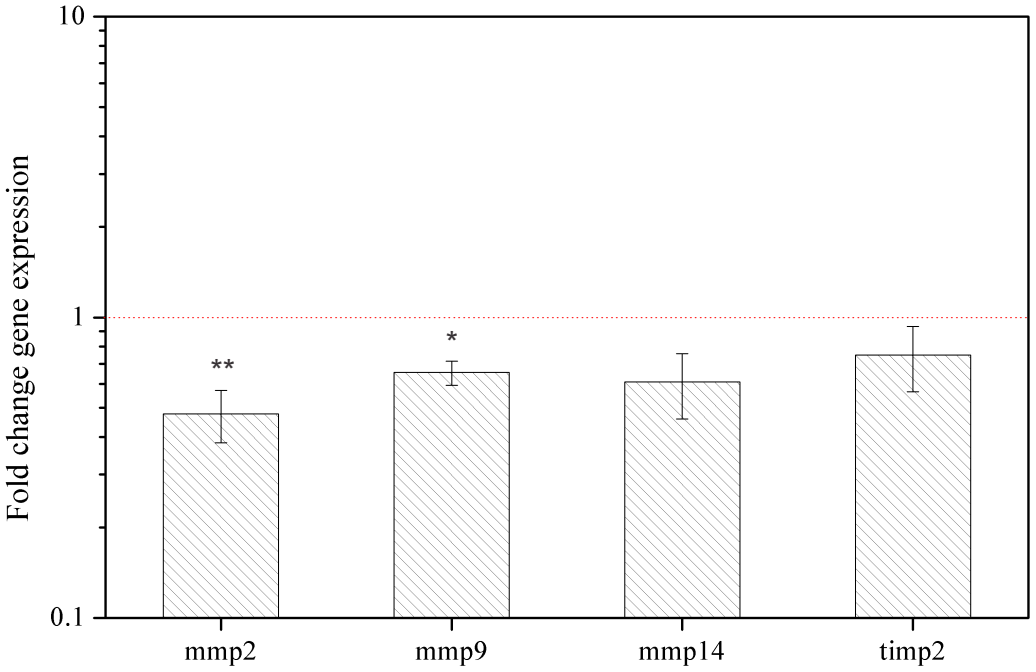

Figure 5.2B: Mean fold change ($2^{-\Delta\Delta Cq}$) in expression of MMP genes in etoricoxib group animals with respect to controls at blastema stage. Dashed red line indicates gene expression level of control group samples taken as 1.

Scale bars represent standard errors of means; *p<0.05, **p<0.01 as compared to controls

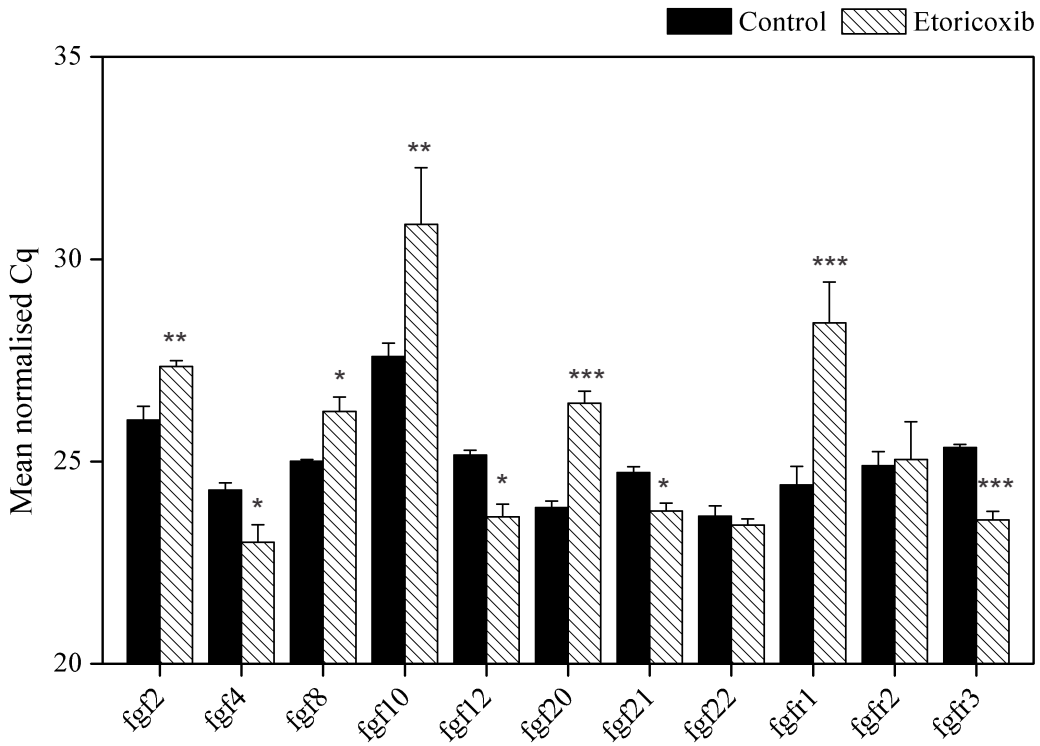

Figure 5.3A: Mean normalised Cq values for FGF genes in control and etoricoxib group animals at blastema stage. Cq value is inversely related to gene expression levels.

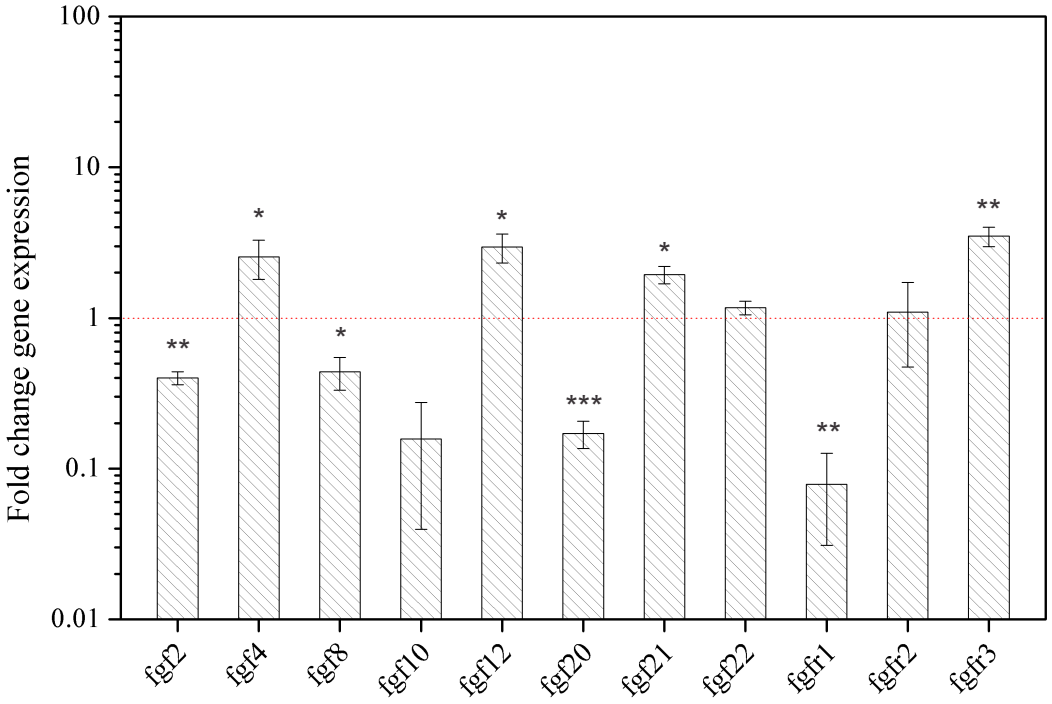

Figure 5.3B: Mean fold change ($2^{-\Delta\Delta Cq}$) in expression of FGF genes in etoricoxib group animals with respect to controls at blastema stage. Dashed red line indicates gene expression level of control group samples taken as 1.

Scale bars represent standard errors of means; *p<0.05, **p<0.01, ***p<0.001 as compared to controls

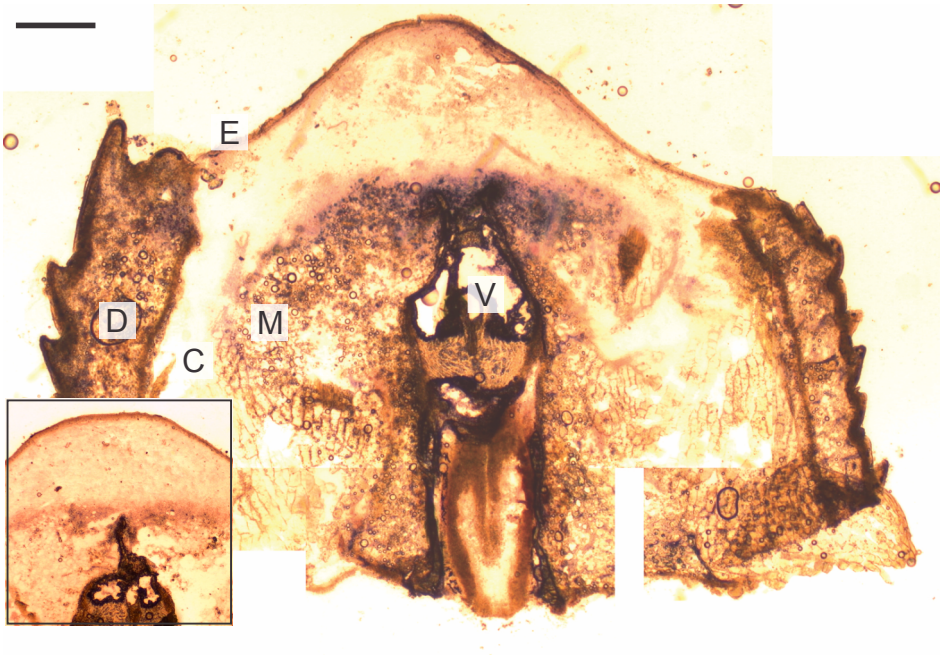

Figure 5.4: Immunohistochemical localisation of β-Catenin in a longitudinal section of blastema stage regenerate. Blue stained region indicates presence of β-Catenin protein. Depicted here is a composite image of multiple micrographs. Negative control for the primary antibody is shown inset. Scale bar represents 500 μm.

C: Connective tissue; D: Dermis; E: Wound epithelium; M: Muscle; V: Vertebra.

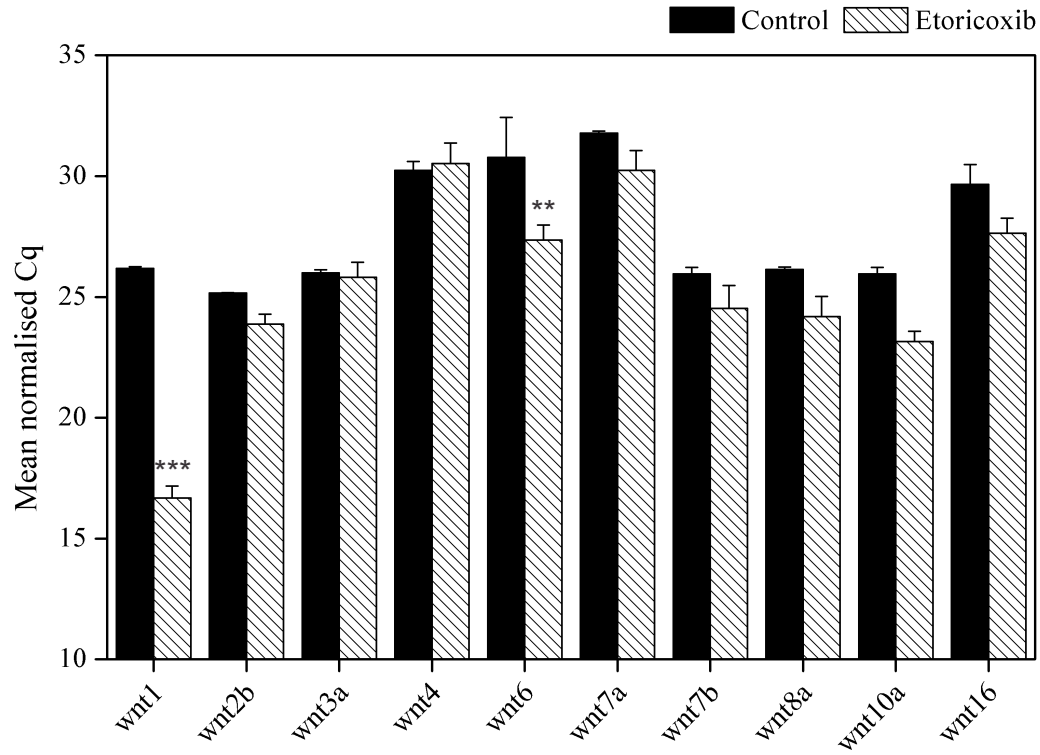

Figure 5.5A: Mean normalised Cq values for Wnt genes in intact (un-amputated) tail and blastema stage regenerates. Cq value is inversely related to gene expression levels.

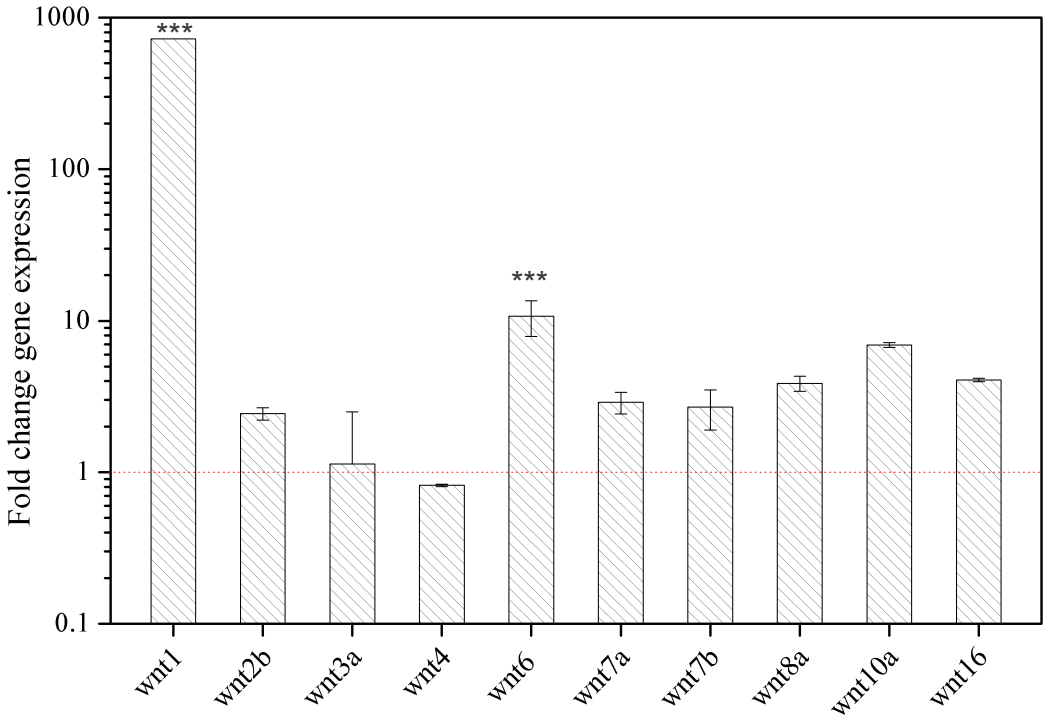

Figure 5.5B: Mean fold change ($2^{-\Delta\Delta Cq}$) in expression of Wnt genes in blastema stage regenerates with respect to intact tail tissue. Dashed red line indicates gene expression level of control group samples taken as 1.

Scale bars represent standard errors of means; **p<0.01, ***p<0.001 as compared to resting tail

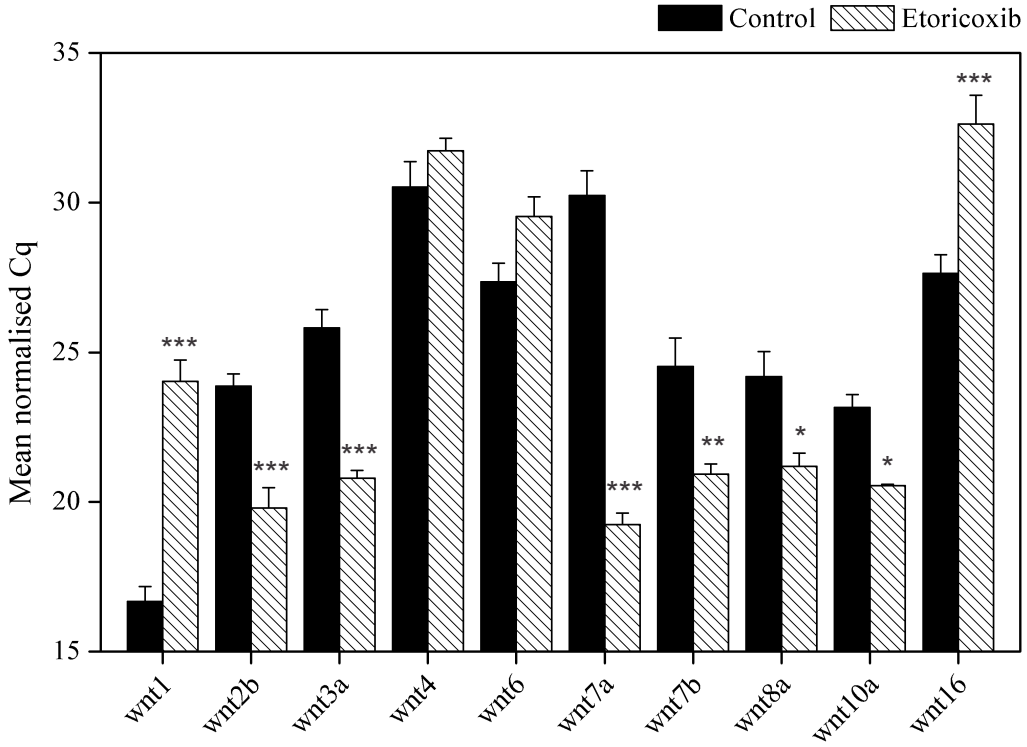

Figure 5.6A: Mean normalised Cq values for canonical Wnt ligand genes in control and etoricoxib group animals at blastema stage. Cq value is inversely related to gene expression levels.

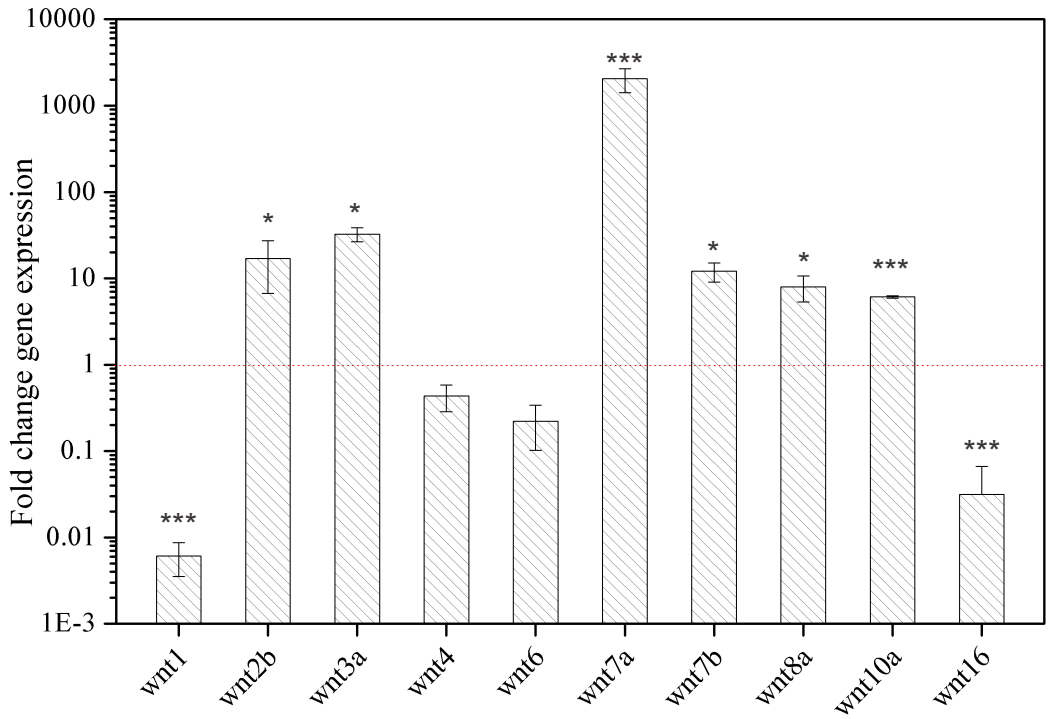

Figure 5.6B: Mean fold change ($2^{-\Delta\Delta Cq}$) in expression of canonical Wnt ligand genes in etoricoxib group animals with respect to controls at blastema stage. Dashed red line indicates gene expression level of control group samples taken as 1.

Scale bars represent standard errors of means; *p<0.05, **p<0.01, ***p<0.001 as compared to controls